รายงานวิจัยฉบับสมบูรณ์

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และสำนักงานกองทุนสนับสนุนการวิจัย

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกอ. และสกว. ไม่จำเป็นต้องเห็นด้วยเสมอไป)

สัญญาเลขที่ BRG5480003

คำนำ

ผู้วิจัยขอขอบคุณ นางสาวนพมาศ พิพัฒน์ไพบูลย์, อ.ดร. วาสนา ปรัชญาสกุล, นางรจนา ทาวัน ในฐานะผู้ช่วยวิจัยที่ช่วยดำเนินการทดลองและให้ความช่วยเหลือในทุกทางตลอดโครงการ นอกจากนี้ ขอขอบคุณศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ (CERT CENTER) ภายใต้การดูแลของ ศาสตราจารย์ (เชี่ยวชาญพิเศษ) ดร. นายแพทย์ นิพนธ์ ฉัตรทิพากร ผู้อำนวยการศูนย์วิจัยและฝึกอบรมสาขาโรคทางไฟฟ้าของหัวใจ คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ที่ให้ความอนุเคราะห์ในการใช้เครื่องมือสำหรับงานวิจัยครั้งนี้ การศึกษานี้ได้รับการสนับสนุนจากกองทุนวิจัยจาก ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา (วุฒิเมธีวิจัย สกว.) (BRG5480003)

สิริพร ฉัตรทิพากร

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สารบัญ

สารบัญรูปภาพ

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สารบัญตาราง

หน้า

สัญญาเลขที่ BRG5480003

บทคัดย่อภาษาไทย

จากการศึกษาของคณะเราก่อนหน้านี้แสดงให้เห็นว่าการบริโภค อาหารไขมันสูง(HFD) ไม่เพียงแต่ทำให้เกิดภาวะดื้อต่ออินซูลิน แต่ยังทำให้เกิดภาวะดื้อต่ออินซูลินในสมองด้วย นอกจากนี้การบริโภคอาหารไขมันสูง ได้มีการแสดงให้เห็นว่าจะทำให้เกิด mitochondrial dysfunction ทั้งในกล้ามเนื้อลาย และ ตับ แต่อย่างไรก็ตาม การบริโภคอาหารไขมันสูงมีผลอย่างไรต่อการทำงานของ brain mitochondria ยังไม่มีการศึกษามาก่อน นอกจากนี้ Rosiglitazone จัดเป็น a peroxisome proliferator-activated receptor-γ ligand ที่ได้มีการพัฒนาเป็นยาที่ใช้ในการรักษาโรคเบาหวานชนิดที่ 2 การศึกษาล่าสุด ชี้ให้เห็นว่า Rosiglitazone สามารถปรับปรุงการเรียนรู้ และหน่วยความจำ ทั้งในการศึกษาของมนุษย์และสัตว์ แต่ผลของ Rosiglitazone กับ ภาวะดื้อต่ออินซูลินในสมอง และ brain mitochondrial function หลังการบริโภคอาหารไขมันสูง ยังไม่มีการศึกษามาก่อน ดังนั้นเราจึงมีการทดสอบสมมติฐานที่ว่า การบริโภคอาหารไขมันสูง สามารถเหนี่ยวนำให้เกิดภาวะดื้อต่ออินซูลินในสมองร่วมกับภาวะ brain mitochondrial dysfunction และ ยา Rosiglitazone ช่วยเพิ่มความไวในการตอบสนองของอินซูลินในสมอง และเพิ่มประสิทธิภาพในการทำงานของ brain mitochondria โดยทำให้เกิดการเพิ่มขึ้นของ insulin-induced long term depression และ brain insulin signaling จากการศึกษานี้ พบว่า การบริโภคอาหารไขมันสูงเป็นเวลา 12 สัปดาห์ ทำให้เกิดภาวะดื้อต่ออินซูลินในสมอง และ brain mitochondrial dysfunction การให้ Rosiglitazone (5 mg/kg ต่อวัน เป็นเวลา 14 วัน) ในหนูมีภาวะดื้อต่ออินซูลินจากการบริโภคอาหารไขมันสูงทำให้เกิดมีการปรับตัวดีขึ้นอย่างมีนัยสำคัญ ต่อ peripheral insulin sensitivity Insulin-induced long-term depression และ increased brain insulin signaling นอกจากนี้ยังพบว่า Rosiglitazone สามารถป้องกันการทำงานที่ผิดปกติของ brain mitochondria โดยสามารถป้องกัน mitochondrial conformational changes และ ลดลง brain mitochondrial swelling, brain mitochondrial membrane potential changes, and brain mitochondrial ROS production โดยการศึกษานี้แสดงให้เห็นว่า ภาวะดื้อต่ออินซูลินในสมองและการผิดปกติในการทำงานของ brain mitochondriaที่เกิดจาก การบริโภคอาหาร HFD เป็นเวลา12 สัปดาห์สามารถกลับมาเป็นปกติได้ด้วย Rosiglitazone

นอกจากนี้ ผลของฮอร์โมนเอสโตรเจนในการป้องกันภาวะดื้อต่ออินซูลินและภาวะดื้อต่ออินซูลินในสมองหลังจากการได้รับอาหารไขมันสูงยังไม่มีการศึกษามาก่อน ดังนั้นในการศึกษานี้จึงศึกษาหนูเพศผู้และเมียที่ได้รับการบริโภคอาหารอาหารไขมันสูงและมีการพัฒนาเป็นภาวะดื้อต่ออินซูลิน โดยที่มีน้ำหนักที่เพิ่มขึ้น มีไขมันเพิ่มขึ้น และมีค่าพลาสม่าอินซูลินสูงและดัชนี HOMA เพิ่มขึ้น หลังจากหนูทั้งหมดได้รับฮอร์โมนเอสโตรเจนทำให้มีการลดลงของพารามิเตอร์เหล่านั้น แสดงให้เห็นฮอร์โมนเอสโตรเจนสามารถ เพิ่มความไวของอินซูลินทั้งในเพศผู้และเพศเมีย นอกจากนี้การบริโภคอาหารอาหารไขมันสูงยังก่อให้เกิดภาวะดื้อต่ออินซูลินในสมอง โดยทำให้เกิดการบกพร่องของ Insulin-induced long-term depression ในฮิบโปแคมบัส และลดการทำงานของการส่งสัญญาณตัวรับอินซูลินในสมองในหนูเพศผู้

และเมีย การรักษาโดยใช้เอสโตรเจน จะทำให้เกิดลดความผิดปกติของสมองในหนูเพศเมียจากการบริโภคอาหารไขมันสูงเป็นเวลานาน โดยกระตุ้นการทำงานของเอสโตรเจนรีเซ็บเตอร์ จะทำให้เพิ่มความไวของอินซูลิน ของ peripheral part ทั้งในหนูเพศผู้และเพศเมีย แต่ประโยชน์ของเอสโตรเจนในสมองจะพบได้เฉพาะในหนูเพศเมีย

Keywords: อาหารไขมันสูง, ภาวะดื้อต่ออินซูลิน, PPAR gamma agonist, เอสโตรเจน

ABSTRACT

We previously demonstrated that a high-fat diet (HFD) consumption can cause not only peripheral insulin resistance, but also neuronal insulin resistance. Moreover, the consumption of an HFD has been shown to cause mitochondrial dysfunction in both the skeletal muscle and liver. Rosiglitazone, a peroxisome proliferator-activated receptor-γ ligand, is a drug used to treat type 2 diabetes mellitus. Recent studies suggested that rosiglitazone can improve learning and memory in both human and animal models. However, the effects of rosiglitazone on brain insulin resistance and brain mitochondria after the HFD consumption have not yet been investigated. Therefore, we tested the hypothesis that rosiglitazone improves neuronal insulin resistance caused by a HFD via attenuating the dysfunction of neuronal insulin receptors and brain mitochondria. We found that rosiglitazone (5 mg/kg per day for 14 days) significantly improved peripheral insulin resistance and insulin-induced long-term depression and increased brain Akt/PKB-ser phosphorylation in response to insulin. Furthermore, rosiglitazone prevented brain mitochondrial conformational changes and attenuated brain mitochondrial swelling, brain mitochondrial membrane potential changes, and brain mitochondrial ROS production. Our data suggest that neuronal insulin resistance and the impairment of brain mitochondria caused by a 12-wk HFD consumption can be reversed by rosiglitazone.

In addition, the effects of estrogen on the prevention of impaired insulin-induced long-term depression in the hippocampus and brain insulin signaling caused by high-fat diet (HF) were studied in male and female rats. Both male and female rats fed with HF developed peripheral insulin resistance as indicated by increased body weight, visceral fat, plasma insulin and HOMA index. Estrogen administration decreased those parameters, indicating improved peripheral insulin sensitivity, in both male and female HF rats. HF diet consumption also caused impaired insulin-induced long-term depression in hippocampus and impaired brain insulin receptor function and signaling, indicating brain insulin resistance, in both male and female rats. Estrogen treatment could attenuate these brain impairments only in HF female rats. The activation of the estrogen pathway could preserve insulin sensitivity in the peripheral tissue in both male and female rats. In brain, however, the benefit of estrogen could be found only in female rats.

Keywords: High-fat diet, Insulin resistance, PPAR gamma agonist, Estrogen

EXECUTIVE SUMMARY

ชื่อโครงการ:

"ผลของยาพีพีเออาร์แกมมาอะโกนิสต์ (PPAR-gamma agonist) และเอสโตเจน ต่อภาวการณ์ดื้อต่ออินซูลินของเซลล์สมองต่อการทำงานของไมโตรคอนเดรียของเซลล์สมองในหนูที่กินอาหารไขมันสูง"

"Effect of PPAR-gamma agonist and estrogen on neuronal insulin resistance and neuronal mitochondrial function in long-term high-fat diet fed rats"

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CHAPTER I

INTRODUCTION

Statement and significant of the problem

A high-fat (HF) diet has been shown as the major cause of obesity and insulin resistance (Riccardi et al., 2004). Despite the dramatic rise of insulin resistance throughout the developed nations (Fujimoto, 2000; Zimmet et al., 2001) and the growing interest in the role of insulin within the central nervous system (CNS) (Craft and Watson, 2004; Greenwood and Winocur, 2005; Winocur and Greenwood, 2005; Zhao and Alkon, 2001), there have been only a few studies examining the effects of this insulin impairment in the CNS. It has been shown that insulin is required to produce memory improvement in elderly people as well as patients with Alzheimer's disease (Craft et al., 2000; Craft et al., 2003). Furthermore, clinical studies have shown that cognitive impairments are often found in association with increased peripheral insulin resistance (Cosway et al., 2001; Mooradian et al., 1988; Ryan and Geckle, 2000). Our previous study has demonstrated that a significant modification of important brain insulin receptor signaling can be induced by a fat-enriched diet. Fed for 12 weeks, the high fat (HF) diet clearly induces brain insulin resistance, which is identified as a significant reduction in the ability of insulin to induce long-term depression (LTD), and a reduction in the stimulated phosphotyrosine activity of insulin receptor (IR), IR substrate (IRS-1) and Akt/PKB in brain slices. Twelve-week HF feeding not only causes brain insulin resistance in rats, but also leads to neuronal stress, as indicated by increased neuronal corticosterone level, and neuronal loss, as indicated by decreased nNOS-positive neurons. Since the defective insulin receptor signaling has been shown to associate with the pathogenesis of Alzheimer's disease (Watson and Craft, 2004), cognitive impairment (Greenwood and Winocur, 1990; Greenwood and Winocur, 1996; Greenwood and Winocur, 2005; Winocur and Greenwood, 2005) and the presence of cognitive impairment in patients with type II diabetes (Gispen and Biessels, 2000), the neuronal insulin resistance developing after 12-week HF consumption could be responsible for the impairment of cognition through the glucocorticoid-mediated effect in this animal model.

Increasing data support the idea that mitochondrial function declines with aging and in age-related disorders, including Diabetes (Gerbitz et al., 1996). It has been shown that chronic hyperglycemia impairs the mitochondrial respiration and induces oxidative damage through the production of reactive oxygen species (ROS) and causes mitochondrial dysfunction (Mastrocola et al., 2005). However, the brain mitochondrial function in cases of brain insulin resistance occurring in the obesity caused by HF consumption, which may have the impairment of cognition, has never been studied. Therefore, in this proposal, we aim to test the first hypothesis that the brain insulin resistance condition caused by long-term HF diet consumption in rats is associated with brain mitochondrial dysfunction.

Numerous studies revealed the beneficial effects of PPAR-gamma agonist, such as rosiglitazone (thiazolidinediones), on peripheral insulin resistance model via the improvement of insulin sensitivity, the reduction of tumor necrosis factor (TNF-α), lowering of lipid and cholesterol levels, and the reduction of the risk for development of type 2 diabetes(Pathan et al., 2008). In addition, several studies showed the beneficial effects of PPAR-gamma agonist in improving memory function in human and animal models (Guo and Tabrizchi, 2006; Risner et al., 2006). However, the effects of PPAR-gamma agonist on brain insulin signaling in long-term HF fed rats have not been investigated. Therefore, we aim to test the second hypothesis in this proposal that PPAR-gamma agonist can improve brain insulin resistance in long-term HF fed rats by increasing brain insulin receptor function (insulin-induced LTD), brain insulin signaling, and can prevent brain mitochondrial dysfunction under this condition

It has been known that the loss of ovarian hormones (i.e., during menopause or ovariectomy) leads to a significant increase in visceral fat deposition and in insulin resistance, which can then be easily developed into type 2 diabetes (Alonso and Gonzalez, 2008). Some evidence has shown that hormone replacement therapy can lead to a reduction in those incidence (Palin et al., 2001). A number of studies also demonstrated the beneficial effects of estrogen replacement on insulin resistance model, including improving peripheral insulin sensitivity, increased the rate of IRS-1 and Akt phosphorylation of insulin receptors, the reduction of central body fatness, lowering lipid and cholesterol levels and reduced risk of the development of type 2 diabetes (Palin et al., 2001). Nevertheless, there were only 2 studies

that demonstrated those beneficial effects of estrogen in HF-fed rats. Both studies have shown that the estrogen treatment exerted anti-diabetic and anti-obesity effects in rats which received HF diet (Bryzgalova et al., 2008). Growing evidence has also revealed the beneficial effects of estrogen on improving memory function in human and animal models (Daniel et al., 1997; Luine et al., 1998). It has been shown that synaptic plasticity processes in the hippocampal CA1 region are disrupted by acute and chronic estrogen deprivation during menopause or ovariectomy. The disruption, in turn, was ameliorated by chronic estrogen replacement (Day and Good, 2005). Nevertheless, the effects of estrogen administration on hippocampal synaptic plasticity and brain insulin signaling in HF-fed rats have never been investigated. Therefore, in this proposal we aim to test the third hypothesis that estrogen can reverse the impairment of brain insulin signaling, insulin-induced LTD in hippocampus and brain mitochondrial dysfunction in ovariectomized HF-fed rats. In addition, a previous clinical study has shown that the peripheral insulin sensitivity in menopause women with obesity was improved following the administration of PPAR-gamma agonist (Ramirez-Salazar et al., 2008). However, the effect of PPAR-gamma agonist on the brain insulin resistance in menopause female with obesity has not yet been investigated. Therefore, we aim to test the fourth hypothesis in this proposal that estrogen can improve brain insulin sensitivity in the ovariectomized HF fed rats.

Literature Review

Obesity, high fat dietary and insulin resistance

Obesity has reached epidemic proportions in many countries around the world. The increase in prevalence and severity of obesity has occurred too fast to be attributed only to genetics. In this case, the environmental factors must be implicated. In humans, high-fat (HF) diet is the major cause of hypertrophic obesity due to adipocyte hypertrophy, which is closely linked to major health issues such as diabetes, hyperlipidemia, hypertension and cardiovascular diseases (Fung et al., 2001). The prevalence of hypertrophic obesity, which often develops in adulthood, is increasing sharply under a HF diet. Insulin resistance, which is usually associated with hypertrophic obesity, is believed to be the major underlying mechanism of those pathological conditions. Obesity is central to the development of insulin resistance; hence increasing obesity escalates the risk of insulin resistance. Moreover, weight gain from

overfeeding significantly induces insulin resistance (Ravussin and Smith, 2002) and weight loss by calories restriction reverses insulin resistance (Henry et al., 1986). High circulating levels of free fatty acids released from adipocytes promotes insulin resistance in the liver and muscle, in a phenomenon known as “lipotoxicity”. Furthermore, the adipose tissue derived hormone, adiponectin, which increases insulin sensitivity, is the second connection between obesity and insulin resistance. In obese people, adiponectin is secreted less than in lean people (Faraj et al., 2004). The adipocyte derived hormone, resistin, has also been implicated in causing insulin resistance in hepatic tissue (Faraj et al., 2004). Other putative mechanisms through which obesity may cause insulin resistance includes the secretion of adipocytes cytokines, such as tumor necrosis factor alpha, interleukin-6 and interleukin-1(Boden, 1997). Insulin resistance appears to contribute to weight gain in adults and children, particularly with regards to the development of abdominal obesity. In human, insulin resistance develops slowly and remains undiagnosed for years. Many insulin resistant individuals maintain near normal levels of plasma glucose by compensatory hyperinsulinemia (DeFronzo, 1992). Although the majority of insulin resistant individuals are able to maintain the level of hyperinsulinemia, the combination of insulin resistance and hyperinsulinemia greatly increases the risk of having some degrees of glucose intolerance, high plasma triacylglycerol and low high-density lipid-cholesterol (HDL-C) concentrations and essential hypertension. These abnormal series in individuals are predisposed to be insulin resistance followed by the development of increased risk of type II diabetes, which are modifiable risk factors for cognitive decline and dementia (Areosa and Grimley, 2002).

