The effect of triterpene saponins on hypothalamic and cortical leptin sensitivity in obese mice

Yizhen Wu

University of Wollongong

UNIVERSITY OF WOLLONGONG
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The effect of triterpene saponins on hypothalamic and cortical leptin sensitivity in obese mice

YIZHEN WU

Supervisors:
Dr. Yinghua Yu, Professor Xu-Feng Huang

This thesis is presented as part of the requirement for the conferral of the degree: DOCTOR OF PHILOSOPHY

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The University of Wollongong
School of Medicine

March 2018
Abstract

Obesity is a worldwide health problem due to its epidemic proportions and high risk for other metabolic disorders, such as type 2 diabetes, cardiovascular diseases and certain cancers. It is generally accepted that a chronic low-grade inflammatory state is at the pathogenic core of obesity. High-fat diet-induced obesity features of hyperleptinemia, hyperinsulinemia, fat deposition, glucose intolerance, central and peripheral inflammation, as well as central and peripheral leptin resistance and hypothalamic leptin signaling compromised. Obesity also features of the impairment of prefrontal cortex, and impairment of leptin-induced regulation of brain-derived neurotrophic factor (BDNF) expression and synaptogenesis, which has been considered to be associated with the incidence of neuronal degenerative diseases, cognitive decline, and depression. Some plant-derived triterpene saponins are anti-inflammatory and inhibit the NF-κB signaling. Those triterpene saponins are potential therapeutic agents for the obesity-associated inflammation to be determined. Natural extracted triterpene saponins, ginsenoside Rb1 and teasaponin, are used in this PhD research project. Ginsenoside Rb1, extracted from Ginseng, has anti-inflammation effect and anti-obesity effect. However, it is unclear whether it has the effect of anti-obesity-associated inflammation and whether it can address the above listed features issue of obesity. Teasaponin is an active compound of tea extract, has been demonstrated to ameliorate obesity, reduce inflammation and improve central leptin sensitivity in obese mice in our previous study. Tea consumption improves cognition and increases brain activation in the prefrontal cortex. However, little is known on the ability of teasaponin on recognition memory and its effect on leptin signaling in the prefrontal cortex of high-fat diet-induced obese mice.

The aim of Chapter 2 was to investigate the effects of ginsenoside Rb1 on treating obesity, obesity-associated inflammation and central leptin resistance, as well as hypothalamic leptin signaling. Results demonstrated that ginsenoside Rb1 treatment
reduced food intake, prevented body weight gain and fat deposition, improved glucose tolerance, as well as improved hyperleptinemia in high-fat diet-induced obese mice. Furthermore, ginsenoside Rb1 treatment reduced obesity-associated peripheral inflammation and hypothalamic inflammation, indicating by the decrease expression of TNFα, IL-6, IL-1β and pIkK. Ginsenoside Rb1 treatment also decreased the protein expression of SOCS3 and PTP1B, the negative regulators of leptin signaling. The reduced central inflammation and downregulation of SOCS3 and PTP1B by Rb1 treatment, contributed to the improvement of central leptin resistance and restore leptin signaling in the mediobasal hypothalamus of obese mice treated with Rb1. Reduced peripheral inflammation contributed to the improvement of glucose intolerance. Besides, the increased expression of circulating PYY, and hypothalamic POMC upregulation and AgRP downregulation extend the mechanism on suppressing food intake by Rb1.

The aim of chapter 3 was to examine the effects of ginsenoside Rb1 on central leptin effects on BDNF expression and synaptogenesis in the prefrontal cortex during obesity via an in vivo and an in vitro model. Ginsenoside Rb1 chronic treatment improved central leptin sensitivity, leptin-JAK2-STAT3 signaling and leptin-induced regulation of BDNF expression in the prefrontal cortex of high-fat diet induced obese mice. In cultured prefrontal cortical neurons, palmitic acid, the saturated fat, impaired leptin-induced BDNF expression, reduced the immunoreactivity and mRNA expression of synaptic proteins, and impaired leptin-induced neurite outgrowth and synaptogenesis. Importantly, Rb1 significantly prevented these pernicious effects induced by palmitic acid. These results indicate that Rb1 reverses central leptin resistance and improves leptin-BDNF-neurite outgrowth and synaptogenesis in the prefrontal cortical neurons.

The aim of chapter 4 was to evaluate the effects of teasaponin on recognition memory and leptin signaling in the prefrontal cortex of obese mice, and on leptin effects on neurogenesis in the saturated fatty acid, palmitic acid, treated primary cortical neurons. Oral teasaponin treatment significantly improved memory deficit in high-fat diet (HFD) fed mice assessed by the novel object recognition test. Furthermore, teasaponin treatment...
(10mg/kg, intraperitoneal) for 21 days improved downstream leptin signaling (JAK2, and STAT3), and leptin’s effect on BDNF, in the prefrontal cortex of HFD fed mice. In the *in vitro* study, teasaponin reversed the impairment caused by palmitic acid on the leptin-mediated BDNF expression, leptin-mediated neurite outgrowth (MAP2 mRNA, neurite branching and length), and synaptogenesis (PSD95 expression). These results indicate teasaponin improves obesity associated recognition memory deficit and central leptin effects on BDNF, neurite outgrowth and synaptogenesis in the prefrontal cortex.

In conclusion, this thesis demonstrated two natural triterpene saponins, ginsenoside Rb1 and teasaponin, as the potential therapeutic agents for obesity and obesity-associated neuronal degenerative diseases, cognitive decline, and depression. Those therapeutic effects benefit from the properties of these two triterpene saponin targeting on anti-obesity-associated inflammation, positive regulation of central leptin signaling, and reversal of central leptin resistance. Overall, both ginsenoside Rb1 and teasaponin supplementation may be used to treat obesity, and prevent obesity-associated neurodegeneration and improve cognitive function.
Acknowledgments

I wish to thank the School of Medicine (as well as the former School of Health Science), and the Illawarra Health and Medical Research Institute (IHMRI), University of Wollongong (UOW) for their academic support through my PhD studies. I also appreciate financial support of IPTA (2011-2014) and DPTA (2014-2018) tuition award from University of Wollongong, and the Matching scholarship offered from my supervisors, Professor Xu-Feng Huang and Dr. Yinghua Yu. I also benefit from travel grants from the Australian Neuroscience Society and the Faculty of Science, Medicine and Health, UOW.

I wish to express my sincerely gratitude to my supervisors Dr. Yinghua Yu and Professor Xu-Feng Huang for your knowledge, dedicated support, inspiration, encouragement, and your kindness and understanding my two years (yes two) leave of absence taking care of my two babies. I would like to express my heartfelt thank you for your patience and great supports through the whole periods of my candidature, especially in those difficult time and slow progress of paper preparation, revised and published. Your great knowledge and timely guidance since the development of ideas through to the completion of my papers and thesis was greatly appreciated and will never be forgotten. I feel fortunate and honored to have both of you to be my supervisors.

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Finally, special memory to my father-in-law, hopefully god has given you peaceful heaven for resting.

Thank you
Certification

I, Yizhen Wu, declare that this thesis submitted in fulfilment of the requirements for the conferral of the degree DOCTOR OF PHILOSOPHY, from the University of Wollongong, is wholly my own work unless otherwise referenced or acknowledged. This document has not been submitted for qualifications at any other academic institution.

Yizhen Wu

26th March 2018
STATEMENT FOR THE STYLE OF THE THESIS

In accordance with the University of Wollongong thesis committee “Guidelines for Preparation and Submission of HDR Theses” (2014), and “Higher Degree Research (HDR) Thesis by Compilation Guidelines” (11 Sep 2017), this PhD thesis is presented in “Journal Article Compilation Style Format”. It is comprised of a series of original studies published in peer-reviewed journals, of which I am the first author. I hereby declare that I am the primary designer of these studies, and have carried out all experiments, data analysis, data interpretation and manuscript preparation.

Yizhen Wu
2018

I consent to the presentation of this PhD in “Journal Article Compilation Style Format” and I acknowledge the above statement pertaining to student contribution to be correct.

Dr. Yinghua Yu, principal Supervisor
Prof. Xu-Feng Huang, Supervisor
2018 2018
PUBLICATIONS

The following publications and presentations have arisen directly from work contained within this thesis.

Publications in Refereed Journals:

- **Wu, Y., X.-F. Huang, and Y. Yu.** The progressive effects on central and peripheral inflammation by the high-fat diet in DIO and DR mice model *(Manuscript preparation)*
- **Wu, Y., X.-F. Huang, and Y. Yu.** Low grade inflammation shown in the prefrontal cortex in chronic diet-induced obese but not in diet-resistant mice, was negative associated with synaptogenesis markers *(Manuscript preparation)*

Publications in conference Proceedings:

- **Wu, Y., X.-F. Huang, and Y. Yu.** 2013. Teasaponin improves central leptin sensitivity in
high-fat diet-induced obese mice. In Australian Neuroscience Society 33rd Annual Meeting, Melbourne, Australia. pp144-144.


Additional Publications:

The following publications and presentations have arisen as a result of other projects I have been involved in during my doctoral studies:

Publications in Refereed Journals:


Publications in conference Proceedings:

• Wu, Y., Y. Yu, Z. Wu, C. Patch, and X.-F. Huang. 2012. DHA supplementation prevents the
increase of cannabinoid-1 receptor in specific brain area of rats fed a high-fat diet. In Australian Neuroscience Society 32nd Annual Meeting, Gold Coast, Queensland, Australia. pp135-135.


## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti gene-related peptide</td>
</tr>
<tr>
<td>AMPK</td>
<td>AMP-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve for glucose</td>
</tr>
<tr>
<td>BDNF</td>
<td>brain-derived neurotrophic factor</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>Ct</td>
<td>Threshold cycle value</td>
</tr>
<tr>
<td>DIO</td>
<td>Diet-induced obesity</td>
</tr>
<tr>
<td>DR</td>
<td>Diet resistant</td>
</tr>
<tr>
<td>FOXO1</td>
<td>Forkhead box protein O1</td>
</tr>
<tr>
<td>HF</td>
<td>High-fat</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostasis model assessment</td>
</tr>
<tr>
<td>icv</td>
<td>intracerebroventricular</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin 6</td>
</tr>
<tr>
<td>ip</td>
<td>intraperitoneally</td>
</tr>
<tr>
<td>IPGTT</td>
<td>Intraperitoneal glucose tolerance test</td>
</tr>
<tr>
<td>JAK2</td>
<td>Janus Kinase 2</td>
</tr>
<tr>
<td>LC</td>
<td>lab chow diet</td>
</tr>
<tr>
<td>LH</td>
<td>Lateral Hypothalamus</td>
</tr>
<tr>
<td>MAP2</td>
<td>microtubule-associated protein 2</td>
</tr>
<tr>
<td>MH</td>
<td>Mediobasal Hypothalamus</td>
</tr>
<tr>
<td>NF-Kb</td>
<td>Nuclear factor-κ-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>pACC</td>
<td>Phosphorylated Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>pAMPK</td>
<td>Phosphorylated AMP-activated protein kinase</td>
</tr>
<tr>
<td>pAkt</td>
<td>Phosphorylated Protein kinase B</td>
</tr>
<tr>
<td>PFC</td>
<td>Prefrontal Cortex</td>
</tr>
<tr>
<td>pFOXO1</td>
<td>Phosphorylated Forkhead box protein O1</td>
</tr>
<tr>
<td>pGSK3β</td>
<td>Phosphorylated Glycogen synthase kinase-3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>pIKK</td>
<td>phosphorylated inhibitory κB kinase</td>
</tr>
<tr>
<td>pIkBα</td>
<td>phosphorylated inhibitor κBα</td>
</tr>
<tr>
<td>pJAK2</td>
<td>Phosphorylated Janus Kinase 2</td>
</tr>
<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
</tr>
<tr>
<td>PSD95</td>
<td>post-synaptic density protein 95</td>
</tr>
<tr>
<td>pSTAT3</td>
<td>Phosphorylated Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>PTP1B</td>
<td>Protein Tyrosine Phosphatase 1B</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus</td>
</tr>
<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>Rb1</td>
<td>Ginsenoside Rb1</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Suppressor of Cytokine Signaling 3</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>SYN</td>
<td>synaptophysin</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor α</td>
</tr>
<tr>
<td>TS</td>
<td>Teasaponin</td>
</tr>
</tbody>
</table>
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CHAPTER ONE

1.1 Introduction

The prevalence of obesity is a worldwide health problem, which affects one third of the world’s population (Hruby and Hu, 2015; Ng et al., 2014; Stevens et al., 2012) and causes great economic burden (Hruby and Hu, 2015). It is an important risk factor for the development of type 2 diabetes, cardiovascular disease, and certain cancer, as well as depression, and neurodegenerative diseases (Hruby and Hu, 2015). Increasing evidence indicated that a chronic low-grade inflammatory state during obesity is the core pathogenic of obesity and type 2 diabetes (Cai et al., 2005; Lumeng and Saltiel, 2011). The pro-inflammatory response includes elevated pro-inflammatory, such as tumor necrosis factor alpha (TNF-α), interleukin 6 (IL-6), and interleukin 1 beta (IL-1β), and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, including inhibitor kappa B alpha (IκBα) and IκB kinase (IKK) (Osborn and Olefsky, 2012; Thaler et al., 2012). The activation of pro-inflammatory cytokines and NF-κB signaling pathway induce insulin and leptin resistance in the peripheral tissue and the central nervous system (Hayden and Ghosh, 2008; Zabolotny et al., 2002; Zhang et al., 2008). Obesity-associated inflammation in the liver and white adipose tissue leads glucose intolerance, insulin resistance and metabolic dysfunction (Cai et al., 2005; Stanton et al., 2011b; Xu et al., 2003). Over-nutrition and obesity also leads to hypothalamic inflammation, which results in the central leptin resistance, hepatic insulin resistance, a reduction in thermogenesis, and cardiovascular disorders (Arruda et al., 2011; Cai and Liu, 2011; Thaler et al., 2010; Thaler and Schwartz, 2010; Wisse and Schwartz, 2009). Therefore, to treat obesity and metabolic syndrome, it is quite necessary to block peripheral and central inflammation. Furthermore, obesity impairs prefrontal cortex, which is a major regulatory center for cognitive function (Frith and Dolan, 1996; Le et al., 2006). Obesity impairs leptin-induced regulation of BDNF expression, neurite
outgrowth and synaptogenesis, which has been considered to be associated with the incidence of neuronal degenerative diseases, cognitive decline, and depression (Gupta et al., 2013; Vanevski and Xu, 2013; Zuccato and Cattaneo, 2009). Hence, it is essential to attenuate the obesity-associated impairment of cortical leptin-BDNF, neurite outgrowth and synaptogenesis to prevent the risk of development obesity-associated metabolic disorder and neurodegeneration.

Studies showed that some triterpene saponins are naturally derived inhibitors of NFκB signaling and have anti-inflammatory potential (Salminen et al., 2008b). Triterpene saponin ginsenoside Rb1, one of the active compounds extracted from ginseng, has been reported to have anti-inflammation effect, anti-obesity effect and neuroprotective effect (Kim et al., 2014; Li et al., 2011b; Lin et al., 2013; Liu et al., 2011a; Nam et al., 2013; Wang et al., 2008; Xiong et al., 2010; Xue et al., 2006; Yuan et al., 2007). Reports also indicated, tea and tea extract (including teasaponin) has anti-inflammatory effect (Park et al., 2011; Ren et al., 2018). However, it is unknown whether Rb1 can improve obesity-associated inflammation and leptin resistance, and also unknown the effects of Rb1 on leptin-BDNF, neurite outgrowth. It is also unclear the effects of teasaponin on obesity-associated leptin resistance, leptin-BDNF-neurite outgrowth and neurogenesis, as well as obesity-associated recognition impairment. The present PhD series studies were designed to investigate the chronic treatment effects of Rb1 on body weight change, energy intake, obesity-associated peripheral and hypothalamic inflammation, glucose intolerance and central leptin resistance, cortical leptin sensitivity and synaptogenesis of high-fat diet-induced obese mice, as well as effects of teasaponin on obesity-linked recognition impairment and cortical leptin sensitivity. These series PhD studies will provide new potential anti-obesity therapeutic agents that modulate obesity-associated inflammation, central leptin sensitivity and prevent obesity-associated neurodegeneration.
1.2 Literature Review

1.2.1 Obesity

The prevalence of overweight and obesity has been reached the worldwide epidemic proportion. In 2013, the proportion of overweight and obese adults increased to 36.9% in men, and 38% in women worldwide; the proportion of overweight and obese children and adolescents in developed countries increased to 23.8% in boys and 22.6% in girls (Ng et al., 2014). As the established health risks and the substantial globally increase in prevalence, obesity has become a major global health challenge and a heavy burden on financial of health care systems worldwide. Globally, it is estimated that overweight and obesity caused 3.4 million deaths, 3.9% of the years of life lost and 3.8% of disability-adjusted life-years in 2010 (Ng et al., 2014). In Australia, about 27% of children and adolescents and 63% of adults were overweight or obese in 2014-2015(AIHW, 2017). In Australia in 2011, 7% of the total health burden was attributed to overweight and obesity, 63% of which was fatal burden. It is estimated that obesity has cost $8.6 billion of the Australian economy in 2011-2012(AIHW, 2017). Also, obesity leads to higher likelihood of chronic diseases such as cardiovascular disease, type 2 Diabetes Mellitus, stoke, osteoarthritis, respiratory, sleep problems, hypertension and certain types of cancers (Dakin et al., 2004; Daling et al., 2001; Huang and Chen, 2009). The issue of obesity will lead to both increased mortality and morbidity (Frezza, 2004; Kojima et al., 2009). Therefore, due to the medical complication and the associated tremendous economic consequences, the alarming growth rate undeniably makes obesity an urgent and critical issue to be addressed (Devlin et al., 2000; Marx, 2003; Wisse et al., 2007).

Obesity results from a sustained energy imbalance—when the energy intake is greater than energy expenditure. It can be attributed by both genetic predisposition and lifestyle factors such as overconsumption of high energy density diet, sedentary lifestyles and reduction in physical activity (Brown et al., 2009; Challis et al., 2003; Hill and Peters, 1998; Perusse and Bouchard, 2000). Epidemiological studies have shown that the
consumption of high-fat diets is positively associated with body weight gain and obesity in human population (Bray and Popkin, 1998; Koegler et al., 1999; Macdiarmid et al., 1996; Smyth and Heron, 2006).

1.2.2 Dietary fat consumption and high-fat diet-induced obese mouse model

Excessive energy intake by overconsumption of diet that rich in saturated fat contributes to energy imbalance and weight gain, leading to overweight and obesity (Brooks et al., 2013; Hill et al., 2000). In a human study, a diet with >35% energy as fat, is classified as a high-fat diet (HFD), and promotes body weight gain both in men and women (Donnelly et al., 2008). Strong evidence suggests greater intake of energy-enrich diet is associated with bigger body fat mass among children and higher body weight among adults (AIHW, 2017). Therefore, to address the obesity issue, high-fat diet is used to establish high-fat diet-induced obesity (DIO) animal models to mimic the most common cause of obesity in humans.

In a DIO mouse model, the development of chronic high-fat diet-induced obesity is a progressive process varying in energy intake, body weight gain and leptin sensitivities at different stages. It can be divided into Early Response (Stage1-DIO, < 5 wks), Middle Regulatory (Stage 2-DIO, from 5 to 15 wks), and Late Failure (Stage 3-DIO, > 15 wks) Stages (Lin et al., 2000b). It can be summarized as in the early stage the body responds to high-fat diet, while in the middle stage the body tries to prevent excessive fat stores by activation of its regulatory system, and finally chronic consumption of high-fat diet eventually fails the regulatory system and results in obesity (Table 1). In parallel with these whole-body measures of energy balance, our lab have further demonstrated as well the progressive dysregulation of leptin secretion, peripheral and then central leptin insensitivity, central leptin receptor gene expression, and the downstream neurotransmitter expressions (Table 1).

The impairment of the potential molecular pathways behind the central nerves system, which may links to the dysregulation of food intake and body weight, may mainly
attribute to the development of DIO and related leptin insensitivities. This study used the DIO mouse model to address the metabolic disorders.

