

Original Article

Effects of $\alpha 7$ nAChR agonist on the tissue estrogen receptor expression of castrated rats

Feng Ma, Fan Gong, Jinhan Lv, Jun Gao, Jingzu Ma

Department of Orthopedics, Ningxia People's Hospital, Yinchuan 750002, Ningxia, China

Received August 27, 2015; Accepted September 28, 2015; Epub October 1, 2015; Published October 15, 2015

Abstract: Osteoporosis is one common disease in postmenopausal women due to depressed estrogen level. It has been known that inflammatory factors are involved in osteoporosis pathogenesis. One regulator of inflammatory cascade reaction, $\alpha 7$ -nicotinic acetylcholine receptor ($\alpha 7$ nAChR), therefore, may exert certain role in osteoporosis. This study thus investigated this question on an osteoporosis rat model after castration. Rats were firstly castrated to induce osteoporosis, and then received $\alpha 7$ nAChR agonist (PNU-282987), diethylstilbestrol or saline via intraperitoneal injection. After 6 or 12 weeks, bone samples were collected for counting osteoblast number, bone density and estrogen receptor (ER α and ER β) expression, in addition to the serum laboratory of inflammatory factors. Bone density, osteoclast number, ER α and ER β expression level were significantly depressed in model group, and were remarkable potentiated in the drug treatment group ($P < 0.05$). The levels of BGP and PTH in drug treatment group were decreased compared to diethylstilbestrol group, while E2 and IGF-1 showed up-regulation. Agonist of $\alpha 7$ nAChR can up-regulate estrogen receptor expression and may prevent the occurrence and development of osteoporosis.

Keywords: $\alpha 7$ nAChR agonist, osteoporosis, estrogen receptor

Introduction

With population aging, osteoporosis has become a severe public health issue to draw the interests of researchers and clinicians worldwide. As one bone disease, osteoporosis is typically manifested with compromised bone strength and elevated bone fracture risk. Estrogen has been known to work as one critical hormone during bone formation and growth, as it can accelerate the maturation of bones and maintain normal bone volume. The deficient of estrogen thus disrupt the dynamic balance between bone formation and reabsorption, causing higher incidence of osteoporosis. One factor mediating inflammatory cascade reaction, $\alpha 7$ nAChR, may prevent osteoporosis via the binding with acetylcholine. This study thus generated an animal model with osteoporosis and targeted estrogen receptor, in an attempt to identify the potential effect of $\alpha 7$ nAChR agonist in osteoporosis.

Materials and methods

Animals and treatment

A total of 40 female SD rats (4 months old, body weight: 220~250 g) were provided by

Laboratory Animal Center of Ningxia Medical University. All animals were randomly divided into 4 groups. After intraperitoneal injection of 2% pentobarbital sodium (0.2 mL/100 g) for general anesthesia, the peritoneal cavity was exposed by a midline incision. Bilateral ovaries were then removed. The control group only underwent the surgical procedure but without the ovary removal. Penicillin sodium (0.5 mL per animal) was intraperitoneal injected before suture. Two days after the surgery, penicillin sodium was given daily. Rats were kept in cages with food and water *ad libitum*.

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of Ningxia Medical University.

The successful generation of osteoporosis model was deduced by bone density quantification after 2 month. At 10 weeks after the surgery, animals received $\alpha 7$ nAChR agonist (PNU-282987, Santa Cruz, US) at 2.4 mg/kg daily via intraperitoneal injection, or diethylstilbestrol (0.05 mg/kg daily, Jinyao, China) via subcutaneous injection or equal volumes of saline by intraperitoneal injection. The drug intervention lasted for 12 weeks.

AChR agonist and estrogen receptor

Table 1. Bone density of all rats

Group	N	Bone density			
		At week 6		At week 12	
PNU-282987	10	0.21±0.12 ^{*,#,&}	5	0.18±0.07 ^{*,#,&}	5
Diethylstilbestrol	10	0.17±0.09 ^{*,&}	5	0.15±0.05 ^{*,&}	5
Saline	10	0.12±0.05 ^{&}	5	0.09±0.02 ^{&}	5
Control	10	0.31±0.17	5	0.31±0.16	5

Note: *, P<0.05 compared to saline group; #, P<0.05 compared to diethylstilbestrol group; &, P<0.05 compared to control group.