Role of insulin in the brain

Insulin is a small protein with a molecular weight of about 6000 Da and is synthesized in significant quantities in the beta-cells of the pancreas. Insulin binds to insulin receptor to regulate the uptake of glucose from the circulation by inducing the translocation of glucose transporters from the cytoplasm towards the plasma membrane(van der Heide et al., 2006). Although previous study showed that insulin did not seem to influence glucose metabolism and transport into the brain, there have evidence suggested the region specific effect on glucose metabolism and transporter in the brain(Doyle et al., 1995). Previous study showed that

insulin-sensitive Glucose transporter type (GLUT) 4 and GLUT8 were selectively distributed in the brain. GLUT4 expressed in the cerebellum, sensorimotor cortex, hippocampus, pituitary and hypothalamus, whereas GLUT8 expressed in hippocampus and hypothalamus(Brant et al., 1993). The overlapping distribution of insulin, insulin receptor and GLUT are consistent with the possibility of insulin-stimulated glucose uptake and metabolism in specific brain region. Previously, it was believed that the central nervous system (CNS) was unresponsive to peripheral insulin level. Later, there has been more evidence that insulin could be produced in the CNS, where its receptors have also been found. Insulin receptors are widely distributed in the brain with highest concentrations in the olfactory bulb, hypothalamus, cerebral cortex, cerebellum and hippocampus (Horsch and Kahn, 1999). Moreover, the downstream effectors of insulin, such as insulin receptor substrate (IRS) proteins and PI3K isoforms, show their distinct patterns of downstream signal expression in the CNS that partly overlap with the expression of insulin receptor (IR)(Horsch and Kahn, 1999). When insulin binds to insulin receptor, it activates the membrane-bound insulin receptor tyrosine kinase, which consists of two α-subunits and two β-subunits (Figure 1-1).

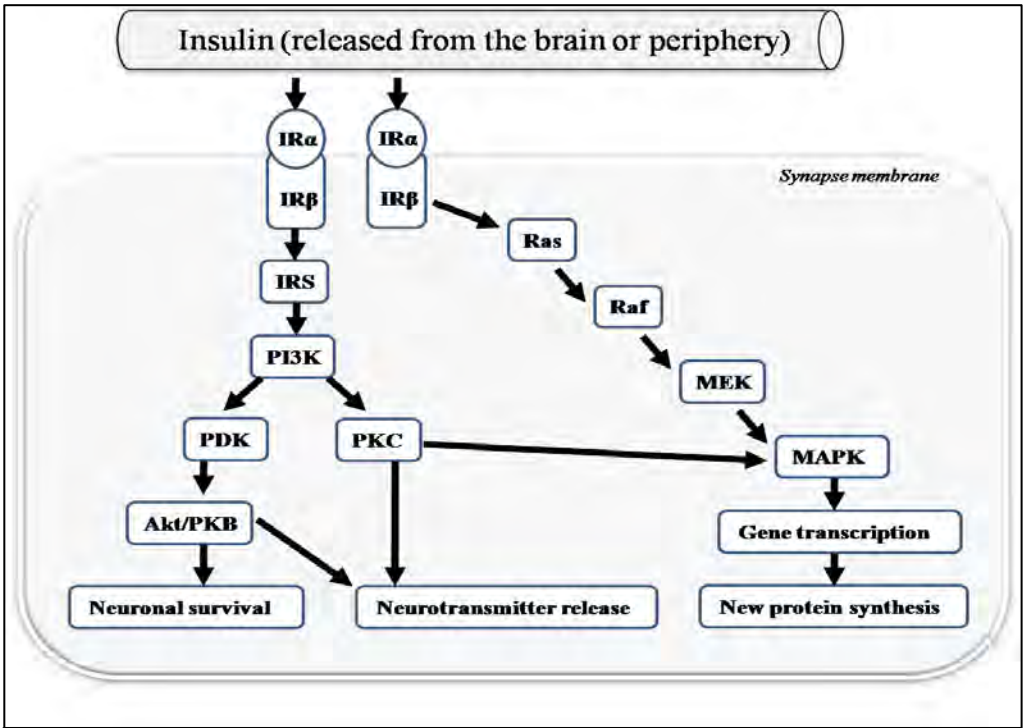


Figure. 1-1: Insulin signaling in the brain (Modified from Craft and Watson, 2004)

The activation of the tyrosine kinase activity of the β-subunits results in the auto-phosphorylation of insulin receptor and subsequently in the phosphorylation of intracellular IRS proteins on tyrosine residues. The phosphorylated IRS proteins then cause the activation of downstream signals, such as the activation of Ras-Raf-MAPK cascade or the activation of serine/threonine kinases downstream of PIP3. Those signals finally result in the diverse biological effects of insulin signaling in the CNS, such as the inhibition of apoptosis, tau phosphorylation, the regulation of amyloid precursor protein (APP) secretion, modulation of CNS concentrations of neurotransmitters, such as acetylcholine and norepinephrine, and the regulation of gene transcription, in which these effects are involved in learning and memory process (as summarized in Figure 1-1).

The role of insulin in brain synaptic plasticity

Insulin signaling in the brain has just emerged as a new field of research. Malfunctions in insulin signaling have been linked to numerous neurodegenerative diseases and impairments in learning and memory (Frolich et al., 1998). In fact, the expression of the insulin receptor and the insulin itself in the hippocampus implies a role for brain insulin in hippocampal processes such as learning and memory. Impairments in insulin signaling pathway in the periphery and in the brain have been implicated in Alzheimer's disease, diabetes and aging (Frolich et al., 1998). In Alzheimer's disease, the reduction in cerebral insulin levels appears to be accompanied by functional disturbances of insulin receptor (insulin-resistant brain state) (Frolich et al., 1998).

One of the critical functions for insulin in the brain is its role in synaptic plasticity. Two opposite forms of activity-dependent synaptic modifications have been identified; long-term potentiation (LTP) and long-term depression (LTD). In LTP, the brief high-frequency afferent activity leads to a long lasting increase in the strength of synaptic transmission, whereas in LTD, the prolonged low-frequency activity results in a persistent reduction in synaptic strength. Both LTP and LTD are the primary experimental models for investigating the synaptic basis of learning and memory. Several studies showed that a brief bath application of insulin can induce a long-lasting depression of excitatory synaptic transmission in hippocampal CA3 region (insulin-induced LTD). The proposed mechanisms of insulin induced LTD may be related to several mechanisms such as involving in an activation of Ca^{2+} influx through L-type voltage-

activated Ca^{2+} channel, the release of Ca^{2+} from postsynaptic intracellular calcium storage, phosphatidylinositol 3-kinase signaling and clathrin-mediated endocytotic removal of postsynaptic AMPA receptors(Huang et al., 2004). In recent years, these insights from behavioral, cellular and molecular neuroscience have been successfully applied to rodent models of diabetes. Biessels and colleagues have investigated the effect of prevented or reversed LTP with insulin treatment in STZ induced diabetic rats (Biessels et al., 1998). Insulin treatment was administered through a subcutaneous sustained-release insulin implant at dose of 2-4 i.u. per day at the onset of diabetes in a prevention experiment, and at 10 weeks after diabetic induction in a reversal experiment. After 10 weeks of treatment, hippocampal LTP in diabetic rats were impaired but the administration of insulin commencing at the onset of the diabetes could prevent this impairment. However, in the reversal experiment, insulin treatment failed to reverse the deficit of LTP in the STZ-induced diabetic rats (Biessels et al., 1998). It was concluded that insulin treatment may prevent but not reverse the deficit of LTP in the STZ-induced diabetic rats. In contrast to this finding, Izumi and colleagues demonstrated that insulin treatment could reverse the LTP impairment in the 2-week STZ induced diabetic rats (Izumi et al., 2003). In this study, insulin was administered at the start of the brain dissection, and incubated for 2-3 hours until the time of experiment (Izumi et al., 2003). In the existence of insulin and 10 mmol/l glucose, LTP was consistently induced in the brain slices of STZ-induced diabetic rats, and the degree of LTP was not significantly different from that in the control slice, which was incubated with 1 μmol/l insulin(Izumi et al., 2003). Discrepancies from these two studies were attributed mainly to the duration of diabetes. Although the mechanism of insulin-induced LTP was unknown, some investigations demonstrated that insulin has several effects on synaptic function, such as inhibiting spontaneous firing of pyramidal neurons (Palovcik et al., 1984), increasing functional postsynaptic GABA receptors (Wan et al., 1997), and rapidly potentiating NMDA receptors mediate EPSPs (Belanger et al., 2004). Thus, these mechanisms may be related to insulin-induced LTP. No alteration in LTP was observed in type-2 diabetes animal model. In a ZDF rat model, LTP was generated by applying theta burst stimulation (10 bursts of 4 stimulation pulses at 100 Hz) to Schaffer collateral-commissural projection in area CA1 of the hippocampus. It was shown that there was no significant decrease in the

magnitude of LTP in area CA1 hippocampal slices that are prepared from the ZDF rats, when compared to the normal rats (Belanger et al., 2004). Similarly, there was no alteration in LTP in Syrian Golden hamsters that were placed upon a high-fructose diet model. The type 2 diabetes in the animal models showed signs of obesity, dyslipidemia and decreases in peripheral insulin sensitivity (Carpentier et al., 2002).

In contrast to LTP, the expression of LTD was enhanced in the CA1 field after low-frequency stimulation of hippocampal slices of diabetic rats. Kamal and colleagues used extracellular recording technique to investigate the effect of LTP and LTD on the CA1 hippocampal slices in STZ diabetic rats, in which each effect was induced by high frequency stimulation and low frequency stimulation, respectively. They found that the 12-week induction of diabetes by STZ enhanced the degree of LTD but impaired LTP(Kamal et al., 1999). In addition, the incidence underlying the diabetes-induced changes in synaptic plasticity did not only alter the degree but also the thresholds of LTD and LTP. This study showed that LTD was persistently facilitated while LTP was inhibited, owing to the simultaneous leftward and rightward shift in membrane potential (Vm) that induced LTD and LTP, respectively. The shift in Vm that induced synaptic plasticity associated with diabetes might be through mechanisms involved in metaplasticity (Artola et al., 2005). It has been proposed that the persistent facilitation of LTD and the inhibition of LTP might contribute to learning and memory impairments associated with diabetes mellitus. In addition to these findings, several previous studies found that the change of LTD in diabetic rats is related to age. Young adult (5 months) and aged (2 years) rats were examined after 8 weeks of STZ-induced diabetes. Synaptic plasticity was test *ex vivo*, in hippocampal slices, in response to trains of stimuli at different frequencies. LTD in both young and aged diabetic rats was enhanced in the brain slices compared with the controls. In addition, the significantly different of LTD in the young diabetic rats was performed only at 1 Hz stimulation, whereas the expression of LTD in the aged diabetic rat was significantly enhanced after 1, 5, 10 Hz stimulation(Kamal et al., 2000). Theses result indicated that both diabetic and aging affect hippocampal synaptic plasticity. Little work has been done using animal models of insulin resistance to investigate the changes of LTD.

Mitochondrial dysfunction in diabetes

Brain requires the large energy to drive all neuronal demands, such as energy to maintain ion gradients across the plasma membrane. Mitochondria are known as subcellular organelles that are essential for generating the energy for the normal cellular function and also for monitoring cellular health in order to initiate a programmed cell death. The maintenance of cellular energy reserves and intact cellular function are closely dependent upon mitochondrial biology. Oxidative stress can trigger the opening of the mitochondrial membrane permeability transition pore (mPTP) and lead to a significant loss of mitochondrial NAD+ stores and subsequent apoptotic cell injury (Lemasters et al., 1998). Mitochondria are also a significant source of superoxide radicals that are associated with oxidative stress (Hirsch et al., 1997).

Oxidative stress also is believed to modify a number of the signaling pathways within a cell that can ultimately lead to insulin resistance. The biological markers of oxidative stress and peripheral insulin resistance suggest that ROS contributes to the pathogenesis of hyperglycemia-induced insulin resistance(Zorzano et al., 2009). Furthermore, hyperglycemia can lead to increased production of ROS in endothelial cells, liver and pancreatic β-cells(Zorzano et al., 2009).

Mitochondrial dysfunction has been reported in skeletal muscle of insulin resistance and of type 2 diabetic patients (Zorzano et al., 2009). Reduced mitochondrial capacity contributes to the accumulation of lipid intermediates, desensitizing insulin signaling and leading to insulin resistance. Although brain mitochondrial dysfunction have been demonstrated in type I diabetes (Mastrocola et al., 2005; Moreira et al., 2007; Verkhratsky and Fernyhough, 2008), its function in the condition of neuronal insulin resistance caused by HF consumption is not clearly understood.

Insulin resistance and PPARγ agonist

Peroxisome proliferator-activated receptors (PPAR) are nucleus hormone receptors. They are classified in 3 groups; PPARα, PPARβ and PPARγ. The binding of retinoid x receptor to PPARs is called heterodimerization. PPARα plays a role in the regulation of fatty acids, while PPARβ has been implicated to be involved in some disorders such as cancer,

infertility, and dyslipidemia (Guo and Tabrizchi, 2006). PPARγ does not only regulate glucose and fatty acids metabolisms, but also improves insulin sensitivity (Guo and Tabrizchi, 2006). The ligands of PPARγ are not only endogenous substances, including saturated fatty acid or polyunsaturated fatty acid, but also exogenous substances such as ibuprofen, pioglitazone, teoglitazone and rosiglitazone (Guo and Tabrizchi, 2006). The adipose tissues are one of etiology to cause insulin resistance because they can secrete protein and peptides, such as adipocyte complement-related protein, TNF-alpha, macrophage migration inhibitory factor, and plasminogen activator inhibitor type-1(PAI-1)(Guo and Tabrizchi, 2006). Several of these peptides are involved in pathogenesis of insulin resistance(Guo and Tabrizchi, 2006). Several studies suggested that PPARγ agonist can reduce TNF-alpha, leptin, interleukin-6, plasminogen activator inhibitor type-1, resistin, and 11-beta-hydroxysteroid dehydrogenase type-1 (Guo and Tabrizchi, 2006; Pathan et al., 2008).

Previous study found that PPARγ agonist can improve insulin resistance by increasing GLUT 4 translocation to the adipocytes and by increasing adipocytokines such as adiponectin, in which both processes can activate fatty acid oxidation in skeletal muscles and livers (Guo and Tabrizchi, 2006). These findings suggested that PPARγ could help to improve peripheral insulin resistance via the improvement of glucose transporter and to control the fatty acid metabolism (Guo and Tabrizchi, 2006).

Role of rosiglitazone (PPARγ agonist) on learning and memory

Several studies have shown that insulin resistance and insulin dysfunction are indicators of type 2 Diabetes and are associated with the risk of memory decline and Alzheimer's disease (Guo and Tabrizchi, 2006; Pathan et al., 2008). The increased level results in synaptic loss, hippocampus atropy, and cognitive decline(Stranahan et al., 2008). PPARγ can be found in the brain, including in hypothalamus, basal ganglia and hippocampus (Inestrosa et al., 2005).

Rosiglitazone is one of the PPARγ agonists, which plays roles in lipid, glucose and energy metabolism, anti-inflammation and increase of insulin sensitivity (Pathan et al., 2008). Previous studies showed that amyloid plaques in Alzheimer's disease are caused by the stimulation of microglia that can secret pro-inflammatory cytokines (Inestrosa et al., 2005). The

effect of rosiglitazone as anti-inflammation helps to reduce the pathogenesis of Alzheimer's disease(Watson and Craft, 2003). It has been shown from a culture method that rosiglitazone can increase dendritic spine(Brodbeck et al., 2008). Moreover, Tg2576 mice model of Alzheimer's disease have shown that rosiglitazone can reduce amyloid a peptide Aβ1-42 in the brain. Furthermore, rosiglitazone can improve learning and memory as demonstrated in Morris Water Maze test (Pedersen et al., 2006). In addition, a previous study showed that rosiglitazone cannot penetrate blood brain barrier (Landreth et al., 2008). Therefore, the improvement in learning and memory of rosiglitazone may be caused by the indirect effect of an improvement in peripheral insulin sensitivity (Pathan et al., 2008). However, the effect of this PPARγ agonist on the brain insulin resistance has never been investigated.

