Characteristics of the state of bone tissue in genetically modified mice with impaired enzymatic regulation of steroid hormone metabolism

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Abstract

Introduction: The aim was to evaluate the structural and functional changes of bone tissue in mice with null expression of 11β-HSD2 or both 11β-HSD2 and Apolipoprotein E.

Materials and methods: The experimental study was performed in 60 male mice, weighting 24–30 g. The animals were kept in accordance with the rules of laboratory practice for preclinical studies on the territory of the Russian Federation. Mice lacking 11β-HSD2 (Hsd2-/-) and male mice lacking 11β-HSD2 and Apolipoprotein E (Hsd2-/-/ApoE-/-) were used in the study. We studied and characterized the state of bone tissue, indicators of bone density, microcirculation in bone tissue, endothelial dysfunction coefficient, width of bone trabeculae, as well as serum concentrations of bone alkaline phosphatase, hydroxyproline, deoxypyrolinol, and expression levels of p53, Bcl2, Bax, eNOS genes.

Results and discussion: We showed that mice with the Hsd2-/- genotype with no expression of 11β-HSD2 by the 6th month of life showed a statistically significant decrease in bone density, which progresses to the 7th and 8th months of life. At the 8th month of animal life, a decrease in bone density is accompanied by a statistically significant decrease in the level of microcirculation in the bone and an increase in the coefficient of endothelial dysfunction. Taking into account the relationship of endothelial dysfunction, atherogenesis and disorders in the processes of bone remodeling, in the framework of this study, we also assessed the state of bone tissue in double transgenes with the genotype Hsd2-/ApoE-/-, which lack the expression of both 11β-HSD2 and Apolipoprotein E. In this study, we also saw increased activation of processes leading to disruption of bone remodeling processes. In the group of the animals with the genotype Hsd2-/ApoE-/-, we found statistically significant differences from the mice with no expression of 11β-HSD2 in bone density and microcirculation, and the width of bone trabeculae. Also, a statistically significant increase in hydroxyproline and deoxypyrolinol was found in the group of double transgenes, in the absence of significant changes in the concentration of bone alkaline phosphatase. This fact indicates a pronounced activation of bone resorption processes in the absence of activation of osteosynthesis processes, which leads to the detected violation of bone remodeling processes.

Conclusion: Thus, we have shown that a violation of the metabolic regulation of steroid hormone metabolism in animals with null expression of the 11β-HSD2 (Hsd2-/- genotype) leads to the development of signs of osteoporosis – bone density decreases, which is accompanied by a decrease in the width of bone trabeculae, the level of microcirculation in bone tissue decreases simultaneously with an increase in the coefficient of endothelial dysfunction. The additional null expression of ApoE gene in double transgenes with the genotype Hsd2-/-/ApoE-/- leads to an increase in the severity of changes associated with a violation of bone remodeling processes and, in addition to a more pronounced change in
bone tissue density, bone trabecular width, microcirculation and the coefficient of endothelial dysfunction leads to an increase in the concentration of biochemical markers of bone resorption. These changes indicate the important role of the enzyme 11ß-hydroxysteroid dehydrogenase type 2 in the processes of bone remodeling disorders.

Graphical abstract

Keywords

osteoporosis, bone density, steroid hormone metabolism, transgenic animals, 11b-HSD2, Aop, Hsd2−/−, Hsd2−/−/Aop−/−.

Introduction

The World Health Organization has officially identified osteoporosis (OP) as one of the ten most important chronic diseases of mankind, because it is very widespread, has a clear definition, diagnostic methods, and opportunities for prevention and treatment. OP – a systemic skeletal disease from the group of metabolic osteopathies – is characterized by a decrease in bone mass and a violation of the microarchitectonics of bone tissue, which leads to a decrease in bone strength and, as a consequence, to an increased risk of fractures. Being one of the most frequent causes of pathological disabling fractures, osteoporosis significantly limits the quality and duration of life and aggravates the course of concomitant diseases (World Health Organization 2003; Sözen et al. 2017; Korokin et al. 2022).