**Table 1.1. Characteristics of three stages of diet-induced obesity**

<table>
<thead>
<tr>
<th></th>
<th>Early stage (week 1)</th>
<th>Middle stage (week 8)</th>
<th>Late stage (week 19)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>ND</td>
<td>↑11%</td>
<td>↑30%</td>
<td>(Lin et al., 2000b)</td>
</tr>
<tr>
<td>Energy intake</td>
<td>↑Slightly (ND)</td>
<td>↓16%</td>
<td>↑15%</td>
<td>(Lin et al., 2000a)</td>
</tr>
<tr>
<td>RVB</td>
<td>ND</td>
<td>↑45%</td>
<td>↑84%</td>
<td>(Lin et al., 2000a)</td>
</tr>
<tr>
<td>Leptin sensitivity</td>
<td>2ug/g i.p.</td>
<td>Yes</td>
<td>No</td>
<td>(Lin et al., 2000b)</td>
</tr>
<tr>
<td></td>
<td>0.1ug/g icv</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Arc</td>
<td>LR mRNA</td>
<td>ND</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Huang et al., 2003a; Huang et al., 2003b; Lin et al., 2000a)</td>
</tr>
<tr>
<td></td>
<td>POMC mRNA</td>
<td>ND or ↑</td>
<td>ND</td>
<td>↓</td>
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1.2.3 Leptin signaling and central leptin resistance

1.2.3.1 Leptin and leptin signaling

Leptin, a 16 kDa peptide hormone consisting of 167 amino acid residues, is produced by adipocytes in proportion to fat stores. Accumulating evidences show that leptin acts on brain to regulate energy balance via activating leptin receptor and related signaling (Bjorbaek and Kahn, 2004; Fruhbeck, 2006). Leptin receptors, members of the class I cytokine receptor family, exert its function through JAKs (Janus kinases) and STATs (signal transducers and activators of transcription). Leptin receptor b, the full-length isoform, is considered to be the functional receptor to activate the JAK/STAT signal transduction pathway (Fruhbeck, 2006). It is clear that leptin receptor b is highly expressed in multi-regions of hypothalamus, especially in the arcuate nucleus of the hypothalamus (Arc) area (Bjorbaek and Kahn, 2004). The ARC region contains two key populations of neurons, NPY and POMC neurons, which produce orexigenic peptides and anorectic peptides, respectively (Cone, 2005). Leptin has been reported to modulate the activity of both neurons, inducing the inhibition of NPY/AgRP mRNA expression and the elevation of POMC mRNA expression (Enriori et al., 2006). In addition, leptin activates leptin receptor b signaling in hypothalamus, including JAK2/STAT3 and insulin receptor substrate/ phosphoinositide 3-kinase/protein kinase B (IRS/PI3K/Akt), to regulate the feeding behavior (Hill et al., 2008; Sahu, 2004; Wauman and Tavernier, 2011). Additionally, AMP-activated protein kinase (AMPK), fuel-sensing
signaling has been suggested to involve in the regulation of leptin’s anorectic effect (Blanco Martinez de Morentin et al., 2011; Gao et al., 2007; Morris and Rui, 2009). Leptin receptor is also distributed in other regions of the brain, such as hippocampus and prefrontal cortex (Ates et al., 2014). Recently, leptin-STAT3 has been reported to be involved in the neuroprotection of cerebral ischemia via promoting neuronal survival in vitro and in vivo (Amantea et al., 2011; Tang, 2008). Leptin signaling in the hypothalamic and prefrontal cortex in obese were investigated in this PhD study.

1.2.3.2 Central leptin resistance and its underlying mechanism

It is well recognized that adequate circulating leptin provides a signal of energy stores to central nervous system (CNS), enable the brain to adjust energy balance via suppressing food intake and increasing the energy expenditure (Bates and Myers, 2003; Friedman and Halaas, 1998). Conversely, lack of leptin signaling, such as attributing to mutation of leptin (ob/ob mice) or leptin receptor (db/db mice) in rodents or human, leads to hyperphagia and suppression of energy expenditure accompanied by increased expression and secretion of orexigenic peptides (NPY and AgRP) (Ahima et al., 1996; Bates and Myers, 2003; Lord et al., 1998). However, most obese humans and rodents do not have lower circulating leptin. In fact, they usually have extremely high level of plasma leptin, which still fails to reduce food intake and body weight and fails to increase energy expenditure, showing the resistance to leptin effect.

The definition of leptin resistance is characterized by the failure of elevated circulating leptin to suppress feeding and weight gain which in turn exacerbates obesity in general (Enriori et al., 2006; Myers et al., 2008b). Recently researches produced a high-fat diet-induced obesity (DIO) model in several strains of rodents focusing on the issue of leptin resistance (Hariri and Thibault, 2010; Howard et al., 2004; Judge et al., 2008; Levin and Dunn-Meynell, 2002; Lin et al., 2000b; Metlakunta et al., 2008). The development of high-fat diet-induced obesity and leptin resistance in C57BL/6J mice can be divided into 3 stages: early stage, middle stage and late stage. In the early stage, mice on HFD gain
weight and remain response to peripheral leptin injection. In the middle stage, mice elevate plasma leptin and fails to response to peripheral leptin but still sensitive to central leptin administration. Finally, in the late stage, DIO mice with highly increasing body adiposity and extreme high level of plasma leptin show central leptin resistance, failing to suppress food intake and body weight in response to icv leptin administration (Lin et al., 2000b).

Interestingly, Protein Tyrosine Phosphatase 1B (PTP1B), a cellular protein, has been shown to implicate in the regulation of cellular leptin signaling. PTP1B dephosphorylates JAK2, the initial tyrosine kinase mediating leptin signaling. Neuronal PTP1B knockout mice have reduced body weight, food intake and enhanced leptin sensitivity (Bence et al., 2006). Furthermore hypothalamic PTP1B is increased during HFD-induced leptin resistance (White et al., 2009). The i.p. injection of PTP1B inhibitor Trodusquemine (MSI-1436) decreased food intake and body weight in diet-induced obese mice (Lantz et al., 2010). These results suggest PTP1B plays an important role in central leptin insensitivity (Cheng et al., 2002; Myers et al., 2008b; Sahu, 2004).

Additionally, low-grade pro-inflammatory is at the pathogenic core of obesity. The activation of pro-inflammatory cytokines and NF-κB signaling pathway mediate the transcription of the suppressor of cytokine signaling 3 (SOCS3) and PTP1B, which will induce insulin and leptin resistance in peripheral tissue and the central nervous system. To alleviate the central leptin resistance, study 1 was carried out to investigate the effect of chronic ginsenoside Rb1 treatment. Study 2 and study 3 were addressing leptin resistance issue in the prefrontal cortex caused by the saturated fat.

1.2.4 Obesity-associated inflammation and its detrimental effects on leptin resistance and cognitive decline

Chronic low-grade pro-inflammatory state is at the pathogenic core of obesity and type 2 diabetes (Cai et al., 2005; Lumeng and Saltiel, 2011). This inflammatory response
includes elevated levels of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6), and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, including inhibitor kappa B alpha (IκBα) and IκB kinase (IKK) (Osborn and Olefsky, 2012; Thaler et al., 2012). The activation of pro-inflammatory cytokines and NF-κB signaling pathway mediate the transcription of the suppressor of cytokine signaling 3 (SOCS3) and protein-tyrosine phosphatase 1B (PTP1B), negative regulators of insulin and leptin signaling, which induce insulin and leptin resistance in peripheral tissues and the central nervous system (Hayden and Ghosh, 2008; Zabolotny et al., 2002; Zhang et al., 2008). Obesity associated inflammation in white adipose tissue and the liver leads to glucose intolerance, insulin resistance and metabolic dysfunction (Cai et al., 2005; Stanton et al., 2011b; Xu et al., 2003). Over-nutrition and obesity also leads to hypothalamic inflammation and stimulation of local proinflammatory NF-κB signaling, resulting in the dysfunction of hypothalamic neurons (Thaler et al., 2012; Zhang et al., 2008). Furthermore, recent studies have shown that induction of inflammation in the hypothalamus results in experimental obesity, resistance to the anorexigenic hormone leptin, peripheral insulin resistance and defective regulation of food intake and energy expenditure (Arruda et al., 2011; Calegari et al., 2011; Milanski et al., 2012).

Neuroinflammation in obesity could contribute to cognitive decline and dementia. Pro-inflammatory cytokines, such as IL-6 and IL-1β have been reported to disrupt neural circuits involved in cognition and memory (Gemma and Bickford, 2007; Jankowsky and Patterson, 1999; Nguyen et al., 2014). For instance, juvenile HFD intake promotes LPS-stimulated IL-1β and TNFα expression in the hippocampus, which is likely to be contributed to a selective impairment of consolidation that impaired long-term spatial memory but not short-term memory (Boitard et al., 2014a). Besides, in children, intake of saturated fatty acids impairs both relational and item memory (Nguyen et al., 2014). Obesity-associated inflammation in hypothalamus and hippocampus has been reported (Arruda et al., 2011; Boitard et al., 2014a; Cai and Liu, 2011). While at this
Stage the prefrontal cortex is to be investigated. As obesity-associated inflammation deleterious leptin resistance and neurodegeneration, it is valuable to pursue therapeutic agents that have anti-obesity, anti-inflammation and alleviating neurodegenerative effects.

1.2.5 BDNF and neurite outgrowth and synaptogenesis regulation by leptin

Brain-derived neurotrophic factor (BDNF) plays an important role in neurite outgrowth (ie, elongation and branching), synaptic plasticity, synaptogenesis and the regulation of energy balance (Noble et al., 2011; Yu et al., 2009), through the activation of its receptor tropomyosin-related kinase B (TrkB). Aberrant BDNF/TrkB signaling is associated with obesity, cognitive impairment, and other neurodegenerative diseases (Gupta et al., 2013; Vanevski and Xu, 2013). Moreover, the BDNF gene mutation is associated with increasing early-onset obesity and cognitive impairment in BDNF-haploinsufficient patients (Gray et al., 2006). The BDNF protein level in the prefrontal cortex is decreased in rodents fed a chronic high-fat (HF) diet (Kanoski et al., 2007b). Furthermore, leptin, secreted from adipocytes, is a key regulator of BDNF expression in the hypothalamus (Liao et al., 2012) and brain stem (Bariohay et al., 2005a), promoting negative energy balance. Leptin increases hippocampic BDNF in normal mice, but not in diet-induced obese (DIO) mice, which exhibit depressive behaviour (Yamada et al., 2011), suggesting central leptin resistance in the obese mice. Thus the impairment of leptin-regulated BDNF in the prefrontal cortex by saturated fat may play an important role in central nervous system dysfunction in obese subjects. Improving leptin-regulated BDNF is imperative to improving obesity-associated brain dysfunction, including cognitive impairment.

Leptin involves in the regulation of the cellular events including neurogenesis, axon growth, and synaptogenesis (Bouret, 2010). Leptin has been shown to regulate neurogenesis, such as increasing axonal growth cone size in cortical neurons (Valerio et al., 2006). Recently, leptin-STAT3 has been reported to be involved in the
neuroprotection of cerebral ischemia via promoting neuronal survival in vitro and in vivo (Amantea et al., 2011; Tang, 2008). It is known that leptin signaling molecules, JAK2 and STAT3, distribute around the postsynaptic sites in the cerebral cortex (Murata et al., 2000). An immunocytochemistry study also shows BDNF and TrkB lie in the postsynaptic densities in the cerebral cortex of adult rats (Aoki et al., 2000). These findings suggest that leptin-JAK2-STAT3 signaling and BDNF/TrkB might regulate synaptogenesis and synaptic plasticity in the prefrontal cortex. Study 2 and study 3 focus on the hypothesis that leptin will regulate BDNF, neuritogenesis and synaptogenesis in the prefrontal cortex.

1.2.6 Obesity-associated impairment of prefrontal cortex and neurodegeneration

Apart from hippocampus, the prefrontal cortex is another important brain region which regulates cognition. Impairment in the prefrontal cortex has been identified in obesity, mood disorders and neurodegenerative diseases. For example, obese women have a lower activation in the prefrontal cortex in response to a meal than lean women examined by positron emission tomography (Le et al., 2007). The abnormal structure and dysfunction in the prefrontal cortex is associated with depression and anxiety-like behaviours and Alzheimer’s disease (Drevets et al., 2008). Cross-sectional study on obesity and brain structure showed that higher levels of BMI were associated with lower global brain volume, and dorsolateral prefrontal cortex (DLPFC) in obese individuals was preferentially vulnerable to atrophy (Bischof and Park, 2015; Brooks et al., 2013; Ward et al., 2005).

Obesity is associated with the neurodegenerative diseases. It is reported that obese rodents induced by a high-saturated fat diet have cognitive impairment, depressive behaviour, and central leptin resistance (Farr et al., 2008; Kanoski and Davidson, 2011; Yamada et al., 2011). Alzheimer’s disease has been linked to a HFD and neuronal leptin
resistance (Bonda et al., 2014; Hanson et al., 2013). Overweight/obesity individuals at varying degrees of risk for bipolar disorder exhibit greater cognitive impairment (McIntyre et al., 2017). A cross-sectional study revealed early cognitive decline in the obese participants, indicated by their normal but significantly lower performance on attention and more impulsive compared to the normal weight participants (Cook et al., 2017). Since obesity increase the neurodegeneration, the research on potential therapeutic agents to treat obesity-associated neurodegenerative disorders is quite essential.

1.2.7 Triterpene saponins

Plant triterpene saponins, biologically, are considered defensive compounds against pathogenic microbes and herbivores. These saponins also have beneficial properties for humans. For instance, Panax plant, well-known traditional herbal medicines, contains saponins (including ginsenosides) with various pharmacological effects (Sawai and Saito, 2011; Shibata, 2001). Triterpene saponins are groups of pant secondary metabolites with structurally composed of a lipid soluble aglycone and a water soluble sugar residues (Abid Ali Khan et al., 2012). The amphiphilic nature of the triterpene saponins suggests that they intercalate into the cell membrane (Abid Ali Khan et al., 2012) to mediate the cellular events. Some plant-derived triterpene saponins are anti-inflammatory and inhibit the NF-κB signaling pathway (Salminen et al., 2008b).

In this study, triterpene saponins ginsenoside Rb1 and teasaponin, derived from ginseng and tea respectively, were used on the animal models to treat obesity-associated metabolic dysfunctions, such as leptin resistance.

1.2.7.1 Ginsenoside Rb1

Ginseng, a traditional herbal medicine, is reported to be adaptogen that enhances body stability against physical loads, and is also suggested to improve cognitive function (Heo et al., 2011). Ginsenosides, the mainly active constituents that are found in extracts of
varies species of ginseng, attribute to the pharmacological properties of ginseng (Attele et al., 1999b). Ginsenoside Rb1 (Rb1) is one of the major bioactive components of ginseng saponins. It has been well chemically characterized, which has the structure of a tetracyclic triterpenoid with a molecular weight of 1109.26 and a molecular formula of $C_{54}H_{92}O_{23}$ (Figure 1.1) (Cho et al., 2004). Rb1 has an amphiphilic nature that enables it to intercalate the plasma membrane, affect membrane function, and elicit a cellular response (Abid Ali Khan et al., 2012). This compound inhibits inflammation in in vitro and in vivo models, including anti-inflammatory effects on aortic smooth muscle exposed to TNF-α (Li et al., 2011a), the colon of mice with colitis (Joh et al., 2011), and brain tissue in an cerebral ischemia animal model (Zhu et al., 2012). Both human and animal studies have demonstrated various physiological effects of Ginsenosides in increasing satiety and improving glucose metabolism. Recent studies suggested that ginsenosides has anorexigenic effect and anti-obesity effect (Etou et al., 1988; Shang et al., 2008; Xiong et al., 2010). Rats acute intraperitoneal administrated of Rb1 (10mg/kg) significantly decreased food intake during 4-10 hours after treatment. Chronic treatment of Rb1 (ip) for 4 weeks significantly reduced food intake, body weight, plasma leptin, fasting glucose and hypothalamic NPY mRNA expression level in DIO rats after 13 weeks’ HFD feeding. Furthermore Rb1 (10µmol/L) treated on primary hypothalamic neurons activated Akt, leptin signaling, peaking at 3h after treatment (Xiong et al., 2010). Ginseng berry extract has been used to treat leptin receptor deficient obese mice (ob/ob), showing decreased food intake and body weight, reduced hyperglycemia and improved glucose intolerance (Attele et al., 2002). Similarly, the saponins of ginseng can inhibit food intake, decrease body weight and blood glucose and improve insulin sensitivity in diet-induced obese rats and mice (Kim et al., 2005; Lin et al., 2013). Furthermore, oral administration of ginseng can increase insulin sensitivity and improve glucose intolerance in patients suffering Type 2 diabetes (Vuksan et al., 2008). Recent animal and cell models show that Rb1 has neuroprotective (Gao et al., 2010; Zhu et al., 2012) and anti-obesity effects (Xiong et al., 2010). For example, Rb1 increases
BDNF expression in rats with cerebral ischemia (Gao et al., 2010). Clinical data shows that ginseng treatment for 24 weeks improves cognitive function for up to 2 years in patients with Alzheimer’s disease (Heo et al., 2011).

However, it is unknown whether Rb1 can improve obesity-associated inflammation, central leptin resistance and obesity-associated impairment of neurite outgrowth and synaptogenesis. Study 1 and 2 focused on those issues.

Figure 1.1 Chemical structure of ginsenoside Rb1. Adapted from (Cho et al., 2004)

1.2.7.2 Teaaponin

Tea has been widely used as a healthy drink worldwide from ancient times. The earliest use of tea for medicinal purposes occurred in China, in roughly 2700 BC during the time of Emperor Shen Nung (Weisburger, 1997). During that time, it was believed to have health promoting properties, and was frequently used as a fluid supply for patients with infectious diseases. Evidence from clinical and animal studies show that tea has anti-obesity effects (Beresniak et al., 2012a; Wolfram et al., 2006), prevent abnormal glucose and lipid metabolism (Beresniak et al., 2012a; Park et al., 2011), anti-inflammation (Henning, 2012; Park et al., 2012) and improves cognitive function (Kuriyama et al.,
Treatment with 1% tea extract in diet for 6 weeks decreased body weight, adipose mass, hepatic TNF-α protein and adipose TNF-α mRNA, and attenuated hepatic steatosis in genetically obese (ob/ob) mice (Park et al., 2011). Supplementation of HFD with tea extract significantly reduced HFD induced p-IκBα and NF-κB activity in liver and adipose tissue (Park et al., 2012). Increased consumption of green tea was associated with a lower prevalence of cognitive impairment in a cross-sectional study in 1003 Japanese (Kuriyama et al., 2006), and longitudinal analysis of data from 1438 Chinese subjects (Ng et al., 2008). Phenolics and saponin are the two major active components extracted from tea. Phenolics in tea have been widely investigated in previous studies, while teasaponin has received little attention. Teasaponins, an important bioactive ingredient naturally extracted from varies of teas, can reduce the elevation of the rat plasma triacylglycerol level caused by an oral administration of a lipid emulsion (Han et al., 2005). Study showed that oral 0.5% teasaponin with HFD for 11 weeks significantly decreased body weight, parametrial adipose tissue weight and reduced the diameters of adipocyte (Han et al., 2001). Chemically teasaponin belongs to the oleanane-type pentacyclic triterpene saponins, whose amphiphilic nature enables them to intercalate into the cell membrane and interact with cell membrane molecules to regulate downstream signaling cascades (Abid et al., 2012; Attele et al., 1999a). Recently teasaponin (10mg/kg ip injection) showed significant anti-inflammatory properties, by inhibiting paw oedema induced by carrageenan in rats (Sur et al., 2001). In the current study, we investigated whether teasaponin could improve central inflammation and leptin sensitivity in the hypothalamus and prefrontal cortex of obese mice. We further investigated the effect of teasaponin on neurite outgrowth in cultured primary prefrontal cortical neurons in response to leptin and the saturated fatty acid, palmitic acid.
1.3 Aims and Hypotheses

1.3.1 Aims

1.3.1.1 General Aim

To evaluate the effects of triterpene saponins (Ginsenoside Rb1 or Teasaponin) on high-fat diet-induced obesity, obesity-associated inflammation, hypothalamic leptin signaling, central leptin sensitivity, and cortical leptin signaling, as well as leptin-induced BDNF expression and neurogenesis in the prefrontal cortex during obesity via an *in vivo* and an *in vitro* model.

1.3.1.2 Specific Aims

The specific aims of this research were to:

1. Investigate the effect of ginsenoside Rb1 on high-fat diet-induced obesity, glucose intolerance, obesity-associated inflammation, central leptin resistance and central leptin signaling in the high-fat diet-induced obese mouse model.

2. Examine the effects of ginsenoside Rb1 on central leptin effects on BDNF expression and synaptogenesis in the prefrontal cortex during obesity via an *in vivo* and an *in vitro* model.

3. Evaluate the effect of teasaponin on obesity, obesity-associated central inflammation and leptin sensitivity in the hypothalamus and prefrontal cortex of obese mice, and on leptin effects on BDNF and neurite outgrowth in cultured primary prefrontal cortical neurons in response to leptin and the saturated fatty acid, palmitic acid.

1.3.2 Hypotheses

1. Ginsenoside Rb1 will ameliorate high-fat diet-induced obesity, obesity-associated inflammation, glucose intolerance, and restore central leptin sensitivity, as well as central
leptin signaling in the high-fat diet-induced obesity.

2. Ginsenoside Rb1 will improve central leptin effects on BDNF expression and synaptogenesis in the prefrontal cortex during obesity in *in vivo* and *in vitro*.

3. Teasaponin will ameliorate obesity, central inflammation; improve memory and leptin sensitivity in the hypothalamus and prefrontal cortex of obese mice; and to improve leptin effects on BDNF and neurite outgrowth in the palmitic acid pre-treated cortical neurons.

