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A synergistic interaction of 17-β-estradiol with specific cannabinoid receptor type 2 antagonist/inverse agonist on proliferation activity in primary human osteoblasts

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Abstract. The bone remodeling process is influenced by various factors, including estrogens and transmitters of the endocannabinoid system. In osteoblasts, cannabinoid receptors 2 (CB-2) are expressed at a much higher level compared to CB-1 receptors. Previous studies have shown that estrogens could influence CB-2 receptor expression. In the present study, the possible interactions of a specific CB-2 agonist and a specific CB-2 antagonist/inverse agonist with 17-β-estradiol were investigated in primary human osteoblasts (HOB). HOB cells were cultured in phenol red-free osteoblast growth medium (37˚C, 5% CO_2). In their 5th passage, HOB were exposed to different concentrations of i) 17-β-estradiol (1, 10 and 100 nM); ii) a specific CB-2 agonist (R,S)-AM1241 (1 and 7.5 μM); and iii) a specific CB-2 antagonist/inverse agonist AM630 (10 μM) and to selected combinations of the substances. After 24 and 48 h of incubation, HOB proliferation activity was measured using a WST-8 assay. Alkaline phosphatase activity was also evaluated using spectrophotometry. Concomitant exposure of HOB to 17-β-estradiol (10 nM) and to specific CB-2 antagonist/inverse agonist (10 μM) showed similar HOB proliferation activity to HOB incubated with 17-β-estradiol only at a 100 nM concentration. By contrast, concomitant incubation of HOB with 17-β-estradiol (10 nM) and specific CB-2 agonist (7.5 μM) resulted in decreased HOB proliferation activity as compared to HOB incubated with 17-β-estradiol only (10 nM). Similar findings were observed after 24 and 48 h of incubation. In all the experiments, HOB successfully passed the alkaline phosphatase differentiation test. In conclusion, for the first time a synergistic interaction between 17-β-estradiol and specific CB-2 antagonist/inverse agonist was observed in HOB. Understanding the molecular pathways of this interaction would be of great importance in developing more efficient and safer drugs for treating or preventing bone diseases.

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Introduction

Bone remodeling is the main process in maintaining the integrity of bone structure. Throughout life, bones undergo three phases: Phase of rapid skeletal growth and increasing bone mineral density, sustenance phase and phase of predominant bone resorption causing bone loss. The bone remodeling process is influenced by numerous factors, including estrogens and the endocannabinoid system. Misbalance of bone remodeling mechanisms causes one of the most common degenerative diseases in developed countries, osteoporosis (1). It is estimated that >200 million people worldwide suffer from osteoporosis. The costs of health care services in the European Union as consequences of osteoporotic complications are already considerable and, if current trends continue, the costs are predicted to double by 2050 (2).

Osteoblasts are influenced by estrogens at the cellular and molecular level. Estrogens bind to nuclear estrogen receptors (ERs) in osteoblasts, the ERs dimerize and act as transcription factors modulating the expression of specific DNA sequences (3). Estrogens increase collagen I and osteoprotegerin gene expression and certain evidence indicates inhibitory effects of estrogens on osteoblast apoptosis (4,5).

Cannabinoids bind and activate cannabinoid receptors 1 and 2 (CB-1 and -2 receptors), as well as non-CB-1/CB-2 receptor GPR55 and vanilloid type 1 receptor (TRPV1) (6-8). CB-1 receptors are predominantly located in the central nervous system, whereas CB-2 receptors are expressed in the immune system, cirrhotic liver, arteriosclerotic plaques, gastrointestinal mucosa and during brain inflammation (9-11). CB-2 receptors have also been reported to have a significantly higher expression in osteoblasts, osteoclasts and osteocytes compared to CB-1 receptors (12-14). Therefore, specific CB-2 agonists/antagonists could be involved in the regulation of bone remodeling as a result of their effects on osteoblasts and osteoclasts. In addition, CB-2 receptor specific ligands do not

induce psychoactive adverse effects, making them more suitable for potential clinical use (15). The CB1 and CB2 receptors inhibit adenylyl cyclase activity, which is linked to a variety of secondary messengers, including p42/44 mitogen-activated protein kinase (16-18), p38 mitogen-activated protein kinase (19), c-Jun N-terminal kinase (20), activator protein 1 and Ca^{2+} transients (18,21). The role of GPR55 receptor in bone formation has not been extensively studied and appears to be minor (7). The TRPV1 receptor is also considered to be important in the pathogenesis of osteoporosis (22,23).