It is known that a decrease in osteoreparative processes and an increase in osteoresorption are often associated with a violation of steroid hormone metabolism. In this regard, the main risk factors for the development of osteoporosis are the postmenopausal period and long-term therapy with glucocorticoids (Laurent et al. 2022). Therefore, one of the most obvious directions for the search for molecular predictors and new targets for pharmacotherapy of osteoporosis is the system of tissue metabolism of steroid
hormones. The skeleton is one of the classic targets of glucocorticoid hormones. Corticosteroid activation in hypercorticism, prolonged treatment with corticosteroid hormones or aldosteronism is associated with a decrease in bone density (Frenkel et al. 2015; Kuipers et al. 2016).

The enzyme 11b-hydroxysteroid dehydrogenase (11b-HSD), represented by two isoforms, performs the mutual conversion of cortisone and cortisol in tissues. Using the methods of reverse genetics, the systemic consequences of knockout of both isoforms were established. Convincing evidence demonstrates that both enzymes are involved in the pathogenesis of osteoporosis, with type 11b-HSD type 2 apparently performing an osteoprotective role. In addition, since animals with type 11b-HSD deficiency are characterized by pro-inflammatory activation of the endothelium, we assume that the study of the interaction between the endothelium and bone tissue is of particular interest. Due to the anatomical features of the structure of the vascular bed of the skeleton, the function of the endothelium is critical for bone homeostasis (Korokin et al. 2022).

Here we aimed to evaluate the structural and functional changes of bone tissue in mice with null expression of 11β-HSD2 or both 11β-HSD2 and Apolipoprotein E.

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Materials and methods

The experiment was performed at the Center for Preclinical and Clinical Studies of Belgorod State National Research University according to The Rules of Laboratory Practice, approved by Order No.708n of the Ministry of Health of the Russian Federation of 23.08.2010, in strict compliance with The European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes (Directive2010/63/EU). The experimental studies were approved by the Bioethical Commission of Belgorod State National Research University (minutes №15/10 of 29.10.2021). Vivisection was performed in compliance with the ethical principles of treating laboratory animals outlined in The European Convention for the Protection of Vertebral Animals Used for Experimental and Other Scientific Purposes (CETS No.123).

Experimental animals

The experimental study was performed in 60 male mice, weighting 24–30 g. The animals were kept in accordance with the rules of laboratory practice for preclinical studies on the territory of the Russian Federation. The animals were kept under the standard conditions corresponding to the sanitary rules on the organization, equipment and maintenance of experimental biological clinics (vivariums) No. 1045-73, approved by the Ministry of Health of the USSR on 06.04.1973 and GOST R 53434-2009, in the individually ventilated cages (Tecniplast S.p.A., Italy) designed for keeping small laboratory animals. The bedding was sawdust, sterilized by ultraviolet irradiation. Special pellet feed for small laboratory rodents and pre-treated water disinfected with UV irradiation were used. In each cage, microclimate was created and supported by an individual ventilation system. All the animals had been acclimatized and quarantined for at least 10 days before the experiment.

Experimental groups

The experimental groups included:

1 – 20 male C57Bl/6J mice (WT);
2 – 20 male mice lacking 11β-HSD2 (Hsd2−);
3 – 20 male mice lacking 11β-HSD2 and Apolipoprotein E (Hsd2−/Apoe−).

Study of bone density

The study of bone density was carried out at the beginning of the experiment, when the age of the animals was 6 months, and then at two time points – at the age of 7 and 8 months, using the In-Vivo FX PRO system (Bruker, USA). In the In-Vivo FX PRO system, the principle of software evaluation of bone density is based on testing bone mineralization in an experimental animal by modeling cylindrical symmetry on an analytical X-ray image of a segment of a long tubular bone with appropriate calibration based on a Ca3(PO4)2 sample immersed in water. Measurements of the density of long tubular bones suggest that the selected bone segment is a flat projection of a cylinder in cross section, where the outer shell of the cylinder is a homogeneous substance of higher density (bone tissue), and the inner substance of lower density (bone marrow). It is assumed that the medium in which the cylinder is immersed is water and may have zero depth (air). The software performs a “cylindrical fitting” procedure to extract important parameters of the selected bone segment from a high-resolution X-ray image. Based on the program evaluation, data characterizing various indicators of the selected bone segment is generated. The “Bone Column Density” and “Marrow Column Density” indicators characterize the degree of attenuation of X-ray radiation per unit depth of the material. This is an analogue of the “optical density”, often used to characterize the attenuation of light passing through a colored substance. The indicators provide a fairly accurate assessment of the density of the bone column, which directly depends on the measured basic contrast of the digital radiograph. The "Bone Density" indicator is determined by the density of the bone column and is calculated from the “Bone Column Density” indicator, taking into account the depth of the tissue sample and the effect of this depth on the density of a similar Ca3(PO4)2. The indicator is expressed in g/cm3. And finally, the "Bone Surface Density" indicator characterizes the average “coverage” of the material projected onto a flat surface. It is calculated based on the
values of the density of the column of bone tissue and radii adjusted for the depth of the tissue and the effect of this depth on the density of a similar column Ca₃(PO₄)₂.