### 1.3.3 Significance

Obesity has been reached epidemic proportions and is an important risk factor for the development of type 2 diabetes, cardiovascular disease and cancer. It is generally accepted that a large component of obesity-associated pathophysiology may stem from a low-grade pro-inflammatory inflammation that occurs during obesity. The pro-inflammatory response includes elevated pro-inflammatory cytokines and activation of NFκB signaling pathway. The activation of pro-inflammatory cytokines and NFκB signaling pathway induce insulin and leptin resistance in the peripheral tissue and the central nervous system. Obesity-associated inflammation in white adipose tissue and the liver leads glucose intolerance, insulin resistance and metabolic dysfunction. Over-nutrition and obesity also leads to hypothalamic inflammation. Hypothalamic inflammation results in the central leptin resistance, hepatic insulin resistance, a reduction in thermogenesis, and cardiovascular disorders. Furthermore, obesity impairs prefrontal cortex, which is a major regulatory center for cognitive function. Obesity impairs leptin-induced regulation of BDNF expression, neurite outgrowth and synaptogenesis, which has been considered to be associated with the incidence of neuronal degenerative diseases, cognitive decline, and depression. Therefore, strategies to attenuate the obesity-associated peripheral and hypothalamic inflammation, central leptin resistance and impairment of cortical leptin-BDNF, neurite outgrowth and synaptogenesis may help to prevent the risk of development obesity-associated metabolic disorder and neurodegeneration.
Studies showed that some triterpene saponins are naturally derived inhibitors of NFκB signaling and have anti-inflammatory potential. Ginsenoside Rb1, one of the triterpene saponins, has been reported to have anti-inflammation effect, anti-obesity effect and neuroprotective effect. Reports also indicated, tea and tea extract (including teasaponin) has anti-inflammatory effect. However, it is unknown whether Rb1 can improve obesity-associated inflammation and leptin resistance, and also unknown the effects of Rb1 on leptin-BDNF, neurite outgrowth. It is also unclear the effects of teasaponin on obesity-associated hypothalamic inflammation, obesity-associated leptin resistance, leptin-mediated BDNF expression and leptin-mediated neurite outgrowth, as well as obesity-associated recognition impairment. This research will explore chronic treatment effects of Rb1 on obesity-associated inflammation and central leptin resistance, cortical leptin sensitivity and synaptogenesis, as well as effects of teasaponin on obesity-associated hypothalamic inflammation, obesity-linked recognition impairment, and hypothalamic and cortical leptin sensitivity. This research will provide new potential anti-obesity therapeutic agents that will modulate obesity-associated inflammation, central leptin sensitivity and prevent obesity-associated neurodegeneration.

1.4 General Methods and Materials

1.4.1 Ethics statement

All procedures of animal studies included in this study were approved by the Animal Ethics Committee of the University of Wollongong, NSW, Australia (Application Approval#: AE10/08), and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes, which is in accordance with the International Guiding Principles for Biomedical Research Involving Animals. All efforts have been made to minimize animal numbers, animal stress and prevent suffering.

1.4.2 Animals care and Drug Treatments
C57Bl/6 male mice were obtained from the Animal Resources Centre (Perth, Western Australia) and housed in environmentally controlled conditions (temperature 22 °C, 12 hour light/dark cycle). All animals were fed a lab chow (LC) diet (5% fat, Vella Stock Feeds, Doonside, NSW, Australia) *ad libitum* to acclimatize for one week. Throughout the study, LC was served as the low fat control diet and was provided *ad libitum* except where noted.

**1.4.2.1 Acute Ginsenoside Rb1 treatment for central leptin sensitivity test**

In study 1 (chapter 2), after acclimatization, mice were fed a HFD for 8 weeks to generate the moderate obesity mice (The HFD contained 40% of energy as fat, SF11-095, Specialty Feeds, Western Australia). Then an intracerebroventricular cannula was implanted (detailed in section 1.3.4). After 5 days of recovery, the obese mice were then acute treated with Rb1 by intraperitoneal injected with Rb1 for two consecutive days. Central leptin sensitivity was then carried out on the mice (detailed in section 1.4.4). The dosage of Rb1 was 14 mg/kg, based on a Rb1 dose (10mg/kg) described previously in rats (Xiong et al., 2010), and using a body surface area ratio of 0.14 from rat to mouse (Paget and Barnes, 1964). Rb1 purified by high-performance liquid chromatography (HPLC) to ≥98% was purchased from Jilin University in China.

**1.4.2.2 Chronic Ginsenoside Rb1 treatment**

In study 1 (chapter 2) and study 2 (chapter 3), after acclimatization, mice were then fed a HFD for 16 weeks to induce the late stage of obesity. After 16 weeks of HFD, obese mice were randomized into two groups (n=16 per group) and chronic treated with either daily intraperitoneal (ip) injections of Rb1 (14 mg/kg) or vehicle (saline) for 21 days. This study also included a parallel control group of age-matched mice fed a LC diet. During Rb1 treatment the animal’s food intake and body weight were recorded daily. On day 18 of Rb1 treatment, an intraperitoneal glucose tolerance test was performed (detailed in
section 1.4.3). On day 21 of Rb1 treatment, an intracerebroventricular cannula was implanted and central leptin sensitivity test was performed (detailed in section 1.4.4). Figure 1.2 was the flow chart of triterpene saponin chronic treatment on experimental obese mice.

This part of chronic Rb1 treatment was both included in chapter 2 and chapter 3.

![Flow chart of triterpene saponin chronic treatment on experimental obese mice.](image)

**Figure 1.2 Flow chart of triterpene saponin chronic treatment on experimental obese mice.**

Wks: weeks; D: day; GTT: glucose tolerance test; BW: body weight; FI: food intake; h: hour (s); icv: intracerebroventricular.

### 1.4.2.3 Chronic Teasaponin treatment

In study 3 (chapter 4), mice were placed on the HFD for 16 weeks. The animals were then randomized into two groups, and administered either teasaponin (10 mg/kg) or vehicle (saline) i.p. injections daily for 21 days. Age-matched, LC diet control mice were maintained on the lab chow diet. Body weight and food intake were measured daily. Teasaponin (96%, C_{57}H_{90}O_{26}, MW=1200) was purchased from the Aladdin Chemistry Co. Ltd, China. On day 18 of TS treatment, an intraperitoneal glucose tolerance test was performed (detailed in section 1.4.3). On day 21 of TS1 treatment, an intracerebroventricular cannula was implanted and central leptin sensitivity test was performed (detailed in section 1.4.4). Figure 1.2 showed the flow chart experiment.

This part of chronic teasaponin treatment was included in chapter 4.

### 1.4.3 Intraperitoneal glucose tolerance test (IPGTT)

On day 18 of Rb1 (or Teasaponin) treatment, the mice were injected intraperitoneally
with glucose at a dose of 0.5 g/kg after an overnight fasting. Blood samples were taken from the tail vein, and blood glucose concentration determined using a glucometer (Freestyle; Abbott Diabetes Care, Alameda, CA) at 0 (fasting), 30, 60 and 120 minutes after glucose injection.

1.4.4 Central leptin sensitivity

Mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. An intracerebroventricular (icv) cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) (Paxinos and Franklin, 2002). Five days after implantation the mice were fasted for 6 hours, and either leptin (0.1 µg/3 µl) or saline (3 µl) was injected into the lateral ventricle through the cannula. Food intake was measured for 1, 4, and 24 hours, and body weight was measured 24 hours after the leptin or vehicle injection.

The accuracy of the cannula placement was histologically confirmed by the methylene blue which was icv injected into the pre-experimental test animal before decapitated. The cannula track of each animal was confirmed when sliced the brain. Figure 1.2 showed the confirmation of correct placement of cannula.
Figure 1.3 A pre-experimental histologically confirmation of the correct cannula placement at the lateral ventricle and the correct location of injection were carried out using a Methylene Blue injection (A). The accuracy of the cannula placement of the experimental animals was confirmed by examining the cannula track in the brain section of each animal. Sections were compared with the corresponding section from the mice brain atlas (B).

1.4.5 Blood and Tissue collection

Following a further four day interval after examining central leptin sensitivity, mice were fasted for 6 hours, administered an icv injection of either leptin (0.1 μg/3 μl) or saline (3 μl) and then euthanized 1 hour later for tissue collection. Blood, white adipose tissue, liver and brain tissue were collected. The plasma was collected after centrifugation at 3000rpm for 15 minutes. Plasma and other tissues were stored at -80 °C for further analyses.

Using a standard mouse brain atlas (Paxinos and Franklin, 2002), 500 µm frozen brain sections were cut from Bregma -1.22 mm to -2.72 mm (for mediobasal hypothalamus), Bregma 2.8-1.98 mm (for prefrontal cortex) using a cryostat at a temperature of -18 °C. The mediobasal hypothalamus were dissected and then collected using a Stoelting Brain Punch (#57401, 0.5 mm diameter, Wood Dale, Stoelting Co, USA) in an overlapping pattern over the 3rd ventricle (White et al., 2009). The prefrontal cortex was also dissected and collected by the stoelting brain punch.

1.4.6 Determination of plasma leptin, insulin, peptide YY (PYY) and adiponectin

Plasma leptin, insulin and PYY were measured using the mouse metabolic magnetic bead panel kit (Merck Millipore, MA), and adiponectin was assayed with the mouse single plex adiponectin kit (Merck Millipore).

1.4.7 Histological analysis and morphometry

Epididymal fat was fixed in 10% buffered formaldehyde and then embedded in paraffin. Tissue sections (5 μm) were cut and mounted onto polysine slides. The sections were stained with hematoxylin and eosin and photographed at 100× magnification. Using the
image analysis software Image J 1.46r (http://rsbweb.nih.gov/ij/download.html), two fields per section and six sections per fat mass were analyzed to quantify the area and number of adipocytes.

1.4.8 Western blot analysis

As described in our previous study (du Bois et al., 2012), tissue protein from liver (study 1, chapter 2), epididymal fat (study 1, chapter 2), mediobasal hypothalamus (study 1, chapter 2) and prefrontal cortex (study 2, chapter 3; and study 3, chapter 4) was extracted using NP-40 Lysis Buffer. The following antibodies were used: TNF-α (sc-8301), IL-1β (sc-7884), IL-6 (sc-7920), pJAK2 (sc-21870), BDNF (sc-20981), TrkB (sc-), and pAkt (sc-) from Santa Cruz Biotechnology (Dallas, TX); pTrkB 95 from Sigma-Aldrich (St. Louis, MO, USA); and p-ΙκBα (#2859), p-IKK (#2697), p-STAT3 (#9145), SOCS3 (#2932), and p-FOXO1 (#9461), pGSK3β (#) from Cell Signaling Technology (Beverly, MA). Bands corresponding to the proteins of interest were analyzed using the automatic imaging analysis system Quantity One (Bio-Rad Laboratories, Hercules, CA). All quantitative analyses were normalized to β-actin as described in our previous study (du Bois et al., 2012).

1.4.9 Novel object recognition test

In animal study 3 (chapter 4), novel object recognition test was performed. This test is based on the innate tendency of rodents to differentially explore novel objects over familiar ones as previously described (Arqué et al., 2008), with minor modifications. In brief, the experimental procedure consisted of habituation, training, and retention sessions. On day 1, for habituation, mice were placed into an open-field box (55 × 55 cm × 35 cm high) for 10 min with a 40 W light bulb in a sound proof room. On day 2, during the training session, two identical objects (A) were placed at opposing corners of the box, 5 cm from the adjacent wall. Each mouse was then placed in the middle of the open-field box and left to explore the objects for 10 min. Ninety minutes later, in the retention
session, one familiar object (A) was replaced with one novel object (B). Each mouse was placed in the middle of the open-field box, and left to explore for another 10 min. The exploration time for the familiar and the new objects was recorded. Memory was operationally defined by the discrimination index for the novel object (DI) as the proportion of time animals spent investigating the novel object minus the proportion spent investigating the familiar one in the testing period in the retention session [Discrimination Index = (Novel Object Exploration Time/Total Exploration Time) – (Familiar Object Exploration Time/Total Exploration Time) × 100].

1.4.10 Primary prefrontal cortical neuronal cultures and treatment

Primary prefrontal cortical neuronal cultures was applied both in study 2 (Chapter 3) and study 3 (Chapter 4). Mouse prefrontal cortical neuronal cultures were prepared from pups at postnatal day 1-2 (C57Bl/6J mice, Perth, Western Australia) as described previously (Hilgenberg and Smith, 2007). Dissociated cells were seeded at an approximate final density of 1 x 10^5 cells/cm² into a Poly-D-Lysine-coated 24-well plate (1.9 cm²/well) for qRT-PCR measurement. For neuronal morphology measurement, the neurons were cultured on Poly-D-Lysine-coated glass coverslips. In study 2 (Chapter 3), at 7 days in vitro, Rb1 (40 µM, refer to (Liao et al., 2002)) was used to treat the cultures simultaneously in the presence of palmitic acid (final concentration of 10 µM, P5585, Sigma-Aldrich, St Louis, MO, USA). Palmitic acid was dissolved following the method described previously (Ross et al., 2010). Four hours after palmitic acid and/or Rb1 pre-exposure, leptin (100 ng/mL, refer to (Valerio et al., 2006), Cat#: 450-31, Peprotech, Rocky Hill, NJ, USA) or vehicle was added to the cultures for another 44 hours before the cells were harvested.

In study 3 (Chapter 4), at 7 days in vitro, 20 µM or 40 µM teasaponin was used to treat the cultures simultaneously in the presence of palmitic acid (10 µM). Four hours after palmitic acid and/or TS pre-exposure, leptin (100 ng/mL, refer to (Valerio et al., 2006), Cat#: 450-31, Peprotech, Rocky Hill, NJ, USA) or vehicle was added to the cultures for
another 44 hours before the cells were harvested.

1.4.11 Quantitative real-time PCR (qPCR)

Total mediobasal hypothalamic RNA (study 1, Chapter 2), and total RNA from cultured cells (study 2, Chapter 3; and study 3, Chapter 4) were extracted using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) and reverse-transcribed to first-strand complementary DNA with the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, Carlsbad, CA), according to the manufacturer’s instructions. qPCR was performed in a 20 µl final reaction volume using SYBR green I master in a Lightcycler 480 (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Primers used are listed in the table of each chapter. Amplification was carried out with 45 cycles of 95 °C for 10 seconds, 60 °C for 30 seconds and 72 °C for 30 seconds. The mRNA expression levels for hypothalamic neuropeptides (study 1, Chapter 2), MAP2, PSD95, SYN and BDNF (study 2, Chapter 3; and study 3, Chapter 4) were normalized to gamma actin, which served as the internal control. Experiments were performed in triplicate. The level of expression for each gene was calculated using the comparative threshold cycle value (Ct) method, using the formula $2^{-\Delta\Delta Ct}$ as described previously (Livak and Schmittgen, 2001; Wilusz et al., 2008).

1.4.12 Immunofluorescence

Immunofluorescence and image analysis were applied in the study 2 (Chapter 3) and study 3 (Chapter 4). For immunocytochemical staining, neurons were washed three times in phosphate-buffered saline (PBS), and then fixed with 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) for 30 min at room temperature. After rinsing with PBS, the neurons were permeabilized with 0.3% Triton-X (Sigma-Aldrich) in PBS (i.e. PBST) for 10 min, and blocked with 5% goat serum in PBST for 1 hour at room temperature. Then anti-BDNF antibody, anti-microtubule-associated protein 2 (MAP2) antibody, anti-
synaptophysin (SYN) antibody, and anti-post-synaptic density protein 95 (PSD 95) antibody were applied overnight at 4°C. MAP2 was visualized by goat anti-mouse secondary antibody conjugated with Alexa Fluor 594. BDNF, SYN, and PSD95 were visualised with isotype-specific donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 488. The details of origin and concentration of antibodies were given in the Table S1. A fluorescence microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) with an attached digital camera was used to obtain MAP2 immunofluorescence images for neuronal morphology analysis. A confocal microscope (Leica TCS SP5 Advanced System, Wetzlar, Germany) equipped with a digital camera was used to obtain images for immunoreactivity analysis of BDNF, PSD95, and SYN.

1.4.13 Neurite length and branching analysis

Neurite length and branching analysis were applied in the study 2 (Chapter 3) and study 3 (Chapter 4). The analysis was performed by the NeuriteQuant program (Dehmelt et al., 2011), which is an open source toolkit “NeuriteQuant V1.23” (http://www.ccb.tudortmund.de/groups/CB/bastiaens/dehmelt/Neurite Quant/) that to be installed as a plugin for image J 1.40g (http://rsbweb.nih.gov/ij/download.html). The quantification of neuronal morphology included: average neurite length, neurite length per cell, neurite number per cell, branches per neurite, and branches per cell. Experiments were independently performed three times, with each time repeated in triplicate (n=9). The image analysis was based on these nine repeats.

1.4.14 Immunoreactivity analysis for BDNF, PSD95, and SYN

In study 2 (Chapter 3), software Image J 1.40g was used to quantify the immunoreactivity of BDNF, PSD95, and SYN. Images were converted into a 16-bit scale for analysis. The mean intensity of the fluorescence of these biomarkers in the cell body was measured. Approximately 1-2 areas (around 10x10 μm² per area) of the cell body per
neuron were analyzed and 7-8 neurons per image were randomly selected. A range of 6-7
images per group from three independent experiments were analyzed. For PSD95 and
SYN, dendrite immunoreactivity was also analyzed. In each dendrite, 2-4 areas (around
10x10 μm² per area) were measured, and 2-4 dendrites per neuron were analyzed.

1.4.15 Statistical analysis

Data were analyzed using the SPSS 19 statistical package (SPSS, Chicago, IL). The two-
tailed student’s t-test was used to compare food intake, adipose tissue histology and
weight, inflammatory markers in epididymal adipose tissue and liver, and hypothalamic
neuropeptides in study 1 (Chapter 2). One-way analysis of variance (ANOVA) was
followed by the post hoc Tukey–Kramer honestly significant difference (HSD) test was
used to analyze final body weight gain, body weight, energy intake, epididymal fat,
visceral fat, liver weight, glucose tolerance test, novel recognition test, plasma cytokines,
central inflammatory markers, central leptin sensitivity, as well as biomarkers of the in
vitro study and neuronal morphology data (study 1-3). A p<0.05 was regarded as
statistically significant, and p<0.10 were considered a trend. Values are expressed as
mean ± SEM.
CHAPTER TWO

Central Inflammation and Leptin Resistance Are Attenuated by Ginsenoside Rb1 Treatment in Obese Mice Fed a High-Fat Diet

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Statement from co-authors

This is to attest that the PhD candidate, Yizhen Wu, contributed significantly to the investigation


Yizhen Wu designed and performed the experimental work, analysed the data, interpreted data, and wrote the manuscript. Y. Yu and X.-F. Huang are supervisors, who have provided comments on experimental design, data analysis, and results interpretation. A. Szabo, and M. Han contributed to the discussion.

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Central Inflammation and Leptin Resistance Are Attenuated by Ginsenoside Rb1 Treatment in Obese Mice Fed a High-Fat Diet

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Abstract

A low-grade pro-inflammatory state is at the pathogenic core of obesity and type 2 diabetes. We tested the hypothesis that the plant terpenoid compound ginsenoside Rb1 (Rb1), known to exert anti-inflammatory effects, would ameliorate obesity, obesity-associated inflammation and glucose intolerance in the high-fat diet-induced obese mouse model. Furthermore, we examined the effect of Rb1 treatment on central leptin sensitivity and the leptin signaling pathway in the hypothalamus. We found that intraperitoneal injections of Rb1 (34 mg/kg, daily) for 21 days significantly reduced body weight gain, fat mass accumulation, and improved glucose tolerance in obese mice on a HF diet compared to vehicle treatment. Importantly, Rb1 treatment also reduced levels of pro-inflammatory cytokines (TNF-α, IL-6 and/or IL-1β) and NF-κB pathway molecules (p-IKKα and p-IκBα) in adipose tissue and liver. In the hypothalamus, Rb1 treatment decreased the expression of inflammatory markers (IL-6, IL-1β and p-p65) and negative regulators of leptin signaling (SOCS3 and PTP1B). Furthermore, Rb1 treatment also restored the anorectic effect of leptin in high-fat fed mice as well as leptin pSTAT3 signaling in the hypothalamus. Ginsenoside Rb1 has potential for use as an anti-obesity therapeutic agent that modulates obesity-induced inflammation and improves central leptin sensitivity in HF diet-induced obesity.


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Introduction

Obesity has reached epidemic proportions and is an important risk factor for the development of type 2 diabetes, cardiovascular disease and cancer. It is generally accepted that a low-grade pro-inflammatory state is at the pathogenic core of obesity and type 2 diabetes [1,2]. This inflammatory response includes elevated levels of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β) and interleukin 6 (IL-6), and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway, including inhibitor kappa B alpha (IKKα) and IkB kinase (IKK) [3,4]. The activation of pro-inflammatory cytokines and NF-κB signaling pathway mediate the transcription of the suppressor of cytokine signaling 3 (SOCS3) and protein-tyrosine phosphatase 1B (PTP1B), negative regulators of insulin and leptin signaling, which induce insulin and leptin resistance in peripheral tissues and the central nervous system [5,6,7]. Obesity-associated inflammation in white adipose tissue and the liver leads to glucose intolerance, insulin resistance and metabolic dysfunction [8,9]. Over-nutrition and obesity also leads to hypothalamic inflammation and stimulation of local pro-inflammatory NF-κB signaling, resulting in the dysfunctions of hypothalamic neurons [4,5]. Furthermore, recent studies have shown that induction of inflammation in the hypothalamus results in experimental obesity, resistance to the anorexigenic hormone leptin, peripheral insulin resistance and defective regulation of food intake and energy expenditure [10,11,12].

Targeted deletion of certain genes important for mediating inflammatory responses protects against the development of hyperglycemia, insulin resistance and obesity in obese mouse models. Disruption of the gene encoding IKKα and the innate immune system receptor Toll-like receptor 4 (TLR4) in mice confers protection from insulin and leptin resistance, and obesity in obese mouse models [5,13]. Also, inhibition of NF-κB signaling using high-dose salicylates confers protection from obesity-induced inflammation and insulin resistance in mice [14]. Activation of hypothalamic NF-κB by central injection of a constitutively active IKKα lentiviral vector interrupts central leptin and insulin signaling, while genetic or viral vector mediated suppression of IKK within the mediobasal hypothalamus protects against obesity and glucose intolerance in mice [5]. Therefore, compounds that attenuate the peripheral and hypothalamic inflammation associated with obesity may prove useful in the management of patients with obesity and type 2 diabetes.