Effect of estrogen on insulin resistance

Estrogen is steroid hormone which mainly synthesis from ovary. After synthesis, estrogen is secreted to the blood stream, where it diffuse into target tissues to exert their specific genomic or non-genomic effects such as neuro-protection, cardio-protection, maintenance of bone density, growth and differentiation of sex organs or increased production of liver proteins (Gruber et al., 2002). Moreover, there is increasing evidence both in human and animal linking the endogenous estrogen to the maintenance of glucose homeostasis.

In human, the loss of ovarian function associated with the menopause is associated with the reduction in whole body insulin-mediated glucose uptake (Proudler et al., 1992), and the changes that accompany menopause may further develop visceral obesity and insulin resistance and are at increased risk for type 2 diabetes mellitus (Strotmeyer et al., 2003). Therefore, hormone replacement therapy leads to a reduction in the incidence of diabetes (Salpeter et al., 2006). In addition several studies showed beneficial effects of estrogen replacement on insulin resistance such as improves insulin sensitivity, reduces central body fatness, lowers lipid and cholesterol levels, and decreases the risk for development of type 2 diabetes (Ryan et al., 2002). These beneficial effects of estrogen depend on dose, route of administration, and duration of treatment (Karjalainen et al., 2001). However, there are studies that have shown no effect on impairment of insulin sensitivity upon estrogen treatment

(Sherwin, 2005). Moreover, early work in animal model showed that administration of estrogen increased insulin content of the pancreas in rat (Gruber et al., 2002). Later, Houssay and colleagues used a partially pancreatectomised rat model to show that administration of estrogen was associated with hypertrophy and regeneration of islets (Houssay et al., 1954). Furthermore, in various spontaneous rodent models of type 2 diabetes, female rodents are protected against hyperglycemia unless they are ovariectomized, and E2 perfusion reverses diabetes in male rodents (Karjalainen et al., 2001). Recently, the study of transgenic mice and mice with genetic alteration of E2 secretion or E2 action has shed light on the antidiabetic properties of E2 at a tissue-specific level (Louet et al., 2004). Previous studies demonstrated the beneficial effect of estrogen on peripheral insulin signaling. It has been shown that low concentrations of estradiol could induce an increase in the rate of IRS-1 phosphorylation, promote the association between IRS-1 and the subunit of PI3-k, p85alpha, cause a decrease in the rate of IRS-1 serine phosphorylation and increase the rate of Akt phosphorylation(Gonzalez et al., 2008). Despite these beneficial effects of estrogen on peripheral insulin resistance in animal model, there have been only 2 studies demonstrated this beneficial effect for insulin sensitivity on HF fed rat model. None, however, has investigated the effect of estrogen on neuronal insulin signaling. Bryzgalova and colleagues showed that estrogen treatment exerts anti-diabetic and anti-obesity effects in rat, which received 34.9 g% fat for 10 months (Bryzgalova et al., 2008). Riant and colleagues showed that E2 treatment reduced HF-induced peripheral insulin resistance by 50% during hyperinsulinemic-euglycemic clamp studies, and improved insulin signaling (Akt phosphorylation) in insulin-stimulated skeletal muscles (Riant et al., 2009). Up until now, there is no study investigating the effects of estrogen on neuronal insulin resistance in HF-fed rat model. Understanding this effect in HF-induced neuronal insulin resistance should provide much fundamental knowledge to future therapeutic purpose in insulin resistance and obese patients.

Effect of estrogen on synaptic plasticity

Although the effect of estrogen on reproductive behavior in the brain is well known, its effects on the other behaviors including mood and cognition have recently been recognized. Numerous studies have revealed the beneficial effects of estrogen on improving memory function in human and animal models (Asthana et al., 1999; Asthana et al., 2001). Estrogen replacement therapy in non-demented menopausal women and in healthy adult surgically menopausal women can improve verbal and spatial memory. In animal model, estrogen enhanced performance on hippocampal dependent task in female rats(Daniel et al., 1997), mice (Luine et al., 1998) and rhesus monkeys (Godsland, 2005). The mechanism of estrogen which enhanced cognition is related to the alteration of synaptic morphology and synaptic plasticity. In female rodents, elevated levels of estrogen are associated with a variety of alteration in synaptic structure, including increased dendritic spine and synapse density, increased synaptic protein, and enhanced neurogenesis (Godsland, 2005). In addition, estrogen is associated with two different types of synaptic plasticity (both LTP and LTD). In rats, estrogen deprivation by ovariectomized disrupted LTP and LTD in hippocampal CA1 region (Day and Good, 2005). Furthermore, when the ovariectomized rats were treated with estrogen, it showed a facilitation for the induction of hippocampal LTP (Day and Good, 2005). Similarly to LTP, many studies showed that estrogen enhanced the induction of LTD in ovariectomized rats (Day and Good, 2005). In addition, LTD was very sensitive to 17β-estradiol treatments in hippocampal slices from adult male rats. This study showed a significant rapid enhancement of LTD by 1–10 nM estradiol in CA1, CA3 and dentate gyrus (Day and Good, 2005). Although previous studies support the beneficial effect of estrogen on the improvement of synaptic plasticity, its effects in insulin resistance model especially HF-induced insulin resistance model has never been investigated.

Objective of the study

***Specific Aim 1*:** To determine the occurrence of brain mitochondrial dysfunction in male rats with neuronal insulin resistance caused by the consumption of long-term HF diet via measuring ROS, mitochondrial swelling and mitochondrial membrane potential in brain mitochondria of both HF-diet and normal diet-fed rats.

***Specific Aim 2*:** To determine whether the administration of PPAR-gamma agonist, rosiglitazone, into male rats with neuronal insulin resistance induced by long term HF diet consumption can improve the brain insulin resistance, by increasing neuronal insulin receptor function (insulin-induced LTD), brain insulin signaling and brain mitochondrial function

Specific Aim 3: To determine whether the administration of estrogen into both genders of animals with neuronal insulin resistance induced by HF diet consumption can improve the neuronal insulin resistance condition by increasing neuronal insulin receptor function in hippocampus (insulin-induced LTD) and brain insulin signaling

Specific Aim 4: To determine whether estrogen administration in long term HF-fed ovariectomized (female) rats can improve brain insulin resistance by increasing neuronal insulin receptor function in hippocampus (insulin-induced LTD) and brain insulin signaling

CHAPTER II

MATERIALS AND METHODS

General Methods

All experiments were conducted in accordance with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Male Wistar rats weighing ~200 g were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were individually housed in a temperature-controlled environment with a 12:12 light-dark cycle. One week after arrival, rats were randomly assigned to one of the two dietary groups. The normal-diet (ND) group received a standard laboratory chow, in which 19.77% of total energy (%E) was from fat, with energy content calculated at 4.02 kcal/g (Mouse Feed Food No.082, C.P. Company, Bangkok, Thailand; as shown in Table 1-1). The HF group consumed a diet containing fat, mostly from lard (59.28% E; as shown in Table 1-2), with energy content calculated at 5.35 kcal/g, for 12 weeks. The animals were maintained in individual cages with unrestricted access to food and water. Body weight and food intake were recorded daily. Blood samples were collected from the tail at weeks 12 and the end of experimental protocol after fasting for at least 5 hours. The oral glucose tolerance test (OGTT) was investigated. Plasma was separated and stored at -80oC for subsequent biochemical analyses. At the end of each experimental period, animals were deeply anesthetized and decapitated. The brain was rapidly removed for brain slice preparation and one lobe of liver as well as visceral fat were removed, weighed and stored at -80^{o}C for further biochemical analysis.

Table 1-1. Composition of Normal Diet

Composition	Normal diet (CP 082) Mouse Feed No.082, C.P. Company (Bangkok, Thailand)		
	g	kcal	%E
Carbohydrate	495.30	1981.20	51.99
Fat	83.70	753.30	19.77
Protein	269.00	1076.00	28.24
Vitamins	65.40	-	-
Fiber	34.30	-	-
Total	947.70	3810.50	100
Kcal/g	4.02 kcal/g		

Table 1-2. Composition of High-Fat Diet

Composition	High-fat diet (HFD) Modified from Srinivasan et al.		
	g	kcal	%E
Carbohydrate	190.76	763.04	14.27
Fat	342.24	3080.16	57.60
Protein	353.60	1414.40	26.45
Cholesterol	10	90	1.68
Vitamins	85.19	-	-
DL-Methionine	3	-	-

Fiber	13.21		
Yeast powder	1	-	-
Sodium chloride	1	-	-
Total	1000	5347.60	100
Kcal/g		5.35 kcal/g	

Diets ingredients and nutrient analyses were modified from Srinivasan *et al.* (Srinivasan et al., 2005). Energy (Kcal) per gram: carbohydrate 4; fat 9; protein 4.

Ovariectomy

Female rats were anesthetized with xylazine (0.15 ml/kg) and zoletil (50mg/kg). Bilateral ovariectomy was preceded by a midline dorsal skin incision. The incision was centered between the bottom of the rib cage and the front of the hind limb. The skin was separated from the underlying muscle. Ligation of the blood vessels and the cutting of the connection between the uterine tube and uterine horn inside peritoneal cavity were carried out. After that, the ovary was removed and the incision was closed.

Determination of glucose

Plasma glucose concentrations were determined using colorimetric assay by commercially available kit (Biotech, Bangkok, Thailand).

Determination of cholesterol

Plasma cholesterol concentrations were determined using colorimetric assay by commercially available kit (Biotech, Bangkok, Thailand

Determination of insulin

Plasma insulin levels were measured by Sandwich ELISA (LINCO Research, MO, USA) (Pratchayasakul et al., 2011b).

Determination of insulin resistance (HOMA index)

Insulin resistance was assessed by using Homeostasis Model Assessment (HOMA) (Appleton et al., 2005; Haffner et al., 1997) as a mathematical model describing the degree of insulin resistance, calculated from fasting plasma insulin and fasting plasma glucose concentration. A higher HOMA index indicates a higher degree of insulin resistance.

Oral glucose tolerance test (OGTT)

OGTT was performed after fasting overnight (12 h). Then the rats were received bolus of glucose (2.0 g/kg body weight) via gavage feeding and blood sample was corrected from tail vein at 0, 30, 60, 90 and 120 minutes after glucose administration in NaF microcentrification tube after that it was centrifuged at 10,000 rpm for 5 min at 4°C. Plasma glucose was estimated by colorimetric assay using a commercially available kit (Biotech, Bangkok, Thailand) (Pratchayasakul et al., 2011b).

Preparation of brain slices and insulin stimulation

The rats were anesthetized with isoflurane and decapitated by guillotine. Whole brain was rapidly removed. After that, the brain was immersed in ice-cold “high sucrose” artificial cerebrospinal fluid (aCSF), containing (mM): NaCl 85; KCl 2.5; $MgSO_4$ 4; $CaCl_2$ 0.5; NaH_2PO_4 1.25; $NaHCO_3$ 25; glucose 25; sucrose 75; kynurenic acid 2; ascorbate 0.5, saturated with 95%O_2/5%CO_2 (pH 7.4). This solution was enhancing neuronal survival during the slicing procedure. The hippocampal slices was cut using a vibratome (Vibratome Company, MO, USA). Following a 30-minute post-slice incubation in high sucrose aCSF, the slices were transferred to a standard aCSF solution containing (mM): NaCl 119; KCl 2.5; $CaCl_2$ 2.5; $MgSO_4$ 1.3; NaH_2PO_4 1; $NaHCO_3$ 26; and glucose 10, saturated with 95% O_2, 5%CO_2 (pH 7.4) for an additional 30 minutes at room temperature (22-24°C). For insulin stimulation, slices were placed into either aCSF or aCSF plus 500 nM insulin (Humulin R, Eli Lilly, Germany) for 5 minutes. These slices were prepared as homogenate for immunoblotting (Pratchayasakul et al., 2011b).

Extracellular recording of hippocampal slices for insulin-induced LTD

To examine insulin-mediated long term depression (LTD), the brain slices were transferred to a submersion recording chamber and continuously perfused at 3-4 ml/min with standard aCSF warmed to 25-28°C. Field excitatory postsynaptic potentials (fEPSPs) was evoked by stimulating the Schaffer collateral-commissural pathway with a bipolar tungsten electrode, while the fEPSPs recordings were taken from the stratum radiatum of the hippocampal CA1 region with micropipettes (3 MΩ) filled with 2M NaCl. The stimulus frequency was at 0.033 Hz and the stimulus intensity was adjusted to yield a fEPSP of 0.8-1.0 mV in amplitude, which produces < 50% of maximal monophasic responses. The brain slices were perfused with aCSF (as baseline condition) for 10 minutes and then perfused with aCSF plus 500 nM of insulin (as insulin-induced LTD) for additional 10 minutes. Thereafter, the slices were perfused with aCSF again for a further 50 minutes and recorded. Data were filtered at 3 kHz, digitized at 10 kHz, and stored in a computer using pClamp 9.2 software (Axon Instruments, CA, USA). The initial slope of the fEPSP was measured and plotted against time (Pratchayasakul et al., 2011b).

Immunoblotting

To detected Akt and insulin-mediated Akt Ser473 phosphorylation, homogenated brain from each dietary and treatment group was boiled at 95°C, for 5 mins. Then, the proteins were separated by electrophoresis on 10% polyacrylamide gels (Bio-Rad Laboratories, CA, USA) SDS-Page and transferred into PDVF membranes. After blocking with 5% non-fat milk/TBST, immunoblotting was conducted with Akt, Akt Ser473 antibody (rabbit polyclonals, 1:2000 in TBST, Santa Cruz, CA, USA) overnight. Membranes were incubated with a secondary goat anti-rabbit antibody, conjugated with horseradish peroxidase (1:8000 in TBST, Bio-Rad Laboratories, CA, USA), and the protein bands were visualized on Amersham hyperfilm ECL (GE Healthcare, Buckinghamshire, UK) using Amersham ECL Western blotting detection reagents system (GE Healthcare, Buckinghamshire, UK). Band intensity was quantified by Scion Image program and the results are shown in average signal intensity (arbitrary units) (Pratchayasakul et al., 2011b).

Determination of plasma and brain estrogen concentrations

Plasma and neuronal estrogen concentrations were measured by using a competitive enzyme immunoassay (EIA) kit (Cayman Chemical Company, MI, USA).

Preparation of brain mitochondria

The brain was removed into ice-cold MSE solution (225 mM mannitol, 75 mM sucrose, 1mM EGTA, 5mM HEPES, 1 mg/ml BSA, pH 7.4) for wash out blood rapidly. Taken together, the brain was transferred to 10 ml of ice-cold MSE-nagarse solution (0.05% nagarse in MSE solution) and homogenized by homogenizer. Next, the tissue was minced and centrifuged at 2,000×g for 4 min and the supernatant was collected after that centrifuged at 12,000×g for 11 min. Then mitochondria pellet were collected and resuspended in 10 ml ice-cold MSE-digitonin solution (0.02% digitonin in MSE solution) and the brown pellet was collected (Kulawiak et al., 2008). Finally, mitochondrial pellet were mince with respiration buffer (150 mM KCl, 5 mM HEPES, 5 mM $K_2HPO_4.3H_2O$, 2 mM L-glutamate, 5 mM Pyruvate sodium salt). Mitochondria protein were determined by the BCA assay (Thummasorn et al., 2011).

The Bicinchoninic Acid (BCA) assay for protein quantitation (Standard assay)

Mitochondrial protein was determined by the Bicinchoninic Acid (BCA) Assay which composed that reagent A and reagent B. Reagent A was composed of 0.1 g sodium bicinchoninate, 2.0 g $Na_2CO_3.H_2O$, 0.16 g sodium tartrate (dihydrate), 0.4 g NaOH, 0.95 g and $NaHCO_3$ in 100 ml of deionize water. The pH was adjusted to 11.25 with $NaHCO_3$ or NaOH, if necessary. Reagent B was composed of 0.4 g CuSO4.5H2O in 10 ml of deionized water. After that the standard working reagent (SWR) was mixed with 100 ml. of reagent A and 2 ml. of reagent B. The solution was apple green color and stable at room temperature for 1 wk. SWR (1 ml) was added to mitochondrial protein (50 µl) and incubated at 60°C for 30 min. The sample was cooled to room temperature then the absorbance was measured at 562 nm using a spectrophotometer. A calibration curve was constructed using dilutions of a stock 1 mg/ml solution of bovine serum albumin (BSA) (Thummasorn et al., 2011).

