RESEARCH

Palmitic acid causes insulin resistance in granulosa cells via activation of JNK

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Abstract

Obesity is a worldwide health problem with rising incidence and results in reproductive difficulties. Elevated saturated free fatty acids (FFAs) in obesity can cause insulin resistance (IR) in peripheral tissues. The high intra-follicular saturated FFAs may also account for IR in ovarian granulosa cells (GCs). In the present study, we investigated the relationship between saturated FFAs and IR in GCs by the use of palmitic acid (PA). We demonstrated that the glucose uptake in cultured GCs and lactate accumulation in the culture medium were stimulated by insulin, but the effects of insulin were attenuated by PA treatment. Besides, insulin-induced phosphorylation of Akt was reduced by PA in a dose- and time-dependent manner. Furthermore, PA increased phosphorylation of JNK and JNK blockage rescued the phosphorylation of Akt which was downregulated by PA. These findings highlighted the negative effect of PA on GCs metabolism and may partially account for the obesity-related reproductive disorders.

Introduction

Obesity is a global public health problem, which acts as the predominant risk factor for IR, type 2 diabetes and other metabolic disorders including hypertension and dyslipidemia (Ozcan et al. 2004, Stumvoll et al. 2005, Zeyda & Stulnig 2009). Besides, obesity has a negative influence on female fertility. Obesity is associated with poor oocyte quality, impaired spontaneous ovulation, low pregnancy rate in assisted reproductive technologies as well as high risk of miscarriages (Shah et al. 2011, Mitchell & Fantasia 2016). Although it has been recognized that a variety of risk factors such as leptin, TNF-α and free fatty acids (FFAs) are involved in the pathogenesis of obesity-related disorders (Uysal et al. 1997, Boden 1998, Hotamisligil 1999, Mitchell et al. 2005), the mechanisms by which obesity affects female fertility remain obscure (Pasquali et al. 2003, Crujeiras & Casanueva 2015).

It has been widely reported that obesity-associated IR exists in insulin target tissues, such as skeletal muscle...
(Ropelle et al. 2013), adipose tissue (Nguyen et al. 2005) and liver (Solinas et al. 2006). Recent studies have demonstrated that IR may also be present in the ovary in obesity. High-fat diet-induced obesity promoted IR in mouse ovary (Akamine et al. 2010) and insulin-induced glucose uptake was downregulated by obesity in mouse granulosa cells (GCs) (Purcell et al. 2012). These results indicated the existence of IR in ovarian GCs in obesity. The impaired glucose metabolism in GCs may reduce the supply of energy substrates for the oocyte and then affect oocyte development and maturation (Sugiura et al. 2005).

Saturated FFAs are elevated in plasma of obese individuals (Boden 2008) and considered as one of the important causes of metabolic disorders in obesity (Boden 1997, Bruce & Febbraio 2007). It has been reported that saturated FFAs affect insulin sensitivity and suppress insulin signaling by activating the c-Jun NH2-terminal kinase (JNK) pathway in various cell types, including hepatocytes and pancreatic β-cells (Solinas et al. 2006, Nakamura et al. 2009). Because saturated FFAs were also increased in follicular fluid of obese women (Yang et al. 2012), it is likely that the elevated levels of saturated FFAs could impair insulin-induced glucose metabolism and cause IR in ovarian GCs.

In the present study, we used palmitic acid (PA), which is a long-chain saturated fatty acid and the major component of saturated FFAs in serum and follicular fluid (Valckx et al. 2014), to elucidate the effects of saturated FFAs on insulin-induced glucose metabolism and insulin metabolic signaling in GCs.

Materials and methods

Animals

Swiss mice in the study were purchased from Laboratory Animal Center of Shandong University. This study was conducted in conformity to ‘the National Institute of Health Guide for the Care and Use of Laboratory Animals revised 1996’. All procedures were approved by the Ethics Review Board of the Center for Reproductive Medicine of Shandong University.