Some plant-derived triterpenoids are anti-inflammatory and inhibit the NF-κB signaling pathway [15]. The tetracyclic triterpenoid ginsenoside Rb1 (Rb1) is the major bioactive compound extracted from ginseng [16,17]. This compound inhibits inflammation in in vivo and in vitro models, including anti-inflammatory effects on the smooth muscle exposed to...
TNF-α [18], the colon of colitis mice [19], and brain tissue in an cerebral ischemia animal model [20]. In high-fat diet-induced obese rats, RB1 significantly reduces food intake and body weight gain [21]. A study by Lin et al. [22] shows that in high-fat diet-induced obese mice, RB1 significantly reduces weight gain, blood glucose, and total cholesterol. However, it is unknown whether RB1 can improve obesity-associated inflammation and central leptin resistance.

Materials and Methods

Animal care and treatment

C57Bl/6 male mice (6 weeks old, average body weight of 19.6 ± 1.4 g) were obtained from the Animal Resources Centre (Perth, Western Australia) and housed in environmentally controlled conditions (temperature 22°C, 12 hour light/dark cycle). All animals were fed a lab-chow (LC) diet (5% fat, Vella Stock Feeds, Doncaster, NSW, Australia) ad libitum for one week and then fed a high-fat (HF) diet for 16 weeks (The HF diet contained 40% of energy as fat, with the fat content consisting of half hard and half sunflower oil. The proportion of saturated fat, n-6 polyunsaturated fat, n-3 polyunsaturated fat and monounsaturated fat were 12%, 16%, 0.4% and 11% respectively. SFIJ-09S, Specialty Feeds, Western Australia). After 16 weeks of HF diet, obese mice (average body weight of 44.1 ± 2.6 g) were randomized into two groups (n = 16 per group) and treated with either daily ip injections of RB1 (14 mg/kg, based on a RB1 dose [10 mg/kg] described previously in rats [21]), and using a body surface area ratio of 0.14 from rat to mouse [23]) or vehicle (saline) for 21 days. This study also included a parallel control group of 4 weeks. Mice fed a LC diet (RB1) purified by high-performance liquid chromatography (HPLC) to >98% was purchased from Jinlin University in China. During RB1 treatment the animals’ food intake and body weight were recorded daily. All procedures were approved by the Animal Ethics Committee of the University of Wollongong, NSW, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The approval ID for this study is AE10/08.

Glucose tolerance test (GTT)

On day 18 of RB1 treatment, the mice were injected intraperitoneally with glucose at a dose of 0.5 g/kg after an overnight fast. Blood samples were taken from the tail vein, and blood glucose concentration determined using a glucometer (Freely, Abbott Diabetes Care, Alameda, CA) at 0 (fasting), 30, 60, and 120 minutes after glucose injection.

Central leptin sensitivity

Central leptin sensitivity was examined in moderate and severely obese mice. For moderate obesity, mice were fed a HF diet for 8 weeks followed by an acute treatment of RB1 (14 mg/kg/day, ip) for 2 days. For severe obesity, the mice were fed a HF diet for 16 weeks and then administered RB1 (14 mg/kg/day, ip) for 21 days. The central leptin sensitivity test was performed as follows. Mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. An intracerebroventricular (icv) cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) [24]. Five days after implantation the mice were fasted for 6 hours, and either leptin (0.1 μg/5 μl) or saline (5 μl) was injected into the lateral ventricle through the cannula. Food intake and body weight were measured for 24 hours after the leptin or vehicle injection.

Blood and Tissue collection

Following a further four day interval after examining central leptin sensitivity, mice were fasted for 6 hours, administered an icv injection of either leptin (0.1 μg/5 μl) or saline (5 μl) and then euthanized 1 hour later for tissue collection. Blood, white adipose tissue, liver and brain tissue were collected. The plasma from mice receiving saline icv injections was collected after centrifugation at 3000rpm for 15 minutes. Plasma and other tissues were stored at −80°C for further analyses.

Using a standard mouse brain atlas [25], 50 μm frozen brain sections were cut from Bregma −2.22 mm to −2.72 mm using a cryostat at a temperature of −18°C. The mediobasal hypothalamus was dissected and then collected using a Stoelting Brain Punch (57401, 0.5 mm diameter, Wood Dale, Stoelting Co, USA) in an overlying pattern over the 3rd ventricle [25].

Determination of plasma leptin, insulin, peptide YY (PYY) and adiponectin

Plasma leptin, insulin and PYY were measured using the mouse metabolic bead panel kit (Merck Millipore, MA), and adiponectin was assayed with the mouse singleplex adiponectin kit (Merck Millipore).

Histological analysis and morphometry

Epithyroid fat was fixed in 10% buffered formaldehyde and then embedded in paraffin. Tissue sections (5 μm) were cut and mounted onto polyvinyl slides. The sections were stained with hematoxylin and eosin and photographed at 100 x magnification. Using the image analysis software Image J 1.44e [http://rsweb.nih.gov/ij/download.html], two fields per section and six sections per fat mass were analyzed to quantify the area and number of adipocytes.

Western blot analysis

As described in our previous study [26], tissue proteins were extracted using NP-40 Lysis Buffer. The following antibodies were used: TNF-α (sc-8301), IL-1β (sc-7884), and IL-6 (sc-7920) from Santa Cruz Biotechnology (Dallas, TX); and p-ERK1/2 (4370), p- JNK1/2/3 (9251), p38 (8690), p-STAT3 (4690), SOCS3 (2833), and p-FOXO1 (#9461) from Cell Signaling Technology (Beverly, MA). Bands corresponding to the proteins of interest were analyzed using the automatic imaging analysis system Quantity One (Bio-Rad Laboratories, Hercules, CA). All quantitative analyses were normalized to β-actin as described in our previous study [26].

Quantitative real-time PCR (qPCR)

Total mediobasal hypothalamic RNA was extracted using the Aurum total RNA mini kit (Bio-Rad Laboratories, Hercules, CA) and reverse-transcribed to first-strand complementary DNA with the high-capacity cDNA reverse transcription kit (AB Applied Biosystems, Carlsbad, CA), according to the manufacturer’s instructions. qPCR was performed in a 20 μl final reaction volume using SYBR green I master in a Lightcycler 480 (F. Hoffmann-La Roche Ltd, Basel, Switzerland). Primers used are listed in Table S1. Amplification was carried out with 45 cycles of 95°C for 10 seconds, 60°C for 30 seconds and 72°C for 30 seconds. The mRNA expression levels for hypothalamic neuro-peptides were normalized to gamma-actin, which served as the internal control. Experiments were performed in triplicate. The level of expression for each gene was calculated using the comparative threshold cycle value (Ct) method, using the formula 2−ΔΔCt as described previously [27,28].

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Figure 1. Effect of chronic administration of Rb1 on food intake, body weight and fat in obese mice fed a HF diet for 16 weeks. Rb1 treatment reduced food intake (A), final body weight gain (C), and visceral and inguinal fat mass (D) \( n = 8 \). Panel B: Body weight of obese mice with or without Rb1 chronic treatment for 21 days. Panel E: Photographs of epididymal fat tissue, and hematoxylin and eosin staining of epididymal fat tissue, scale bar 100 \( \mu m \). Panel F: Adipocyte area in epididymal fat. Panel G: Frequency distribution of adipocyte surface area in epididymal fat, \( *p < 0.05 \) vs. HF control group, \( \dagger p < 0.05 \) vs. LC lean control.

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Statistical analysis

Data were analyzed using the SPSS 19 statistical package (SPSS, Chicago, IL). The two-tailed student’s t-test was used to compare food intake, adipose tissue histology and weight, inflammatory markers in epididymal adipose tissue and liver, and hypothalamic neuropeptides. One-way analysis of variance (ANOVA) was followed by the post hoc Tukey-Kramer honestly significant difference (HSD) test was used to analyze final body weight gain, plasma cytokines, central inflammatory markers, and central leptin sensitivity. A \( p < 0.05 \) was regarded as statistically significant, and \( p < 0.10 \) were considered a trend. Values are expressed as mean \( \pm \) SEM.

Results

Rb1 treatment lowered food intake and prevented weight gain and fat deposition in obese mice on a HF diet. Overall, Rb1 treatment reduced average food intake by 11\% \((p < 0.05)\) in HF diet fed mice, and a reduction of food intake was
observed on days 7, 11, 10, 12, 14, 19 and 20 of Rb1 treatment (all p<0.05, Fig. 1A) compared with HF control group. Rb1 treatment significantly reduced body weight gain (Fig. 1C) and visceral and subcutaneous (inguinal) fat deposition (Fig. 1D and Table 1) in mice maintained on a HF diet. Rb1 treatment also decreased the size of adipocytes (an indication of fat stegrowth), with adipocytes from epididymal visceral fat pads being significantly smaller in response to Rb1 treatment (Fig. 1E and F). The distribution of adipocytes by cell surface area showed a higher proportion of small-sized cells (1,000 µm²) and a lower proportion of larger-sized cells (3,000–7,000 µm²) in the Rb1-treated group compared to the HF group (Fig. 1G).

| Table 1. Weight of fat pads in HF diet-induced obese mice with and without ginsenoside Rb1 treatment. |
|----------------------------------|--------|-------|----------|
|                                | HF     | Rb1   | p-value  |
| Visceral fat (g)                | 2.78±0.17 | 2.75±0.12 | <0.001 |
| Epinephelial fat (g)            | 2.10±0.06 | 1.91±0.06 | <0.001 |
| Perirenal fat (g)               | 0.85±0.06 | 0.58±0.04 | <0.001 |
| Mesenteric fat (g)              | 0.33±0.06 | 0.71±0.06 | 0.236  |
| inguinal fat (g)                | 1.50±0.08 | 0.81±0.05 | <0.001 |

HF: high-fat diet-induced obese mice; Rb1: high-fat diet-induced obese mice treated with ginsenoside Rb1. Visceral fat includes epididymal, perirenal and mesenteric.

*P<0.05 vs. HF group. Data are presented as Mean±SEM.

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Figure 2. Chronic administration of Rb1 improved plasma profiles and glucose tolerance in obese mice fed a HF diet for 16 weeks. Rb1 decreased plasma leptin (A) and insulin (B), improved glucose tolerance (C) and AUC (D), and increased adiponectin (E) and PYY (F) in obese mice (n=8) fed a HF diet for 16 weeks. *P<0.05 vs. HF control group; **P<0.05 vs. LC diet control group. Data are presented as mean ± SEM. Area under the curve for glucose (AUC/glucose) was calculated using the trapezoidal rule.

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Rb1 treatment improved blood hormone profiles for energy balance regulation

HF diet-induced hyperleptinemia was significantly decreased by Rb1 treatment (Fig. 2A). Plasma insulin was elevated in HF diet-induced obese mice, but Rb1 did not significantly reverse hypothyroidism in these animals (Fig. 2B). To evaluate the functional outcome of Rb1 treatment on glucose homeostasis, we conducted a glucose tolerance test (GTT). Blood glucose was reduced by Rb1 treatment at the 30 and 60 minute time points of the GTT (Fig. 2C). The blood glucose area under the curve (AUC) after glucose injection was reduced in Rb1-treated mice compared to HF mice without Rb1 treatment (Fig. 2D). Rb1 also increased plasma adiponectin in HF diet-induced obese mice (Fig. 2E). Circulating concentrations of the anorexigenic peptide PYY were significantly increased in the Rb1 treatment group compared with HF mice (Fig. 2F).

Rb1 treatment decreased inflammation in adipose tissue and the liver

Given the anti-inflammatory properties of Rb1 in aortic smooth muscle, colon and brain [10, 19, 20], we investigated whether Rb1 could reduce low-grade inflammation of adipose and liver tissue in HF diet-induced obese mice. In the epididymal adipose tissue of HF mice treated with Rb1, we found significantly reduced expression of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β, -35%; p<0.05; 0.001), as well as the inflammatory signaling molecule p-IKK (−44%; p<0.001), compared to HF control mice (Fig. 3A). In a statistical trend, Rb1 treatment lowered p-IRβ2 expression in epididymal adipose tissue (−21%; p=0.06, Fig. 3A). For the liver of HF mice treated with Rb1, the expression of TNF-α and IL-6 (−28% and −20%; p<0.05) was also significantly reduced compared to HF mice without Rb1 treatment (Fig. 3B). Rb1 treatment lowered SOCS3 expression in the liver (−34%; p=0.08, Fig. 3B) compared to HF control mice, in a statistical trend. However, no difference was found in the hepatic expression of IL-1β and p-IKK in HF mice with or without Rb1 treatment (Fig. 3B).

Rb1 treatment attenuated hypothalamic inflammation and negative regulators of leptin signaling

A HF diet stimulates pro-inflammatory cytokine mRNA expression in the hypothalamus of rodents [4], and here we investigated if Rb1 treatment could attenuate this inflammation. Using western blot analysis, we confirmed that protein levels of IL-6, TNF-α and p-IRβ2 increased in the mediobasal hypothalamus of HF diet-induced obese mice compared with LC diet mice (Fig. 4A, C and D). The protein levels of SOCS3 and PTPIB, negative regulators of leptin signaling, also increased in the mediobasal hypothalamus of HF fed mice (Fig. 4E and F). Importantly, Rb1 treatment significantly decreased the expression of IL-6, IL-1β, p-IKK, SOCS3 and PTPIB (−44%, −31%, −15%, −20% and −14% respectively; p<0.05). Fig. 4D in the hypothalamus compared with HF control mice.

Rb1 treatment improved central leptin sensitivity and leptin signaling

To evaluate if Rb1 treatment improved central leptin sensitivity in conjunction with the inhibition of hypothalamic inflammation, central leptin sensitivity was examined at two stages in the development of obesity, at 8 and 16 weeks of HF diet. First, we demonstrated that ivc injection of leptin increased energy intake (−31%; p<0.05, Fig. 5A) and body weight gain (p<0.05, Fig. 5D) compared with saline injection in lean LC fed mice. Second, after 8 weeks of HF diet leptin did not suppress energy intake and body weight gain in HF control mice (Fig. 5B and E), while acute Rb1 treatment (2 days) restored leptin sensitivity, evidenced by a 41% reduction in energy intake and a very significant reduction in body weight gain following leptin ivc injection compared to saline ivc injection (p<0.05, Fig. 5B and E). Furthermore, acute Rb1 treatment did not significantly suppress overall food intake and body weight (Table S2). In severely obese control mice fed a HF diet for 16 weeks, leptin ivc injection did not significantly decrease energy intake and body weight gain compared with saline (p>0.05, Fig. 5C and F). With the addition of chronic Rb1 treatment, ivc leptin injections significantly decreased energy intake by −22% and decreased body weight gain by −25% compared to leptin injections in obese mice not treated with Rb1 (p<0.05, Fig. 5C and F). This suggests that the Rb1 chronic treatment increased the ability of leptin to inhibit energy intake and body weight gain. To clarify the mechanism by which chronic Rb1 treatment improved leptin sensitivity, protein expression of the leptin signaling molecules p-STAT3 and p-FOXO1 was measured in the mediobasal hypothalamus. Ivc injection of leptin increased p-STAT3 (53%; p<0.05) in mice fed LC diet, while these responses were not observed in HF diet-induced obese mice (Fig. 6A). After
Figure 4. Chronic Rb1 treatment reduced hypothalamic inflammation and negative regulators of leptin signaling in obese mice.

Chronic treatment of Rb1 significantly decreased the level of IL-6 (A), IL-1β (B), p-IkBα (D), SOCS3 (E) and PTP1B (F) in the mediobasal hypothalamus of obese mice (n=6-8) fed a HF diet for 16 weeks without leptin injection. Panel C: HF diet significantly increased the protein levels of TNFα in the mediobasal hypothalamus of mice. *p<0.05 vs LF group, †p<0.05 vs LC lean control. Data are presented as mean ± SEM.

Rb1 treatment, the response of leptin signaling molecules was restored, with a 42% increase in p-STAT3 (p<0.001) following leptin administration to Rb1-treated HF mice (Fig. 6A). Leptin also increased phosphorylation of FOXO1 in the mediobasal hypothalamus of LF diet fed mice, a response that was blunted in HF mice (Fig. 6B). However, in this case Rb1 treatment did not restore the leptin-induced increase in p-FOXO1 (Fig. 6B). Therefore, in the mediobasal hypothalamus, Rb1 acted on the STAT3 pathways rather than the FOXO1 pathway to restore leptin signaling.

Rb1 treatment affected the hypothalamic neuropeptides regulating energy balance

The effect of Rb1 treatment on hypothalamic neuropeptides expression was examined to investigate the mechanisms by which this compound suppressed food intake and body weight gain. Rb1 treatment significantly increased anorexigenic pro-opio melanocortin (POMC, +75%; p<0.05) and decreased orexigenic agouti-related protein (AgRP, -24%; p<0.05) mRNA expression in the mediobasal hypothalamus of HF mice, but had no effect on orexigenic neuropeptide Y (NPY) mRNA levels (Table 2).

Discussion

In the current study, Rb1 prevented body weight gain and reduced food intake in obese mice fed a HF diet. Rb1 also decreased average food intake during the course of this study. This is similar to the study by Xiong and colleagues, which showed that Rb1 has an anti-obesity effect in rats and its suppression of food intake is not due to malaise, as attested by a conditioned taste aversion test [21]. Importantly, our study extends the mechanism of Rb1 in suppressing food intake. Rb1 treatment increased the anorexigenic hormone, peptide YY (PYY), in the blood and modulated
hypothalamic neuropeptides, specifically by increasing anorexic POMC and decreasing orexigenic AgRP mRNA expression in HF diet-induced obese mice. Rodent models of HF diet-induced obesity are characterized by inflammation in both peripheral tissues and in the hypothalamic regions critical for energy homeostasis [1,8], which is considered an important mechanism linking obesity to glucose intolerance, insulin resistance and leptin resistance. This study has demonstrated that ginsenoside Rb1 treatment provides an anti-obesity-associated inflammatory effect, with the pronounced reduction of peripheral and hypothalamic inflammation and improvement of glucose tolerance and central leptin resistance in HF diet-induced obese mice.

Overnutrition and obesity induce inflammatory responses in peripheral metabolic tissues, which decreases insulin sensitivity in target cells (adipocytes and hepatocytes) and contributes to glucose intolerance and the development of type 2 diabetes [2,8,9,29]. For example, in obese rodents fed a HF diet macrophages infiltrate the liver and increase the mRNA expression of the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 [29]. These cytokines activate NF-κB signaling in hepatocytes, causing hepatic insulin resistance and glucose intolerance [2,3]. In the current study, Rb1 decreased the level of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and the inflammatory signaling molecule (p-IκB) in the adipose tissue and liver, which may have contributed to the improved glucose tolerance observed in Rb1 treated diet-induced obese mice. Furthermore, it is well-documented that adiponectin ameliorates insulin resistance and reduces fatty acid levels in rodents [30], by decreasing hepatic gluconeogenesis and increasing lipid oxidation in muscle [31,32]. In this study, the increased adiponectin levels after Rb1 treatment may contribute to the improved glucose tolerance and reduced fat accumulation that we observed in obese mice treated with this compound.

Our study demonstrated that chronic treatment with Rb1 suppressed inflammation in the mediobasal hypothalamus of diet-induced obese mice, as shown by decreased protein expression of IL-6, IL-1β and p-IκB in this region. In accordance with our results, another study demonstrated that acute oral administration of Rb1 significantly reduced IL-6, IL-1β and TNF-α mRNA expression in mouse brain tissue, and inhibited morphological activation of microglia following intraperitoneal injections of lipopolysaccharide-endotoxin [33]. Recently, Thaler and colleagues demonstrated that mice and rats fed a HF diet had increased TNF-α and IL6K/NEFα mRNA expression in the hypothalamus [4]. Hypothalamic inflammation is considered a key pathology of obesity in rodents and humans [34], leading to central leptin resistance through activation of the negative regulators of leptin signaling, SOCS5 and PTP1B [5,12]. Our results demonstrate that Rb1 decreased the upregulation of SOCS5 and PTP1B in the hypothalamus of HF diet-induced obese mice. Therefore, the inhibition of SOCS5 and PTP1B and attenuation of hypothalamic inflammation, contributes to the therapeutic effect of Rb1 on central leptin resistance observed in our mouse model.

The adipocyte-derived hormone leptin promotes negative energy balance through various signaling pathways (STAT3 and FOXO1) in the hypothalamus. Constitutive activation of the inflammatory signaling molecule IκBα in the hypothalamus of mice impaired STAT3 phosphorylation in response to central...
The anti-inflammatory and leptin sensitizing effects of Rb1 treatment could be due to multiple factors. It is likely that reduced food intake leads to a reduction in fat deposition that in turn reduces pro-inflammatory cytokines, glucose intolerance and improves leptin sensitivity. However, a direct effect of Rb1 of inhibiting inflammation cannot be completely excluded [18,21]. For example, it has been reported in an in vitro study that Rb1 directly inhibited inflammatory responses in rat aortic smooth muscle cells [18]. Furthermore, in a rat study by Xiong et al, paired rats with comparable food intake to Rb1 treatment rats did not show the improved glucose tolerance evident in Rb1 treated animals, suggesting a direct effect of Rb1 in improving glucose metabolism [21].

Peptide YY (PYY) is a gut-brain anorexogenic hormone that promotes negative energy balance by reducing appetite [38]. Peripheral infusion of PYY reduces food intake in rodents [39], and transgenic mice overexpressing PYY have increased plasma PYY concentrations, and are protected against diet-induced obesity [38]. In the present study, the increased plasma PYY in Rb1 treated HF diet-induced obese mice may have contributed to the negative energy balance, lower body weight gain and fat accumulation in these animals. The mechanism by which Rb1 treatment increased circulating PYY levels remains to be determined. However, PYY is predominantly secreted by intestinal L cells located in the distal gastrointestinal tract [40], and it has been reported that the PYY levels are decreased in patients with inflammatory bowel disease [41]. In addition, three days of oral Rb1 treatment potently inhibited the expression of EN1β and EN1β in the inflamed colon of mice with colitis [19]. Since the colon of obese mice overexpresses pro-inflammatory cytokines [42], an anti-inflammatory effect of Rb1 in the gastrointestinal tract may have increased PYY secretion in obese mice.