Brain mitochondrial ROS assay

Isolated mitochondria were stained by dichlorohydro-fluorescein diacetate (DCFHDA) that can pass cell membrane before hydrolysis by intracellular esterase. ROS can oxidize DCFH to dichlorodihydro-fluorescein (DCF), which is highly fluorescent at λex 485 nm and λem 530 nm. ROS causes an increase in fluorescence. The isolated mitochondria 0.4 mg/ml were stained with 2 μM DCFDA and incubated at room temperature for 20 min. The fluorescence was determined using a fluorescent microplate reader (Bio-Tek Instruments, Inc. Winooski, Vermont USA) (Thummasorn et al., 2011).

Brain mitochondrial membrane potential ($\Delta\psi$m) assay

The mitochondria membrane potential were measured by fluorescent dye 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1) which can accumulated in mitochondria. JC-1 dye, a monomer dye can access to mitochondria and produce a green fluorescent (emission wavelength at 530 nm.) at lower membrane potential whereas at high membrane potential, JC-1 dye changes to JC-1 aggregate dye that produced a red fluorescent (emission wavelength at 590 nm.).

The mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The isolated mitochondria 0.4 mg/ml were strained with JC-1 at 37 °C for 15 min then the fluorescence intensity was determined using a fluorescent microplate reader (Bio-Tek Instruments, Inc. Winooski, Vermont USA). The JC-1 monomer (green) fluorescence is excited at λex 485 nm and the emission is detected at λem 530 nm. JC-1 aggregate (red) fluorescence is excited at λex 485 nm and emission fluorescence is detected at λem 590 nm (Thummasorn et al., 2011).

Brain mitochondrial swelling assay

The isolated mitochondrial swelling was assessed by measuring the changes in the absorbance of the suspension at 540 nm by using a microplate reader (Bio-Tek Instruments, Inc. Winooski, Vermont USA). Mitochondria (0.4 mg/ml) were incubated in 2 ml respiration buffer (containing150 mM KCl, 5 mM HEPES, 5 mM K2HPO4.3H2O, 2 mM L-glutamate, 5 mM

Pyruvate sodium salt). The decreases absorbance means mitochondrial swelling (Thummasorn et al., 2011).

Data analysis

The data for each experiment were presented as mean ± SEM. For all comparisons, the significance of the difference between the mean were calculated by one-way ANOVA followed by LSD post hoc tests. $P<0.05$ was considered to be statistically significant.

Methodological approach for research specific aims

***Specific Aim 1*:** To determine the occurrence of brain mitochondrial dysfunction in male rats with neuronal insulin resistance caused by the consumption of long-term HF diet via measuring ROS, mitochondrial swelling and mitochondrial membrane potential in brain mitochondria of both HF-diet and normal diet-fed rats.

***Specific Aim 2*:** To determine whether the administration of PPAR-gamma agonist, rosiglitazone, into male rats with neuronal insulin resistance induced by long term HF diet consumption can improve the brain insulin resistance, by increasing neuronal insulin receptor function (insulin-induced LTD), brain insulin signaling and brain mitochondrial function

Protocol 1: Effects of rosiglitazone on brain insulin receptor function.

A total of twenty-four male Wistar rats weighing 180-200 g from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand, were used for this study. All experiments were conducted with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. The animals were randomized into 2 groups: control group (n=12) consumed normal diet (ND), standard laboratory chow, which has energy content of 4.02 kcal/g, and 19.77% of total energy (%E) of the food was from fat (Mouse Feed Food No. 082, C.P. Company (Bangkok, Thailand) and high-fat group (n=12) that received high-fat diet (HFD) which had energy content of 5.35 kcal/g and contains fat mostly from lard (59.28% E) for a period of 12 weeks. The animals were given free access to diet and drinking water. Body weight were measured every other week and blood sampling from tail vein was performed at week 12 after fasting for 5-6 hours to measure glucose, cholesterol and insulin levels. Blood samples for glucose assay were kept

on cold-ice in NaF microcentrification tube whereas blood samples for insulin and cholesterol assay were kept on cold-ice in EDTA microcentrification tube. Plasma was stored at -80oC for subsequent biochemical analysis.

After 12 weeks, ND and HFD groups were divided into 2 subgroups (n=6/subgroup). Each subgroup was received either rosiglitazone 5 mg/kg/day (dissolved in normal saline 2 ml/kg/day) for 14 consecutive days or vehicle (normal saline, 2 ml/kg/day) for 14 consecutive days. Rosiglitazone was retrieved from Glaxo Wellcome S.A. (Aranda de Duero, Spain). At the end of experiment, all animals were deeply anesthetized and decapitated. After that, the brains were rapidly removed for preparation of brain slices to determine effect of rosiglitazone on brain insulin resistance (insulin-induced LTD) and brain insulin signaling. Moreover, all of the visceral fat were removed, weighed and stored at -80 oC for further biochemical analysis.

Protocol 2: Effects of rosiglitazone on brain mitochondrial function.

A total of twenty-four male Wistar rats weighing 180-200 g from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand, were used for this study. All experiments were conducted with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. The animals were randomized into 2 groups: control group (n=12) that consumed ND, standard laboratory chow, which had energy content of 4.02 kcal/g, and 19.77% of total energy (%E) of the food is from fat (Mouse Feed Food No. 082, C.P. Company (Bangkok, Thailand) and high-fat group (n=12) that receives HFD which had energy content of 5.35 kcal/g and contains fat mostly from lard (59.28% E) for a period of 12 weeks. The animals were given free access to diet and drinking water. Body weight was measured every other week and blood sampling from tail vein was performed at week 12 after fasting for 5-6 hours to measure glucose, cholesterol and insulin levels. Blood samples for glucose assay were kept on cold-ice in NaF microcentrification tube whereas blood samples for insulin and cholesterol assay were kept on cold-ice in EDTA micro-centrification tube. Plasma was stored at -80 ^{o}C for subsequent biochemical analyses.

After 12 weeks, ND and HFD groups were divided into 2 subgroups (n=6/subgroup). Animals in each subgroup were received either rosiglitazone 5 mg/kg/day (dissolved in normal

saline 2 ml/kg/day) for 14 consecutive days or vehicle (normal saline, 2 ml/kg/day) for 14 consecutive days. Rosiglitazone was retrieved from Glaxo Wellcome S.A. At the end of the experimental, animals were determined OGTT and then anesthetized with thiopental (80 mg/kg) by intraperitoneal injection and open chest for gavage perfusion via the heart and then were killed by decapitation. After that, the brains were rapidly removed for determination of brain ROS production, brain mitochondrial membrane potential changes.

***Specific Aim 3*:** To determine whether the administration of estrogen from both genders of animals onto neuronal insulin resistance, that was caused by the consumption of high-fat diets, would improve the neuronal insulin resistance by increasing neuronal insulin receptor function in hippocampus (insulin-induced LTD), and brain insulin signaling.

Protocol 3: all experiments were conducted in accordance with an approved protocol from the Faculty of Medicine, Chiang Mai University Institutional Animal Care and Use Committee, in compliance with NIH guidelines. Male (n=40) and female (n=40) Wistar rats weighing ~ 180-200 g were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were individually housed in a temperature-controlled environment with a 12:12 light-dark cycle. One week after arrival, all rats were randomly assigned to one of the two dietary groups (n=40 in a HF diet group, 20 male: 20 female and n=40 in a normal diet group, 20 male: 20 female). The normal-diet (ND) group received a standard laboratory chow, in which 19.77% of total energy (%E) was from fat, with energy content calculated at 4.02 kcal/g (Mouse Feed Food No. 082, C.P. Company, Bangkok, Thailand). The HF group consumed a fat-enriched diet, containing fat, mostly from lard (59.28% E), with energy content calculated at 5.35 kcal/g, for 12 weeks. The animals were maintained in individual cages with unrestricted access to food and water. Body weight and food intake were recorded daily. Blood samples were collected from the tail at weeks 0 and 12 after fasting for at least 5 hours and kept at -80oC for subsequent biochemical analyses, such as plasma glucose, triglyceride, and insulin assay. After 12 weeks, each dietary group was divided into two subgroups and each subgroup was given either vehicle (90% sesame oil + 10% ethanol) or 17-β estradiol (50 μg/kg) subcutaneously for 30 days (Bryzgalova et al.,

2008). This dose and duration was chosen since it has been shown to improve the peripheral insulin resistant condition (Bryzgalova et al., 2008). At the end of the experimental periods, animals were deeply anesthetized with isoflurane after fasting for at least five hours and decapitated. The brain was rapidly removed for brain slice preparation. Visceral fat was removed and weighed. Plasma collected from animals was stored at -80oC for further biochemical analysis.

Specific Aim 4: To determine whether the ovariectomized female rats develop the brain insulin resistance by decreasing in neuronal insulin receptor function in hippocampus (insulin-induced LTD), and the estrogen administration in ovariectomized female rats is able to improve neuronal insulin resistance by increasing neuronal insulin receptor function in hippocampus (insulin-induced LTD)

Protocol 4: Forty female Wistar rats weighing ~ 180-200 g were obtained from the National Animal Center, Salaya Campus, Mahidol University, Thailand. All animals were individually housed in a temperature-controlled environment with a 12:12 light-dark cycle. One week after arrival, these female rats are randomly assigned as either sham-operated (Sham) or ovariectomized (OVX). One week after the surgery, these rats were fed with normal diet (ND; 19.77% E fat). At week13, the rats were divided into four subgroups (n=10/group): Sham+ND+V, Sham+ND+E2, OVX+ND+V and OVX+ND+E2. The subgroups with either +V or +E2 were given vehicle (V; 90% sesame oil + 10% ethanol) or 50 μg/kg estrogen (E2; 17-β estradiol), subcutaneously, every day for 30 days. This dose and duration was chosen since it has been shown to improve the peripheral insulin resistance condition (Bryzgalova et al., 2008). The animals were maintained in individual cages with free access to food and water. Body weight and food intake were recorded daily. Blood samples were collected from the tails 30 days before and after the estrogen administration. Plasma were separated and stored at -80oC for subsequent biochemical analyses such as plasma glucose, triglyceride, insulin and estrogen assay. At the end of the experimental periods, animals were deeply anesthetized with isoflurane after fasting for at least five hours and decapitated. The brain was rapidly removed for brain slice preparation. Visceral fat was removed and weighed.

CHAPTER III

RESULTS

Rosiglitazone reduced peripheral insulin resistance in HFD-fed rats.

The effects of rosiglitazone on body weight, visceral fat and plasma biochemical parameters were shown in Table 3-1. Fourteen-week HFD-fed rats had significantly increased body weight, visceral fat, plasma cholesterol level, plasma insulin level, and HOMA index, compared to ND-fed rats ($p<0.05$). Rats treated with rosiglitazone in both dietary groups (5mg/kg/day, 14 days) had significantly increased body weight and visceral fat, compared with the vehicle groups ($p< 0.05$). In HFD-fed rats, rosiglitazone treatment significantly decreased plasma cholesterol level, plasma insulin level and HOMA index ($p<0.05$). However, rosiglitazone significantly decreased plasma glucose level in both dietary groups ($p< 0.05$).

In the glucose tolerance test (OGTT), rosiglitazone improved glucose tolerance in both dietary groups, compared with the vehicle treatment. Mean of area under the curve (AUC) of the vehicle-treated HFD subgroup (HFV) was significantly greater than that of the vehicle-treated ND subgroup (NDV) (Table 3-1). The administration of rosiglitazone significantly decreased AUC in both dietary groups

($p<0.01$). These findings suggest that rosiglitazone ameliorated peripheral insulin resistance in both dietary groups.

Table 3-1. Effects of rosiglitazone on body weight, visceral fat, plasma cholesterol level, plasma glucose level, plasma insulin level, HOMA index and OGTT AUC in rats fed with ND and HFD.

Parameter	Normal diet Vehicle	Normal diet Rosiglitazone	High-fat diet Vehicle	High-fat diet Rosiglitazone
Body Weight (g)	431.50±5.68	460.33±4.13*	546.25±12.10*,†	603.3±9.86*,†,‡
Visceral fat (g)	25.22 ±1.11	32.57 ±1.26*	47.94± 2.88*,†	61.90 ±3.61*,†,‡
Cholesterol (mg/dl)	234.78 ±6.38	227.67 ±6.82	301.45±9.81*,†	251.55±8.24†,‡
Glucose (mg%)	130.88±4.26	101.39±3.25*	140.68±3.65†	122.01±2.14†,‡
Insulin (ng/ml)	1.72±0.13	1.84±0.14	3.85±0.18*,†	2.05±0.08‡
HOMA index	12.60±0.59	11.87±0.83	32.03±1.67*,†	14.36±0.74†,‡
OGTT AUC (a.u.)	45,814±789	39,875±895*	63,020±1026*,†	49,722±935*,†,‡

a.u.: arbitrary unit; NDV: normal diet-fed subgroup treated with vehicle (normal saline); NDR: normal diet-fed subgroup treated with rosiglitazone; HFV: high-fat diet-fed subgroup treated with vehicle (normal saline); HFR: high-fat diet-fed subgroup treated with rosiglitazone. * $p<0.05$ vs. NDV, † $p<0.05$ vs. NDR, ‡ $p<0.05$ vs. HFR

Rosiglitazone improved brain insulin receptor function in HFD-fed rats.

In the NDV subgroup, the application of insulin (500 nM) onto hippocampal slices led to long-term-depression of fEPSP or called insulin-induced LTD, and the mean of percentage insulin-induced LTD in NDV group was 74.43% of pre-insulin treatment, whereas rosiglitazone treatment in the ND-group had no effect on insulin-induced LTD (80.79% of pre-insulin treatment) (Figure 3-1A). In HFD groups, the insulin-induced LTD in the vehicle-treated HFD group was significantly reduced, with the mean percentage of insulin-induced LTD in the vehicle-treated HFD group at 5.7±4.6% of pre-insulin treatment (Figure 3-1B). This finding suggested that the impairment of insulin-induced LTD occurred in HFD group. However, rosiglitazone treatment completely reversed the impairment of insulin-induced LTD in the HFD group (insulin-induced LTD: 64.3±4.8% of pre-insulin treatment, Figure 3-1B). These findings suggest that HFD consumption impairs the neuronal insulin receptor function or brain insulin resistance and rosiglitazone can reduce that impairment.

To confirm whether rosiglitazone can reverse or enhance brain insulin receptor signaling of brain insulin resistance following HFD, the Ser473 phosphorylation of Akt/PKB between both dietary groups, either with or without rosiglitazone treatment, was investigated. The serine 473 phosphorylation of Akt/PKB was used as a marker for brain insulin receptor signaling activity, since we previously showed that brain insulin resistance following HFD consumption led to the reduction of serine 473 phosphorylation of Akt/PKB (Pratchayasakul et al., 2011b). In the present study, we found that the protein levels of Akt/PKB and the serine phosphorylation of Akt/PKB without insulin stimulation in all treated subgroups, i.e. ND with vehicle treatment (NDV), ND with rosiglitazone treatment (NDR), HFD with vehicle treatment (HFV), and HFD with rosiglitazone treatment (HFR), were not significantly different (Figure 3-2A and 3-2B, respectively). However, after insulin stimulation, the Akt/PKB serine phosphorylation of HFV subgroup was significantly decreased compared to the NDV subgroup ($p<0.05$, Figure 3-2B). The administration of rosiglitazone significantly increased Akt/PKB serine phosphorylation after insulin stimulation in only the HFD group ($p<0.05$, Figure 3-2B). All of these findings indicate that rosiglitazone attenuates the brain insulin resistance induced by HFD consumption by preserving both brain insulin receptor function and brain insulin receptor signaling.