Increased expression of CB-2 receptors was found on osteoclasts when they were treated with 17-β-estradiol (24). Our new hypothesis is that 17-β-estradiol could also influence the CB-2 receptor expression in osteoblasts and is based on the 17-β-estradiol action on ERs and changes in gene transcription.

Accordingly, a possible interaction between 17-β-estradiol and specific CB-2 agonist and/or antagonist was considered and, to the best of our knowledge, for the first time the hypothesis was experimentally tested in primary human osteoblasts (HOB).

Materials and methods

Primary HOB growing and testing. Proliferating HOB were purchased from PromoCell (Heidelberg, Germany). The donor was a healthy 60-year-old Caucasian female. The cells were grown in phenol red-free cell growth medium (PromoCell) at 37˚C and 5% CO_2 (CO_2-incubator; Sanyo, Moriguchi, Japan). Trypsinization was performed following the PromoCell's subcultivation instructions. The experiment started with HOB in their 5th passage. After 24 and 48 h of HOB exposure to different concentrations of 17-β-estradiol (1, 10 and 100 nM), to specific CB-2 agonist (R,S)-AM1241 (1.0 and 7.5 μM), to specific CB-2 antagonist/inverse agonist AM630 (10 μM) and to their selected combinations, the colorimetric cell viability test WST-8 (PromoKine, Milpitas, CA, USA; cat. no. PK-CA705-CK04-100) and alkaline phosphatase activity colorimetric assay (BioVision, Milpitas, CA, USA; cat. no. K412-500) were performed in triplicates, strictly following the manufacturer's instructions. (R,S)-AM1241 (cat. no. A6478-5MG), AM630 (cat. no. SML0327-5MG) and 17-β-estradiol (cat. no. E2758-1G) were all obtained from Sigma (St. Louis, MO, USA). The samples with HOB exposed to 1 nM concentration of 17-β-estradiol, which approximately corresponds to physiological estrogen levels in healthy females (25), were treated as controls.

Statistical analysis. All the data are presented as means ± standard deviation from three experimental samples. Statistical differences between the groups were determined using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA), with independent samples t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Proliferation activity. In detail, the results on HOB proliferation activity are presented in Figs. 1-3.

Influence of a combination of 17-β-estradiol and specific CB-2 antagonist/inverse agonist on HOB proliferation activity. After 24 h, the highest proliferation activity was observed in HOB exposed to the specific CB-2 agonist (7.5 μM; Fig. 3), 100 nM concentration of 17-β estradiol and in HOB exposed to a combination of 10 nM concentration of 17-β estradiol with 10 μM of the specific CB-2 antagonist/inverse agonist. The interaction between 17-β-estradiol and specific CB-2 antagonist/inverse agonist on HOB proliferation activity thus appears to be synergistic.

Influence of 17-β-estradiol on HOB proliferation activity. The influence of 17-β-estradiol (1, 10 and 100 nM) on HOB proliferation activity was dose-dependent; HOB proliferation activity increased with increasing 17-β-estradiol concentrations (Figs. 1 and 2).

Influence of specific CB-2 agonist on HOB proliferation activity. Similar dose-dependent findings were observed in HOB treated with specific CB-2 agonist; higher concentration of specific CB-2 agonist (7.5 μM) resulted in increased HOB proliferation activity compared to the lower concentration of the specific CB-2 agonist (1 μM) (Fig. 3).

Influence of a combination of 17-β-estradiol and specific CB-2 agonist on HOB proliferation activity. HOB exposed only to specific CB-2 agonist (7.5 μM) had higher proliferation activity compared to HOB exposed to a combination of specific CB-2 agonist (7.5 μM) and 17-β-estradiol (10 nM) (Figs. 1 and 3). HOB proliferation activity of the combination was similar to that found in HOB treated only with 10 nM 17-β-estradiol. The interaction between 17-β-estradiol and specific CB-2 agonist on HOB proliferation activity thus appears to be antagonistic.