The melanocortin system comprises anorexogenic POMC expressing neurons and orexigenic AgRP expressing neurons in the arcuate nucleus of the mediodbasal hypothalamus [43]. α-melanocortin-stimulating hormone (α-MSH), a post-translational product of the POMC gene, binds to the melanocortin receptor 1 (MC1R) and triggers an anorectic signal in the hypothalamus, while AgRP (an inverse agonist of MC1R) prevents α-MSH binding to MC1R. Chronic Rb1 treatment significantly increased POMC and inhibited AgRP mRNA expression in high-fat diet fed mice with central leptin resistance. The inhibition of leptin-induced FOXO1 phosphorylation in obese mice with central leptin resistance indicates the impairment of leptin signaling occurs at the step of FOXO1 phosphorylation or upstream. In the current study, treatment of obese mice with Rb1 restored leptin induced activation of STAT3 phosphorylation, but not phosphorylation of FOXO1 in the mediobasal hypothalamus. Therefore, in the hypothalamus, Rb1 acted on the STAT3 signaling pathway rather than the FOXO1 pathway to restore leptin signaling and sensitivity, although the precise mechanisms need further examination.

Figure 6. Chronic Rb1 treatment improved central leptin signaling in obese mice fed a HF diet for 16 weeks. The phosphorylation of STAT3 (A) and FOXO1 (B) in the mediobasal hypothalamus 1 hour after the iv injection of leptin or saline in mice fed a LC diet and obese mice fed a HF diet for 16 weeks with or without chronic treatment of Rb1 (n = 6–8). *p < 0.05 vs. vehicle + saline group; **p < 0.05 vs. vehicle + leptin group; ***p < 0.05 vs. LC + saline group. Data are presented as mean ± SEM.

Table 2. Relative mRNA expression of neuropeptides in the mediobasal hypothalamus in obese mice fed a HF diet for 16 weeks with or without Rb1 chronic treatment.

<table>
<thead>
<tr>
<th>Neuropeptide</th>
<th>HF (n = 5)</th>
<th>Rb1 (n = 5)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>POMC</td>
<td>1.01 ± 0.14</td>
<td>1.75 ± 0.23</td>
<td>0.004</td>
</tr>
<tr>
<td>AgRP</td>
<td>1.00 ± 0.05</td>
<td>0.76 ± 0.07</td>
<td>0.046</td>
</tr>
<tr>
<td>NPY</td>
<td>1.07 ± 0.04</td>
<td>0.90 ± 0.09</td>
<td>0.464</td>
</tr>
</tbody>
</table>

HF: High-fat diet-induced obese mice; Rb1: high-fat diet-induced obese mice treated with ginsenoside Rb1. *p < 0.05 vs. HF group. Data are presented as Mean ± SEM. doi:10.1371/journal.pone.0029018.t002

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Yizhen WU
mice, implying that Rb1 exerts an ameliorative action at least partially by targeting the melanocortin system, the POMC and AgRP neurons. It is known that these neurons located in the mediodorsal hypothalamus receive and integrate the signaling of various gut and adipokine hormones, including PYY, leptin and insulin. In our study, the effect of Rb1 treatment on the hypothalamic melanocortin system may be due to increased plasma PYY, and the improvement of hyperglycemia and central leptin sensitivity after Rb1 treatment.

In summary, this study has demonstrated that ginsenoside Rb1 treatment inhibits inflammation in the adipose tissue, liver and hypothalamus of HF diet-induced obese mice. Treatment with Rb1 resulted in the improvement of glucose tolerance, central leptin sensitivity and hypothalamic leptin signaling (p-STAT3). We have also shown that Rb1 treatment increased the circulating concentrations of the ameliorative hormone PYY and regulated melanocortin POMC/AgRP neuropeptides in the mediodorsal hypothalamus, which contribute to negative energy balance. Ginsenoside Rb1 has the potential for use as an antiobesity therapeutic agent that functions by modulating obesity-induced inflammation and improving central leptin sensitivity in HF diet-induced obesity.

References

Supporting Information
1. Table S1: The primers used in qPCR for neuropeptide mRNA measurement.
2. Table S2: Effects of acute Rb1 administration (14 mg/kg, ip, two days) on body weight and food intake in obese mice after a high-fat diet feeding for 8 weeks.

Acknowledgments
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Author Contributions
Conceived and designed the experiments: YZW YHY XHF. Performed the experiments: YZW YHY. Analyzed the data: YZW YHY. Contributed reagents/materials/analysis tools: YZW YHY. Wrote the paper: YZW YHY AZ MTH XHF.


Table S1 The primers used in qPCR for neuropeptide mRNA measurement

<table>
<thead>
<tr>
<th>GENE</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>NCBI reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>ATACTACTCCGC</td>
<td>GTGTCTCAGGGCTG</td>
<td>NM_023456.2</td>
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<tr>
<td></td>
<td>TCTGCGAC</td>
<td>GATCT</td>
<td></td>
</tr>
<tr>
<td>AgRP</td>
<td>AGTTGTGTCTCTG</td>
<td>CTGATGCCCTTCAG</td>
<td>NM_007427.2</td>
</tr>
<tr>
<td></td>
<td>CTGTTGGC</td>
<td>TGGAG</td>
<td></td>
</tr>
<tr>
<td>POMC</td>
<td>CCATAGATGTGT</td>
<td>CCAGCGAGAGGTC</td>
<td>NM_008895.3</td>
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<td></td>
<td>GGAGCTGG</td>
<td>GAGTT</td>
<td></td>
</tr>
<tr>
<td>γ-actin</td>
<td>GCTAACAGAGA</td>
<td>CAGATGCATAAGA</td>
<td>NM_009609.2</td>
</tr>
<tr>
<td></td>
<td>GAAGATGACG</td>
<td>GACAGC</td>
<td></td>
</tr>
</tbody>
</table>
Table S2 Effects of acute Rb1 administration (14 mg/kg, ip, two days) on body weight and food intake in obese mice after a high-fat diet feeding for 8 weeks

<table>
<thead>
<tr>
<th></th>
<th>HF</th>
<th>HF + Rb1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BW before Rb1 treatment (g)</td>
<td>29.84 ± 0.49</td>
<td>29.59 ± 0.47</td>
</tr>
<tr>
<td>BW after Rb1 treatment (g)</td>
<td>30.12 ± 0.47</td>
<td>29.89 ± 0.43</td>
</tr>
<tr>
<td>FI of 24 hours after Rb1 treatment (g)</td>
<td>3.39 ± 0.16</td>
<td>3.44 ± 0.10</td>
</tr>
</tbody>
</table>

HF: high-fat diet-induced obese mice; Rb1: ginsenoside Rb1 treatment; BW: body weight; FI: food intake. Data are presented as mean ± SEM.
CHAPTER THREE

Ginsenoside Rb1 Improves Leptin Sensitivity in the Prefrontal Cortex in Obese Mice

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Statement from co-authors

This is to attest that the PhD candidate, Yizhen Wu, contributed significantly to the investigation


Yizhen Wu designed and performed the experimental work, analysed the data, interpreted data, and wrote the manuscript. Y. Yu and X.-F. Huang are supervisors, who have provided comments on experimental design, data analysis, and results interpretation. C. Bell contributed to the discussion.

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Ginsenoside Rb1 improves leptin sensitivity in the prefrontal cortex in obese mice

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Summary

Aim: Obesity impairs leptin-induced regulation of brain-derived neurotrophic factor (BDNF) expression and synaptogenesis, which has been considered to be associated with the incidence of neuronal degenerative diseases, cognitive decline, and depression. Ginsenoside Rb1 (Rb1), a major bioactive component of ginseng, is known to have an obesity effect and improve cognition. This study examined whether Rb1 can improve central leptin effects on BDNF expression and synaptogenesis in the prefrontal cortex during obesity using an in vivo and an in vitro model.

Result: Ginsenoside Rb1 (Rb1) chronic treatment improved central leptin sensitivity, leptin-JAK2-STAT3 signaling, and leptin-induced regulation of BDNF expression in the prefrontal cortex of high-fat diet-induced obese mice. In cultured prefrontal cortical neurons, palmitic acid, the saturated fat, impaired leptin-induced BDNF expression, reduced the immunoreactivity and mRNA expression of synaptic proteins, and impaired leptin-induced neurite outgrowth and synaptogenesis. Importantly, Rb1 significantly prevented these pernicious effects induced by palmitic acid.

Conclusion: These results indicate that Rb1 reverses central leptin resistance and improves leptin-BDNF-neurite outgrowth and synaptogenesis in the prefrontal cortical neurons. Thus, Rb1 supplementation may be a beneficial avenue to treat obesity-associated neurodegenerative disorders.

Keywords
BDNF, ginsenoside Rb1, leptin, neurite outgrowth, prefrontal cortex, synaptogenesis

1 INTRODUCTION

Obesity increases the incidence of neuronal degenerative diseases, cognitive decline, and depression,1,2,3 which have been linked to a high intake of dietary fat.4,5 In humans, an increased intake of dietary fat results in cognitive impairment in later life6 and is strongly associated with cognitive decline in women with type 2 diabetes.7 The prefrontal cortex is a major regulatory center for cognitive function.8 Impairment in the prefrontal cortex has been recognized as a feature of obesity.9 Positron emission tomography shows low postprandial activation in the dorsolateral prefrontal cortex in obese individuals.10,11 Furthermore, an increased body mass has been linked to cognitive deficits and decreased prefrontal cortical activity.12 Brain-derived neurotrophic factor (BDNF) promotes neurite outgrowth (ie, elongation and branching) and synaptogenesis, as well as the regulation of energy balance.13,14 Aberrant BDNF signaling is associated with obesity, cognitive impairment, and other neurodegenerative diseases.15,16 Furthermore, leptin, secreted by adipocytes, elevates BDNF expression in the hypothalamus17 and brainstem,18 promoting negative energy balance. Leptin increases hippocampal BDNF in normal mice, but not in diet-induced obese...
mice with depressive-like behaviors, suggesting central leptin resistance in the obese mice. Therefore, these evidences suggest that leptin-induced activation of BDNF in the central nervous system may be involved in cognition and synaptogenesis, while brain leptin resistance could contribute to obesity-related neurodegenerative diseases.

Ginseng, a traditional herbal medicine, is reported to be an adaptogen that enhances body stability against physical loads, and is also suggested to improve cognitive functions. Extracts of ginseng promote neurite outgrowth of spinal cord neurons. Ginsenoside Rb1 (Rb1) is one of the major bioactive saponins in ginseng. Recent animal and cell models show that Rb1 has neuroprotective and antioxidative effects. For example, Rb1 increases BDNF expression in the forebrain of rats with cerebral ischemia. In vitro, Rb1 reduces the neurotoxic effect of glutamate on neurite outgrowth in neurons. Our previous study has demonstrated that Rb1 improves leptin signaling in the hypothalamus of obese mice. The main aim of this study was to examine whether Rb1 can improve central leptin effects on BDNF expression and synaptogenesis in the prefrontal cortex during obesity using an in vivo and an in vitro model. Firstly, we investigated the effect of Rb1 on leptin signaling in the prefrontal cortex of high-fat diet-induced obese mice. Furthermore, we investigated the effect of Rb1 on neurite outgrowth and synaptogenesis in cultured primary prefrontal cortical neurons in response to leptin and palmitic acid, one of the most abundant saturated fatty acids in human diet.

2 MATERIALS AND METHODOLOGY

2.1 Experimental animals and Rb1 treatment

Sixty C57Bl/6J male mice (6 weeks old, body weight: 19.6 ± 1.4 g) were obtained from the Animal Resources Centre (Perth, WA, Australia), and housed in environmentally controlled conditions (temperature 22°C, 12 hour light/dark cycle). All mice were fed a lab chow diet (LC, 5% fat, Vella Stock Feeds, Donnside, NSW, Australia) to acclimate for 1 week. Throughout the study, LC was served as the low fat control diet and was provided ad libitum except where noted. All procedures were approved by the Animal Ethics Committee, University of Wollongong, NSW, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. After acclimatization, the mice were fed a high-saturated fat diet (40% fat in calories) for 16 weeks and developed obesity (average body weight: 44.2 ± 2.6 g). Subsequently, the obese mice were randomized into two groups and intraperitoneally (ip) administered either ginsenoside Rb1 (Rb1, purity ≥98% [HPLC], purchased from Jilin University in China) at a dose of 14 mg/kg/d or vehicle (saline) for 25 days. Body weight and food intake for 24 hours (weight of food placed in hopper) were monitored every 2 days. Energy intake (Kcal/24 hours) was calculated as (food intake) × (energy density of food in Kcal/g). Average energy intake was calculated as [total energy intake measured] / (number of measurement).

2.2 Intraportaline glucose tolerance test (IPGGT)

On day 18 of Rb1 treatment, the mice were fasted overnight and injected (ip) with glucose at a dose of 0.5 g/kg. Blood samples were taken from the tail vein, and blood glucose measured using a glucometer (Abbott Diabetes Care, Alameda, CA, USA) at 0 (fasting), 30, 60, and 120 minutes following glucose administration.

2.3 Central leptin sensitivity test

After Rb1 treatment for 21 days, the mice were anesthetized by isoflurane inhalation and placed in a stereotactic device. The mice were implanted with an intracerebroventricular (icv) cannula into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm inferior to the surface of the skull). Five days after cannula implantation, the mice were fasted for 6 hours, and then administered with either leptin (0.1 μg/3 μL) or saline (3 μL) into the lateral ventricle through the cannula. Food intake was measured 1 and 4 hours after the leptin or vehicle injection. Body weight gain was recorded 24 hours after icv injection.

2.4 Blood and tissue collection

Following a 4 day interval after measuring central leptin sensitivity, the mice were fasted for 6 hours, and were then given an icv injection of leptin or vehicle. The mice were sacrificed one hour after icv injection. Blood samples were collected in EDTA-coated tubes. Plasma was collected after centrifugation at 845 x g for 15 minutes. Brain tissue was removed and immediately stored in liquid nitrogen. Epididymal fat (left and right sides) was dissected, weighed, and immediately stored in liquid nitrogen. Plasma, brain, and epididymal fat samples were then transferred to a −80°C freezer until further analysis.

2.5 Determination of plasma leptin and insulin

Plasma leptin and insulin were measured using the mouse metabolic magnetic bead panel kit (Merck Millipore, Billerica, MA, USA).

2.6 Western blotting

Prefrontal cortical tissue, equivalent to the prefrontal cortex, was identified and dissected at the level of Bregma 2.8-1.98 mm according to a standard mouse brain atlas. Tissue protein was extracted in an NP-40 lysis buffer as described in our previous study. The antibodies were used as follows: p42/p44 (sc-21870) and BDNF (sc-20981) from Santa Cruz Biotechnology Inc (Santa Cruz, CA, USA); and p53 (2873) from Cell Signaling Technology (Beverly, MA, USA). Bonds corresponding to the proteins of interest were scanned, and their density analyzed using the Quantity One automatic imaging analysis system (Bio-Rad Laboratories, Hercules, CA, USA). All quantitative analyses were normalized to β-actin, based on our previous study.
FIGURE 1 Chronic Rb1 prevented weight gain, fat deposition, and improved glucose tolerance in obese mice fed a high-saturated fat diet for 16 wk. Glibenclamide Rb3 (Rb3) treatment reduced average energy intake (A), prevented body weight gain (B) since day 9; reduced epidymal fat mass (C); as well as lowered blood glucose at time point 30 min and 60 min in the glucose tolerance test (D). LC, lab chow fed mice: Ob., high-saturated fat diet-induced obese mice without treatment; Ob + Rb1, high-saturated fat diet-induced obese mice chronic treated with glibenclamide Rb1. Data are presented as mean ± SEM (n = 6-8). *P < 0.05 vs LC, **P < 0.05 vs Ob.

2.7 | Primary prefrontal cortical neuronal cultures and treatment

Mouse prefrontal cortical neuronal cultures were prepared from pups at postnatal day 1-2 (C57Bl/6J mice, Perth, Western Australia) as described previously. Dissociated cells were seeded at an approximate final density of 1 x 10^5 cells/cm^2 into a Poly-D-Lysine-coated 24-well plate (1.2 cm^2/well) for qRT-PCR measurement. For neuronal morphology measurement, the neurons were cultured on Poly-D-Lysine-coated glass coverslips. At 7 days in vitro, Rb1 (40 μM/mL, refer to) was used to treat the cultures simultaneously in the presence of palmitic acid (final concentration of 10 μM/mL, P5585, Sigma-Aldrich, St Louis, MO, USA). Palmitic acid was dissolved following the method described previously. Four hours after palmitic acid and/or Rb1 pre-exposure, leptin (100 ng/mL, refer to), Cat# 450-31, Peprotech, Rocky Hill, NJ, USA) or vehicle was added to the cultures for another 44 hours before the cells were harvested.

2.8 | Immunofluorescence staining

For immunocytochemical staining, neurons were washed three times in phosphate-buffered saline (PBS), and then fixed with 4% paraformaldehyde (Sigma-Aldrich) for 30 minutes at room temperature. After rinsing with PBS, the neurons were permeabilized with 0.3% Triton-X (Sigma-Aldrich) in PBS (x, PBSx) for 10 minutes, and blocked with 5% goat serum in PBS for 1 hour at room temperature. Then anti-BDNF antibody, antimicrotubule-associated protein 2 (MAP2) antibody, antisynaptophysin (SYN) antibody, and antipostynaptic density protein 95 (PSD95) antibody were applied overnight at 4°C. MAP2 was visualized by goat anti-mouse secondary antibody conjugated with Alexa Fluor 594. BDNF, SYN, and PSD95 were visualized with isotype-specific donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 488. The details of origin and concentration of antibodies were given in the Table S1. A fluorescence microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) with an attached digital camera was used to obtain MAP2 Immunofluorescence images for neuronal morphology analysis. A confocal microscope (Leica TCS SPS Advanced System, Wetzlar, Germany) equipped with a digital camera was used to obtain images for immunoreactivity analysis of BDNF, PSD95, and SYN.

2.9 | Neurite length and branching analysis

The quantification of neuronal morphology was carried out using the NeuriteQuant program, including the following: average neurite length, neurite length per cell, neurite number per cell, branches per neurite, and branches per cell. Experiments were independently performed three times, with each time repeated in triplicate (n = 9). The image analysis was based on these nine repeats.

2.10 | Immunoreactivity analysis for BDNF, PSD95, and SYN

Software Image J 1.40 g was used to quantify the immunoreactivity of BDNF, PSD95, and SYN. Images were converted into a 16-bit scale for analysis. The mean intensity of the fluorescence of these biomarkers in the cell body was measured. Approximately, 1-2 areas (around 10 x 10 μm² per area) of the cell body per neuron were analyzed, and 7-8 neurons per image were randomly selected. A range of 6-7 images per group from three independent experiments was analyzed. For PSD95 and SYN, dendrite immunoreactivity was also analyzed. In each dendrite, 2-4 areas (around 10 x 10 μm² per area) were measured, and 2-4 dendrites per neuron were analyzed.

2.11 | RNA extraction and qRT-PCR

Total RNA was extracted from the cultured cells using Aurum total RNA mini kits (Bio-Rad Laboratories). Complementary DNA synthesis and quantitative real-time PCR (qPCR) were performed as previously described. The primers used are listed in Table S2. Amplification was carried out with 45 cycles of: 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. The mRNA expression levels for MAP2, PSD95, SYN, and BDNF were normalized to g-actin, which served as the internal control. Experiments were performed in triplicate.
2.12 Statistical analysis

Data were analyzed using the SPSS 19 statistical package (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by the post hoc Tukey-Kramer honestly significant difference (HSD) test was used to analyze the body weight change, energy intake, epididymal fat, glucose tolerance test and leptin signaling of the LC, obese, and Rb1 treatment groups in response to ivc leptin/saline, as well as biomarkers of the in vitro study and neuronal morphology data. A P-value < 0.05 was considered statistically significant. Data are presented as mean ± SEM.

3 RESULTS

3.1 Ginsenoside Rb1 reduced energy intake, prevented weight gain, fat deposition, and improved glucose tolerance in obese mice

As shown in Figure 1A, high-saturated fat diet-induced obese mice showed higher average energy intake than LC lean mice, while Rb1 treatment decreased the average energy intake. Meanwhile, Rb1 treatment significantly reduced body weight gain in obese mice from day 9 to the end of the treatment (Figure 1B). Ginsenoside Rb1 (Rb1) also reduced the epididymal fat mass in obese mice, although it was still higher than LC mice (Figure 1C). In the IGTTT, the Rb1-treated group showed lower glucose at 30 and 60 minutes after glucose injection compared to the obese group without treatment (Figure 1D). However, at 0, 30, and 120 minutes, blood glucose in Rb1 group was still higher than the LC group.

3.2 Ginsenoside Rb1 restored central leptin sensitivity and significantly reduced hyperleptinemia in obese mice

In the LC lean mice, leptin significantly reduced energy intake for 4 hours after the ivc leptin injection (all P < 0.05, Table S3). However, in the obese mice, the central leptin injection failed to suppress energy intake (all P > 0.05). Importantly, in the Rb1 treatment group, leptin significantly decreased food intake for 4 hours compared with saline in the obese group [Ob + Rb1]/leptin vs [Ob + Rb1]/saline, P < 0.05, suggesting Rb1 improved central leptin sensitivity in the obese mice.