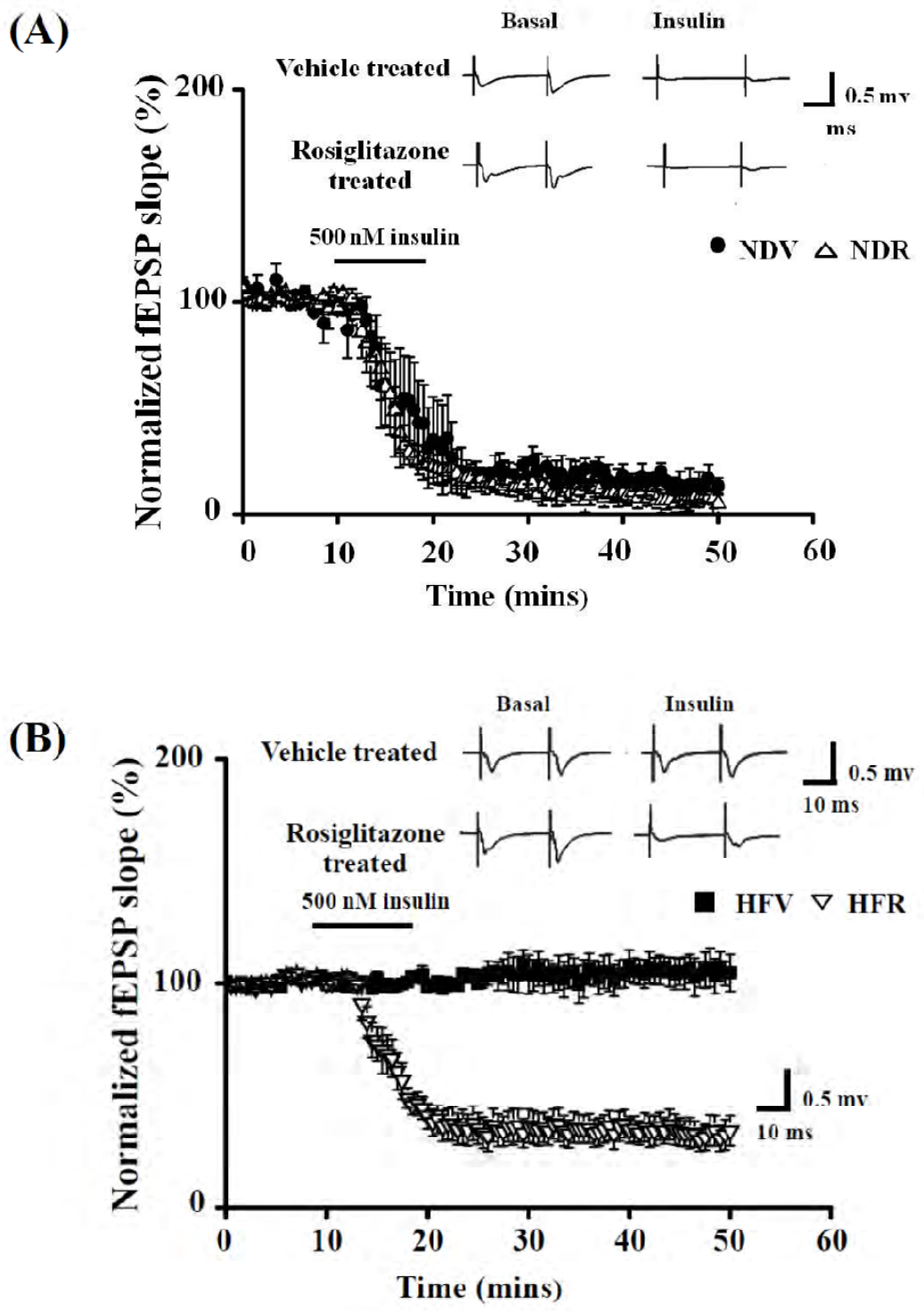


Figure 3-1. Effects of rosiglitazone on insulin-induced LTD. **(A)** HFD feeding significantly diminished the ability of insulin to induce long term depression (LTD) in the CA1 hippocampus. **(B)** Rosiglitazone, 14 days following 12-week HFD, significantly improved the

ability of insulin to induce long term depression (LTD) in the CA1 hippocampus. Panels A and B represent response before and after insulin stimulation in the brain slices from NDV vs. NDR and HFV vs. HFR subgroups, respectively. ***Panel A:*** A summary of averages of normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from NDV (n=6-8 independent slices) and NDR (n=6-8 independent slices) brain slices. ***Panel A inset***: Examples of averages of 20 consecutive traces taken from a slice treated with aCSF (basal) and with 500 nM (insulin). ***Panel B:*** A summary of averages of normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from HFV (n=6-8 independent slices) and HFR (n=6-8 independent slices) brain slices. ***Panel B inset***: Examples of averages of 20 consecutive traces were taken from a slice treated with aCSF (basal) and with 500 nM (insulin). There were no differences between the insulin-induced LTD in NDV and NDR subgroups. However, the HFR subgroup showed a significant increase in insulin-induced LTD compared to the HFV subgroup. NDV: normal diet-fed subgroup treated with vehicle (normal saline); NDR: normal diet-fed subgroup treated with rosiglitazone; HFV: high-fat diet-fed subgroup treated with vehicle (normal saline); HFR: high-fat diet-fed subgroup treated with rosiglitazone.

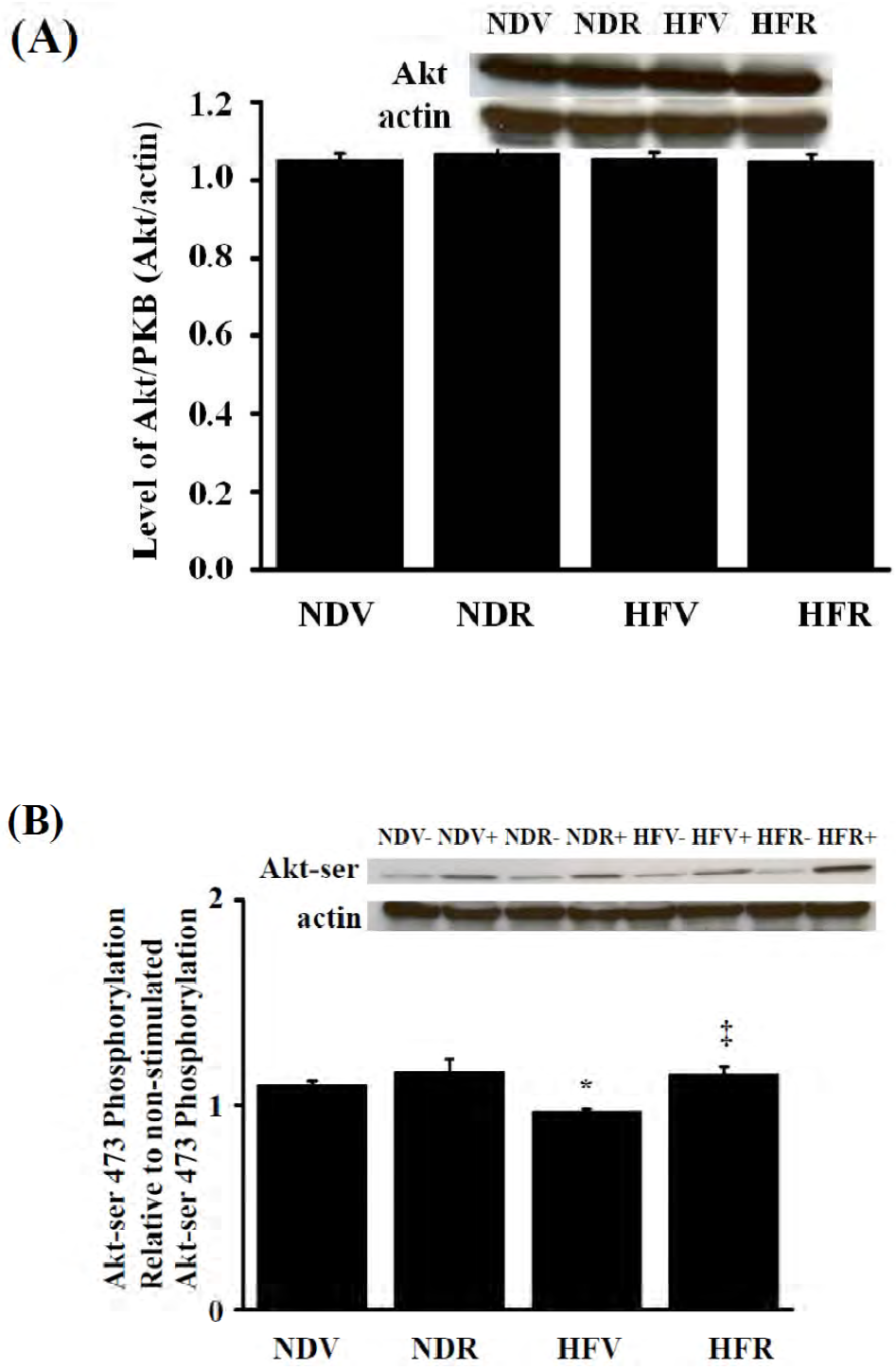


Figure 3-2. Effects of rosiglitazone on brain insulin signaling. **(A)** There was no change in the protein levels of Akt/PKB in any group. All immunoblot lanes were loaded with the same amount of protein (30μg/lane). **(B)** Rosiglitazone significantly improved insulin-induced phosphorylation of Akt/PKB at Ser473 residue in HFD consumption. Insulin-induced phosphorylation of Akt/PKB at Ser473 residue was significantly weakened in HFV group

($p<0.05$). However, insulin-induced phosphorylation of Akt/PKB at Ser473 of the HFR subgroup significantly increased compared to the HFV subgroup ($p<0.01$). All immunoblotting lanes were load with the same amount of protein (60μg/lane). -: no insulin stimulation; +: insulin stimulation * $p<0.05$ vs. NDV, ‡ $p<0.05$ vs. HFR. NDV: normal diet-fed subgroup treated with vehicle (normal saline); NDR: normal diet-fed subgroup treated with rosiglitazone; HFV: high-fat diet-fed subgroup treated with vehicle (normal saline); HFR: high-fat diet-fed subgroup treated with rosiglitazone.

Rosiglitazone attenuated brain mitochondrial dysfunction in HFD-fed rats.

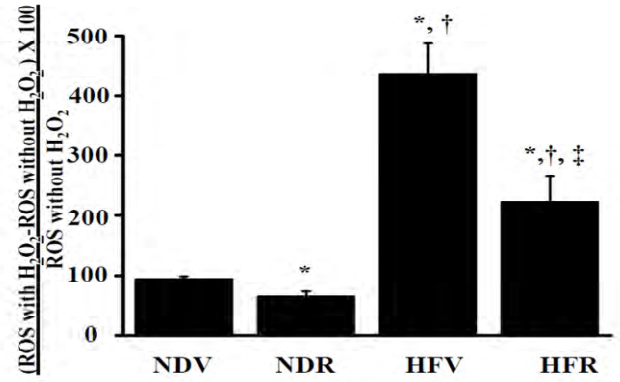
In addition to the effect of rosiglitazone on improving the neuronal insulin resistance induced by HFD consumption, we further investigated whether rosiglitazone can improve brain mitochondrial dysfunction following HFD consumption. We investigated the dysfunction of brain mitochondria by measuring brain ROS production, brain mitochondrial membrane potentials ($\Delta\psi m$) and brain mitochondrial swelling.

We demonstrated that the HFV subgroup had significantly increased ROS levels after H2O2 application, compared to the NDV subgroup (Figure 3-3A). Rosiglitazone treatment significantly reduced brain mitochondrial ROS levels in both dietary groups during oxidative stress stimulation (Figure 3-3A).

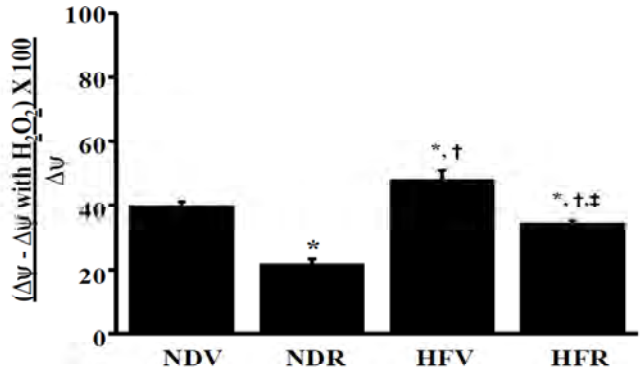
For brain mitochondrial membrane potential change, we found that the rate of $\Delta\psi m$ changes in the HFV subgroup was greater than that in the NDV subgroup after H2O2 application (Figure 3-3B). Rosiglitazone treatment decreased the rate of $\Delta\psi m$ changes in both dietary groups (Figure 3-3B). These findings suggest that brain mitochondria in the HFV subgroup were greater depolarized than in the NDV subgroup during oxidative stress stimulation, and that rosiglitazone can attenuate the mitochondrial membrane depolarization caused by HFD consumption. To investigate the brain mitochondrial swelling, we measured the absorbance of brain mitochondria in all four treatment subgroups (NDV, NDR, HFV and HFR). The absorbance of brain mitochondria in the HFV subgroup was decreased compared to that in the NDV subgroup, indicating that brain mitochondria in the HFV subgroup were swollen (Figure 3-3C). In HFD rats, we found that the absorbance of brain mitochondria in the

rosiglitazone treated (HFR) subgroup was significantly increased, compared to that in the vehicle-treatment (HFV) subgroup (Figure 3-3B). These findings suggest that rosiglitazone can attenuate brain mitochondrial swelling following HFD consumption. All of these mitochondrial findings suggest that HFD consumption can lead to brain mitochondrial dysfunction and that rosiglitazone can attenuate the brain mitochondrial dysfunction caused by HFD consumption.

(A)



(B)



(C)

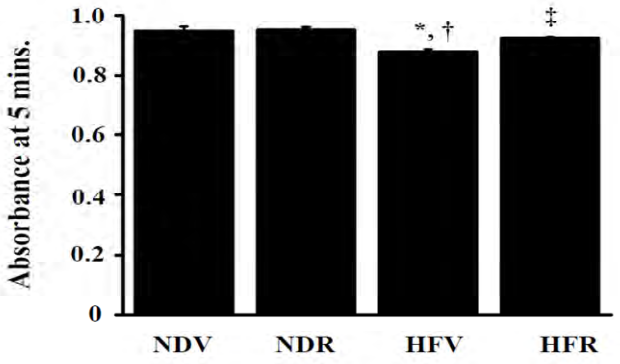


Figure 3-3. Effects of rosiglitazone on brain mitochondrial function. **(A)** Brain mitochondrial ROS production was measured by fluorescent dye during 2 mM H2O2 application onto brain mitochondria. Rosiglitazone significantly reduced ROS production following H2O2 application in both ND and HFD consumption. **(B)** HFD consumption induced an increase in mitochondrial membrane potential change ($\Delta\psi$m) during 2 mM H2O2 application to brain mitochondria, measured by fluorescent dye. Rosiglitazone significantly decreased brain mitochondrial membrane potential change ($\Delta\psi$m) following H2O2 application in both ND and HFD consumption. **(C)** High-fat diet consumption induced mitochondrial swelling as measured by absorbance at 540 nm. Rosiglitazone significantly decreased mitochondrial swelling in both dietary groups. NDV: normal diet-fed subgroup treated with vehicle (normal saline); NDR: normal diet-fed subgroup treated with rosiglitazone; HFV: high-fat diet-fed subgroup treated with vehicle (normal saline); HFR: high-fat diet-fed subgroup treated with rosiglitazone. * $p<0.05$ vs. NDV, † $p<0.05$ vs. NDR, ‡ $p<0.05$ vs. HFV.

Pioglitazone restored dendritic spines density in HFD-fed rats

The administration of pioglitazone in HFD-fed rats decreased plasma cholesterol, plasma glucose, plasma insulin levels and, HOMA index, consistent with rosiglitazone treatment. To investigate the effect of HFD on dendritic spines by using Golgi Stain kit. We found that sixteen-week HFD-fed rats had significant decreased dendritic spines number on secondary and tertiary dendrites in apical dendrite compared with ND-fed rat. Anti-diabetic drug, PPARγ agonist, pioglitazone restored dendritic spines density to normal level in HFD group, but had no effect on ND group (Figure 3-4).

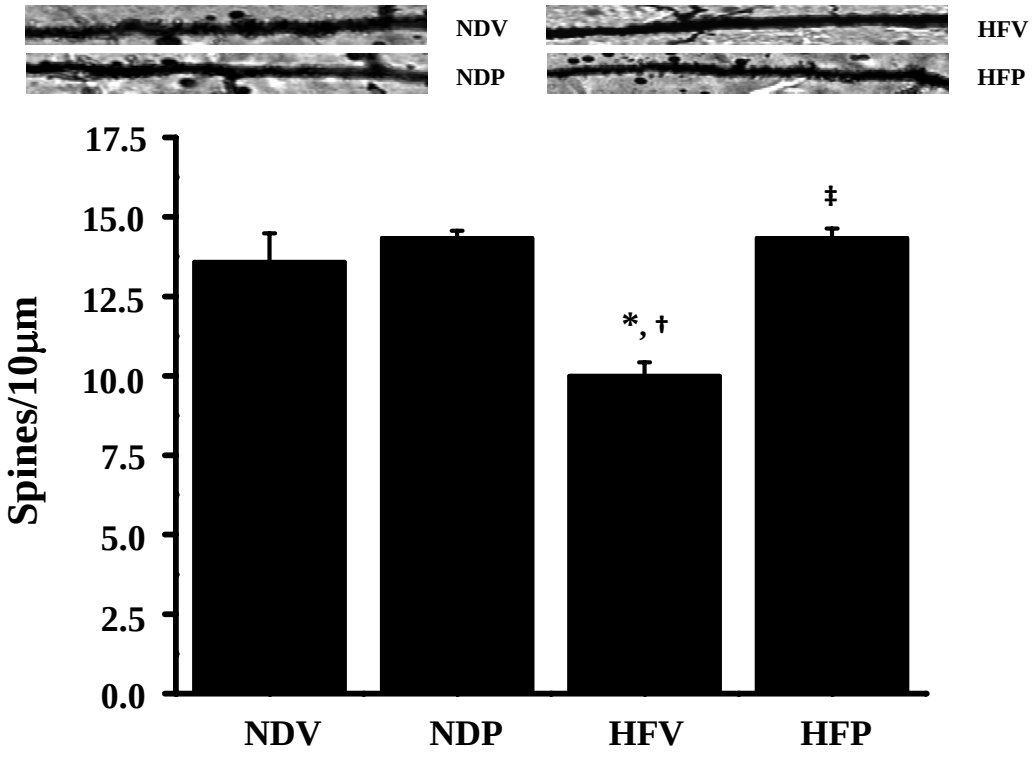


Figure 3-4. Effect of pioglitazone on dendritic spines number. HFD consumption decreased dendritic spine density. Pioglitazone treatment restored dendritic spines density. * $p<0.05$ vs. NDV, † $p<0.05$ vs. NDP, ‡ $p<0.05$ vs. HFV. NDV: normal diet-fed subgroup treated with vehicle (normal saline); NDP: normal diet-fed subgroup treated with pioglitazone; HFV: high-fat diet-fed subgroup treated with vehicle (normal saline); HFP: high-fat diet-fed subgroup treated with pioglitazone.