Influence of specific CB-2 on HOB proliferation activity. Specific CB-2 agonist at 7.5 μM increased HOB proliferation activity more than specific CB-2 antagonist/inverse agonist at 10 μM. These results also show that HOB proliferation activity was higher when the cells were concomitantly treated with a combination of specific CB-2 agonist (7.5 μM) and specific CB-2 antagonist/inverse agonist (10 μM) compared to cell exposure solely to specific CB-2 antagonist/inverse agonist (10 μM).

Similar findings on HOB proliferation activity were also observed after 48 h of incubation; however, the differences after 48 h were less prominent. In all the experiments, HOB successfully passed the alkaline phosphatase differentiation test.

Discussion

To the best of our knowledge, the present study shows for the first time, a probable synergistic interaction between 17-β-estradiol and the specific CB-2 antagonist/inverse agonist AM630, as observed in primary HOB *in vitro*.

The influence of a combination of 17-β-estradiol and specific CB-2 antagonist/inverse agonist on HOB proliferation activity was examined. The results highlighted that the combination of 17-β-estradiol (10 nM) with specific CB-2 antagonist/inverse agonist (10 μM) increases HOB proliferation activity ~32% compared to proliferation activity of HOB exposed solely to the same concentration of 17-β-estradiol (10 nM). To achieve

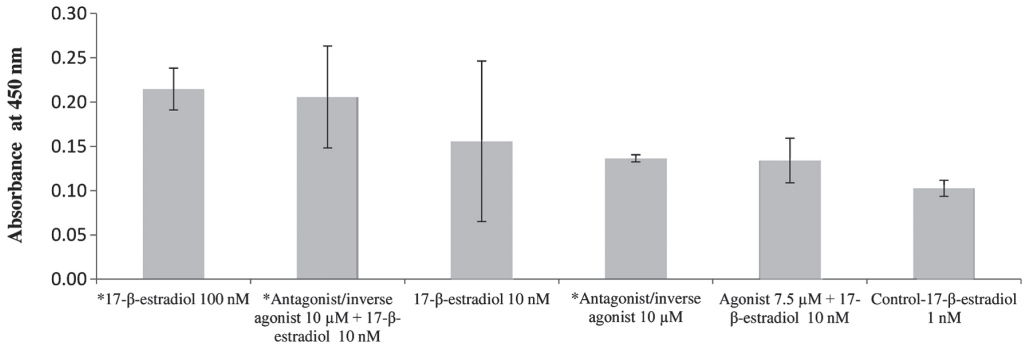

Figure 1. Human osteoblast (HOB) proliferation activity after 24 h of exposure. Average absorbance values of triplicates (which are in linear correlation with HOB proliferation activity) and standard deviations after 24 h of exposure are shown. *Statistically significant differences (P<0.05). *P<0.05, compared to the control (1 nM 17-B-estradiol).

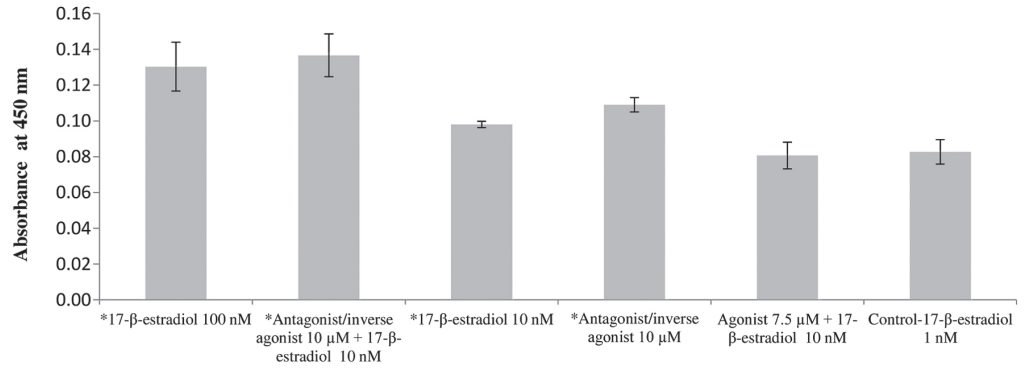

Figure 2. Human osteoblast (HOB) proliferation activity after 48 h of exposure. Average absorbance values of triplicates (which are in linear correlation with HOB proliferation activity) and standard deviations after 48 h of exposure are shown. *Statistically significant differences (P<0.05). *P<0.05, compared to the control (1 nM 17-B-estradiol).