Plasma leptin was significantly higher in the obese mice compared with the LC mice (Table S3). Ginsenoside Rb1 (Rb1) treatment lowered plasma leptin in the obese mice (Ob/saline: 16.9 ± 2.0 ng/mL, [Ob + Rb1]/saline: 8.9 ± 1.1 ng/mL, P < 0.05), but ivc leptin injection did not further decrease plasma leptin.

3.3 Ginsenoside Rb1 improved leptin-JAK2-STAT3 signaling and leptin-induced increase in BDNF in the prefrontal cortex of obese mice

We examined leptin-JAK2-STAT3 signaling in the prefrontal cortex of obese mice induced by high-saturated fat diet with and without Rb1 treatment. Western blot showed that central leptin administration evoked pJAK2 (+40%, P < 0.05) in the prefrontal cortex of the LC lean mice, but not the obese mice (P > 0.05, Figure 2A). Importantly, after Rb1 treatment, leptin administration significantly increased pJAK2 compared with saline (+28%, P < 0.05) in the obese mice. There was a similar result in the pSTAT3, downstream step of leptin/pJAK2

**FIGURE 2** Chronic Rb1 treatment improved leptin-pJAK2-pSTAT3 signaling and leptin-mediated brain-derived neurotrophic factor (BDNF) expression in the prefrontal cortex of obese mice fed a high-saturated fat diet for 16 wk. Protein expression level of pJAK2 (A), pSTAT3 (B), and BDNF (C) responding to ivc leptin or saline in the prefrontal cortex in LC lean mice and high-saturated fat diet-induced obese mice with or without Rb1 treatment. [A] mg/kg. ip 3 wk. LC lab showed mice. Ob, high-saturated fat diet-induced obese mice without treatment; Ob + Rb1, high-saturated fat diet-induced obese mice chronic treated with ginsenoside Rb1. Data are presented as mean ± SEM (n = 7–9).

*P < 0.05 vs LC/saline, †P < 0.05 vs Ob/saline, ‡P < 0.05 vs [Ob+Rb1]/saline
signaling in the LC, obese, and Rb1-treated obese mice (Figure 2B). Leptin significantly increased pSTAT3 by 68% in the prefrontal cortex of LC mice, while in the obese mice pSTAT3 only increased by 23% after the leptin injection. Following chronic Rb1 treatment, pSTAT3 increased by 81% in response to leptin compared with the saline injection, suggesting that Rb1 improved leptin-JAK2-pSTAT3 signaling in the obese mice. We also determined the leptin-stimulated expression of BDNF in the prefrontal cortex. Results showed that in the LC group, leptin significantly increased BDNF expression in the prefrontal cortex by +41% (Figure 2C), but not in the obese mice. Chronic Rb1 treated mice not only increased BDNF expression in the prefrontal cortex, but also facilitated leptin’s action in increasing BDNF expression (P < 0.05).

3.4 | Ginsenoside Rb1 Increased leptin-induced BDNF expression in the prefrontal cortical neurons

Using cultured primary prefrontal cortical neurons, the effect of Rb1 on the leptin-stimulated BDNF expression under the circumstance of saturated fatty acid, palmitic acid exposure was examined. Leptin treatment significantly increased BDNF immunoreactivity by 65% (P < 0.001, Figure 3A-B). However, in neurons pretreated with palmitic acid, leptin failed to increase BDNF immunoreactivity (P > 0.05). Importantly, with Rb1 treatment, leptin increased BDNF immunoreactivity by 40% (P = 0.020) in palmitic acid pretreated neurons, suggesting that Rb1 is able to prevent palmitic acid-induced leptin insensitivity. Consistent with the immunocytochemistry, a similar pattern of BDNF mRNA expression was observed (Figure 3C).

3.5 | Palmitic acid reduced leptin-stimulated neurite outgrowth in the prefrontal cortical neurons, which can be reversed by Rb1 treatment

Following verification of BDNF, we examined neurite outgrowth, including neurite branching and length, in cultured primary cortical neurons (Figure 4). Leptin treatment significantly increased neurite branching (branches per neurite: +77%, P = 0.001, Figure 4D; branches per cell: +60%, P = 0.001, Figure 4E) compared to the saline treatment in cortical neurons. However, palmitic acid pretreatment significantly suppressed leptin-stimulated branching. Ginsenoside Rb1 (Rb1) significantly ameliorated palmitic acid-induced impairment in leptin-stimulated branching compared with the palmitic acid pretreatment (branches per neurite: +45%, P = 0.018, Figure 4D; branches per cell: +56%, P = 0.001, Figure 4E).

Ginsenoside Rb1 (Rb1) significantly reversed the palmitic acid-induced decrease in average neurite length and total neurite length per cell (P = 0.009 and P = 0.045, respectively, Figure 4G-H). Firstly, leptin significantly increased average neurite length and total neurite length per cell (both P < 0.001) compared to the saline control group. However, after exposing cortical neurons to palmitic acid, leptin did not increase the average neurite length nor the total neurite length per cell (both
Figure 4: Ginsenoside Rb1 (Rb1) reversed the impairment of leptin-mediated neurite elongation and branching in the prefrontal cortical neurons. A, Cultured cortical neurons stained with MAP2 for neuronal analysis; B, Neuronal analysis tracing generated by the NeuriteQuan toolkit. C, Represented MAP2 immunofluorescence staining images in cortical neurons; all parameters (D-I) were automatically analyzed and calculated using the NeuriteQuan protocol. (D) Branches per neuron; (E) Branches per cell; (F) Neurite number per cell; (G) Average neurite length; (H) Neurite length per cell; (I) MAP2 mRNA expression measured by qRT-PCR (n = 5-7). Scale bar = 100 μm. Data are presented as mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

P < 0.05. Ginsenoside Rb1 (Rb1) reversed this impairment, evidenced by the leptin decreased average neurite length by 37% (p < 0.001, Figure 4G) and total neurite length per cell by 44% (p < 0.001, Figure 4H) in palmitic acid and Rb1 co-treated cells compared to palmitic acid pretreated neurons alone. The mRNA expression of MAP2, an important marker of neurite outgrowth, was subsequently measured by qRT-PCR. Ginsenoside Rb1 (Rb1) prevented palmitic acid from inhibiting the leptin-induced stimulation of MAP2 mRNA (Figure 4I).

3.6 | Palmitic acid reduced leptin-mediated synaptogenesis in the prefrontal cortical neurons, which can be reversed by Rb1 treatment

We examined the effect of Rb1, in the presence of palmitic acid, on the postsynaptic density marker 95 (PSD95) in response to leptin in cortical neurons (Figure 5A, B, and Table S4). Leptin significantly increased PSD95 immunoreactivity (p < 0.001) both in dendrites and soma.
5 | DISCUSSION

This study showed that Rb1 can reverse the adverse effects of high-saturated fat on leptin sensitivity in the prefrontal cortex. Chronic Rb1 treatment ameliorated hyperleptinemia, and improved leptin signaling via JAK2-STAT3 and leptin-induced BDNF expression in the prefrontal cortex of obese mice. Moreover, in the cultured primary prefrontal cortical neurons, palmitic acid impaired the effect of leptin on BDNF, neurite outgrowth, and synaptogenesis markers. Ginsenoside Rb1 (Rb1) treatment improved the leptin-mediated neurite outgrowth (branching and length) and synaptic protein (SYN and PSD95) in palmitic acid pretreated prefrontal cortical neurons.

In the present study, we found that leptin activates JAK2-STAT3 signaling in the prefrontal cortex in LC lean mice. The prefrontal cortex is important for cognitive control, and high-fat diet reduces synaptic plasticity in this region leading to learning and memory impairments. Furthermore, in the present study, we found that leptin-JAK2-STAT3 signaling was substantially impaired in the prefrontal cortex of the obese mice. In obese individuals, central leptin insensitivity also exists. The prevalence of dementia and other neurodegenerative diseases was higher in obese subjects with hyperleptinemia, which suggests that high plasma leptin levels unlikely activate signaling pathways in the brain of obese individuals. Therefore, central leptin resistance is a pathology of obesity. Importantly, in the present study, Rb1 treatment improved impairments of the leptin-JAK2-pSTAT3 signaling pathway in the prefrontal cortex, restored central leptin sensitivity, and reduced hyperleptinemia in obese mice. Previously, both clinical and animal studies have shown that ginseng extract or Rb1 treatment improves cognitive function. Therefore, these findings, including ours, suggest that Rb1, in improving the leptin-JAK2-STAT3 pathway in the prefrontal cortex, may contribute to ginseng and Rb1 in improving cognition.
The present study found that leptin elevates BDNF in the prefrontal cortex, as illustrated by protein expression level in the in vivo study, and immunohistochemistry and mRNA in the in vitro study. High-fat diet or palmitic acid altered the ability of leptin to elevate BDNF in the prefrontal cortex, suggesting that a high-saturated fat diet can affect leptin function in prefrontal cortical neurons. In accordance with our results, the impairment of central leptin-regulated BDNF has been demonstrated in obese mice, which is associated with depressive behavior. Moreover, mutation of the BDNF gene is associated with increasing early-onset obesity and cognitive impairment in BDNF haploinsufficient patients. Brain-derived neurotrophic factor (BDNF) in the prefrontal cortex promotes neuronal plasticity and neurogenesis, which are important for learning and memory. Thus, the impairment of leptin-regulated BDNF in the prefrontal cortex by saturated fat may play an important role in central nervous system dysfunction in obese subjects. Along with improving the leptin-PI3K/AKT/pSTAT3 signaling pathway, our results demonstrated a beneficial effect of Rb1 on the upregulation of BDNF by leptin. These results suggest that Rb1-induced activation of leptin signaling may modulate BDNF expression, enhancing neuronal plasticity in the prefrontal cortex.

In this study, we demonstrated the beneficial effect of leptin on neurite outgrowth and synaptogenesis in cultured primary prefrontal cortical neurons. We found leptin increases neurite branching and length, neurite marker MAP2 mRNA expression, and synaptic marker PSD95 and SYN immunoreactivity and mRNA expression. It has been reported that the expression of synaptogenesis markers, SYN and PSD95, depends on BDNF processing. It is known that leptin signaling molecules, JAK2 and STAT3, distribute around the postsynaptic sites in the cerebral cortex. Therefore, leptin may promote neurogenesis and synaptic plasticity via activation of leptin signaling molecules and increasing BDNF, SYN, and PSD95 in cortical neurons. Furthermore, we found that high-saturated fatty acid diet impaired leptin signaling JAK2 and STAT3 in the prefrontal cortex. We confirmed that saturated palmitic acid impaired leptin’s action on neurite outgrowth and synaptogenesis in cultured prefrontal cortical neurons. These findings suggest that saturated fatty acids may impair leptin signaling and induce a leptin insensitivity effect on neurite outgrowth and synaptogenesis in the prefrontal cortical neurons. Importantly, our results demonstrated that this impairment induced by palmitic acid was prevented by the Rb1 treatment. Especially, Rb1 ameliorated palmitic acid-induced impairment on leptin-stimulated SYN immunoreactivity in the dendrite but not in the soma. It is possible that Rb1 may interact with the leptin receptor in the prefrontal dendrites of neurons and activate synaptophysin expression, although this requires further investigation. Clinical data show that gingko treatment for 24 weeks improves cognitive function in patients with Alzheimer’s disease. Overall, these findings suggest that Rb1 treatment in improving leptin-regulated BDNF and synaptogenesis via JAK2-STAT3 signaling may, at least partly, contribute to gingko improvements in cognitive function.

Ginsenoside Rb1 belongs to steroidal saponins, which share structure features with steroid hormones. Due to the steroid-like structure and the amphiphilic nature, Rb1 ginsenosides can intercalate into...
the plasma membrane replacing membrane cholesterol, which increases membrane fluidity and changes the physical nature of cell membrane proteins, such as the GABA receptor.\(^2\)\(^,\)\(^3\)\(^,\)\(^4\) Furthermore, ginsenoside may traverse cell membranes freely and activate intracellular membrane receptors, such as steroid receptors, which may regulate the transcription of target genes.\(^5\)\(^,\)\(^6\) This study demonstrated that RB1 increased leptin sensitivity in the prefrontal cortex of obese mice, and in prefrontal cortical neurons treated with palmitic acid, as well as reversed the alterations of leptin downstream signaling molecules (p-JAK2, p-STAT3, BDNF, and PSD95). However, it remains to be determined if RB1 directly interacts with the leptin receptor. Furthermore, it is reported that hyperleptinemia is required for the development of leptin resistance in diet-induced obese mice.\(^4\)\(^,\)\(^5\) In our study, RB1 significantly attenuates hyperleptinemia in obese mice, which may reduce the overstimulation of the leptin receptor, thereby improving downstream signaling.

In summary, this study has shown ginsenoside RB1 treatment ameliorates alteration of leptin-pJAK2-pSTAT3 and leptin-BDNF in the prefrontal cortex of obese mice and improves hyperleptinemia. Treatment with RB1 also promoted leptin's effect on neurite branching and elongation, and synaptogenesis in prefrontal cortical neurons. As high-fat-diet-induced obesity has been implicated in the progression of neurodegenerative diseases, such as vascular dementia, RB1 treatment may have therapeutic effects in attenuating the progression of cognitive decline in obese patients and reducing the risk of neurodegenerative diseases. This will be the next work to further validate using obese mice model.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Xu-Feng Huang http://orcid.org/0000-0002-5895-2253

REFERENCES

SUPPLEMENTARY DATA

**Table S1. The antibodies used in this study**

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<td>p-STAT3</td>
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<td>PSD95</td>
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Table S2. Primers used in qPCR for mRNA measurement of synaptogenesis markers

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Table S3. Central leptin sensitivity and plasma level of leptin in experimental groups in response to icv leptin administration

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<th>LC Saline</th>
<th>LC Leptin</th>
<th>Obese Saline</th>
<th>Obese Leptin</th>
<th>Obese + Rb1 Saline</th>
<th>Obese + Rb1 Leptin</th>
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<tr>
<td>Central leptin sensitivity</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>EI_1 hr. icv (Kcal)</td>
<td>2.3 ± 0.3</td>
<td>1.6 ± 0.4</td>
<td>1.0 ± 0.1</td>
<td>0.9 ± 0.1</td>
<td>0.5 ± 0.1b</td>
<td>0.4 ± 0.1c</td>
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<td>EI_4 hrs. icv (Kcal)</td>
<td>5.2 ± 0.6</td>
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<td>4.9 ± 0.6</td>
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<td>Leptin (ng/mL)</td>
<td>0.6 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>16.9 ± 2.1a</td>
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<td>8.9 ± 1.1ab</td>
<td>8.3 ± 1.4c</td>
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Central leptin sensitivity: Energy intake (EI) for 1, and 4 hours after icv leptin administration. LC: Lab-chow diet fed mice; obese: obese mice induced by a high-fat diet; obese + Rb1: obese mice treated with Rb1 for 3 weeks.

*p<0.05 vs. LC/saline group; **p<0.05 vs. obese/saline group; ***p<0.05 vs. obese/leptin group; ****p<0.05 vs. (obese + Rb1)/saline group.

Data are presented as mean ± SEM, n=6-8.
Table S4. The levels of PSD95 and SYN mRNA expression in cultured prefrontal cortical neurons

<table>
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<th></th>
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<td>Control</td>
<td>PA</td>
<td>PA + Rb1</td>
<td>Control</td>
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<tr>
<td>PSD</td>
<td>100.0 ± 9.7</td>
<td>105.6 ± 2.8</td>
<td>119.0 ± 3.3</td>
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<td>SYN</td>
<td>100.0 ± 7.4</td>
<td>92.5 ± 6.8</td>
<td>107.3 ± 9.2</td>
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PSD95: post-synaptic density protein 95; SYN: synaptophysin. PA: palmitic acid; Rb1: ginsenoside Rb1. Cultured primary cortical neurons were treated on the 7th day of in vitro, with 10 μM PA or (10 μM PA + 40 μM Rb1) for 4 hours, then supplemented with 100ng/mL leptin or vehicle for 44 hours more. After 48 hours' treatment, neurons were collected to examine the mRNA expression levels of PSD95 and SYN. a p<0.05 vs. respective saline group; b p<0.05 vs. control/leptin; c p<0.05 vs. PA/leptin. Data were presented as Mean ± SEM, n=5-7.
CHAPTER FOUR

Teasaponin improves hypothalamic and cortical leptin sensitivity in diet-induced obese male mice
Teasaponin improves hypothalamic and cortical leptin sensitivity in diet-induced obese male mice

Abstract:
Hypothalamic inflammation is involved in the pathogenesis of obesity and type 2 diabetes. Obesity impairs cognition, and the leptin-induced increase of brain-derived neurotrophic factor (BDNF) and neurogenesis. Tea consumption improves inflammation, cognition and increases brain activation in the prefrontal cortex. This study examined whether teasaponin, an active ingredient in tea, could improve central inflammation and leptin sensitivity in the hypothalamus and prefrontal cortex of obese mice. Teasaponin (10mg/kg, intraperitoneal) for 21 days significantly decreased the food intake and body weight of high-fat (HF) diet-induced obese mice. In the hypothalamus teasaponin decreased both pro-inflammatory cytokines and inflammatory signaling in the mediobasal hypothalamus. Teasaponin treatment also enhanced the anorexigenic effect of central leptin administration, restored leptin p-STAT3 signaling in the hypothalamus. Furthermore, teasaponin improved downstream leptin signaling (JAK2, and STAT3), and leptin’s effect on BDNF, in the prefrontal cortex of HF fed mice. Prefrontal cortical neurons were cultured with teasaponin and palmitic acid (the most abundant dietary saturated fatty acid) to examine their effects on BDNF and neurite outgrowth in response to leptin. Palmitic acid decreased leptin’s effect on BDNF and neurite outgrowth in cultured cortical neurons, which were reversed by teasaponin. Therefore, teasaponin supplementation may be used to prevent obesity-associated neurodegeneration and improve cognitive function.

Keywords: Teasaponin, anti-inflammation, leptin sensitivity, diet-induced obesity
4.1 Introduction

Obesity has reached epidemic proportions and is an important risk factor for the development of type 2 diabetes, cardiovascular disease, and some forms of cancer. There is compelling evidence that a large component of obesity-associated pathophysiology may stem from the low-grade inflammation that occurs during obesity. This includes increased production of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6), and activation of the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) inflammatory signaling pathway in adipose tissue, liver and the hypothalamus of the brain (Osborn and Olefsky, 2012; Thaler et al., 2012).

Over nutrition induces inflammatory responses in peripheral metabolic tissues, including the infiltration of pro-inflammatory cytokine secreting macrophages into the adipose tissue of obese mice and humans (Osborn and Olefsky, 2012; Weisberg et al., 2003; Xu et al., 2003). Recent research has identified a similar type of low-grade inflammation in the hypothalamus of rodents (Thaler et al., 2012), where TNF-α, IL-6 and IκB kinase (IKK) mRNA expression are increased within a week of starting a HFD. IKK along with inhibitor kappa B alpha (IκBα) are components of the NF-κB signaling pathway, and commonly used to investigate inflammatory signaling events. Hypothalamic inflammation results in central leptin resistance, hepatic insulin resistance, a reduction in thermogenesis and cardiovascular disorders (Cai and Liu, 2012). Therefore, blocking the peripheral and central inflammation induced by HFD has the potential to treat obesity and metabolic syndrome. This could be achieved using novel therapies incorporating effective natural agents, particularly agents with the dual properties of preventing inflammation and controlling body weight.

Recent epidemiological studies have demonstrated that obesity increases the incidence of cognitive decline in neurodegenerative diseases, such as vascular dementia (Hassing et al., 2002; Singh-Manoux et al., 2012). Empirical evidence has linked high-fat diet (HF)-induced obesity with impairments in learning and memory, including a decline in recognition memory (Boitard et al., 2014b; Camer et al., 2015; Valladolid-Acebes et al., 2011). For example, HF for
8 weeks impairs spatial learning in the eight-arm radial maze test, prior to metabolic alterations linked to obesity developing and without significant changes in plasma glucose and insulin levels (Valladolid-Acebes et al., 2011). HF treatment of juvenile rodents impairs long-term spatial reference memory in the Morris water maze, without affecting acquisition or short-term memory (Boitard et al., 2014b). Furthermore, in our previous study, recognition memory was impaired in chronic HF fed mice as assessed by performing a novel object recognition test (Camer et al., 2015). The prefrontal cortex plays an important role in higher cognitive function (Cole et al., 2012a). Abnormal structure and dysfunction in the prefrontal cortex is associated with cognitive decline and the pathogenesis of mood disorders (Fumagalli et al., 2003; Kanoski et al., 2007b; Weickert et al., 2003). Furthermore, positron emission tomography has shown that obese women have reduced activation in the prefrontal cortex in response to a meal than lean women (Le et al., 2007). In preclinical animal studies, a high-fat diet reduces synaptic plasticity in the prefrontal cortex (Val-Laillet et al., 2011b), which leads to learning and memory impairments (Laroche et al., 2000a). Moreover, obese patients are leptin resistant and have significantly higher levels of plasma leptin, which correlates with body fat mass (Heymsfield et al., 1999). Our previous study showed that in rodents a high saturated fat diet induces body weight gain followed by the development of obesity, which is accompanied by peripheral and then brain leptin resistance (Lin et al., 2000b). Despite this, therapeutic interventions targeting HF-induced cognitive impairment and central leptin resistance are lacking.

