Effect of High Fat Diet Induced-obesity and Testosterone Deprivation on Insulin Resistance, Osteoblast Proliferation and Osteoblastic Insulin Signaling

ผลของความอ้วนจากการได้รับอาหารไขมันสูงและขาดฮอร์โมนเทสโทสเตอโรน ต่อภาวะดื้ออินซูลิน การเจริญเติบโต และสื่อสัญญาณอินซูลินของเซลล์กระดูก

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ABSTRACT

The present study aimed to investigate osteoblastic insulin signaling and osteoblast function in testosterone deprived rats with or without obesity. Wistar rats were divided into 4 groups; normal diet with sham operation (NDS, group 1) and with bilateral orchiectomy (NDO, group 2), high fat diet with sham operation (HFS, group 3) and with orchiectomy (HFO, group 4). At the end of week 12, blood was corrected and osteoblasts were isolated from tibia. Osteoblastic cell proliferation was performed using alamarBlue viability assay. Osteoblastic insulin signaling was determined by measuring the phosphorylation of insulin receptor and Akt protein using western blot analysis. We found that peripheral insulin resistance indicated by increased HOMA index was occurred in HFS and HFO groups. Proliferation and osteoblastic insulin signaling was significantly decreased in NDO, HFS and HFO groups, compared to NDS group. These findings indicated that either testosterone deprivation or obesity alone impaired osteoblastic insulin signaling and osteoblastic cell proliferation, but obesity did not aggravate those deleterious effects in the testosterone-deprived rats.

บทคัดย่อ

การวิจัยนี้มีจุดประสงค์เพื่อศึกษาสื่อสัญญาณอินซูลินและการเจริญของเซลล์กระดูก ในภาวะขาดฮอร์โมนเทสโทสเตอโรนร่วมกับการมีหรือไม่มีภาวะอ้วนร่วมด้วยโดยหนูแรทสายพันธุ์วิสต้า ถูกแบ่งเป็น 4 กลุ่มคือ กลุ่มควบคุม ได้รับอาหารปกติ กลุ่มที่ 2 ได้รับอาหารปกติและถูกเอาอัณฑะออกทั้งสองข้าง กลุ่มที่ 3 ได้อาหารไขมันสูง และกลุ่มที่ 4 ได้อาหารไขมันสูงและถูกตัดอัณฑะออกทั้งสองข้าง หลังจากนั้น 12 สัปดาห์ ทำการตรวจเลือดเพื่อวิเคราะห์ภาวะดื้อต่ออินซูลิน จากนั้น แยกกระดูกขาเพื่อนำมาสกัดเป็นเซลล์กระดูกและประเมินการเจริญเติบโตด้วย alamarBlue cell viability assay ศึกษาสื่อสัญญาณอินซูลินโดยวัดการเติมฟอสเฟตที่อินซูลินรีเซพเตอร์และโปรตีน Akt ผลจากการวิเคราะห์โปรตีนพบว่า ภาวะดื้อต่ออินซูลินที่บ่งชี้จากค่า HOMA ที่เพิ่มขึ้น เกิดเฉพาะในหนูกลุ่มที่ได้รับอาหารไขมันสูงเท่านั้น การเจริญเติบโตของเซลล์กระดูก และสื่อสัญญาณต่ออินซูลินลดลงในหนูกลุ่มที่ 2, 3 และ 4 เปรียบเทียบกับกลุ่มควบคุม การศึกษานี้แสดงให้เห็นว่าไม่ว่าจะขาดฮอร์โมนเทสโทสเตอโรนหรือความอ้วนสามารถทำให้เกิดการดื้อต่ออินซูลินภายในเซลล์กระดูก แต่ความอ้วนไม่ได้ทำให้ภาวะดังกล่าวแย่ลงในหนูที่ขาดฮอร์โมนเทสโทสเตอโรนร่วมด้วย

Key Words: Obesity, Osteoblastic insulin resistance, Testosterone deprivation

คำสำคัญ: อ้วน ภาวะดื้อต่ออินซูลินในเซลล์กระดูก ภาวะขาดฮอร์โมนเทสโทสเตอโรน

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Introduction

Many studies show that obesity from a high-fat diet (HFD) consumption cause insulin resistance in several target tissues, including muscle, fat and brain (Riccardi et al., 2004; Pedersen et al., 1991; Pintana et al., 2012; Pramojanee et al., 2013). Recent studies have demonstrated that insulin resistance in osteoblasts has a negative effect on bone (Fulzele et al., 2010; Ferron et al., 2010). In addition we previously showed that an obese-insulin resistant condition induced by long-term HFD consumption markedly impaired osteoblastic insulin signaling and osteoblast proliferation and decreased osteoblast survival in which result in osteoporosis in the jaw bone (Pramojanee et al., 2013).