Table 2. Osteoblast number of all rats

Group	N	Osteoblast number	
		At week 6	At week 12
PNU-282987	10	29.11±3.11 ^{*,#,&}	27.55±2.37 ^{*,#,&}
Diethylstilbestrol	10	22.03±3.02 ^{*,&}	20.56±2.12 ^{*,&}
Saline	10	21.12±3.08 ^{&}	19.09±2.01 ^{&}
Control	10	33.56±4.58	33.57±4.66

Note: *, P<0.05 compared to saline group; #, P<0.05 compared to diethylstilbestrol group; &, P<0.05 compared to control group.

Sample collection

At 6 weeks or 12 weeks of drug treatment, rats were anesthetized. Bone density of the whole body was firstly quantified by X-ray bone densitometry, with a target area at 0.05 cm² in triplicates. Blood samples were then collected from posterior femoral artery. After incubated for 1 hour, blood was centrifuged at 12000 g for 10 min to collect serum at the supernatants.

Under sterilized condition, bone samples were removed and cut into small pieces. After rinsing in penicillin-streptomycin, bone tissues were digested by 0.1% type I collagenase. Hank's solution was then used to wash isolated cells, which were cultured in DMEM containing 10% fetal bovine serum (DMEM) at 10⁴~10⁵ per mL density. After incubation at 37°C with 5% CO₂ perfusion, fibroblasts were removed. Osteoblasts were collected from the supernatants and quantified under a microscope.

Western blotting

Osteoblasts were collected for extracting total proteins. After quantification, proteins were separated under 8% SDS-PAGE, and were transferred to PVDF membrane. Blocking was performed under room temperature for 1 hour.

Primary antibody, including rabbit anti-ER α (1:200, Santa Cruz, US), goat anti-ER β (1:200, Santa Cruz, US) and goat anti-actin (1:500, Santa Cruz) were applied for overnight incubation at 4°C. After rinsing in TBST, anti-rabbit IgG or anti-goat IgG secondary antibody (1:2 000) was applied for 1-hour incubation at room temperature. Chromogenic substrates (Bio-Rad, US) were then added to develop the color, which was exposed and analyzed for optical density (OD) values.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were added into 96-well plate along with serially diluted standards. Serum level of BGP, estradiol (E2), parathyroid hormone (PTH) and insulin-like growth factor-1 (IGF-1) was quantified by ELISA kits following the manual instruction. Absorbance values at 450 nm were quantified by a microplate reader. The linear regression based on standard samples was used to deduce the sample concentration.

Statistical analysis

SPSS 17.0 software was used to process all collected data, which were presented as mean \pm standard deviation (SD). Enumeration data were analyzed by chi-square test, while measurement data were compared by student t-test. A statistical significance was defined when P<0.05.

Results

Bone density

The osteoporosis model was successfully generated as the bone density of saline group at 6 week and 12 week was significantly lower than that in other two groups (P<0.05, **Table 1**). The drug treatment, however, remarkable increased bone density compared to those undergone diethylstilbestrol or saline treatment (P<0.05, **Table 1**).

Number of osteoblasts

The removal of ovaries significantly suppressed the number of osteoblasts. Drug treatment, however, significantly elevated number of

Table 3. Estrogen receptor expression level in rat osteoblasts

Group	N	At week 6		At week 12	
		ER α	ER β	ER α	ER β
Saline	10	133.71 \pm 4.28 ^{&}	129.82 \pm 4.12 ^{&}	124.32 \pm 4.26 ^{&}	120.12 \pm 4.05 ^{&}
Control	10	191.53 \pm 6.43	190.24 \pm 5.98	190.97 \pm 6.12	188.76 \pm 5.88
PNU-282987	10	172.57 \pm 5.60 ^{*,#,&}	171.86 \pm 5.47 ^{*,#,&}	165.24 \pm 5.12 ^{*,#,&}	163.82 \pm 5.23 ^{*,#,&}
Diethylstilbestrol	10	141.83 \pm 4.12 ^{*,&}	134.75 \pm 4.24 ^{*,&}	139.26 \pm 4.33 ^{*,&}	133.72 \pm 4.11 ^{*,&}

Note: *, P<0.05 compared to saline group; #, P<0.05 compared to diethylstilbestrol group; &, P<0.05 compared to control group.