The adipocyte-secreted hormone leptin has important effects on synaptogenesis and dendritic morphology in the brain, which regulate energy homeostasis and facilitate learning and memory (Bariohay et al., 2005b; Komori et al., 2006; Yamada et al., 2011). For example, in vitro, leptin increases neurite outgrowth marker and synaptogenesis markers in mouse H19-7 HN neural cell lines, as well as stimulating hippocampal neurogenesis by increasing cell proliferation and differentiation (Moon et al., 2013). Leptin replacement at physiological doses for two years substantially increased the rate of development in most neurocognitive domains in individual with leptin gene mutation (Paz-Filho et al., 2008). Brain-derived neurotrophic factor
BDNF plays an important role in synaptic plasticity and neurogenesis (Arancio and Chao, 2007; Noble et al., 2011), cognitive function (Grassi-Oliveira et al., 2008) and energy metabolism (Yu et al., 2009) through the activation of its receptor, tropomyosin-related kinase B (TrkB). BDNF is broadly expressed in the developing and adult mammalian brain, particularly in the cerebral cortex, hippocampus, hypothalamus and brainstem (Noble et al., 2011). Plasma BDNF levels are positively correlated to memory performance in female patients with major depressive disorder (Grassi-Oliveira et al., 2008). Leptin administration significantly increases hippocampal BDNF in control but not HF-induced obese mice suggesting decreased central leptin sensitivity in obese mice (Yamada et al., 2011). Altogether, this evidence suggests that leptin activation of BDNF in the central nervous system may be involved in cognition, while brain leptin resistance could contribute to obesity related cognitive decline.

From ancient times tea has been widely used as a healthy drink worldwide. Recent evidence has emerged that tea can prevent obesity and abnormal glucose and lipid metabolism (Beresniak et al., 2012b; Wolfram et al., 2006). The earliest use of tea for medicinal purposes occurred in China, in roughly 2700 BC during the time of Emperor Shen Nung (Weisburger, 1997). During that time, it was believed to have health promoting properties, and was frequently used as a fluid supply for patients with infectious diseases. Recently, the effect of tea on inflammation has received increasing attention. Six cups of green tea daily for three to eight weeks significantly reduced NF-κB signalling in men with prostate cancer prior to undergoing prostatectomy, compared to the control group (Henning, 2012). Treatment of genetically obese (ob/ob) mice with 1 % tea extract in diet for 6 weeks decreased hepatic TNF-α protein, adipose TNF-α mRNA, and attenuated hepatic steatosis (Park et al., 2011). Increased consumption of green tea was associated with a lower prevalence of cognitive impairment in a cross-sectional study in 1003 Japanese (Kuriyama et al., 2006), and longitudinal analysis of data from 1438 Chinese subjects (Ng et al., 2008). Phenolics and saponin are the two major active components of tea extract. Phenolics in tea have been widely investigated in previous studies, while teasaponin (TS), an important bioactive ingredient of tea extract, has received
little attention. Teasaponin chemically, belongs to the oleanane-type pentacyclic triterpene saponins, which are naturally derived inhibitors of NF-kB signaling and have anti-inflammatory potential (Salminen et al., 2008a). Recently teasaponin (10mg/kg ip injection) showed significant anti-inflammatory properties, by inhibiting paw oedema induced by carrageenan in rats (Sur et al., 2001). In the current study, we expanded these findings to investigate the effect of teasaponin on recognition memory and leptin signaling in the hypothalamus and prefrontal cortex of HF fed mice. We further investigated the effect of teasaponin in cultured primary prefrontal cortical neurons in response to leptin and the saturated fatty acid, palmitic acid.

4.2 Materials and Methods:

4.2.1 Animals

C57Bl/6J male mice (10 weeks old, body weight: 19.6 ± 1.4 g) were obtained from the Animal Resources Centre (Perth, Western Australia), and housed in environmentally controlled conditions (temperature 22 °C, 12 hour light/dark cycle). Lab chow (LC) served as the low-fat control diet (5% fat, Vella Stock Feeds, Doonside, NSW, Australia) and was provided *ad libitum* except where noted. Mice were acclimatised for one week prior to experimentation. All procedures were approved by the Animal Ethics Committee, University of Wollongong, NSW, Australia, and complied with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

4.2.2 Intraperitoneal (ip) teasaponin treatment

Mice were placed on the HF (containing 60% fat by calories, Specialty Feeds, Western Australia) for 16 weeks. The animals were then randomised into two groups, and administered either teasaponin (10 mg/kg) or vehicle (saline) i.p. injections daily for 21 days. Age-matched, LC diet control mice were maintained on the lab chow diet. Body weight and food intake were measured daily. Teasaponin (96%, C_{57}H_{90}O_{26}, MW=1200) was purchased from the Aladdin Chemistry Co. Ltd, China.
4.2.3 Intraperitoneal glucose tolerance test (IPGTT)

On day 18 of the teasaponin treatment, the mice were fasted overnight and given an ip injection of glucose (0.5g/kg) as reported previously (Morton et al., 2013; Wu et al., 2014; Yoshihara et al., 2010). Blood samples were taken from the tail vein and blood glucose measured using a glucometer (Abbott Diabetes Care, Alameda, CA) at 0 (fasting), 30, 60 and 120 min after glucose administration.

4.2.4 Central leptin sensitivity test

After intraperitoneal teasaponin treatment for 21 days, mice were anaesthetised and placed in a stereotactatic device. A intracerebroventricular (icv) cannula was implanted into the right lateral brain ventricle (0.25 mm posterior and 1.0 mm lateral relative to Bregma and 2.5 mm below the surface of the skull) (Paxinos and Franklin, 2002). Five days after implantation, mice were fasted for 6 hours and administered either leptin (0.1 µg/3 µl) or saline (3 µl) injected into the lateral ventricle through the cannula. Food intake and body weight were measured 24 hours after the leptin or vehicle injection to examine central leptin sensitivity.

4.2.5 Blood and tissue collection

Four days after the first central leptin sensitivity test, the mice received a second icv leptin or saline injection and were sacrificed one hour later. Plasma and brain tissue were collected and stored at -80 °C for further analyses as detailed below.

4.2.6 Measurement of plasma leptin and insulin

Plasma leptin and insulin were measured using the mouse metabolic magnetic bead panel kit (Merck Millipore, MA, USA).

4.2.7 Western blot analysis

Protein expression in frozen prefrontal cortex (equivalent to the prelimbic cortex, PrL,
dissected at the level of Bregma 2.8 mm to 1.98 mm) was determined using western blot as described in our previous study (Yu et al., 2013). The following antibodies were used: TNF-α (sc-8301), IL-1β (sc-7884) and IL-6 (sc-7920), BDNF, TrkB 145 and 95, pJAK2 and pAkt (Santa Cruz, CA, USA); pTrkB 95 (Sigma-Aldrich, St Louis, MO, USA); and p-IKK (Ser176/180) (#2697), p-IκBα (Ser32) (#2859), pSTAT3, pGSK3β and pFOXO1 (Cell Signaling Technology, Beverly, MA, USA). Bands corresponding to the proteins of interest were scanned and band density analysed using the Quantity One automatic imaging analysis system (Bio-Rad Laboratories, Hercules, CA, USA). All quantitative analyses were normalised to β-actin, based on our previous studies (Yu et al., 2013). Due to the small amount of tissue in the prefrontal cortex, we used a previously described modified multi-strip western blot (Kiyatkin and Aksamitiene, 2009; Yu et al., 2013).

4.2.8 Oral teasaponin treatment and the novel object recognition test

Mice were placed on a HFD for 8 weeks. The animals were then randomized into two groups, continuation of HFD for 6 weeks, or HFD with 0.5% teasaponin for 6 weeks. Age-matched, LC diet control mice were maintained on the lab chow diet. A novel object recognition test was performed 5 weeks after commencing teasaponin treatment. This test is based on the innate tendency of rodents to differentially explore novel objects over familiar ones as previously described (Arqué et al., 2008), with minor modifications. In brief, the experimental procedure consisted of habituation, training and retention sessions. On day 1, for habituation, mice were placed into an open-field box (55×55cm × 35cm high) for 10 minutes with a 40 W light bulb in a sound proof room. On day 2, during the training session, two identical objects (A) were placed at opposing corners of the box, 5cm from the adjacent wall. Each mouse was then placed in the middle of the open-field box and left to explore the objects for 10 minutes. Ninety minutes later, in the retention session, one familiar object (A) was replaced with one novel object (B). Each mouse was placed in the middle of the open-field box, and left to explore for another 10 minutes. The exploration time for the familiar and the new objects was recorded. Memory was operationally defined by the discrimination index for
the novel object (DI) as the proportion of time animals spent investigating the novel object minus the proportion spent investigating the familiar one in the testing period in the retention session [Discrimination Index = (Novel Object Exploration Time/Total Exploration Time) – (Familiar Object Exploration Time/Total Exploration Time)×100].

4.2.9 Prefrontal cortical neuronal cultures and treatment

Prefrontal cortical neuronal cultures were prepared from postnatal day 1 mice as described previously (Hilgenberg and Smith, 2007). Cells were plated at a final density of $5 \times 10^5$/cm$^2$ into Poly-D-Lysine-coated 24-well culture plates for RT-PCR analysis. For neurogenesis imaging, cortical neurons were cultured on Poly-D-Lysine-coated coverslips. At 7 days in vitro, teasaponin (40µM) and/or palmitic acid (10 µM, P5585, Sigma-Aldrich) were added to the cultures. The method for dissolving palmitic acid was as described by Ross et al (Ross et al., 2010). Four hours after palmitic acid and/or teasaponin pre-exposure, leptin (100 ng/ml) or vehicle was added to the cultures for another 44 hours before the cells were harvested.

4.2.10 Immunofluorescence and image analysis

For immunocytochemical staining, neurons were fixed with 4% paraformaldehyde and 4% sucrose in Dulbecco's PBS for 20 min at room temperature. The samples were incubated with PBS containing 0.2% Triton X-100 for 5 min, and blocked with 10% horse serum in PBS for 1 h at 37 °C. Then, the anti-BDNF antibody and anti-microtubule-associated protein 2 (MAP2) and antibody were applied overnight at 4 °C. BDNF was visualized with isotype-specific donkey anti-rabbit secondary antibody conjugated with Alexa Fluor 488; MAP2 was visualized by goat anti-mouse IgG (H+L) secondary antibody conjugated to Alexa Fluor 594. A fluorescence microscope (Axiovert 200, Carl Zeiss, Oberkochen, Germany) with an attached digital camera was used to obtain MAP2 immunofluorescence images for neuronal morphology analysis. A confocal microscope (Leica TCS SP5 Advanced System, Wetzlar, Germany) equipped with a digital camera was used to obtain images for immunofluorescence image of BDNF.
4.2.11 Neurite outgrowth and branching analysis.

The quantification of neuronal morphology included: average neurite length, neurite length per cell, neurite number per cell, branches per neurite, and branches per cell. Experiments were independently performed three times, with each time repeated in triplicate (n=9). The image analysis was based on these nine repeats.

4.2.12 Statistical analysis

Data were analysed using the SPSS 19 statistical package (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) and the post-hoc Tukey-Kramer honestly significant difference (HSD) test were used to analyse the novel recognition test, body weight, and the glucose tolerance test and leptin signaling of the LC, HF, and teasaponin treatment groups in response to saline and leptin, as well as data from the cell culture experiments. A \( p \) value < 0.05 was regarded as statistically significant. Values are expressed as mean±SEM.

4.3 Results

4.3.1 Teasaponin reduced body weight and food intake in obese mice

When teasaponin was administrated via ip injection to HFD-induced obese mice it significantly reduced body weight and food intake during the 21-day observation period (Fig. 1A and 1B). The final body weight and average energy intake of the teasaponin group was also significantly lower than the HF control group following treatment (final body weight: -11.05%, \( p = 0.004 \); average energy intake: -24%, \( p < 0.001 \), Fig. 1A and 1C).
Fig. 1 Chronic administration of teasaponin (TS, 10 mg/kg ip for 21 days) significantly decreased body weight (A), daily food intake (B) and average energy intake (C) in obese mice (n=16) fed a high-fat (HF) diet for 16 weeks. *p<0.05 vs. HF control group.

4.3.2 Teasaponin improved glucose tolerance, blood hormone profiles and discrimination index

Glucose tolerance tests were performed to assess glucose homeostasis and insulin sensitivity in HFD-induced obese mice treated with teasaponin. Blood glucose levels were lower at the 30 and 60 minute time points in the teasaponin treatment group compared to the HF controls during the glucose tolerance test (Fig. 2A). The teasaponin treatment also decreased HOMA (Fig. 2B), indicating that it reduced insulin resistance in the HFD-induced obese mice. HFD-induced hyperinsulinemia and hyperleptinemia were significantly reversed by teasaponin treatment (Fig. 2C and 2D). Teasaponin attenuated HF-induced decline in discrimination index reflecting recognition memory in the novel object recognition test in mice (Fig. 2E).
Chronic administration of teasaponin improved glucose tolerance (A) and HOMA (B), decreased plasma insulin (C) and leptin (D) in obese mice (n=8) fed a high-fat (HF) diet for 16 weeks. *p<0.05 vs. HF control group. The homeostasis model assessment of insulin sensitivity (HOMA) is calculated by (fasting glucose [mmol/l] multiplied by fasting insulin [U/ml] divided by 22.5). (E) Teasaponin attenuated HF-induced decline in discrimination index reflecting recognition memory in the novel object recognition test in mice. Discrimination Index = (Novel Object Exploration Time / Total Exploration Time) – (Familiar Object Exploration Time / Total Exploration Time) × 100. *p<0.05 vs. HF fed mice.

Data are presented as mean±SEM, n=10. LC: lab chow diet fed mice.

4.3.3 Teasaponin attenuated hypothalamic inflammation and improved central leptin sensitivity and leptin signaling

Western blot analysis showed that in the mediobasal hypothalamus, a HFD elevated TNF-α, IL-6, p-IKK and SOCS3 protein expression, which was significantly decreased by treatment with teasaponin (Fig. 3A, 3B, 3D, and 3E). We also evaluated if leptin signaling in the CNS improved in conjunction with the observed reduction in hypothalamic inflammation. Leptin administered icv significantly decreased energy intake for 24 hours in LC diet fed mice (-40%, p < 0.001; Fig 4A), but not in HFD fed animals (-18%, p = 0.421; Fig. 4B). While energy
intake was significantly decreased in the teasaponin-treated mice receiving icv injections of leptin compared with the icv injection of saline (-31%, \( p=0.023 \); Fig. 4B).

To clarify the mechanisms underlying the enhanced effect of leptin in the teasaponin-treated mice, we measured p-STAT3 protein levels in the mediobasal hypothalamus after the chronic treatment with teasaponin and leptin stimulation. Leptin administered icv significantly increased p-STAT3 in LC diet fed mice (\( p < 0.001 \); Fig. 4C), while p-STAT3 did not significantly increase in response to the leptin injection in control HFD-induced obese mice (Fig. 4D). With the teasaponin treatment, the p-STAT3 level significantly increased after icv leptin injection compared with icv saline (94%, \( p < 0.001 \)) (Fig. 4D).

**Fig. 3** Effect of chronic teasaponin treatment on the protein levels of inflammatory markers in the mediobasal hypothalamus of obese mice fed a high-fat (HF) diet for 16 weeks. Chronic treatment of teasaponin significantly decreased the level of the pro-inflammatory cytokines TNF-\( \alpha \) (A), IL-6 (B) and IL-1\( \beta \) (C), as well as the inflammatory signaling molecules p-IKK (D) and SOCS3 (E). n=6-8, *\( p<0.05 \) vs HF group.
4.3.4 Teasaponin restored leptin-pJAK2-pSTAT3 signaling and improved leptin-mediated BDNF expression in the prefrontal cortex of HF fed mice

Several molecules, including JAK2-STAT3 and Akt-FOXO1/GSK, have been established as downstream mediators of leptin signaling in the hypothalamus (Benzler et al., 2013; Kim et al., 2006; Morton et al., 2005). Here we examined the leptin signaling pathways JAK2-STAT3 and Akt-FOXO1/GSK in the prefrontal cortex. An icv injection of leptin significantly increased pJAK2 in the prefrontal cortex of LC mice ($p<0.001$), but not in HF fed mice ($p>0.05$, Fig. 5A). However, with teasaponin treatment, the leptin injection significantly increased pJAK2 in HF fed mice compared to saline injection ($p<0.05$). There was a similar response in the pSTAT3, downstream step of leptin/pJAK2 signaling in the LC, HF and teasaponin-treated HF mice (Fig. 5B). Leptin significantly increased pSTAT3 by 42% in the prefrontal cortex of LC mice.
mice, while in HF fed mice pSTAT3 only increased by 16% after the leptin injection. Following chronic teasaponin treatment, pSTAT3 increased by 46% in response to leptin compared with the saline injection, suggesting that teasaponin improved leptin-pJAK2-ppSTAT3 signaling in HF fed mice. We also found that leptin significantly increased pAkt, pGSK3β and pFOXO1 in the prefrontal cortex of LC but not in HF fed mice ($p<0.05$, Fig. 5C-E). However, teasaponin treatment did not reverse impaired leptin-pAkt-pGSK3β/pFOXO1 signaling in HF fed mice.

**Fig. 5** Effect of teasaponin treatment (TS, 10mg/kg, ip for 21 days) on leptin-p1AK2-pSTAT3 signaling (A, B), pAkt-pGSK3β/pFOXO1 (C-E) and leptin-BDNF in the prefrontal cortex of high-fat diet (HF) fed mice. *$p<0.05$ vs. saline in individual group. Data are presented as mean±SEM, $n=8–9$. LC: lab chow diet fed mice.

### 4.3.4 Teasaponin improved leptin-mediated BDNF expression in the prefrontal cortex of HF fed mice and cultured prefrontal cortical neuron

After icv leptin injection, the BDNF level in the prefrontal cortex was significantly increased in
LC mice (p<0.05), but not in HF fed mice (Fig. 5F). Importantly, with chronic teasaponin treatment, leptin increased BDNF levels compared to saline in the prefrontal cortex of HF fed mice, suggesting that teasaponin treatment improved the leptin-induced increase in BDNF in the prefrontal cortex. However, teasaponin treatment did not significantly alter basal BDNF protein levels in HF mice (p>0.05).

In cultured prefrontal cortical neurons, immunostaining with anti-BDNF antibody showed a granule distribution of BDNF in MAP2-positive neurons (Fig. 6A). Leptin treatment significantly increased BDNF expression, evidencing by the strong immunoreactivity (Fig. 6B) and substantial increase in mRNA expression (2.5-fold, p<0.05, Fig. 6C). However, following palmitic acid pretreatment, leptin did not increase BDNF expression (Fig. 6B and 6C). This suggests palmitic acid inhibits leptin stimulation of BDNF in prefrontal cortex neurons. Importantly, teasaponin treatment significantly increased leptin-mediated BDNF mRNA expression (p < 0.05, Fig. 6C) and immunoreactivity (Fig. 6B) in palmitic acid pre-treated cortical neurons. Interestingly, teasaponin treatment also induced a significant increase in basal BDNF expression (i.e. without leptin) in cortical neurons (Fig. 6B and 6C).

Fig. 6 Teasaponin improved leptin-mediated BDNF expression in the cultured prefrontal cortical neuron. Immunocytochemistry images showing the typical BDNF-containing vesicles (indicated by white arrowheads), taken by confocal microscope (Nikon) (A); Palmitic acid (PA) decreased leptin-induced BDNF immunoreactivity (B) and mRNA expression (C) in cultured prefrontal cortical neurons, which can be corrected by teasaponin (TS) (B and C); TS and leptin significantly increased BDNF immunoreactivity in cortical neurons (B). Pretreatment with PA significantly inhibited leptin-induced...
BDNF (B and C); TS prevented PA from inhibiting the leptin-induced stimulation of BDNF. Data expressed as mean ± SEM (n = 6, obtained from six independent culture wells), *p < 0.05. Leptin (100 ng/mL) was applied to the neuron cultures 4 hours after pre-treated with PA and/or TS, then lasted for 44-h exposure. Scale bar = 50 μm. TS: teasaponin; PA: palmitic acid.

4.3.5 Teasaponin promoted neurite outgrowth in response to leptin in cultured prefrontal cortical neurons

We examined the effect of teasaponin on neurite outgrowth in cells treated with leptin and palmitic acid (Fig. 7A and 7B). Morphological analyses showed that leptin significantly increased the average neurite length (Fig. 7D) and total neurite length per cell (Fig. 7E) in control cortical neurons, but not in palmitic acid treated neurons. Whereas with teasaponin pre-treatment, leptin increased average neurite length (45%, p<0.05, Fig. 7D) and total neurite length per cell (45%, p<0.05, Fig. 7E) compare to saline, suggesting teasaponin attenuated the effect of palmitic acid on leptin-induced neurite length. However, the neurite number per cell in cortical neurons was not affected by leptin, PA or teasaponin (Fig. 7F). Furthermore, leptin treatment significantly increased neurite branching (branches per neurite: +59%, Fig. 7G, branches per cell: +59%, Fig. 7H) compared with saline treatment in control neurons, but not in palmitic acid treated neurons. With teasaponin pretreatment, leptin significantly increased branches per neurite, but not branches per cell. The mRNA expression of MAP2, a neurite marker, was also examined by RT-PCR. Teasaponin prevented palmitic acid from inhibiting the leptin-induced stimulation of MAP2 mRNA (Fig. 7I).
Fig. 7 Effect of teasaponin (TS) and palmitic acid (PA) on the leptin-induced neurite outgrowth. A: represents fluorescence images obtained under Confocal Microscope (Nikon), scale bar = 50 μm; B: an example of MAP2 immunofluorescence staining image (8 bit) used for neuron morphology analysis, scale bar = 100 μm; C: an example of analysis tracing of neuron morphology quantification by using NeuriteQuant toolkit. All parameters (D-H) used were automatically analysed and calculated by the algorithm of NeuriteQuant in pixels, n=9; D: Average neurite length; E: Neurite length per cell; F: Neurite number per cell; G: Branches per neurite; H: Branches per cell; I: MAP2 mRNA expression measured by qRT-PCR (n=5-7). *p<0.05 vs. saline in individual group. Data are presented as mean ± SEM. Leptin (100 ng/ml) was applied to the neuron cultures 4 hours before 44 hour exposure to TS (20 or 40µM) or palmitic acid (10 µM).