In this study, we operated on the results of the “Bone Density” index (BD, g/cm³) for the proximal metaphysis, diaphysis and distal metaphysis of the femur.

Morphofunctional assessment of the state of bone tissue

Morphofunctional assessment of the development of osteoporosis was evaluated in animals at the age of 8 months by assessing regional microcirculation, conducting vascular tests and histomorphometric examination. The level of microcirculation was assessed in the spongy bone tissue of the proximal metaphysis of the right femur. To obtain bone microcirculation data, BIOPAC Systems (USA) equipment was used: an MP100-150 polygraph with LD-F100C laser flowmetry module and a TSD144 sensor. The LDF results were recorded by the Acq Knowledge software (versions 3.8-4.2); the microcirculation values were expressed in perfusion units (PE). The development of microcirculation disorders was assessed after measuring the intraosseous level of microcirculation, for which vascular tests were performed for endothelium-dependent (acetylcholine intravenously (IV) 40 mcg/kg) and endothelium-independent (sodium nitroprusside IV 30 mcg/kg) vasodilatation. Using the results of vascular tests, the coefficient of endothelial dysfunction (QED) was calculated as the ratio of the area of the triangle above the microcirculation recovery curve in response to the administration of nitroprusside to the area of the triangle above the microcirculation recovery curve in response to the administration of acetylcholine (Sobolev et al. 2018).

Histological examination

To confirm the development of osteoporosis, a morphological study of the proximal metaphyses of the femurs was performed. For this purpose, during sample preparation, decalcification was previously carried out (Zamboni fixative and decalcifatol with a 20% solution of ethylenediaminetetraacetate and 5% sucrose-0.1 M tris at +4 °C), fixation and decalcification was performed. For this purpose, during sample preparation, it was subjected to chloroform cleaning; the sample was collected and washed with isopropyl alcohol and 70% ethyl alcohol. The concentration of the obtained RNA was measured on an IMPLEN-NanoPhotometer spectrophotometer and adjusted to a concentration of 300 ng/µL. Reverse transcription was performed using the MMLVRTSK021 kit in accordance with the manufacturer’s protocol (Evrogen). The mixture was gently mixed and heated at 70 °C for 2 minutes to melt the secondary RNA structures and then anneal the OligoDT primer. After that, the samples were transferred to ice. The entire reaction mixture was incubated for 60 minutes at 40 °C in a T100 Thermal Cycler (Bio-Rad). To stop the reaction, the mixture was heated at 70 °C for 10 minutes (Korokin et al. 2019). The resulting cDNA was diluted to a concentration of 1 ng/µL. The level of gene expression was calculated relative to the values of the Gapdh reference gene. The expression at a specific point was calculated using the formula: Gene expression = [(Ct(Gapdh)/Ct(Gene of Interest)] (Table 1).

Biochemical blood testing

Blood sampling was carried out in the animals aged 8 months from the cavity of the left ventricle of the heart during the euthanasia procedure. The concentrations of bone alkaline phosphatase (a marker of osteosynthesis), hydroxyproline and deoxypyrinoline (a marker of osteoresorption) were determined by enzyme immunoassay. The collected blood was stored at room temperature for 2 hours, centrifuged at 1000 rpm for 20 minutes and the infusion fluid was taken. After that, the concentrations of bone alkaline phosphatase (B-ALP, SEB091Mu, CloudClone Corp), hydroxyproline (Hyp, CEA621Ge, CloudClone Corp) and deoxypyrinoline (DPD, E-EL-M0408, Elabscience) were determined using a Thermo, MULTISKAN MK3, USA, using standard kits for ELISA Biotechnology in accordance with the instructions provided by the manufacturer.