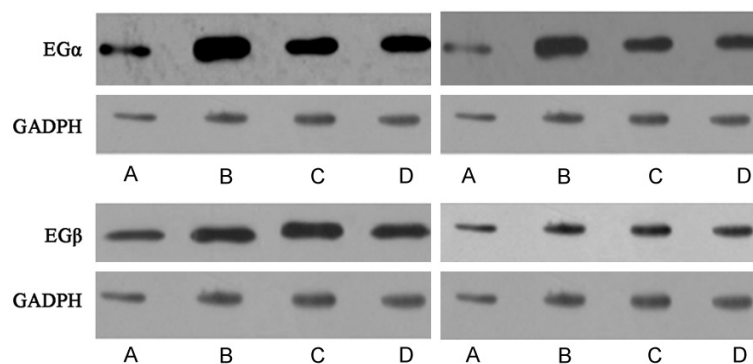


Figure 1. Estrogen receptor ER α (upper panels) and ER β (low panels) expression level in rat osteoblasts. Left, 6 weeks; Right, 12 weeks. Lane A, saline group; Lane B, control group; Lane C, PNU-282987 group; Lane D, diethylstilbestrol group.

osteoblasts when compared to diethylstilbestrol or saline group (P<0.05, **Table 2**).

Estrogen receptor expression level in osteoblasts

Using Western blot to quantify ER α and ER β expression levels in osteoblasts from all rats, we found significantly elevated expression of estrogen receptors in drug intervention group compared to the other two groups, but was still lower than the control group (**Table 3**; **Figure 1**).

Serum BGP, E2, PTH and IGF-1 levels

Using ELISA to quantify serum levels of BGP, E2, PTH and IGF-1 in all rats, we found that BGP and PTH contents in drug treated group were significantly lower than those in diethylstilbestrol group, while E2 and IGF-1 levels were elevated (P<0.05, **Table 4**). All those indexes were higher in drug treatment group compared to control group.

Discussion

Osteoporosis has now become a worldwide healthy issue due to the aging of population [1, 2]. As one systematic bone disease, osteoporosis is usually manifested with decreased bone volume and density, compromised bone strength, along with higher risk of bone fracture [3]. The occurrence of osteoporosis involves multiple factors. The definition of osteon has been suggested to include both osteoblasts and osteoclasts involving in the bone reformation

[4]. Osteon accomplished the bone reformation cycle via bone transformation, which consists of cell activation, bone absorption and formation. As one important component of osteon, osteoblast plays a vital role in bone formation, modulating bone reabsorption, inhibiting differentiation of osteoclasts and accelerate their apoptosis [5]. Estrogen plays an important role in the development and maturation of bones in addition to modulating reproductive functions. For postmenopausal and ovary removal women, the rapid depressed on body's estrogen level may lead to imbalance of bone metabolism, causing the degradation of bone structure, decreased bone volume and bone density, thus making higher incidence of bone fracture [6].

In this study, we generated an osteoporosis rat model by artificial castration. Specific agonist of a7nAChR, PNU-282927, was applied for intervention. We found significantly depressed bone density and osteoblast number in model group, suggesting the occurrence of osteoporosis

Table 4. Serum indexes

Index	PNU-282987 (mmol/l)	Diethylstilbestrol (mmol/l)	Saline (mmol/l)	Control (mmol/l)
At week 6				
BGP	0.74±0.13 ^{*,#,&}	0.98±0.12 ^{*,&}	0.58±0.08 ^{&}	0.63±0.15
E2	0.80±0.16 ^{*,#,&}	0.67±0.12 ^{*,&}	0.55±0.02 ^{&}	0.56±0.11
PTH	0.68±0.11 ^{*,#,&}	0.82±0.08 ^{*,&}	0.52±0.06 ^{&}	0.58±0.09
IGF-1	0.76±0.02 ^{*,#,&}	0.61±0.04 ^{*,&}	0.48±0.02 ^{&}	0.56±0.06
At week 12				
BGP	0.65±0.11 ^{*,#,&}	0.91±0.08 ^{*,&}	0.48±0.06 ^{&}	0.52±0.09
E2	1.29±0.17 ^{*,#,&}	0.98±0.17 ^{*,&}	0.75±0.13 ^{&}	0.93±0.15
PTH	0.61±0.02 ^{*,#,&}	0.72±0.06 ^{*,&}	0.46±0.02 ^{&}	0.50±0.03
IGF-1	1.12±0.05 ^{*,#,&}	0.97±0.08 ^{*,&}	0.81±0.09 ^{&}	0.98±0.07