almost the same effect on HOB proliferation activity as with 100 nM 17-β-estradiol, 10-times lower concentrations of 17-β-estradiol (10 nM) could be used when co-administering 17-β-estradiol with specific CB-2 antagonist/inverse agonist (10 μM). A synergistic interaction between estrogens and specific CB-2 antagonists/inverse agonists could be proposed and explained by the possibility of the increase in CB-2 receptor expression influenced by estrogens and/or by interactions on other molecular levels. 17-β-Estradiol could be assumed to increase the expression of CB-2 receptors, as has been previously observed in osteoclasts *in vitro* (24), or it is possible that estrogens act through other, non-genomic mechanisms (26). Recent studies also show that selective estrogen modulators, raloxifene (27), bazedoxifene and lasofoxifene, behave as CB-2 inverse agonists (28), which may also be true for 17-β-estradiol and would correlate with the present results.

Previous studies showed that CB-2 receptor antagonist/inverse agonist AM630 inhibited osteoclast formation and bone resorption *in vitro* (12,29). Furthermore, it has been shown that the administration of AM630 to ovariectomized wild-type mice prevented ovariectomy-induced bone loss (29). The present results do not entirely support the above findings; HOB proliferation activity after 24 h was higher compared to control cells when the tested cells were exposed to specific CB-2 antagonist/inverse agonist as the only testing substance.

Influence of 17-β-estradiol on HOB proliferation activity. Estrogens have antiresorptive properties and have been in clinical use for treatment of osteoporosis for a long time (30). The results from the present study confirm a dose-dependent estrogen response on HOB proliferation activity. As expected,

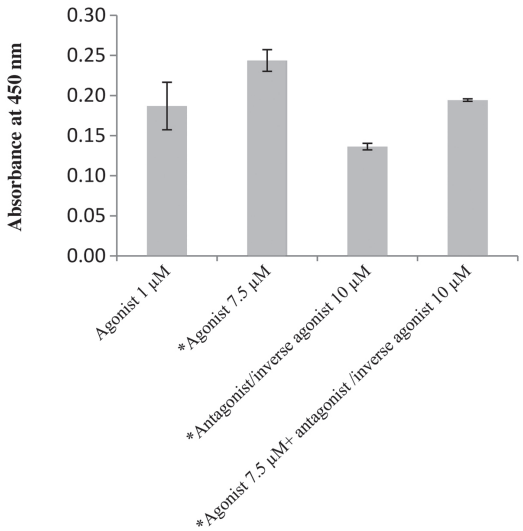

Figure 3. Human osteoblast (HOB) proliferation activity after 24 h of exposure. Average absorbance values of triplicates exposed to cannabinoid receptor 2 antagonist/inverse agonist or agonist (which are in linear correlation with HOB proliferation activity) and standard deviations after 24 h of exposure are shown. *Statistically significant differences (P<0.05). *P<0.05, compared to the control (1 nM 17-B-estradiol).

higher concentrations of 17-β-estradiol resulted in higher HOB proliferation activity compared to lower 17-β estradiol concentrations.

The influence of specific CB-2 agonist on HOB proliferation activity was investigated in the present study. Previous studies have shown that specific CB-2 agonists directly stimulate stromal cells/osteoblasts and simultaneously inhibit monocyte/osteoclasts, directly and indirectly by inhibiting the expression of RANKL in stromal cells/osteoblasts (13). Previous studies have also shown that specific CB-2 agonists induce mitogenic effects in osteoblasts via activation of a Gi protein-cyclin D1 and the extracellular signal-regulated kinase 1/2 pathway (31,32). This was indirectly confirmed in the present study by the increase in proliferation activity of HOB when they were treated solely with the specific CB-2 agonist. As hypothesized, HOB proliferation activity was influenced by the specific CB-2 agonist in a dose-dependent manner; higher concentrations of the specific CB-2 agonist increased HOB proliferation activity more than the lower concentrations.

The influence of a combination of 17-β-estradiol and specific CB-2 agonist were examined on HOB proliferation activity. The results show that in the absence of 17-β-estradiol, the specific CB-2 agonist had a stimulating effect on HOB proliferation activity *in vitro*, whereas the combination of the two substances appeared to have an antagonistic effect on HOB proliferation activity. This result additionally confirms the interplay between estrogens and cannabinoids, as already proposed by a synergistic interaction between 17-β-estradiol and specific CB-2 antagonist/inverse agonist.