Table 1. Primers Used for Quantitative PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>F-primer</th>
<th>R-primer</th>
<th>Product Length (b.p.)</th>
<th>GenBank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tpp35 (p53)</td>
<td>CGACTCAGTGGGGGCCCAC</td>
<td>CCAAGGAGGTCTCCAGTC</td>
<td>95</td>
<td>NM_001127233.1</td>
</tr>
<tr>
<td>Bcl2</td>
<td>TTCACCATCAGCTAGTCAC</td>
<td>TACTCCAGAAGGTGACGC</td>
<td>102</td>
<td>NM_009741.5</td>
</tr>
<tr>
<td>Bax</td>
<td>CCGGGAAGTGTGAGTACCA</td>
<td>GAGGGCTTTCTCCAGTAC</td>
<td>96</td>
<td>NM_007527.3</td>
</tr>
<tr>
<td>Nos3 (eNOS)</td>
<td>AGGCGATCTGCCGAGATGCTA</td>
<td>TAGGGCGCATAGCGTATC</td>
<td>103</td>
<td>NM_008713.4</td>
</tr>
</tbody>
</table>
Statistical data processing

The statistical data were processed using the Statistica 10.0 software. Shapiro-Wilk and Spiegelhalter (norm-test package) normality tests were performed for the obtained data; the equality of variances was assessed using the Levene’s test (lawstat package). Depending on the type of distribution and the equality of variances, the significance of the results obtained was evaluated using parametric (ANOVA) or non-parametric (Kruskal-Wallis test) one-way analyses of variance, and as a post-hoc analysis to identify intergroup differences, the Student’s t-test or the Mann-Whitney test were used, respectively, with the Benjamini-Hochberg correction for multiple tests. The results were considered reliable at \( p \leq 0.05 \).

Results and discussion

In this study, we measured the values of the “Bone Density” index (BD, g/cm³) for the proximal metaphysis, diaphysis and distal metaphysis of the right and left femur (Fig. 1).

As a result of measuring bone density at these points using the In Vivo FX PRO system (Bruker, USA), it was found that in wild-type animals (WT), bone density did not change statistically significantly between the age of 6 and 8 months and was 5.76±0.145 g/cm³ at the age of 6 months, 5.74±0.143 g/cm³ in animals aged 7 months and 5.70±0.14 g/cm³ in animals aged 8 months.

As soon as the age of 6 months, male mice with the Hsd2⁻/⁻ genotype with no expression of 11ß-HSD2 showed a statistically significant decrease in bone density (\( p=0.0018 \) in comparison with wild-type animals). We also found a progression of bone density reduction from the age of 6 to the age of 7 and 8 months in this experimental group (Fig. 2). In the group of genetically modified animals with the genotype Hsd2⁻/⁻/Apoe⁻/⁻ – male double knockout mice with zero expression of 11ß-HSD2 and apolipoprotein E, the most pronounced decrease in bone density was found at the age of 6 months, whereas at the age of 8 months, bone density in this experimental group was significantly (\( p=0.048 \)) below the values in the Hsd2⁻/⁻ group (Fig. 2).

During the study, it was found that the microcirculation level of the proximal metaphysis of the femur of animals with the genotype 11ß-HSD2⁻/⁻ relative to the intact animals significantly decreased from 101.1±2.54 perfusion units (PE) in the intact group to 91.13±2.12 PE (\( p=0.0099 \)) in the HSD2⁻/⁻ group and to 82.76±1.91 (\( p<0.001 \)) in the group of mice with the Hsd2⁻/⁻/Apoe⁻/⁻ genotype (Fig. 3, 8th month LDF). When conducting tests with endothelium-dependent (intravenous administration of acetylcholine) and endothelium-independent vasodilation (intravenous administration of sodium nitroprusside) with calculation of the coefficient of endothelial dysfunction, an increase in QED was found from 1.21±0.025 in the intact animals to 2.065±0.07 (\( p<0.001 \)) in the group of mice with the Hsd2⁻/⁻ genotype and up to 2.37±0.04 (\( p<0.001 \)) in the group of mice with the Hsd2⁻/⁻/Apoe⁻/⁻ genotype (Fig. 3, 8th month CED).