β-Estradiol (E2) administration improved peripheral insulin sensitivity in both male and female HF-fed rats.

After 12-weeks of HF feeding, both male and female rats demonstrated the characteristics of peripheral insulin resistance, such as increased body weight, visceral fat, liver triglyceride, fasting plasma insulin and HOMA index (Table 3-2). After one month of E2 administration, these parameters were significantly decreased in both male and female HF-fed rats, compared to the vehicle-treated group HF ($p<0.05$, Table 3-2), but these parameters were not altered in ND-fed rats (Table 3-2). Furthermore, the circulating estrogen level in the vehicle-treated female HF-fed rats was also significantly reduced, compared to the vehicle-treated female ND rats. This finding is consistent with a previous study demonstrating that high body fat in female rats was associated with decreased estradiol levels (Ziomkiewicz et al., 2008). In contrast; the circulating estrogen levels were not significantly changed between the HF and ND groups that received E2 in either gender ($p= 0.816$ and $p=0.814$, in male and female, respectively, Table 3-2).

Table 3-2 Effect of estrogen administration on peripheral parameters in both male and female HF-fed rats.

Parameters	Vehicle-treated				Estrogen-treated			
	Normal diet		High-fat diet		Normal diet		High-fat diet	
	Male	Female	Male	Female	Male	Female	Male	Female
Body weight (g)	482.50 ± 12.50	282.50± 10.31a	595.00 ± 47.87 *	380.00 ± 4.08 * a	407.50 ± 14.93*	254.00 ± 6.00 a	514.00 ± 6.00 * # a	340.00 ± 17.03 * # a
Visceral fat (g)	22.41 ± 2.72	10.16 ± 0.42 a	43.81 ± 7.95 *	37.71 ± 2.02 *	17.69 ± 2.20	13.33 ± 0.83	40.99 ± 3.76 *	29.93 ± 2.09 *#
Glucose (mg%)	121.70± 4.70	119.78 ± 2.54	134.36 ± 12.72	130.75 ± 19.11	99.43 ± 4.89	102.28 ± 5.86	118.52 ± 9.07	108.58 ± 9.57
Triglyceride (mg%)	86.99 ± 20.58	47.12 ± 1.29	77.16 ± 17.71	128.18 ± 3.18 * a	126.50 ± 24.11	53.75 ± 11.43 a	79.89 ± 7.19	61.25 ± 8.11#
Free fatty acid (mM)	0.44 ± 0.17	0.47 ± 0.07	0.42 ± 0.07	0.46 ± 0.04	0.57 ± 0.13	0.53 ± 0.05	0.55 ± 0.10	0.38 ± 0.01
Insulin (ng/ml)	1.61 ± 0.48	1.32 ± 0.23	4.88 ± 0.34*	3.40 ± 0.25 * a	0.93 ± 0.15	1.29 ± 0.13	2.50 ± 0.46#	2.43 ± 0.42 *#
Liver triglyceride (mg/gm tissue)	39.31 ± 4.39	24.21 ± 1.80	103.00 ± 3.56*	109.28 ± 8.42*	24.37 ± 3.91	23.43 ± 3.53	61.61 ± 6.52* #	75.69± 6.69* # a
HOMA index	11.57 ± 2.87	9.35 ± 1.64	40.04 ± 4.76*	26.41 ± 4.15* a	5.51 ± 0.92	7.69 ± 0.55	16.72 ± 2.69* #	14.89 ± 1.99* #
Plasma Estrogen (pg/ml)	56.66 ± 4.60	133.38 ± 10.32 a	44.80 ± 6.85	112.91 ± 24.43 a	240.23 ± 27.91#	266.89 ± 37.87#	247.74 ± 23.61 #	260.01 ± 12.36 #
Brain Estrogen (pg/mg protein)	64.87 ± 14.17	75.33 ± 7.32	65.11 ± 7.74	82.03 ± 15.44	74.25 ± 6.81	149.06 ± 16.78# a	68.80 ± 9.20	128.54 ± 22.99 # a

*; $p<0.05$ significantly from diet , # ; $p<0.05$ significantly from vehicle, a ; significantly $p<0.05$ from male

E2 administration prevented neuronal insulin resistance in female high fat-fed rats

In ND animals, the degree of the neuronal insulin receptor function or insulin-induced LTD observed from male and female animals were not significantly different (Figure 3-5A). At 30-minute post-insulin stimulation, the percentage reduction of the normalized fEPSP slope from male and female groups were 61.64 $\pm$ 7.89 % and 66.57 $\pm$ 9.47 % of the average slope recording during the baseline level, respectively (n=10-11 independent slices per group, n=10 animals/group, Figure 3-5A). Furthermore, the insulin-induced LTD was impaired following HF consumption in both male and female rats as indicated by the disappearance of insulin-induced LTD in the hippocampus of HF-fed rats (Figure 3-5A). At 30-minute post-insulin stimulation, the percentage reduction of the normalized fEPSP slope from 12-week HF-fed male and female rats were 5.07 $\pm$ 4.00 % and -2.38 $\pm$ 2.07 % of the average slope recording during the baseline level, respectively ($p<0.05$, n=10-11 independent slices, n=10 animals/group, Figure 3-5A).

Interestingly, we found that E2 administration could significantly prevent the impairment of insulin-induced LTD only in female HF rats ($p<0.05$, Figure 3-5B). In E2-treated female HF-fed rats, the percentage depression of fEPSPs after insulin administration was 31.18$\pm$9.93 %, compared to 2.37$\pm$3.18 % from E2-treated male HF-fed rats. Our results indicated that E2 administration reduced the occurrence of HF-induced neuronal insulin resistance in female HF-fed rats, but not in male HF-fed rats. Moreover, we found that E2 administration could not affect the ability of insulin-induced in both gender of ND-fed rats (Figure 3-5B). The percentage reduction of the normalized fEPSP slope from E2-treated male ND-fed rats and E2-treated female ND-fed rats were 73.42$\pm$8.24 % and 82.20 $\pm$ 12.11 % of the average slope recording during the baseline level, respectively (n=10-11 independent slices, n=10 animals/group, Figure 3-5B).

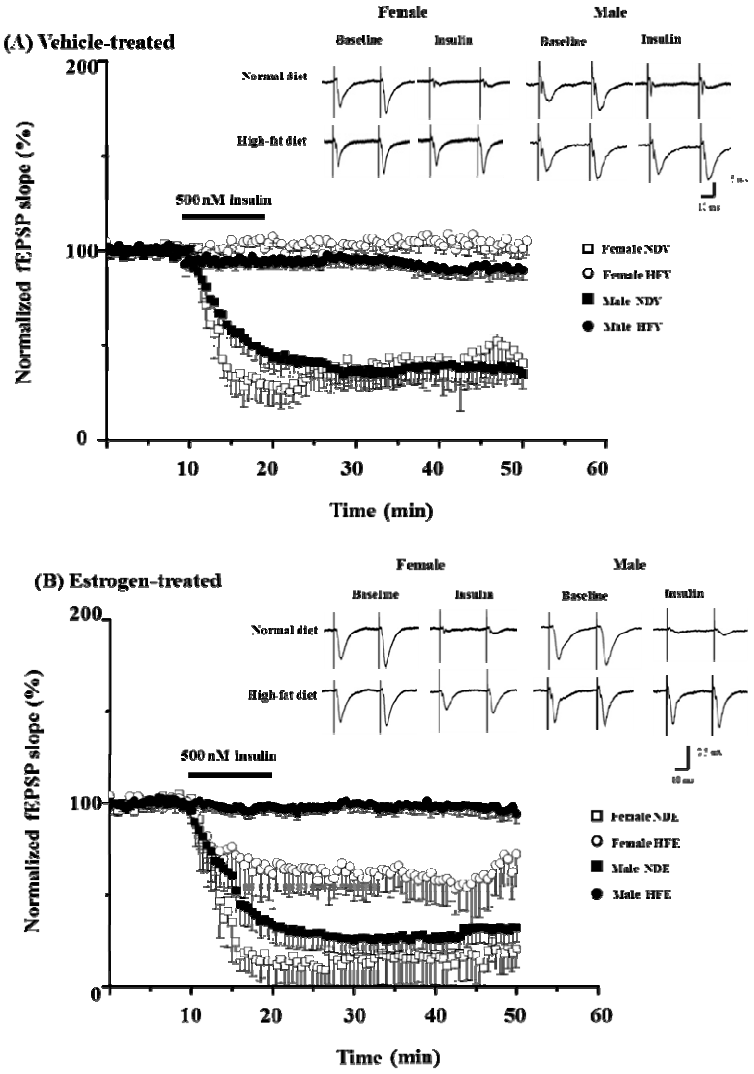


Figure 3-5 E2 administration for 30 days significantly improved the impairment of insulin-mediated long term depression (LTD) in the CA1 hippocampus following HF consumption in female rats. ***Panel A***: A summary of average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from NDV male and female rats (n=10-11 independent slices, n=10 animals/group) and HFV male and female rats (n=10-11 independent slices, n=10 animals/group) brain slices. It shows that bath application of 500 nM insulin for 10 minutes produced a depression of fEPSPs in male and female NDV brain slices and the fEPSPs did not fully recover after washout of insulin. However, 500 nM insulin-mediated LTD was significantly attenuated in both male and female HFV rats. ***Panel B***: summary of average normalized fEPSPs from estrogen-treated ND (NDE) male and female rats (n=10-11 independent slices, n=10 animals/group) and estrogen-treated

HF(HFE) male and female rats (n=10-11 independent slices, n=10 animals/group) brain slices. NDV = normal diet group with vehicle treatment, HFV =high-fat diet group with vehicle treatment, NDE=normal diet group with E2 treatment, HFE= high-fat diet group with E2 treatment.

E2 administration improved neuronal insulin signaling in female high fat-fed rats.

To investigate the mechanism of the beneficial effect of E2 on neuronal insulin resistance, we determined whether the E2 improves insulin signaling, particularly Akt/PKB in the brain. As shown in aim 1, 12-week HF consumption led to deleterious effects on insulin receptor signaling, such as phosphorylated IR, phosphorylated IRS-1 and phosphorylated Akt/PKB. In the present study, the impairment of neuronal insulin signaling was examined using immunoblot Akt/PKB phosphorylation. To address the mechanism of the beneficial effect of E2 in neuronal insulin signaling in both male and female rats, we investigated whether estrogen can improve neuronal insulin signaling, particularly Akt/PKB phosphorylation. Our results demonstrated that the level of Akt/PKB protein was not different in both male and female rats receiving either ND or HF diet. The densitometric quantification of blots illustrated that the Akt/PKB/β-actin protein level in the male ND group was 1.00 ± 0.18, in the female ND group was 0.99 ± 0.17, in the male HF group was 1.00 ± 0.01 and in the female HF group was 0.98 ± 0.16, arbitrary scanning units (n=10/group, Figure 3-6A). Furthermore, E2 administration did not alter Akt/PKB protein concentration in the brain of both male and female rats of either dietary group (1.04 ± 0.06, 1.00 ± 0.02, 0.97 ± 0.01 and 0.98 ± 0.01 arbitrary scanning units for the male NDE, female NDE, male HFE and female HFE groups, respectively, n=10/group, Figure 3-6A). However, the amounts of the phosphorylated form of Akt/PKB at the serine 473 site were significantly decreased in both vehicle-treated male and female rats in the HF group, compared to the vehicle-treated rats in the ND group (Figure 3-9B). However, the amounts of the phosphorylated form of Akt/PKB at the serine 473 site were significantly increased in the brains of E2-treated female rats compared to the vehicle-treated female rats in the HF group, suggesting that E2 could protect neuronal insulin signaling from the deleterious effect of HF feeding in female rats ($p<0.05$, Figure 3-6B). In male brains, the

phosphorylated form of Akt/PKB at the serine 473 site was still decreased after E2 treatment (Figure 3-6B). Furthermore, in the E2 treatment group, the amount of the phosphorylated form of Akt/PKB at the serine 473 site in the ND and HF-fed groups was not different in female rats. However, in male rats that received E2 treatment, the level of phosphorylated Akt/PKB at the serine 473 site of the HF group was significantly reduced compared to that in the ND group ($p<0.05$, Figure 3-6B).

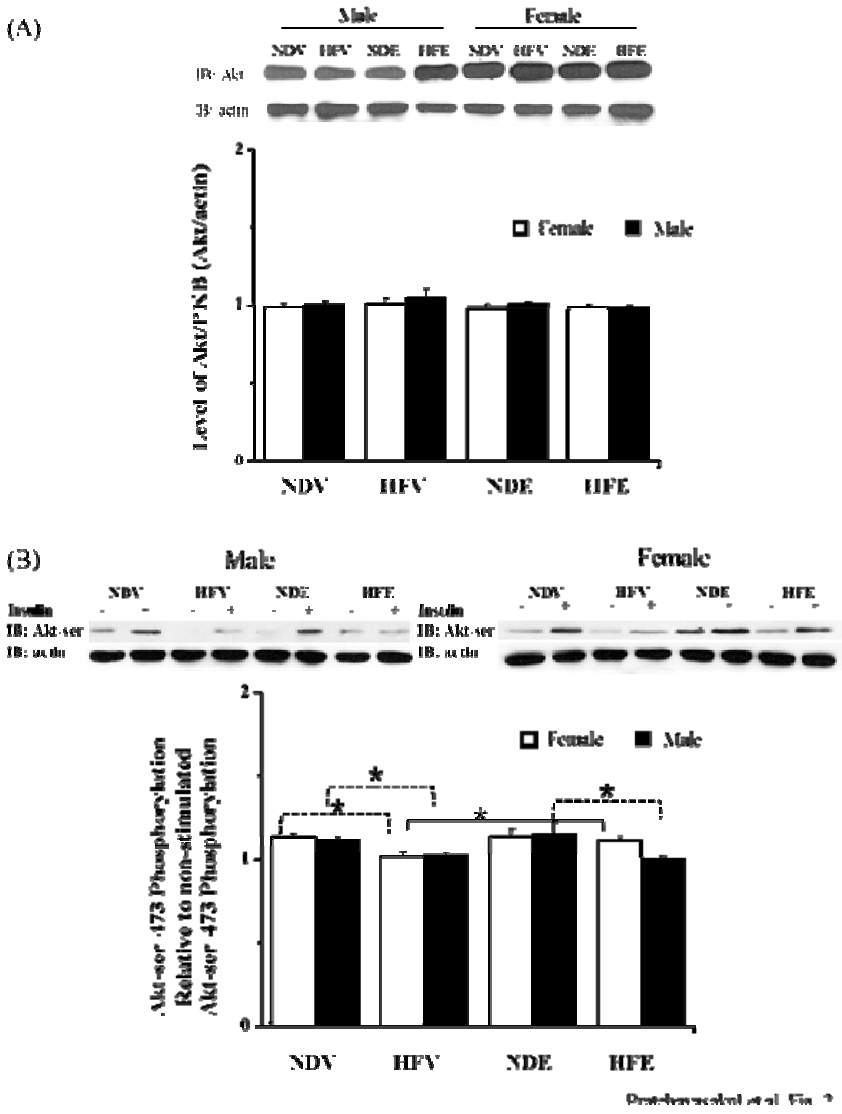


Figure 3-6: Insulin-induced serine phosphorylation of neuronal Akt/PKB was decreased in both male and female HFV groups. ***Panel A:*** Representive blots of protein level of AKt/PKB in brain slices harvested from the NDV, NDE, HFV and HFV groups (n= 10/group) compared between male and female. The densitometric quantitation of blots

from all groups was not different. ***Panels B***: Representive blots of serine 473 kinase of Akt/PKB phosphorylation in brainslices harvested from the NDV, NDE, HFV and HFV groups compared between male and female rats. Densitometric quantitation of blots from insulin-stimulated Akt/PKB in the female HFE group was significantly greater than in the female HFVgroup. All immunoblotting lanes were loaded with equal amounts of protein (40μg/lane). *, p <0.05; NDV = normal diet group with vehicle treatment, HFV =high-fat diet group with vehicle treatment, NDE=normal diet group with E2 treatment, HFE= high-fat diet group with E2 treatment; -, no insulin stimulation; +, insulin stimulation.