Insulin is important for regulating osteoblast function (Fulzele et al., 2010) and bone development (Lac et al., 2008). It increases bone anabolic markers, including collagen synthesis, alkaline phosphatase production (Yang et al., 2010; Pun, Lau, and Ho, 1989). Several studies show that insulin signaling pathway including insulin receptor (IR), insulin receptor substrate-1 (IRS-1) and Akt are important for osteoblastic proliferation and differentiation (Fulzele et al., 2010; Ogata et al., 2000).

Testosterone is an androgenic steroid producing mainly from Leydig cells of testis. Testosterone has direct effects on osteoblastic function (Kung, 2003). It increases osteoblastic survival via decrease apoptosis in orchiectomized rats (Manolagas, 2000; Kousteni et al., 2002). Interestingly, testosterone also inhibits osteoclast formation and bone resorption (Michael et al., 2005). Several studies showed that men with testosterone deficiency increased risk of osteoporosis (Kenny et al., 2000; Jackson, Riggs, and Spiekerman, 1992). In contrast, some studies demonstrated lack of testosterone increased bone turnover rate (Khosla et al., 1998). However, the effects of testosterone on osteoblastic insulin signaling have not been investigated. Moreover, the effects of combined testosterone deprivation and obesity on osteoblastic function and osteoblastic insulin signaling have not been identified. Therefore, this study was design to investigate osteoblastic insulin signaling and osteoblastic proliferation in testosterone deprived rats with or without obesity. We hypothesized that 1) either testosterone deprivation or obesity alone impairs osteoblastic insulin signaling and osteoblastic cell proliferation as well as 2) obesity aggravates those deleterious effects in the testosterone-deprived rats

Objectives of the study

To investigate osteoblastic insulin signaling and osteoblastic proliferation in testosterone deprived rats without or with obesity induced by 12-week HFD

Methodology

Animals and diet

Male Wistar rats (body weight 180-200 g, n= 16) from the National animal center, Salaya campus, Mahidol University, Bangkok were divided into 2 groups (n = 8/group) as sham operated group and bilateral orchiectomy (ORX) group. After 1 week of surgery, rats in each group were divided into 2 sub-groups to receive a normal diet (ND) or a high fat diet (HFD) for 12 weeks (n=4/sub-groups). Rats in ND group were fed by standard laboratory chow which has energy content of 4.02 kcal/g and 19.77% total energy (%E) from fat (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand). Rats in HFD group were fed by high-fat diet which had energy content of

5.35 kcal/g and 59.28 %E form lard. All animals were maintained under an environmentally controlled conditions (25 ± 0.5 °C, 12 h light/12 h dark cycle). At the end of week 12, blood samples were collected from all rats (sham-operated ND rats (NDS), orchiectomized ND rats (NDO), sham-operated HFD rats (HFS) and orchiectomized HFD rats (HFO)). The tibia from each rat was rapidly removed for the preparation of primary osteoblastic culture.

The metabolic parameters

Body weight of each rat was recorded at the end of the week 12. Blood sample was collected from tail vein after fasting for 5-6 hours to measure plasma glucose, cholesterol, insulin and testosterone levels. Plasma was centrifuged at 6,000 rpm, 4°C for 5 minutes and then kept in -80°C until analysis. Plasma glucose and cholesterol levels were determined by colorimetric assay from commercially available kits (Biotech, Bangkok, Thailand). Sandwich ELISA kit (LINCO Research, MO, USA) was used to measure plasma insulin levels. Peripheral insulin resistance was assessed by Homeostasis Model Assessment (HOMA) in which fasting plasma insulin (μU/ml) fasting plasma glucose (mmol/l) / 22.5 (Matthews et al., 1985). Plasma testosterone level was measured by Bio-active Ltd.'s testosterone ELISA Kit.

Cell culture

At week 12, each rat was anesthetized with isoflurance and decapitated. Visceral fat was measured. The tibia was removed and immediately transferred to culture room for osteoblastic isolation by serial enzymatic methods (Ng, 2011). Briefly, tibias were digested for 30 minutes at 37°C in an enzyme solution containing 2% collagenase in DMEM for 5 times. The cells isolated by the last 3 digestions were combined as an osteoblast population and cultured in DMEM containing 10% FBS and 50 mg/ml ascorbic acid. The medium was changed every two days.