Cell culture

The human granulosa tumor cell line KGN (Nishi et al. 2001) was obtained from RIKEN BioResource Center, Ibaraki, Japan. The human granulosa cell line SVOG was immortalized with SV40 large T antigen (Lie et al. 1996) and gifted from Prof. Peter C.K. Leung of University of British Columbia to The Chinese University of Hong Kong-Shandong University (CUHK-SDU) Joint Laboratory on Reproductive Genetics. The human GCs were maintained in DMEM/F12 medium containing 10% fetal bovine serum (Biological Industries, Israel), 100IU/mL penicillin and 100μg/mL streptomycin at 37°C in a humidified 5% CO2 incubator.

Mouse primary GCs were isolated as described by Fan et al. (2009). Briefly, 23-day-old Swiss mice were injected with 5IU of PMSG (Ningbo Sansheng Pharmaceutical, Ningbo, China). Twenty-four hours after injection, the mice were killed and the ovaries were excised. GCs were harvested after puncturing ovaries with needle and then filtered through 70μm cell strainer. Cells were washed twice with PBS and then cultured in the same condition as human GCs.

PA and oleic acid (OA) were dissolved in ethanol at the concentration of 200mM and further diluted with 10% BSA in PBS to the concentration of 40mM. Finally, PA and OA were added to culture medium to obtain the indicated concentrations. GCs were cultured overnight and then incubated with indicated concentrations of PA (Santa Cruz) and/or insulin (Wanbang Biopharmaceuticals, Xuzhou, China) for different times.

MTT assay

KGN cells were cultured at a density of 5×10^4 cells/well in 96-well plates. After overnight culture, the cells were treated with different concentrations of PA (0, 25, 50, 100μM) for 12h, and 10μL of MTT solution (5mg/mL, Beyotime Biotechnology, Shanghai, China) was added to each well to form formazan. After 4h, 100μL of DMSO was added and cells were incubated in 37°C until formazan dissolved completely. The absorbance of each well was measured at 570nm with an iMark Microplate Reader (Bio-Rad). Nine repeated wells were set per experiment and three independent experiments were conducted.

Protein extraction and Western blotting

GCs were treated with various concentrations of PA and/or insulin for different time as indicated, and then lysed with RIPA buffer (Beyotime Biotechnology, Shanghai, China) containing 1mM phenylmethylsulfonyl fluoride and phosphatase inhibitor cocktail (Biotool, USA) for 30min on ice. Lysates were centrifuged at 12000g at 4°C for 10min to remove insoluble materials, and protein concentration of supernatants was determined by BCA method. After heating at 100°C with SDS loading buffer

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(Beyotime Biotechnology) for 10 min, total protein was separated by SDS-PAGE and electrotransferred to PVDF membrane (Millipore). Following blocking in 5% defatted milk in TBST, the membranes were incubated with primary antibodies at 4°C overnight. The primary antibodies used were anti-Caspase 3 (Cell Signaling Technology, CST), anti-Cleaved Caspase3 (Asp175, CST), anti-p-Akt (Ser473, CST), anti-Akt (CST), anti-p-JNK (Thr183/Tyr185, CST), anti-JNK (CST) and anti-β-actin (Sigma-Aldrich). Membranes were subsequently incubated with secondary antibodies and then detected with enhanced chemiluminescence system (Millipore). A ChemiDoc MP System (Bio-Rad) was used to capture images. β-Actin was used as an internal control. Independent experiments were conducted three times.

Glucose uptake assay
KGN cells were cultured at 5 × 10^3 cells/well in 96-well plates. Following 12-h treatment with different doses of PA, the medium was replaced with fresh medium with or without insulin (100ng/mL). After 15 min, the glucose uptake was determined by using a Glucose Uptake Fluorometric Assay Kit (BioVision) including 2-deoxyglucose (2-DG) which was taken up by cells and metabolized to 2-DG-6-phosphate. According to the manufacturer, 10 µL of 10 mM 2-DG was added to each well. After incubation for 20 min, cells were washed three times with PBS and then lysed with 90 µL of extraction buffer. Cell lysates were frozen and thawed once and then heated at 85°C for 40 min, and subsequently neutralized by adding 10 µL of neutralization buffer. After 40-min incubation at 37°C with the reagents mixture containing probe and enzyme that can generates fluorescence with 2-DG-6-phosphate, the fluorescence at 587 nm with excitation at 535 nm was measured by EnSpire Multimode Plate Reader (PerkinElmer). Protein concentrations were determined by the BCA method as internal reference. Three repeated wells were set per experiment, and independent glucose uptake assays were performed in triplicate.