β-Estradiol (E2) administration improved peripheral insulin sensitivity in ovariectomized ND-fed rats.

Interestingly, loss of estrogen hormone by ovariectomy exhibited peripheral insulin resistance in ND feeding, such as increased body weight, fasting plasma glucose, fasting plasma insulin and HOMA index. However, the administration of E2 for one month had significantly positive effects on improving peripheral insulin resistance in ovariectomized normal diet fed rats such as decreased fasting plasma glucose, fasting plasma insulin and HOMA index ($p<0.05$, as shown in Table 3-3).

Furthermore, we confirmed the effect of estrogen deprivation via ovariectomy by determining serum estrogen levels and uterine weight. We found that ovariectomy resulted in decreased serum estrogen levels and uterine weight in ND-fed rats. However, one month of E2 replacement was significantly reversed these effects, compared to the vehicle-treated ND rats ($p<0.05$; Table 3-3).

Table 3-3 Effect of estrogen deprivation on body weight, visceral fat, fasting plasma glucose, triglyceride, insulin, HOMA index and plasma estrogen.

Parameter	Vehicle-treated		Estrogen-treated	
	Sham	**OVX**	**Sham**	**OVX**
Body weight (g)	271.00 ± 6.40	332.00± 19.85†	281.00 ± 10.77	275.00 ± 14.32
Visceral fat (g)	14.97 ± 1.04	18.27 ± 1.46	16.62 ± 1.15	12.86 ± 2.01
Uterus (g)	0.51 ± 0.05	0.14 ± 0.02†	0.73 ± 0.09	0.83 ± 0.18ŧ
Glucose (mg%)	205.86± 3.77	226.93± 11.46†	196.50 ± 5.61	193.36 ± 6.42ŧ
Triglyceride (mg%)	273.80 ± 16.83	299.00± 9.91	286.60 ± 14.85	301.40 ± 6.82
Insulin (ng/ml)	0.98 ± 0.11	1.78 ± 0.14†	1.12 ± 0.10	1.14 ± 0.17ŧ
HOMA index	11.83 ± 1.21	23.09 ± 1.53†	13.03 ± 1.09	14.80 ± 2.25ŧ
Plasma Estrogen (pg/ml)	135.20 ± 12.69	29.53 ± 8.60†	224.84 ± 22.47ŧ	226.92 ± 19.23ŧ

†, p <0.05 compared to Sham group in each treatment; ŧ, p<0.05 compared vehicle-treated group

E2 administration prevented neuronal insulin resistance in ovariectomized ND-fed rats

The neuronal insulin receptor function or insulin-induced LTD was determined by extracellular recording of hippocampal slices. We found that the percentage reduction of the normalized fEPSP slope from Sham-ND rats were 87.82 $\pm$ 2.45 % of the average slope recording during the baseline level (n=10-11 independent slices, n=10 animals/group), while the percentage reduction of fEPSPs of OVX-ND rats were 33.35 $\pm$ 7.42 % of the values recorded before insulin application (n=10-11 independent slices, n=10 animals/group; Figure 3-7A). These result indicated that loss of ovarian hormone by ovariectomy significantly decreased the ability of insulin-induced LTD ($p<0.05$, Figure 3-7A).

However, one month of E2 administration reversed the impairment effect of insulin-induced LTD in OVX group, we found that the percentage reduction of the normalized fEPSP slope from OVX+ND+E2 group was significantly increased to 79.41 $\pm$ 2.76 % of the average slope recording during the baseline level ($p<0.05$, n=10-11 independent slices, n=10 animals/group. Moreover, the degree of insulin-mediated LTD observed from slices of Sham+ND+V, Sham+ND+E2, OVX+ND+E2 animals were not significantly different from each other (87.82 ± 2.45, 83.70 ± 2.98 and 79.41 ± 2.76 % of the average slope recording during the baseline level for Sham+ND+V, Sham+ND+E2, OVX+ND+E2, respectively; n=10-11 independent slices per group, n=10 animals/group, Figure 3-7B). These findings suggest that the loss of estrogen in normal rats can lead to the impairment of neuronal insulin receptor function and estrogen administration can reverse this effect.

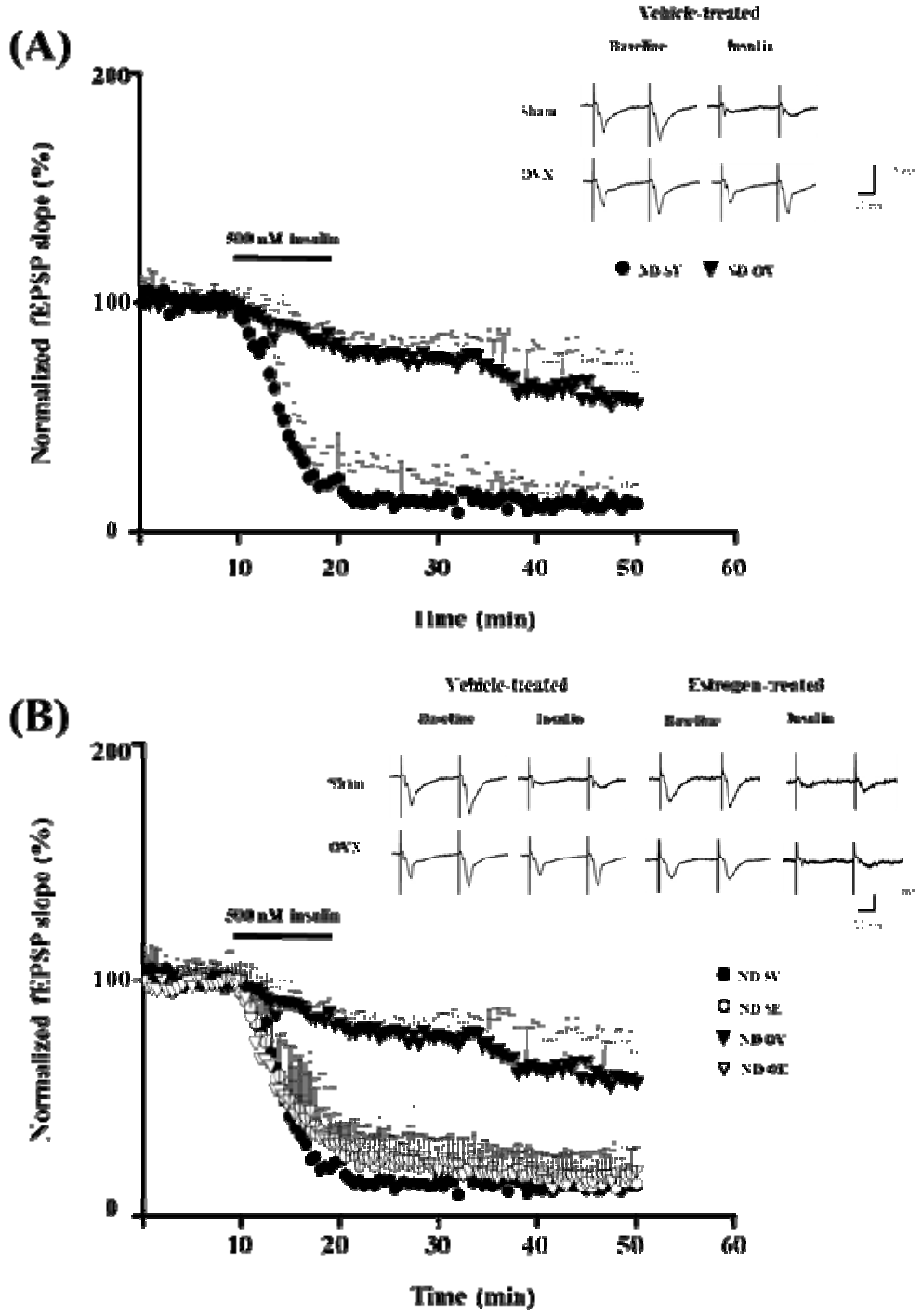


Figure 3-7: Loss of ovarian hormone by ovariectomy caused the impairment of insulin-induced LTD and E2 administration for 30 days significantly improved this impairment. ***Panel A:*** A summary of average normalized fEPSPs (fEPSPt/fEPSPo with fEPSPs being points at which fEPSP slopes stabilized) from Sham+ND+V and OVX+ND+V (n=10-11 independent slices, n=10 animals/group) brain slices. ***Panel B:*** A summary of average

normalized fEPSPs from Sham+ND+V, Sham+ND+E2, OVX+ND+V and OVX+ND+E2 (n=10-11 independent slices, n=10 animals/group) brain slices. Sham+ND+V = normal diet-fed sham rats with vehicle treatment, Sham+ND+E2= normal diet-fed sham rats with estrogen treatment, OVX+ND+V = normal diet-fed ovariectomized rats with vehicle treatment, OVX+ND+E2 = normal diet-fed ovariectomized rats with estrogen treatment

CHAPTER IV

DISCUSSION AND CONCLUSIONS

PPARγ agonist improves brain insulin receptor function, brain mitochondria function and dendritic spines in rats with insulin resistance induced by 12-week HFD consumption.

The major findings of our study are as follows: 1) PPARγ agonist prevents the impairment of neuronal insulin-induced LTD in brain insulin resistant rats caused by HFD consumption; 2) PPARγ agonist improves insulin receptor signaling dysfunction by increasing ser 473 Akt/PKB phosphorylation in brain insulin resistant rats caused by HFD consumption; 3) HFD consumption can cause brain mitochondrial dysfunction; 4) PPARγ agonist reverses the brain mitochondrial dysfunction by preventing brain mitochondrial swelling, decreasing ROS production and attenuating brain mitochondrial membrane potential changes, particularly during oxidative stress stimulation; and 5) PPARγ agonist increases dendritic spine in hippocampus.

Consistent with our previous study (Pratchayasakul et al., 2011b), we also found that 14-week HFD-fed rats (59.28% energy of fat) exhibited not only peripheral insulin resistance, as indicated by excessive body weight gain and visceral fat, hypercholesterolemia, hyperinsulinemia, increased HOMA index and the impairment of oral glucose tolerance (OGTT) test, but also brain insulin resistance indicated by decreased insulin-induced LTD as well as the impairment of brain insulin receptor signaling, decreased Akt/PKB serine phosphorylation. Previous studies have shown that HFD consumption can impair brain function, such as the impairment of spatial learning

in both radial arm maze and Morris Water Maze tests in rats (Pathan et al., 2008; Valladolid-Acebes et al., 2010), the reduction of hippocampal dendritic spines (Stranahan et al., 2008), the reduction of long-term potentiation (LTP) (Stranahan et al., 2008), increased brain malondialdehyde (MDA) (Park et al., 2010), and decreased brain-derived neurotrophic factor (BDNF) (Stranahan et al., 2008). In this study, we have demonstrated,

for the first time, 14-week HFD consumption caused not only brain insulin resistance, but also brain mitochondrial dysfunction. Rats with HFD-consumption for 14-weeks has shown to increased ROS production in kidney, liver, skeletal muscle (Bonnard et al., 2008; Ruggiero et al., 2011), decreased mitochondrial sizes and numbers in liver and muscle (Mao et al., 2011) and reduced mitochondrial membrane potential in liver (Han et al., 2010; Vial et al., 2010). In the present study, HFD consumption altered brain mitochondrial functions, as indicated by increased brain ROS production, brain mitochondrial depolarization and increased brain mitochondrial swelling.

Brain mitochondria are important organelles for maintaining intracellular Ca2+ levels and play an important role in synaptic transmission (Levy et al., 2003). The dysfunction of brain mitochondria has been shown to cause the impairment of synaptic plasticity (Levy et al., 2003). In the present study, the brain mitochondrial dysfunction could be responsible for the impairment of insulin-induced LTD in rats fed with HFD. Increased brain mitochondrial ROS production caused by HFD consumption may cause the opening of mitochondrial permeability transition (mPTP), leading to brain mitochondrial swelling and brain mitochondrial depolarization as been demonstrated in cardiac mitochondria (Sedlic et al., 2010; Thummasorn et al., 2011). These ROS-mediated mitochondrial changes could induce Akt dephosphorylation at Ser-473 (Cao et al., 2009), indicating the disruption of downstream insulin signaling. Therefore, brain insulin resistance induced by HFD consumption might be developed from the brain mitochondrial dysfunction.

PPARγ agonist has been shown to increase the expression of insulin receptors and stimulate tyrosine phosphorylation of insulin receptors in the brown adipocytes of fetal rats (Hernandez et al., 2003). In the present study, PPARγ agonist increased Akt/PKB ser-473 phosphorylation in the HFD group and also tended to increase that in the ND group. An increase in the phosphorylation of Akt/PKB may be responsible for the improvement of insulin-induced LTD in PPARγ agonist-treated HFD rats, since increased Akt/PKB phospholyration can lead to increased intracellular Ca2+ levels, thus restoring the insulin-induced LTD in the brain (Huang et al., 2003).

The present study has also shown that PPARγ agonist protects brain mitochondrial dysfunction caused by HFD by preventing mitochondrial swelling, attenuating ROS production and decreasing mitochondrial membrane potential dissipation. Similar with the present studies, PPARγ agonist has been shown to have neuroprotective effects (Kapadia et al., 2008) and has beneficial effects on brain mitochondria (Dello Russo et al., 2003; Fuenzalida et al., 2007; Jung et al., 2007). The underlying mechanism of PPARγ agonist in the protection of brain mitochondrial dysfunction may be due to 1) PPARγ agonist acts as anti-oxidants. For example A) PPAR γ agonist increases the production of at least two anti-oxidant agents, Cu/Zn-SOD and Mn-SOD, in traumatic brain injury, leading to decreased ROS production (Yi et al., 2008), B) It also increases super oxide dismutase (SOD) activity and decreases lipid peroxidation production (Potenza et al., 2009), resulting in decreased ROS production, and C) PPARγ agonist, in a dose-dependent manner, increase mitochondrial activity by regulating ATP production and transcription of mitochondrial structural proteins and cellular antioxidant enzymes, thus attenuating ROS levels (Rong et al., 2007), 2) the role of PPARγ agonists is to help in mitochondrial biogenesis and the repair of mitochondria during cellular injury or cell death. For example A) PPARγ agonist prevents the depolarization of mitochondrial membrane potential in brain oxygen-glucose deprivation followed by reoxygenation (Miglio et al., 2009; Wu et al., 2009) and B) PPARγ agonist protects hippocampal and dorsal root ganglion neurons against Aβ-induced mitochondrial damage and NGF deprivation-induced apoptosis by up-regulation of Bcl-2 (Fuenzalida et al., 2007). Consistent with those previous studies, our study showed that PPARγ agonist can reduce brain mitochondrial dysfunction caused by 12-week HFD consumption by reducing ROS production and decreasing mitochondrial membrane depolarization. It has also been shown that the reduction of ROS production can prevent the dissipation of mitochondrial membrane potential (Yi et al., 2008). In addition, keeping ROS production at a low level has been shown to improve insulin signaling (Loh et al., 2009). Therefore, the effects of PPARγ

agonist in reducing neuronal mitochondrial dysfunction may be one of the reasons that PPARγ agonist improves neuronal insulin sensitivity in the brain.

Moreover, HFD induced-insulin resistant rats in the present study also demonstrated decreased the number of dendritic spines in CA1 hippocampus. Insulin resistance has been shown to be correlated with T2DM and Alzheimer's disease. Insulin resistance has been shown to induce a reduction of dendritic spines that may decreased the level of brain-derived neurotrophic factor (BDNF) (Joghataie et al., 2007; Malone et al., 2008; Martinez-Tellez et al., 2005; Stranahan et al., 2008). Previous study demonstrated that IR signaling regulated dendritic spines formation through PI3K/Akt signaling pathway in primary cultures of rat hippocampal neurons (Lee et al., 2011). In the present study has been shown that HFD consumption reduced insulin signaling by decreased Akt ser-473 phosphorylation (Pratchayasakul et al., 2011a) that may be the possible mechanism to reduced dendritic spines in the present study. In addition, PPARγ agonist (pioglitazone) also reversed the number of dendritic spines in CA1 hippocampus. The underlying mechanism of PPARγ agonist protecting dendritic spines deterioration following HFD consumption occur through the improvement of brain insulin resistance of the PPARγ agonist treatment.