Alizarin red staining

Alizarin red staining, a stain for calcium deposition, was performed as described (Jacobson, 1995). Shortly, 2 g of Alizarin red S (sigma) was dissolved in 100 ml of de-ionized water and adjusted pH to 4.2. The cells were briefly washed with phosphate-buffered saline (PBS) followed by fixation with 4% paraformaldehyde for 30 minutes.After washing three times with PBS, the cells were incubated with alizarin red for about 10 minutes and then washed with de-ionized water.

Alkaline phosphatase staining

Cells were briefly washed with PBS and then fix with 4% formaldehyde on ice for 10 minute. After washing with PBS three times, ethanol/acetone (50:50 v/v) was added to the cell for 1 minute. Cells were incubated with alkaline phosphatase substrate premixed solution (Wako, Japan) at 37 °C for 45 minutes (Cordell et al., 1984). Deionized water was added to stop reaction. Staining was visualized under light microscopy.

Western blot analysis

The cells were washed with 1% cools PBS followed by addition of cell lysis buffer (20 mM Tris-HCl, 1 mM Na_3VO_4, 5 mM NaF) containing protease inhibitors and extracted in 2x sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. The protein preparations were separated by 10%SDS-PAGE and then transferred to PVDF membranes (Immobilon-P; Millipore, Bedford, MA) for 1 hour at 100V. The membranes were blocked in 5% non-fat milk in TBS containing 0.1%tween-20 for 1 hour at 25°C and then incubated with primary antibodies against phosphorylation of insulin receptor

beta-subunit (1:1000, Santa Cruz, USA), phosphorylation of Akt (Ser 473), Akt (1:1000, Cell Signaling, USA) and beta-actin (1:2000, Santa Cruz, USA) at 25°C for overnight and followed by the incubation of horseradish peroxidase-conjugated anti-rabbit IgG (1:2000, Santa Cruz, USA). The protein bands were visualized by ECL western blotting detection reagent and quantitated using Image J software.

Cell proliferation assay

The osteoblasts were plated in 96-well plates at a density of 1 x 10^4 cells/well and cultured in DMEM supplemented with 10% FBS for 24 hrs. Cell proliferation was measured by alamarBlue fluorometric cell viability assay (Biosource, Carlsbad, CA, USA). Briefly, alamarBlue reagent was added directly to each well and incubated for 4 hours at 37°C 5% CO_2. The change in fluorescent intensity was measured using microplate reader for excitation at 530 nm and emission detection at 590 nm.

Statistical analysis

All data was expressed as median ± SEM. One-way ANOVA followed by LSD post-hoc analysis was used to determine the difference between groups. $p<0.05$ was considered statistical significant.

Results

Obesity induced by HFD caused peripheral insulin resistance

At the end of week 12, blood samples were collected from all rats (sham-operated ND rats (NDS), orchiectomized ND rats (NDO), sham-operated HFD rats (HFS) and orchiectomized HFD rats (HFO)). The metabolic parameter was determined as shown in table 1. We found that body weight and visceral fat were significantly increased in HFD consumption but decreased in orchiectomied group. Only HFS and HFO groups developed peripheral insulin resistance as indicated by increased HOMA index (27.8 ± 3.5, 26.7 ± 4.7, respectively) compared to that of NDS and NDO groups, (14.9 ± 2.2, 16.9 ± 4.2, respectively). The testosterone levels of NDO, HFS and HFO groups were significantly decreased compared to that of NDS group (0.07 ± 0.01, 0.48 ± 0.05, 0.18 ± 0.09 vs 0.74 + 0.1, respectively) (Table 1). This finding suggests that only HFD consumption induces peripheral insulin resistance and testosterone deprivation alone did not cause peripheral insulin resistance.

Osteoblastic proliferation significantly decreased in either HFD group or OVX groups.

After the cells were isolated from tibia and grew in osteogenic medium for a few weeks, alkaline phosphatase and alizarin red staining were performed to characterize osteoblastic cells. Isolated cells from each group showed positive staining for alkaline phosphatase and alizarin red (Fig. 1).

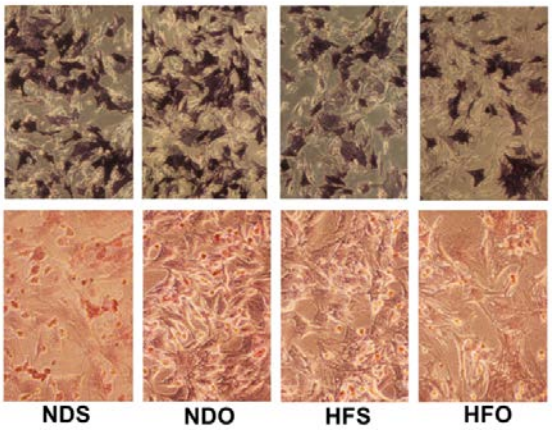

Fig. 1 Alkaline phosphatase activity (upper panel) was performed to evaluate osteoblast activity. Alizarin red was used to stain calcium (bright red, lower panel). The staining were visualized by light microscopy.