Additional notable observations in the present study are that 7.5 μM specific CB-2 agonist increases HOB proliferation activity to a higher extent compared to 10 nM specific CB-2 antagonist/inverse agonist. Furthermore, in the presence of 7.5 μM specific CB-2 agonist and 10 nM specific CB-2 antagonist/inverse agonist, HOB proliferation activity is higher compared to cells treated with 10 nM specific CB-2 antagonist/inverse agonist as the only testing substance. Therefore, it could be speculated that in the absence of estrogens, specific CB-2 agonists may overcome the effects of specific CB-2 antagonists/inverse agonists on HOB proliferation activity. However, further studies with different CB-2 agonists and antagonists/inverse agonists in various concentrations should be tested to clarify the above observations and to evaluate their potential clinical importance.

Another factor in the present study is that all the experiments and treatments, dose- and time-dependency, as well as the controls, showed that HOB in cell cultures maintained their alkaline phosphatase activity. In brief, HOB *in vitro* did not de-differentiate in the controls or when treated with various chemicals, but further quantitative studies should be performed in order to evaluate the degree of differentiation exactly in each single experiment setting.

There are possible clinical applications for the results of the present study. The difference between the actions of cannabinoid antagonists/agonists in the presence or absence of estrogens may lead to specific drug treatment of osteoporosis in elderly patients with specific CB-2 agonists, which are more effective in the presence of low estrogen concentrations and even better as a combination drug for females with CB-2 antagonists/inverse agonists and 17-β-estradiol, which would have a synergistic effect on osteoblasts activity and could provide novel treatment of postmenopausal induced osteoporosis in females. This could lead to a new, possibly gender-dependent strategy in osteoporosis prevention. Firstly, combination treatment of specific CB-2 antagonists/inverse agonists and estrogens may lead to lower doses of estrogens administered to patients with osteoporosis and consequently fewer of their harmful side-effects and minimizing of the serious complications. Notably, it is well-known that long-term use of estrogens can promote carcinogenesis, particularly in breast and endometrial cancer (32-34). Secondly, a number of studies indicate that the endocannabinoid system plays protective roles against the growth and the spreading of several types of carcinomas, including endometrial and breast cancers (35,36). Therefore, a combination of cannabinoids and estrogens may have beneficial effects by inhibiting estrogen-induced carcinogenesis. Thirdly, adding specific CB-2 antagonists/inverse agonists to contraceptive pills could increase maximal bone mineral density in females. Contraceptive pills are most commonly used before the age of 30 years, which corresponds to the phase of rapid skeletal growth and increasing bone mineral density; this makes it ideal for preventing osteoporosis or delaying its development. Furthermore, the involvement of the endocannaboid system is also indicated in cardioprotection (37), which is a particularly important concern in the postmenopausal era. These benefits could potentially be increased by synergistic interactions with estrogens.

Recent pharmacological improvements allow oral application of specific CB-2 agonists (i.e., S-777469) and specific CB-2 antagonists/inverse agonists (i.e., SR 144528); therefore (38,39), their potential medical applications in the treatment of various diseases are becoming even more noteworthy. By contrast, safety and adverse effects of CB-2 agonists/antagonists/inverse agonists should be examined prior to clinical use.

In conclusion, the results of the present study are quite informative and represent the idea of a probable synergistic interaction between 17-β-estradiol and specific CB-2 antagonist/inverse agonist in primary HOB. However, these observations require further investigation in order to clarify and evaluate the clinical applicability of the results. Mineralization, specific osteoblast molecular biomarkers (i.e., runt-related transcription factor 2, osteonectin, osteocalcin, bone sialoprotein and collagen I), molecular pharmacodynamic interactions, expression of ERs α and β, as well as the CB-1 and -2 receptors expression should be performed. As indicated in the introduction, other receptors are also involved in cannabinoid signaling pathways. For this reason, an in depth understanding of how TRPV1 and GPR55 receptors in combination with CB-1 and -2 receptors interact to modify the activity of osteoblasts could be helpful in predicting the efficiency of cannabinoid pharmacotherapy.

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