In conclusion, our findings suggest that PPARγ agonist improves not only peripheral insulin resistance but also brain insulin resistance caused by HFD consumption. The improvement of neuronal insulin sensitivity may occur via the effect of PPARγ agonist on attenuating brain mitochondrial dysfunction and protecting dendritic spines. Therefore, PPARγ agonists may be a useful medicine to ameliorate brain insulin resistance.

Conclusion: PPARγ agonist, an anti-diabetic drug, have beneficial effects in both peripheral insulin resistance and brain insulin resistance caused by HFD consumption, possibly though the improvement of brain mitochondrial functions. In addition, HFD consumption leads to the reduction of dendritic spines, in which could impair the learning and memory. The anti-diabetic drugs treatment in HFD-fed animals can increase the

density of dendritic spines. Therefore, anti-diabetic drugs should have a therapeutic benefit in the improvement of cognition in type 2 DM patients with cognitive decline.

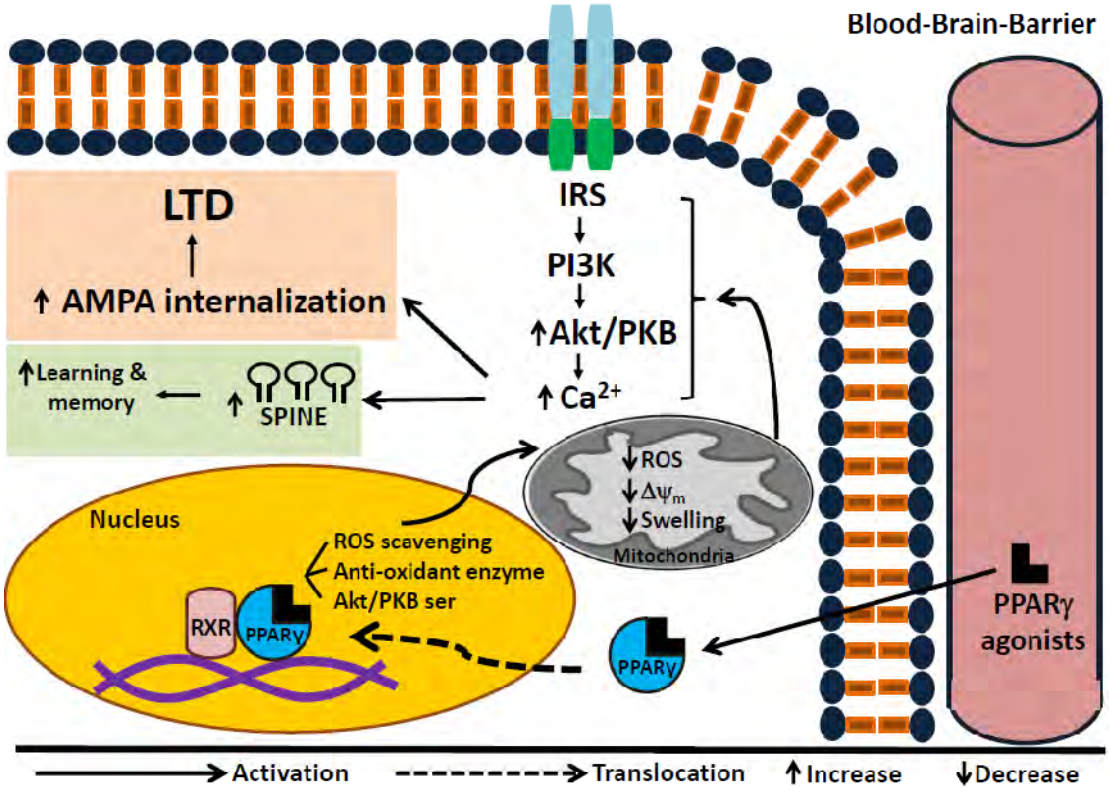


Figure 4-1. The proposed mechanism of PPARγ agonist improved brain insulin resistance.

Our study demonstrated that a 30-day administration of estrogen reduces the occurrence of HF-induced peripheral insulin resistance in male and female rats. Our findings confirmed the therapeutic effect of exogenous estrogen on peripheral insulin resistance induced by HF consumption as shown in previous studies (Kumagai et al., 1993; Riant et al., 2009). Furthermore, the second study was the first to demonstrate the beneficial effect of exogenous estrogen on neuronal insulin resistance in ND- and HF-fed rats. Our results demonstrated that estrogen treatment could attenuate the impairment of neuronal insulin receptors and neuronal insulin signaling dysfunction, particularly the Akt/PKB phosphorylation, caused by HF diet consumption only in female rats. These findings support the hypothesis that estrogen could exert its beneficial effect on neuronal insulin receptor function via improved downstream neuronal insulin receptor signaling. Our

findings also indicated that estrogen could enhance Akt/PKB phosphorylation in insulin-stimulated neuronal tissues, suggesting that estrogen is directly involved in the insulin signaling pathway in neuronal-sensitive tissues. Moreover, the present study also demonstrated an important role of estrogen receptor activation in the central nervous system which could play a crucial role in the metabolic effect of insulin signaling in the brain.

Estrogen has been shown to play an important role in regulating neuronal structure and function. Previous studies have demonstrated that estrogen can either prevent or improve the cognitive deficits of Alzheimer's disease (AD) (Shang et al.,2010; Simpkins et al., 1997) regulate synaptic density in hippocampal regions (Woolley et al., 1996). In addition, chronic estradiol (90 days) treatment in female rats has been shown to induce the activation of the insulin receptor substrate-1 signaling pathway in the cerebral cortex and diencephalon of the brain (Alonso and Gonzalez, 2008). Our findings demonstrated that estrogen could improve the impairment of Akt/PKB phosphorylation. All of these beneficial effects of estrogen help explain the improvement of insulin-induced LTD in CA1 hippocampus in the HF-fed female rats. The finding that estrogen could not improve the impairment of insulin-induced LTD in the HF-fed male rats could be due to the difference in the distribution of estrogen receptors in the hippocampus between male and female rats. Estrogen receptors consist of estrogen receptor alpha (ER-α) and estrogen receptor beta (ER-β). Both ER-α and ER-β are found in rat hippocampus, suggesting that this receptor plays an important role in learning and memory (Shughrue and Merchenthaler, 2000). In addition, previous studies have shown that the ERs are important regulators for metabolic disorders such as insulin resistance (Alonso and Gonzalez, 2008; Godsland, 2005; Palin et al., 2001). Furthermore, a previous study has clearly shown gender differences in the ER-β immunoreactivity in rats (Zhang et al., 2002). In that study, the authors found that the expression of ERβ in the hippocampus of female rats is greater than that in male rats. Therefore, the low numbers of ERs in the hippocampus of male rats could be insufficient for estrogen to act to attenuate the neuronal insulin resistance found in male HF rats.

In summary, the second study demonstrated that the activation of the estrogen pathway could preserve insulin sensitivity in the peripheral tissue in both male and female rats, whereas it could preserve the neuronal insulin sensitivity only in female rats. With the development of selective ER modulators, future use of the beneficial action of estrogen on neuronal insulin receptor function could be clinically significant and the undesired effects of estrogen could be avoided.

In the study of aim 4, we found that the activation of the estrogen pathway could preserve insulin sensitivity in the peripheral and neuronal tissue in female rats, therefore, in third experiment we use ovariectomized model for confirming the effect of estrogen deprivation in ND-fed female rats. Our study demonstrated that OVX rats developed the peripheral and neuronal insulin resistance. The estrogen administration had a beneficial effect on improving of both peripheral and neuronal insulin resistance. Our findings confirmed the therapeutic effects of exogenous estrogen on peripheral insulin resistance which induced by estrogen deprivation as shown in previous studies (Alonso et al., 2006). For example study in ovariectomized model demonstrated that the lower estrogen levels resulted in an overweight, dyslipidemia, impaired glucose tolerance and impaired insulin-mediated glucose uptake (Alonso et al., 2006). However, estrogen replacement improved this peripheral insulin resistance by increased insulin sensitivity, improved glucose tolerance, reduced lipid accumulation (Ropero et al., 2008).

Furthermore, the third study was the first to demonstrate the beneficial effect of exogenous estrogen on neuronal insulin receptor function in ND-fed ovariectomized rats. We found that loss of ovarian hormone by ovariectomized caused the impairment of insulin-induced LTD which estrogen replacement could reverse that impairment. The proposed mechanisms of insulin induced LTD may be related to the activation of moderate and prolong Ca^{2+} influx via insulin signaling. The Ca^{2+} influx initiates the phosphorylation of phosphatase pathway that caused internalization of AMPA receptors at the postsynaptic sites, which led to the depression of excitatory synaptic transmission or LTD (Huang et al., 2003; Huang et al., 2004). In addition, in experiments of aim 2 we found that estrogen could enhance Akt/PKB phosphorylation in insulin-stimulated neuronal tissues, suggesting

that estrogen is directly involved in the insulin signaling pathway in neuronal-sensitive tissues. Therefore the beneficial effect of estrogen on improving insulin-induced LTD in OVX rats may relate to Akt/PKB phosphorylation in insulin signaling. Nevertheless, further study is needed to investigate this proposed mechanism.

In summary, the third study demonstrated that OVX rats developed the peripheral insulin resistance and impaired insulin receptor function, which indicated by the decreasing in ability of insulin-induced LTD with ~ 85 days after OVX. The estrogen administration had a beneficial effect on improving of both peripheral and neuronal insulin resistance in these OVX animals. The propose mechanism of this beneficial effect may be related to neuronal insulin signaling.

In conclusions, the present study demonstrated that peripheral insulin resistance was observed following 8 week of HF feeding. However, neuronal insulin resistance was occurred following 12 weeks of HF feeding, which is identified as a significant reduction in the ability of insulin to induce LTD, and a reduction in the stimulated phosphotyrosine activity of IR, IRS-1 and AKt/PKB in brain slices. Furthermore, the activation of the estrogen pathway could preserve this insulin sensitivity in the peripheral tissue in both male and female rats, whereas it could preserve the neuronal insulin sensitivity only in female rats. In addition, we found that estrogen could enhance Akt/PKB phosphorylation in insulin-stimulated neuronal tissues, suggesting that estrogen is directly involved in the insulin signaling pathway in neuronal-sensitive tissues. Moreover, we used ovariectomized model for confirming the effect of estrogen deprivation in ND-fed female rats. We found that OVX rats caused peripheral insulin resistance and impaired insulin-induced LTD. Estrogen administration could reverse this impairment effects. The propose mechanism of this beneficial effect may be related to neuronal insulin and estrogen signaling.

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กิตติกรรมประกาศ

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OUTPUT

การตีพิมพ์ผลงานทางวิชาการในวารสารระดับนานาชาติ

1. Supakul L, Pintana H, Apaijai N, **Chattipakorn SC**, Shinlapawittayatorn K, Chattipakorn N. Protective effects of garlic extract on cardiac function, heart rate variability, and cardiac mitochondria in obese insulin resistant rats. *Eur J Nutr* 2014;53(3):919-28 (Impact Factor = 3.127)
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กิจกรรมที่เกี่ยวข้องกับการนำผลจากโครงการไปใช้ประโยชน์

การแต่งหนังสือ

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2. นสพ. เกริกเกียรติ จินดา นักศึกษาปริญญาเอก ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ในงานวิจัย เรื่อง “DPP-4 inhibitor improves neuronal insulin receptor function and brain mitochondrial function caused by high-fat diet

consumption.” ซึ่งงานวิจัยเรื่องนี้ได้รับรางวัล The Best Oral Presentation Award of the 42nd Annual Meeting of the Physiological Society of Thailand, 2012

3. ภก. จิรภาส ศรีเพชรวรรณดี นักศึกษาปริญญาเอก ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ในงานวิจัย เรื่อง “Mitochondrial calcium uniporter blocker effectively prevents brain mitochondrial dysfunction caused by iron overload.” ซึ่งงานวิจัยเรื่องนี้ได้รับรางวัล The Best Poster Presentation Award of the 42nd Annual Meeting of the Physiological Society of Thailand, 2012
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6. น.ส. ศิรินาฏ คำฟู นักศึกษาปริญญาเอก ภาควิชาสรีรวิทยา คณะแพทยศาสตร์ มหาวิทยาลัยเชียงใหม่ ในงานวิจัย เรื่อง “Effect of calcium channels and divalent metal transporter1 blockers on cardiac functions, iron deposition and mortality of iron-loaded thalassemia mice.” ซึ่งงานวิจัยเรื่องนี้ได้รับรางวัล The Best Oral Presentation Award of the RGJ-Ph.D. Congress XIII of Thailand annual conference, 2012
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development of cardiometabolic disorders and cardiac mitochondrial impairments in estrogen-deprived rats" ซึ่งงานวิจัยเรื่องนี้ได้รับรางวัล The Best Poster Presentation Award of the 43nd Annual Meeting of the Physiological Society of Thailand, 2014

การเชื่อมโยงกับต่างประเทศ

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ภาคผนวก

ผลงานทางวิชาการ (Articles)

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บทความสำหรับเผยแพร่

ภาวะอ้วนจัดเป็นปัญหาทางสุขภาพที่สำคัญอันหนึ่งของโลก ภาวะอ้วนนั้นเป็นปัจจัยเสี่ยงสำคัญของการเกิดโรคไม่ติดต่อเรื้อรัง โดยเฉพาะอย่างยิ่ง กลุ่มอาการเมตาบอลิซึมที่รวมถึงโรคเบาหวาน ความดันโลหิตสูง ไขมันในเลือดสูง และนำไปสู่โรคระบบหัวใจและหลอดเลือด และอาจนำไปสู่โรคสมองเสื่อมได้อีกด้วย การรับประทานอาหารที่มีไขมันสูงติดต่อกันเป็นเวลานานเป็นปัจจัยส่งเสริมที่ทำให้เกิดภาวะอ้วนตามมา ซึ่งภาวะอ้วนนำไปสู่การเกิดโรคเบาหวานชนิดที่ 2 ได้ โดยคนที่มีภาวะอ้วนจะมีเนื้อเยื่อไขมันเพิ่มมากขึ้น และไขมันที่เพิ่มขึ้นนั้นจะมีส่วนร่วมในการก่อให้เกิดการดื้อต่ออินซูลินในทุกอวัยวะของร่างกาย ซึ่งภาวะดื้อต่ออินซูลินนี้จัดเป็นกลุ่มอาการเมตาบอลิซึมที่ส่งผลต่อเนื่องในการเหนี่ยวนำให้เกิดปัญหาสุขภาพหลายอย่างที่มีเชื่อมโยงกัน ในการศึกษาครั้งนี้พบว่าภาวะอ้วนจากการกินอาหารไขมันสูงนั้นนอกจากก่อให้เกิดการดื้อต่ออินซูลินโดยทั่วๆไปแล้วยังก่อให้เกิดภาวะดื้อต่ออินซูลินในเซลล์สมอง เกิดความเครียดในสมอง และความบกพร่องของการทำงานของไมโตคอนเดรียในสมอง ซึ่งส่งผลตามมาทำให้เกิดการเรียนรู้และความจำที่ลดลงได้ ซึ่งภาวะความผิดปกติในสมองเหล่านี้ สามารถกลับมาทำงานเป็นปกติได้ด้วยการรักษาด้วยยาต้านเบาหวานชนิด ยาพีพีเออาร์แกมมาอะโกนิสต์ นอกจากนี้ ผลของฮอร์โมนเอสโตรเจนยังช่วยในการแก้ไขภาวะดื้อต่ออินซูลินที่เกิดตามหลังการเกิดภาวะอ้วนได้ทั้งในเพศชายและเพศหญิง แต่ประโยชน์ของเอสโตรเจนในสมองจะพบได้เฉพาะในเพศหญิง เท่านั้น นอกจากนี้ การขาดหายไปของฮอร์โมนเอสโตรเจนในเพศหญิงที่ไม่มีภาวะอ้วนร่วมด้วย ยังทำให้ก่อให้เกิดภาวะดื้อต่ออินซูลินในเซลล์สมองได้ และ เมื่อมีการขาดหายไปของฮอร์โมนเอสโตรเจนร่วมกับมีภาวะอ้วนยิ่งทำให้ภาวะดื้อต่ออินซูลินในเซลล์สมองเพิ่มมากขึ้นดังนั้นการหลีกเลี่ยงอาหารไขมันสูงนอกจากจะทำให้ไม่อ้วนแล้ว ยังจะทำให้สมองทำงานได้ดีขึ้นด้วยไม่ว่าจะมีหรือไม่มีฮอร์โมนเพศแล้วก็ตาม