Lactate assay
SVOG cells were seeded at a density of 5 × 10^4 cells/well in 12-well plates and cultured overnight. After treatment with the indicated concentrations of PA in serum-free medium for 12 h, medium was removed and new serum-free medium with or without insulin (100 ng/mL) was added. After 16 h of incubation, lactate level of culture supernatant was measured using Lactate Colorimetric Assay Kit (BioVision). According to the manufacturer, culture supernatants were placed in 50 µL/well in a 96-well plate, and then 50 µL of the mixture containing lactate probe and lactate enzyme was added to each well. After incubation for 30 min at room temperature, lactate was assayed by absorbance measurements at 570 nm using an iMark Microplate Reader (Bio-Rad). Protein of each well was extracted and quantified as described previously. All results were adjusted against cellular protein of each well. Three repeated wells were set per experiment and the assays were done in triplicate independently.

JNK siRNA and transfection
Specific JNK siRNA (JNK1: 5'-GUUCCCCAGGUACAGAU CAUTT-3'; JNK2: 5'-CUGAAGAUCCUUAGACUUUTT-3') and the negative control siRNA (5'-UCUCCGAACGU GUCACGUTT-3') were designed and synthesized by GenePharma Company, China. The mixture of JNK1 and JNK2 siRNA were used to achieve optimal JNK knockdown. KGN cells were plated in 12-well plates (1.0 × 10^5 cells/well) and cultured overnight, and then cells were transfected with 100 nM siRNA using Lipofectamine 3000 (Invitrogen). After 48 h of culture, cells were treated with PA for further analysis.

Statistical analysis
Data were analyzed using the SPSS version 21.0. For independent experiments with repeated measurements (MTT, glucose uptake and lactate assays), data were presented as mean ± S.E.M., and ANOVA of randomized block design followed by Tukey procedure was used to make comparisons. For analysis of results from Western blotting which did not set repeated wells per experiment, data were presented as mean ± S.D., and results for different groups were compared by one-way ANOVA followed by Tukey procedure. P < 0.05 was considered statistically significant.

Results
Low dose of PA has no effect on viability and apoptosis of GCs
The human granulosa tumor cell line KGN maintains most physiological activities of immature GCs, including steroidogenesis and apoptosis as the normal GCs (Nishi et al. 2001), so it was used in most of our experiments. KGN cells were incubated with different doses of PA (0, 25, 50 and 100 µM) for 12 h and then used for MTT assay or Western blotting. As shown in Fig. 1A, no significant
differences in absorbance were observed among four groups in MTT assay, suggesting that low dose of PA did not affect the viability of human GCs. Besides, the expression of cleaved caspase 3 was not induced by different doses of PA (Fig. 1B), indicating no effect of low dose of PA on cell apoptosis.

PA reduces glucose uptake and lactate accumulation induced by insulin in GCs

In order to determine the effect of PA on glucose metabolism in GCs, we treated GCs with different concentrations of PA (0, 25, 50 and 100 μM) for 12 h, and further incubated them with 100 ng/mL of insulin. The glucose uptake and lactate accumulation were detected after 15 min and 16 h respectively. The results showed that insulin had a stimulatory effect on glucose uptake in control group, but PA decreased insulin-stimulated glucose uptake in a dose-dependent manner in KGN cells (Fig. 2). Because KGN is a tumor cell line and has a high level of glycolysis, a non-tumor human granulosa cell line SVOG was used to explore effects of PA on lactate production in GCs. Insulin increased lactate accumulation in SVOG cells, and insulin-induced lactate accumulation in SVOG cells was reduced by PA as well (Fig. 3A). Because OA has also been found at a high concentration in the follicular fluid (Valckx et al. 2014), we further determined the effect of OA and/or PA on lactate production in SVOG cells. As shown in Fig. 3B, OA had no effect on lactate production, but the inhibitory effect of PA on lactate production could be rescued by OA.