Note: ^{*}, P<0.05 compared to saline group; [#], P<0.05 compared to diethylstilbestrol group; [&], P<0.05 compared to control group.

sis at 6 weeks. The drug intervention significantly improved the bone density, and elevated ER α and ER β expression levels when compared to those undergone saline or diethylstilbestrol treatment. These results suggested the hypothalamic inhibition of estrogen receptor expression by castration, while $\alpha 7$ nAChR agonist can increase estrogen receptor expression, thus exerting its anti-osteoporosis role. Serum laboratory results showed elevated BGP and PTH, in addition to depressed E2 and IGF-1 levels in model group. These abnormalities, however, can be alleviated by PNU-282927 treatment. Previous studies have suggested the correlation between inflammatory factors and osteoporosis [7-10]. The nervous stimulation of certain components in cholinergic anti-inflammatory pathway thus can inhibit the production of pro-inflammatory cytokines without adverse effects of central nervous system [11-13]. Inflammation is related with vagus nerve, which consists of afferent and efferent nerves and can modulate body inflammation by hypothalamic-pituitary-adrenal gland axis [14, 15]. The efferent cholinergic activity exerts certain modulatory effects on immune functions [16-18]. Among all agonists of $\alpha 7$ nAChR, acetylcholine can be hydrolyzed by acetylcholine lipase to generate toxic nicotine, making it unfavorable as the drug candidate in clinics. PNU-282927 is one novel high-selective $\alpha 7$ nAChR agonist [19, 20]. This study illustrated the possible correlation between osteoporosis and decreased estrogen level. The agonist of $\alpha 7$ nAChR has been shown to increase both bone density and

osteoblast number, increase estrogen receptor expression, decrease pro-inflammatory cytokine levels and inhibit over-reactive inflammation, thus exerting certain role in preventing and/or treating osteoporosis.

In summary, the occurrence of osteoporosis is correlated with decreased estrogen level. The agonist of $\alpha 7$ nAChR can increase estrogen receptor expression, suppress body's inflammatory factor level, thus may be a candidate for preventing/treating osteoporosis. Results from this study may help us to explore

novel ways of non-medication prevention and treatment of osteoporosis.

Acknowledgements

Ningxia Natural Science Foundation (NZ13188).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Feng Ma, Department of Orthopedics, Ningxia People's Hospital, Room 301, Zhengyuan Street (North), Jinfeng District, Yinchuan 750002, Ningxia, China. Tel: +86-951-2063133; +86-13519514546; Fax: +86-951-2063133; +86-13519514546; E-mail: mafenlive@sina.com

References

- [1] Kim MH, Choi YY, Han JM, Lee HS, Hong SB, Lee SG, Yang WM. Ameliorative effects of Schizandra chinensis on osteoporosis via activation of estrogen receptor (ER)-alpha/-beta. *Food Funct* 2014; 5: 1594-601.
- [2] Soubhye J, Alard IC, van Antwerpen P, Dufrasne F. Type 2 17-beta hydroxysteroid dehydrogenase as a novel target for the treatment of osteoporosis. *Future Med Chem* 2015; 7: 1431-56.
- [3] Ashraf A, Roshanzamir S, Bemana G, Mohammadi A, Jahani N, Naseri M. Sympathetic Skin Response and Vasomotor Symptoms in Postmenopausal Osteoporotic Women. *Int J Community Based Nurs Midwifery* 2015; 3: 227-33.
- [4] Miyakoshi N, Hongo M, Kobayashi T, Abe T, Abe E, Shimada Y. Improvement of spinal align-