We next determined the effect of HFD consumption and testosterone deprivation on cell proliferation using alamarBlue fluorometric cell viability assay. Fluorescent intensity of alamarBlue was measured at 4 hours of day1 and day3 (Fig. 2A). Percentage of cell growth was significantly decrease in NDO, HFS and HFO (72.12 %, 75.26% and 76.68%, respectively) compare to NDS (100%) group (Fig. 2B). No differences were found among NDO, HFS and HFO rats.

Table 1 Metabolic parameters of each animal group. Blood samples were collected from all rats at the end of week 12. Values are mean ± SEM. * $p<0.05$ compared to control (NDS) and # $p<0.05$ compare to HFS

Metabolic parameters	NDS	NDO	HFS	HFO
Body weight (g)	465 ± 6.5	411.3 ± 4.3*,#	571 ± 14.4*	518 ± 15.5#
Visceral fat (g)	28.9 ± 3.4	16.9 ± 1.8 *,#	50.5 ± 5.1*	31.3 ± 2.9 #
Plasma insulin (ng/ml)	1.8 ± 0.2	2.0 ± 0.4	3.0 ± 0.6*	2.9 ± 0.4*
Plasma glucose (mg/dl)	144.6 ± 7.3	143.1 ± 15.2	147.8 ± 7.9	146.8 ± 8.9
HOMA index	14.9 ± 2.2	16.9 ± 4.2	27.8 ± 3.5*	26.7 ± 4.7*
Plasma total cholesterol (mg/dl)	49.4 ± 3.7	50.0 ± 5.7	70.7 ± 3.2*	68.5 ± 8.1*
Plasma testosterone (ng/dl)	0.74 ± 0.1	0.07 ± 0.01*,#	0.48 ± 0.05*	0.18 ± 0.1*,#

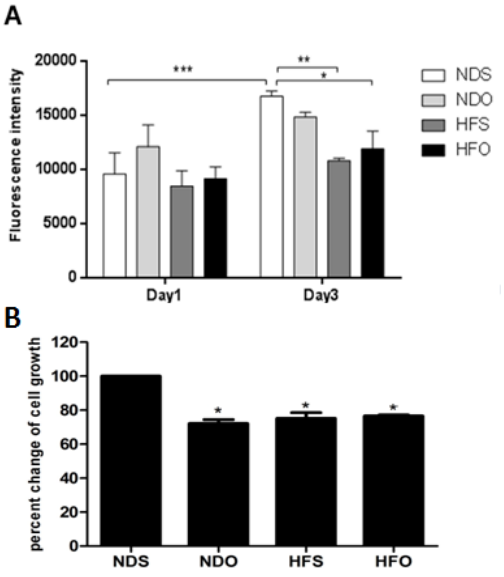

Fig. 2 alamarBlue cell viability assay shown as fluorescence intensity at 4 hours of day 1 and day 3 (A). Percentage change of cell growth was shown in bar graph (B). Values are mean ± SEM of n =4 in triplicate samples. *, **, *** are p <0.05, p <0.01 and p <0.001, respectively, compared to NDS group.

Osteoblastic insulin signaling was impaired in either HFD consumption or testosterone deprivation

The activity of osteoblastic insulin signaling including insulin receptor beta and Akt was determined its phosphorylation by western blot analysis. In control (NDS) group, phosphorylation of insulin receptor was significantly increased 1.3 times after stimulated with insulin 10 nM (compare to the base line) (Fig. 3). Interestingly, insulin mediated phosphorylation of insulin receptor was markedly decreased in NDO, HFS and HFO groups (Fig. 3). In addition, the phosphorylation of Akt protein, the downstream signal molecules was also significantly decreased in NDO, HFS and HFO groups (Fig. 4). However, no differences were found among NDO, HFS and HFO group.