Insulin-induced Akt phosphorylation is downregulated by PA in GCs

Subsequently, we investigated the effect of PA on insulin signaling in GCs. When KGN cells were treated with different doses of PA (0, 25, 50, 100 μM) for 12 h and then stimulated by insulin for 15 min, the phosphorylation of Akt (p-Akt) and total Akt were examined by Western blotting. Results showed that insulin-stimulated p-Akt in control group, but PA decreased insulin-stimulated glucose uptake in a dose-dependent manner in KGN cells (Fig. 2).
L Xu, W Wang et al. PA causes insulin resistance in GCs via JNK

Discussion

Many functions of insulin in ovarian GCs have been revealed, such as regulation of protein synthesis, stimulation of cell growth and differentiation and inhibition of apoptosis (Dupont & Scaramuzzi 2016). Besides, the presence of GLUT4 has been confirmed in rodent and human GCs (Rice et al. 2011, Zhang et al. 2012) and insulin can induce the translocation of intracellular GLUT4 to the cell membrane in GCs (Rice et al. 2011). Furthermore, GLUT4s can be regulated by gonadotropins, and cross-talk between insulin and gonadotropins takes place at some critical nodes such as MAPK and Akt (Kodaman & Behrman 1999, Taniguchi et al. 2006). Moreover, it has been reported that insulin-stimulated glucose metabolism was impaired in GCs from...
patients with polycystic ovary syndrome (PCOS) (Lin et al. 1997, Fedorcsak et al. 2000, Wu et al. 2003, Rice et al. 2005). Therefore, although ovarian GCs can uptake and metabolize glucose without insulin, these results suggest that GCs are insulin sensitive and insulin can stimulate glucose uptake and metabolism in ovarian GCs.

It has been reported that high dose (>200μM) of saturated FFA such as PA can induce apoptosis in ovarian GCs, which may contribute to the ovarian dysfunction in obesity (Mu et al. 2001, Xie et al. 2012). However, the concentration of PA in human follicular fluid was less than 100μM in obese women (Valckx et al. 2014). Therefore, we treated GCs with low doses of PA within this concentration range in our study. While no obvious effect was found on cell viability and apoptosis, the low dose of PA impaired glucose metabolism in ovarian GCs as in peripheral tissues (Boden et al. 2002).

In GCs, anaerobic glycolysis plays a key role in energy metabolism (Lin et al. 1997, Sugiuira et al. 2005). Therefore, glucose uptake as well as lactate accumulation induced by insulin act as suitable indicators of energy metabolism in GCs (Rice et al. 2005). Our previous study has demonstrated that insulin could stimulate lactate accumulation in ovarian GCs (Zhao et al. 2016). In the present study, while insulin significantly increased glucose uptake and lactate accumulation in control groups, PA treatment attenuated the stimulatory effects of insulin in a dose-dependent manner, suggesting the existence of IR in GCs. Furthermore, as another major component of FFAs in the follicular fluid, OA showed no effect on insulin-induced lactate production in GCs, but we found that OA rescued PA-induced downregulation of lactate accumulation. While PA is a saturated FFA and can inhibit insulin signaling in peripheral cells, monounsaturated FFAs such as OA can attenuate PA-induced toxicity (Malhi et al. 2006, Ruddock et al. 2008, Ricchi et al. 2009).

Our results also showed the protective role of OA in ovarian GCs.