For further morphological studies, bone biomaterial was taken. Histological sections of proximal femoral bones of animals were subjected to microscopy and histomorphometry. Osteoporotic changes in skeletal bones were histologically confirmed in mice with the genotype Hsd2⁻/⁻ and double transgenics Hsd2⁻/⁻/Apoe⁻/⁻. Microscopy revealed pathological changes in the spongy bone tissue of the thigh, the thinning of the lattice network of bone trabeculae, as well as the thinning and perforation of bone plates. Also, we found a decrease in the average width of bone trabeculae in the spongy tissue of the proximal metaphysis of the femur. Thus, the average width of bone trabeculae in this localization in the intact animals was 62.58±7.23 microns, whereas in mice with the genotype Hsd2⁻/⁻ the width of the bone trabeculae was 53.74±4.27 microns (\( p = 0.001 \)) compared to WT. Width of bone trabeculae in double transgenes Hsd2⁻/⁻/Apoe⁻/⁻ was 48.61±4.7 microns, which is significantly lower in comparison with both the group of the intact animals (\( p = 0.001 \)) and the group Hsd2⁻/⁻ (\( p = 0.0265 \)) (Fig. 4A). Micrographs of the histological picture of the bone tissue of animals of experimental groups at >200 magnification are shown in Fig. 4B–D).

Figure 1. Example of bone density measurement using the In-Vivo FX PRO system (Bruker, USA). Note: 1 – proximal epiphysis of the right femur; 2 – diaphysis of the right femur; 3 – distal epiphysis of the right femur; 4 – proximal epiphysis of the left femur; 5 – diaphysis of the left femur; 6 – distal epiphysis of the left femur.
Further, the concentrations of bone alkaline phosphatase (a marker of osteosynthesis), hydroxyproline and deoxyprinoline (a marker of osteoresorption) were determined for the biochemical analysis of bone metabolism processes. It was found that there was no statistically significant change in bone alkaline phosphatase in the experimental groups (Fig. 5, 8 months B-ALP). In the group of double transgenes, the animals with the genotype Hsd2−/−/Apoe−/−, a statistically significant increase in the concentration of hydroxyproline ($p = 0.019$) was found (Fig. 5, 8 months Hyp). A statistically significant ($p = 0.0178$) increase in the concentration of another marker of bone resorption, deoxyproline, in comparison with the control group, was also found in the group of animals with the genotype Hsd2−/−/Apoe−/− (Fig. 5, 8 months DPD).
Gene expression assay revealed that mutant animals are characterized by increased expression of mRNA of proapoptotic factors p53 and Bax along with a decreased expression of mRNA of antiapoptotic factor Bcl2 and eNOS (Fig. 6). As can be seen from the heatmap, these changes (except eNOS) were more pronounced in double knockout mice consistent with morphological, X-ray and biochemical data.

**Conclusion**

The pleiotropy of the biological effects of steroid hormones and the complexity of their metabolic pathways cause difficulties in finally understanding the pathogenetic aspects of the development and progression of osteoporosis.
In tissues expressing receptors for steroid hormones, a complex cascade of enzymatic transformations functions, which limits the interaction of hormones with their intracellular ligands (Manolagas 2013).

The discovery of steroid hormone receptors on osteocytes led scientists to believe that steroid hormones should modulate their biosynthetic activity. Soon after, it was shown that steroid hormones not only affect the functional state of existing osteocytes, but actually are powerful regulators of their formation and life expectancy. In 1992–1995, cytokine-mediated mechanisms of regulation of osteoclastogenesis by estrogens and androgens were shown (Wiegertjes et al. 2020; Schoppa et al. 2022).