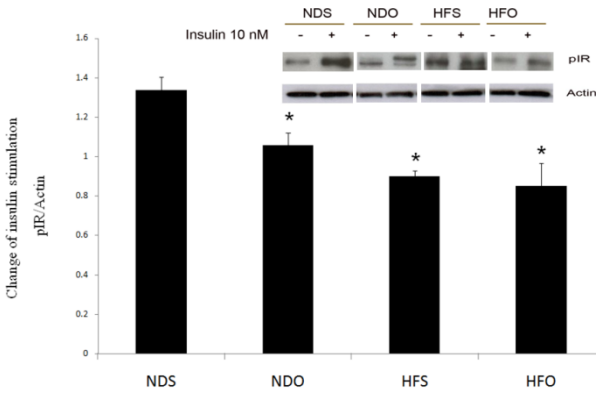

Fig. 3 Western blot of insulin receptor phosphorylation (pIR) of cells stimulated with or without 10 nM insulin. The bar graph represents the insulin-stimulated change of pIR after normalizing with actin. Values are mean ± SEM. * $p<0.05$ compared to control (NDS).

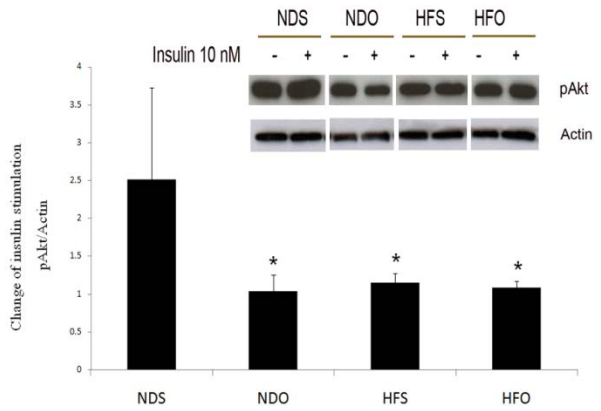

Fig. 4 Western blot of Akt phosphorylation (pAkt) of the cells stimulated with or without 10 nM insulin. The bar graph represents the insulin-stimulated change of pAkt after normalizing with actin. Values are mean ± SEM.* $p<0.05$ compared to control (NDS).

Discussion and Conclusion

Major findings of the present study are that 1) testosterone deprivation causes peripheral insulin resistance 2) either testosterone deprivation alone or obesity alone lead to osteoblastic insulin resistance 3) either testosterone deprivation alone or obesity alone decrease osteoblastic cell proliferation and 4) obesity does not aggravate osteoblastic insulin resistance and decreased osteoblastic proliferation in testosterone deprived condition.

Insulin resistance in osteoblasts and hypogonadism are among the risk factors of developing osteoporosis (Pramojanee et al., 2013; Hanayama et al., 2009). Our previous study found that 12-week feeding high fat diet induced obesity and peripheral insulin resistance in rat model (Pintana et al., 2012). In consistent with our previous study, obesity alone caused not only peripheral insulin resistance, but also osteoblastic insulin resistance and impaired osteoblastic cell proliferation.

Surprisingly, testosterone deprivation alone in the present study did not lead to peripheral insulin resistance. In contrast to previous study, it showed that male Sprague–Dawley rats developed peripheral insulin resistance and had high fasting glucose levels after 10 week orchiectomy (Xia et al., 2013). The differences in species of rats using in previous study and the present study might explain different findings.

In addition, we showed that testosterone level was significantly decreased in HFD-fed rats. Previous studies also show that obese men with type 2diabetes have lower testosterone levels (Barrett-Connor, 1992; Andersson et al., 1994; Grossmann, 2013). These findings suggest that peripheral insulin resistance may have adverse effects on testosterone production.

Although ND-fed OVX rats did not show peripheral insulin resistance, their osteoblastic insulin signaling and osteoblastic proliferation was impaired. This finding suggests that testosterone may play a role in osteoblastic insulin signaling. However, obesity did

not aggravate this defect in testosterone deprived rats. These findings suggest that testosterone deprivation caused osteoblastic insulin resistance and decreased osteoblastic proliferation at the maximum levels; therefore, further peripheral insulin resistance following obesity might not have further effects on osteoblastic insulin signaling and osteoblastic cell proliferation.

It is widely accepted that insulin, an anabolic bone regulator, enhances bone formation by activation of osteoblast proliferation and differentiation (Yang et al., 2010). Therefore, the defect of osteoblastic insulin signaling in testosterone-deprived rats or HFD-fed rats resulted in decreased osteoblast proliferation. Decreased osteoblastic proliferation may finally diminish bone formation and eventually cause osteoporosis. However, we cannot speculate from this study, whether the osteoporosis occurs. Therefore, the further investigation about bone quality is still needed.

In conclusion, this study demonstrated that either testosterone deprivation or obesity alone can impair osteoblastic insulin signaling and cell proliferation. However, obesity does not aggravate those deleterious effects in the testosterone-deprived rats.

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