Insulin binds to its receptor and subsequently stimulates phosphorylation of insulin-receptor substrate-1 (IRS-1) on tyrosine residues, and then activates multiple downstream signaling pathways, including the PI3-K/Akt pathway, which mainly mediates insulin-induced glucose metabolism in GCs by stimulating glucose uptake and lactate accumulation. Furthermore, we treated GCs with low doses of PA within this concentration range in our study. While no obvious effect was found on cell viability and apoptosis, the low dose of PA impaired glucose metabolism in ovarian GCs as in peripheral tissues (Boden et al. 2002).

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in GCs. It has been reported that ceramide derived from PA could inhibit insulin signaling via activation of JNK in muscle cells (Hage Hassan et al. 2016). Therefore, PA may also increase the level of ceramide to activate JNK and further impair insulin signaling in GCs. Besides, it has been revealed that PA affected insulin signaling pathway by increasing the expression of sterol regulatory element-binding protein-1c (SREBP-1c), which was able to bind to the promoter and repress the gene transcription of IRS-1 (Bi et al. 2014) or by activating IkB kinase β (IKKβ) that could phosphorylate IRS-1 at Ser 312 (Gao et al. 2002). Furthermore, the increased intracellular fatty acid-derived metabolites such as diacylglycerol and fatty acyl-CoA might activate the protein kinase C-theta, which induced serine phosphorylation of IRS-1 and IRS-2, leading to decreased activation of PI3-K (Kim et al. 2001). However, the exact mechanisms of IR induced by PA in GCs need to be further determined.

The JNKs activated by PA can impair insulin signaling pathway by phosphorylating IRS-1 and -2 at serine residues in Chinese hamster ovary cells (Aguirre et al. 2000). Sustained activation of JNK is associated with decreased insulin sensitivity and glucose metabolic disorder in peripheral tissues (Hirosumi et al. 2002). In the present study, we found that p-JNK was increased in a dose-dependent manner after PA treatment, indicating that JNK may also be involved in PA-induced IR in GCs. Furthermore, we explored the relationship between PA-induced JNK activation and PI3-K/Akt pathway, using specific JNK inhibitor and siRNA against JNK. Our results clearly showed that blocking JNK could reverse the negative effects of PA on p-Akt in GCs, which was consistent with the results in hepatocytes (Nakamura et al. 2009), suggesting that the activation of JNK was, at least partially, involved in PA-induced IR in GCs. Moreover, our study suggested that the selective JNK inhibitor might be a candidate for therapeutic strategy to improve ovarian IR.

In this study, we used PA to highlight the important role of saturated FFAs in IR in ovarian GCs, which may account for the diminished oocyte quality in obesity. It has been reported that PA could inhibit cell proliferation and stimulate estradiol-17β production in bovine GCs (Vanholder et al. 2005). Besides, PA could activate endoplasmic reticulum stress pathway in mouse cumulus-oocyte complexes, which affected protein secretion, mitochondrial activity and oocyte developmental competence (Wu et al. 2012). Furthermore, saturated FFAs such as PA decreased lipid storage in the maturing oocyte and reduced oocyte developmental competence (Aardema et al. 2011). Taken together, all these studies revealed the
association between high saturated FFAs and the impaired oocyte quality in obesity. Interestingly, PCOS, the most common endocrine abnormality among women of reproductive age, also exhibits features of obesity and metabolic disturbances including ovarian IR (Rice et al. 2005, Barber et al. 2006). We previously demonstrated that metabolic actions of insulin in ovarian GCs were not affected by hyperandrogenism directly (Zhao et al. 2016). Because it has been reported that PA levels were increased in serum and follicular fluid in PCOS patients (Niu et al. 2014), the elevated PA may result in ovarian IR in PCOS patients.

In summary, our study provided in vitro evidence that PA could impair glucose metabolism and interfere with the classical PI3-K/Akt pathway in ovarian GCs via activation of JNK pathway. Our results showed the direct action of PA on ovarian GCs which may partially account for the obesity-related reproductive disorders. Our results also indicated the significant role of JNK pathway in ovarian IR in obesity, which might represent a new therapeutic target. However, the effects of PA on GCs metabolism in vivo need to be further explored.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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