AChR agonist and estrogen receptor

- ment and quality of life after corrective surgery for spinal kyphosis in patients with osteoporosis: a comparative study with non-operated patients. *Osteoporos Int* 2015; 26: 2657-64.
- [5] Kootala S, Ossipov D, van den Beucken JJ, Leeuwenburgh S, Hilborn J. Bisphosphonate-functionalized hyaluronic acid showing selective affinity for osteoclasts as a potential treatment for osteoporosis. *Biomater Sci* 2015; 3: 1197-207.
- [6] Siddapur PR, Patil AB and Borde VS. Comparison of Bone Mineral Density, T-Scores and Serum Zinc between Diabetic and Non Diabetic Postmenopausal Women with Osteoporosis. *J Lab Physicians* 2015; 7: 43-8.
- [7] Kusec V, Virdi AS, Prince R, Triffitt JT. Localization of estrogen receptor-alpha in human and rabbit skeletal tissues. *J Clin Endocrinol Metab* 1998; 83: 2421-8.
- [8] Mundy GR. Osteoporosis and inflammation. *Nutr Rev* 2007; 65: S147-51.
- [9] Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, Wang H, Abumrad N, Eaton JW, Tracey KJ. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000; 405: 458-62.
- [10] Pavlov VA and Tracey KJ. **Controlling inflammation: the cholinergic anti-inflammatory pathway.** *Biochem Soc Trans* 2006; 34: 1037-40.
- [11] Rosas-Ballina M and Tracey KJ. Cholinergic control of inflammation. *J Intern Med* 2009; 265: 663-79.
- [12] Beekwilder JP and Beems T. Overview of the clinical applications of vagus nerve stimulation. *J Clin Neurophysiol* 2010; 27: 130-8.
- [13] Huston JM, Gallowitsch-Puerta M, Ochani M, Ochani K, Yuan R, Rosas-Ballina M, Ashok M, Goldstein RS, Chavan S, Pavlov VA, Metz CN, Yang H, Czura CJ, Wang H, Tracey KJ. Transcutaneous vagus nerve stimulation reduces serum high mobility group box 1 levels and improves survival in murine sepsis. *Crit Care Med* 2007; 35: 2762-8.
- [14] La Marca R, Nedeljkovic M, Yuan L, Maercker A, Elhert U. Effects of auricular electrical stimulation on vagal activity in healthy men: evidence from a three-armed randomized trial. *Clin Sci (Lond)* 2010; 118: 537-46.
- [15] Thayer JF. Vagal tone and the inflammatory reflex. *Cleve Clin J Med* 2009; 76 Suppl 2: S23-6.
- [16] de Jonge WJ, van der Zanden EP, The FO, Bijlsma MF, van Westerloo DJ, Bennink RJ, Berthoud HR, Uematsu S, Akira S, van den Wijngaard RM, Boeckstaens GE. Stimulation of the vagus nerve attenuates macrophage activation by activating the Jak2-STAT3 signaling pathway. *Nat Immunol* 2005; 6: 844-51.
- [17] Huston JM, Ochani M, Rosas-Ballina M, Liao H, Ochani K, Pavlov VA, Gallowitsch-Puerta M, Ashok M, Czura CJ, Foxwell B, Tracey KJ, Ulloa L. Splenectomy inactivates the cholinergic anti-inflammatory pathway during lethal endotoxemia and polymicrobial sepsis. *J Exp Med* 2006; 203: 1623-8.
- [18] Ghia JE, Blennerhassett P, Kumar-Ondiveeran H, Verdu EF, Collins SM. The vagus nerve: a tonic inhibitory influence associated with inflammatory bowel disease in a murine model. *Gastroenterology* 2006; 131: 1122-30.
- [19] Johnston GR and Webster NR. Cytokines and the immunomodulatory function of the vagus nerve. *Br J Anaesth* 2009; 102: 453-62.
- [20] Iwasaki K, Hatip-Al-Khatib I, Egashira N, Akiyoshi Y, Arai T, Mishima K, Takagaki Y, Inui K, Fujiwara M. Ovariectomy combined with amyloid beta (1-42) impairs memory by decreasing acetylcholine release and alpha 7nAChR expression without induction of apoptosis in the hippocampus CA1 neurons of rats. *Neurotox Res* 2004; 6: 299-309.