The activation of osteoclasts caused by glucocorticoids and the inactivation of osteoblasts leads to a negative balance of calcium in the bones. By blocking Wnt signaling and expression of the transcription factor Runx2 in osteoblasts, glucocorticoids stop osteogenesis and osteoporilation (Frenkel et al. 2015). Work in this direction naturally led to an understanding of the important role of tissue enzymes of steroid metabolism in the development of osteoporosis. Both 11ß-HSD enzymes are present in human and rodent bone tissue, but isoform 1 is expressed only in osteoblasts and osteoclasts, and isoform 2 is expressed only in osteoblasts (de Kloet et al. 2014; Fenton et al. 2022).

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In (Arampatzis et al. 2005), a reasonable assumption was proved that an imbalance in the activity of isoforms 11ß-HSD leads to the accumulation of cortisol in bone tissue and stimulation of osteoclast activity with further demineralization of bone tissue.

Mice with knockout 11ß-HSD 1, created and characterized in Edinburgh under the leadership of Yuri Kotel'levtsev in 1997, demonstrate metabolic tolerance to high doses of glucocorticoids, which logically results from the function of this enzyme (Kotel’levtsev et al. 1997). Recently, a study was conducted confirming that this line is resistant to the development of osteoporosis when exposed to high doses of glucocorticoids (Fenton et al. 2019). Mice with type 11ß-HSD knockout, obtained by the same team, are prone to hypertension, heart failure, hypokalemia and develop a clinical picture similar to hyperaldosteronism syndrome (Kotel’levtsev et al. 1999; Holmes et al. 2001). However, despite a deep phenotypic analysis conducted for over 20 years, there is practically no information about the state of bone tissue in this line. The closest work to this direction was carried out in 2004 by lines of mice with the expression of 11ß-HSD 2 in osteoclasts, which showed resistance to osteoporosis when administered with high doses of glucocorticoids (Wang et al. 2022). Also in 2017, a paper was published where it was demonstrated that the overexpression of 11ß-HSD 2 in osteoblasts led to a decrease in the expression of pro-apoptotic factors Fas and caspase-8 in vitro (Zhang et al. 2017).

Taking into account the relationship of endothelial dysfunction, atherosclerosis and disorders in the processes of bone remodeling, in the framework of this study, we also assessed the state of bone tissue in double transgenes with the genotype Hsd2-/-/ApoE-/-, which lack the expression of both 11ß-HSD2 and Apolipoprotein E. Previous studies have shown that mice with this genotype have a significant increase in pro-inflammatory processes in the endothelium of mice (Deuchar et al. 2010). In the present study, we also saw increased activation of processes leading to disruption of

![Figure 6. Heatmap representing gene expression of the transcriptomic markers related to apoptosis (Tp53, Bcl2, Bax) and function of endothelial cells (Nos3). Note: WT – wild-type C57Bl/6J males; Hsd2−/− – knockout male mice with null expression of the 11β-HSD2; Hsd2−/−/ApoE−/− – double knockout male mice with null expression of the 11β-HSD2 and Apolipoprotein E.](image-url)
bone remodeling processes. In the group of animals with the genotype Hsd2-/Apoe-/-, we found statistically significant differences from mice with no expression of 11β-HSD2 in bone density and microcirculation, and the width of bone trabeculae. Also, a statistically significant increase in hydroxyproline and deoxypyroline was found in the group of double transgenics, in the absence of significant changes in the concentration of bone alkaline phosphatase. This fact indicates a pronounced activation of bone resorption processes in the absence of activation of osteosynthesis processes, which leads to the detected violation of bone remodeling processes.

Thus, we have shown that a violation of the metabolic regulation of steroid hormone metabolism in animals with null expression of the 11β-HSD2 (Hsd2-/- genotype) leads to the development of signs of osteoporosis – bone density decreases, which is accompanied by a decrease in the width of bone trabeculae, and the level of microcirculation in bone tissue decreases simultaneously with an increase in the coefficient of endothelial dysfunction. The additional null expression of Apoe gene in double transgenics with the genotype Hsd2-/-Apoe-/- leads to an increase in the severity of changes associated with a violation of bone remodeling processes and, in addition to a more pronounced change in bone tissue density, bone trabecular width, microcirculation and the coefficient of endothelial dysfunction lead to an increase in the concentration of biochemical markers of bone resorption. These changes indicate the important role of the enzyme 11β-hydroxysteroid dehydrogenase type 2 in the processes of bone remodeling disorders.

References


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