4.4 Discussion:

This study demonstrated that teasaponin treatment reduced obesity, hypothalamic inflammation, and central leptin resistance in high-fat (HF) diet-induced obese mice. The anti-
inflammatory effects of teasaponin were associated with an improved glycemic status in the treated animals, evidenced by improved glucose tolerance, HOMA and fasting plasma insulin. Furthermore, teasaponin decreased pro-inflammatory cytokines and inflammatory signaling in the mediobasal hypothalamus, and enhanced the anorexigenic effect of central leptin administration as demonstrated by the restoration of p-STAT3 signaling in the mediobasal hypothalamus. Furthermore, chronic teasaponin treatment enhanced object recognition memory, and improved leptin signaling and leptin-induced BDNF expression in the prefrontal cortex of HF fed mice. Moreover, in cultured primary prefrontal cortical neurons, palmitic acid impaired the effect of leptin on neurite growth, and teasaponin ameliorated these palmitic acid induced effects.

Leptin promotes negative energy balance by signaling in the brain, and the hypothalamus is a key region for the control of food intake by this hormone. This negative feedback loop becomes disrupted in most obese individuals, resulting in a state known as central leptin resistance. In this study we confirmed central leptin resistance in HFD induced obese mice. The icv injection of leptin significantly decreased food intake in LC control mice, but not in HF obese mice with hyperleptinemia. There are two mechanisms that explain central leptin resistance, hyperleptinemia (Knight et al., 2010) and hypothalamic inflammation (Thaler et al., 2012; Zhang et al., 2008). Firstly, it is reported that hyperleptinemia is required for the development of leptin resistance in diet-induced obese mice (Knight et al., 2010). In the current study, teasaponin significantly reduced body fat and dramatically ameliorated hyperleptinemia induced by HFD-induced obesity. This may have reduced the overstimulation of the leptin receptor and downstream signaling, thus improving central leptin sensitivity. Secondly, recent studies have revealed that hypothalamic inflammation can mediate central leptin resistance in HFD-induced obese rodents (Thaler et al., 2012; Zhang et al., 2008). Constitutive activation of IKKβ in the hypothalamus of mice induced central leptin resistance and impaired leptin signaling through p-STAT3 (Zhang et al., 2008). In contrast, a genetic or pharmacological blockade of inflammatory signaling in the hypothalamus improved leptin sensitivity and elevated p-STAT3 (Milanski et al., 2012; Zhang et al., 2008). In mice with an IKK knockout in
hypothalamic AgRP neurons, the level of p-STAT3 was significantly increased in response to icv administered leptin in animals fed a HFD (Zhang et al., 2008). In this study, teasaponin decreased the expression of the hypothalamic pro-inflammatory cytokines and inflammatory signaling molecules, such as TNF-α, IL-6, IL-1β and p-IKK. This may have contributed to the improved leptin sensitivity and hypothalamic leptin signaling via p-STAT3 following the teasaponin treatment in the diet-induced obese mice.

SOCS3 has been identified as a negative regulator of central leptin signaling. The overexpression of SOCS-3 results in the inhibition of leptin signaling through JAK2/STAT3 (Bjørbæk et al., 1999). Negative feedback in response to excessive hormone stimulation is a classical mechanism of hormone resistance. It is known that leptin stimulates the expression of SOCS3, which directly inhibits leptin signaling in the hypothalamus (Bjørbæk et al., 1998). Furthermore, overexpression of IKK in the hypothalamic neurons of mice increases SOCS3 mRNA expression and protein levels in the hypothalamus (Zhang et al., 2008). Therefore in the current study, hyperleptinemia and hypothalamic inflammation in diet-induced obese mice may activate a common negative regulator of leptin signaling, SOCS3, and contribute to central leptin resistance. Upregulation of SOCS3 in POMC neurons leads to impairment of STAT3 signaling, with subsequent leptin resistance, obesity, and glucose intolerance (Reed et al., 2010). In contrast, hypothalamic SOCS3-deficient rats exhibited enhanced leptin-induced STAT3 activation, decreased body weight gain and improved metabolic parameters when exposed to a high-fat diet (Liu et al., 2011b). In the present study, teasaponin significantly decreased the level of SOCS3 in the hypothalamus of HF induced obese mice, suggesting that SOCS3 is a potential target for teasaponin’s therapeutic intervention during obesity.

In the present study, central acute administration of leptin significantly increased its downstream signaling molecule, pJAK2 and pSTAT3 in the prefrontal cortex of control mice. In accordance with our results, another study demonstrated that systemic administration of leptin activated STAT3 phosphorylation in cortical neurons, in a rat model of cerebral ischemia (Amantea et al., 2011). We also have demonstrated that leptin activates pAkt, pFOXO1 and pGSK3β, another leptin signaling pathway, in the prefrontal cortex of LC mice. In obesity
plasma leptin is increased, which theoretically will prevent overeating and obesity. However, this is not the case as leptin resistance is a pathology of obesity (Myers et al., 2008a). Furthermore, obese subjects with hyperleptinemia have an increased prevalence of dementia and other neurodegenerative disease (Carpenter et al., 2000; Carter et al., 2000; Lieb et al., 2009b). Therefore, it is unlikely that high plasma leptin levels will appropriately activate signaling pathways in the brain of obese individuals with leptin resistance. Indeed, our results showed that, in HF fed obese mice, both the leptin-pJAK2-pSTAT3 and pAkt-pGSK3β/pFOXO1 signaling pathways were impaired in the prefrontal cortex. Importantly, teasaponin treatment corrected alteration of the leptin-pJAK2-pSTAT3 signaling pathway in the prefrontal cortex of HF fed mice. It has been reported that leptin replacement improves cognition in leptin-deficient patients with a delay or decline of cognitive function (Paz-Filho et al., 2008). The prefrontal cortex is important for cognitive control (Cole et al., 2012a), and HF reduces synaptic plasticity in this region (Val-Laillet et al., 2011b) leading to learning and memory impairments (Laroche et al., 2000a). Therefore, teasaponin may attenuate HF induced impairments of leptin signaling in the prefrontal cortex, leading to improved recognition memory.

In the prefrontal cortex, BDNF promotes neuronal plasticity and neurogenesis, which are important for learning and memory (Kanoski et al., 2007b; Sakata et al., 2013a). HFD reduced BDNF in the prefrontal cortex and impaired discrimination reversal learning in rats (Kanoski et al., 2007b). A continuous icv infusion of antisense BDNF oligonucleotide resulted in an impairment of spatial learning in rats, with a significant reduction of BDNF mRNA and protein levels in the brain (Mizuno et al., 2003). In the clinic, it is reported that BDNF levels in plasma are positively correlated to memory performance in patients with depression (Grassi-Oliveira et al., 2008). Leptin administration has been shown to increase BDNF expression in the hippocampus of mice, which can be blocked by pre-treatment with K252a, a blocker of the BDNF receptor (Yamada et al., 2011). In this study, we observed that leptin stimulated BDNF expression in the prefrontal cortex of control mice, but not in HF fed obese mice. In cultured primary prefrontal cortical neurons, leptin increased neurite outgrowth, however, this effect
was impaired by palmitic acid. Along with improving the leptin-pJAK2-pSTAT3 signaling pathway, our results demonstrated a beneficial effect of teasaponin on the upregulation of BDNF and neurite outgrowth by leptin. These results suggest that teasaponin-induced activation of leptin signaling may have modulated BDNF expression, enhancing neurite outgrowth in the prefrontal cortex and contributing to an improvement in recognition memory.

In summary, we have demonstrated that chronic teasaponin treatment significantly reduced food intake and body weight in HFD-induced obese mice. Treatment with teasaponin significantly ameliorated central inflammation by reducing pro-inflammatory cytokines and inflammatory signaling molecules in the hypothalamus. Therefore, teasaponin has important effects in improving glucose tolerance, central leptin sensitivity and hypothalamic leptin signaling. These results identify a novel role for teasaponin as an anti-obesity and anti-inflammatory agent. Furthermore, teasaponin improved recognition memory in the HF fed mice. Through in vivo and in vitro studies we revealed that leptin activated leptin-pJAK2-pSTAT3 and pAkt-pFOXO1/pGSK3β signalling, stimulated BDNF expression and neurite outgrowth in the prefrontal cortex. However, these prefrontal cortex responses are impaired when mice are fed a high-saturated fat diet, or neurons exposed directly to saturated fatty acid, i.e. palmitic acid in vitro. Teasaponin treatment ameliorates alteration of leptin-pJAK2-pSTAT3 and leptin-BDNF in the prefrontal cortex of HF fed mice. Treatment with teasaponin also promoted leptin’s effect on neurite outgrowth in prefrontal cortical neurons. Since high fat diet-induced obesity has been implicated in the progression of neurodegenerative diseases, such as vascular dementia, teasaponin supplementation may have beneficial effects in attenuating the progression of cognitive decline in obese patients and reducing the risk of neurodegenerative diseases. The dose of teasaponin obtained by frequently drinking tea is significantly lower than the doses used in this rodent study. In order to reach the effect observed in this study, a supplemental delivery of teasaponin (oral or injection) would be needed. Clinical trials would also be required to determine the optimum dose in humans. Furthermore, the potential toxicity of long-term teasaponin exposure should be investigated before teasaponin supplementation can be recommended.
Declaration

I would like to declare that this chapter is my own work. I have designed the study, carried out all animal experimentations and primary cell culture, performed biomarker detection and analysis, interpreted data and wrote this chapter. These works have been used in two publications. The first was in Molecular Nutrition and Food Research (Yu, Y. #, Y. Wu #, A. Szabo, S. Wang, S. Yu, Q. Wang, and X.-F. Huang, Molecular Nutrition and Food Research, 2015, 59:2371-2382), in which I was a shared first author. The second paper was “Teasaponin Reduces Inflammation and Central Leptin Resistance in Diet-Induced Obese Male Mice” published in Endocrinology, 2013, 154:3130-3140, in which I was the second author.

I hereby declared the above declaration of my own works is true.

Yizhen Wu

Statement from supervisors:
We hereby declare that the above declaration from the candidate, Yizhen Wu, about the contribution to this work is correct.

Dr. Yinghua Yu

Prof. Xu-Feng Huang
References:


Yu, Y., Y. Wu, A. Szabo, Z. Wu, H. Wang, D. Li, and X.-F. Huang. 2013. Teasaponin Reduces Inflammation...

## SUPPLEMENTARY DATA

**Table S1.** The antibodies used in this study

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<th>Peptide/protein target</th>
<th>Name of Antibody</th>
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### Table S2. The primers used in qPCR for mRNA measurement of neurogenesis markers

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CHAPTER FIVE

5.1 Overall Discussion and conclusions

The prevalence of obesity is a worldwide health problem, and it is an important risk factor for the development of type 2 diabetes, cardiovascular disease, and certain cancer, as well as depression, and neurodegenerative diseases. Obesity features as weight gain, fat deposition, and energy homeostasis imbalance, obesity-associated central and peripheral inflammation, central and peripheral leptin resistance, blunt leptin signaling, as well as obesity-associated neurodegeneration and cognitive decline. Addressing these features issues will help to anti-obesity, and reduced the risks of obesity-linked diseases. A chronic low-grade inflammatory state during obesity is at the pathogenic core of obesity and type 2 diabetes. Therefore, it is essential to develop novel therapeutic agents that have the ability of anti-obesity-associated inflammation. Triterpene saponins, are groups of pant secondary metabolites with structurally composed of a lipid soluble aglycone and a water soluble sugar residues. The amphiphilic nature of the triterpene saponins suggests that they can intercalate into the cell membrane to mediate the cellular events. Some plant-derived triterpene saponins are anti-inflammatory and inhibit the NF-κB signaling pathway. In this study, ginsenoside Rb1, one of the triterpene saponins, has been reported to have anti-inflammation and neuroprotective effect, has been implicated to treat the central leptin resistance, obesity-associated inflammation, and glucose intolerance, as well as to investigate whether it could improve leptin effects on BDNF, synaptogenesis in the prefrontal cortical neurons of obese, using an in vivo and in vitro model. Teasaponin, active ingredient derived from tea, has recently been showed anti-inflammation properties. This study was to evaluate its effects on obesity, obesity-associated hypothalamic inflammation, hypothalamic and cortical leptin sensitivity of the high-fat diet-induced obese mice, as well as leptin-mediated BDNF expression and neurite outgrowth in the prefrontal cortical neurons, using an in vivo and in vitro model.

► Central Inflammation and Leptin Resistance Are Attenuated by Ginsenoside Rb1
Treatment in Obese Mice Fed a High-Fat Diet

A low-grade pro-inflammatory state is at the pathogenic core of obesity and type 2 diabetes. We tested the hypothesis that the plant terpenoid compound ginsenoside Rb1 (Rb1), known to exert anti-inflammatory effects, would ameliorate obesity, obesity-associated inflammation and glucose intolerance in the high-fat diet-induced obese mouse model. Furthermore, we examined the effect of Rb1 treatment on central leptin sensitivity and the leptin signaling pathway in the hypothalamus. We found that intraperitoneal injections of Rb1 (14mg/kg, daily) for 21 days significantly reduced body weight gain, fat mass accumulation, and improved glucose tolerance in obese mice on a HFD compared to vehicle treatment. Importantly, Rb1 treatment also reduced levels of pro-inflammatory cytokines (TNF-α, IL-6 and/or IL-1β) and NF-κB pathway molecules (p-IKK and p-IκBα) in adipose tissue and liver. In the hypothalamus, Rb1 treatment decreased the expression of inflammatory markers (IL-6, IL-1β and p-IKK) and negative regulators of leptin signaling (SOCS3 and PTP1B). Furthermore, Rb1 treatment also restored the anorexic effect of leptin in high-fat fed mice as well as leptin pSTAT3 signaling in the hypothalamus. Ginsenoside Rb1 has potential for use as an anti-obesity therapeutic agent that modulates obesity-induced inflammation and improves central leptin sensitivity in HFD-induced obesity.

► Ginsenoside Rb1 improves leptin sensitivity in the prefrontal cortex in obese mice

Obesity impairs leptin-induced regulation of brain-derived neurotrophic factor (BDNF) expression and synaptogenesis, which has been considered to be associated with the incidence of neuronal degenerative diseases, cognitive decline and depression. Ginsenoside Rb1 (Rb1), a major bioactive component of ginseng, is known to have an anti-obesity effect and improve cognition. This study examined whether Rb1 can improve central leptin effects on BDNF expression and synaptogenesis in the prefrontal cortex during obesity using an in vivo and an in vitro model. Rb1 chronic treatment improved central leptin sensitivity, leptin-JAK2-STAT3 signaling and leptin-induced regulation of BDNF expression in the prefrontal cortex of high-fat diet induced obese mice. In cultured prefrontal cortical neurons, palmitic acid, the saturated fat, impaired leptin-induced BDNF expression, reduced the immunoreactivity and mRNA
expression of synaptic proteins, and impaired leptin-induced neurite outgrowth and synaptogenesis. Importantly, Rb1 significantly prevented these pernicious effects induced by palmitic acid. These results indicate that Rb1 reverses central leptin resistance and improves leptin-BDNF-neurite outgrowth and synaptogenesis in the prefrontal cortical neurons. Thus, Rb1 supplementation may be a beneficial avenue to treat obesity-associated neurodegenerative disorders.

► Teasaponin improves hypothalamic and cortical leptin sensitivity in diet-induced obese male mice

Hypothalamic inflammation is involved in the pathogenesis of obesity and type 2 diabetes. Obesity impairs cognition, and the leptin-induced increase of brain-derived neurotrophic factor (BDNF) and neurogenesis. Tea consumption improves inflammation, cognition and increases brain activation in the prefrontal cortex. This study examined whether teasaponin, an active ingredient in tea, could improve central inflammation and leptin sensitivity in the hypothalamus and prefrontal cortex of obese mice. Teasaponin (10mg/kg, intraperitoneal) for 21 days significantly decreased the food intake and body weight of high-fat (HF) diet-induced obese mice. In the hypothalamus teasaponin decreased both pro-inflammatory cytokines and inflammatory signaling in the mediobasal hypothalamus. Teasaponin treatment also enhanced the anorexigenic effect of central leptin administration, restored leptin p-STAT3 signaling in the hypothalamus. Furthermore, teasaponin improved downstream leptin signaling (JAK2, and STAT3), and leptin’s effect on BDNF, in the prefrontal cortex of HF fed mice. Prefrontal cortical neurons were cultured with teasaponin and palmitic acid (the most abundant dietary saturated fatty acid) to examine their effects on BDNF and neurite outgrowth in response to leptin. Palmitic acid decreased leptin’s effect on BDNF and neurite outgrowth in cultured cortical neurons, which were reversed by teasaponin. Therefore, teasaponin supplementation may be used to prevent obesity-associated neurodegeneration and improve cognitive function.

This chapter will provide a general discussion and conclusions of the present PhD research project undertaken. The main findings of this thesis are summarized in Table 5.1. Detailed discussion of each study has been included in the discussion section at the end of chapter 2-4.
while overall discussion of the findings is included in section 5.1.1. The proposed potential treatment mechanisms of triterpene saponins are included in section 5.1.2 (Figure 5.1). The conclusions of the thesis are presented in section 5.1.3. Based on the research outcomes of the present project and the related existing knowledge, recommendations of future research are included in section 5.1.4.

Table 5.1 The main finding of this project (Chapter 2-4)  
--------Effects of Triterpene Saponins treatment

<table>
<thead>
<tr>
<th>Parameters/ Reference</th>
<th>Effect of Rb1 (Wu et al., 2018; Wu et al., 2014)</th>
<th>Effect of Teasaponin (Yu et al., 2015; Yu et al., 2013)</th>
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<td><strong>In vivo (DIO mice)</strong></td>
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<td>Food Intake (g)</td>
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<tr>
<td>Body Weight Gain</td>
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<tr>
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<td>pIKK</td>
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<td>pIkBα</td>
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<tr>
<td>SOCS3</td>
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<td>NSD</td>
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Table 5.1 (Continued)

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<td>pIKK</td>
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<td>pIκBα</td>
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<td>pIKK</td>
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<tr>
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<tr>
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<td>Length</td>
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<tr>
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<tr>
<td>Leptin-mediated synaptogenesis</td>
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<tr>
<td>PSD95 Immuneactivity</td>
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<tr>
<td>PSD95 mRNA</td>
<td>↑</td>
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</tr>
<tr>
<td>SYN Immuneactivity</td>
<td>↑ (in dendrite)</td>
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<tr>
<td>SYN mRNA</td>
<td>NSD</td>
<td>NSD</td>
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Abbreviations: Rb1: ginsenoside Rb1; HFD: vs.: versus; high-fat diet; DIO: high-fat diet-induced obese; NSD: no significant difference; PYY: peptide YY; POMC: Proopiomelanocortin; AgRp: Agouti gene-related peptide; NPY: Neuropeptide Y; GTT: glucose tolerance test; BG: blood glucose; IL-6: Interleukin
6: IL-1β: Interleukin-1β; TNFα: Tumor necrosis factor α; pIκK: pIκBα: ; SOCS3: Suppressor of Cytokine Signaling 3; PTP1B: Protein Tyrosine Phosphatase 1B; EI: energy intake; BWG: body weight gain; Ob: high-fat diet-induced obese mice; pJAK2: Phosphorylated Janus Kinase 2; pSTAT3: Phosphorylated Signal Transducer and Activator of Transcription; pAkt: Phosphorylated Protein kinase B; pGSK3β: Phosphorylated Glycogen synthase kinase-3; pFOXO1: Phosphorylated Forkhead box protein O1; PA: palmitic acid; TS: teasaponin; BDNF: brain-derived neurotrophic factor; PSD-95: ; SYN: synaptophysin.

5.1.1 Overall discussion of findings

In chapter 2, we reported Rb1 treatment decreased food intake, final body weight and reduced fat deposition in the high-fat diet-induced obese mice. These results are consistent with a previous report, which showed that Rb1 has an anti-obesity effect in rats and its suppression of food intake is not due to malaise, as attested by a conditioned taste aversion test (Xiong et al., 2010). Rb1 reduced fat deposition mainly attributed by the decreased in the adipocyte size and the increased the number of small adipocytes and reduced the number of large adipocytes. Importantly, this study also extends the mechanism of Rb1 in suppressing food intake. Rb1 treatment increased the anorexigenic hormone, peptide YY (PYY), in the blood and modulated hypothalamic neuropeptides, specifically by increasing anorexigenic POMC and decreasing orexigenic AgRP mRNA expression in the HFD-induced obese mice. Furthermore, this study reported Rb1 decreased the protein level of pro-inflammatory cytokines (TNFα, IL-6 and IL-1β) and inflammation signaling (pIKK) in the peripheral metabolic tissue, epididymal fat tissue and liver. Since peripheral inflammatory responses induced by over nutrition and obesity has been linked to decreases insulin sensitivity in target cells and contributed to glucose intolerance and development of type 2 diabetes (Cai et al., 2005; Stanton et al., 2011a; Weisberg et al., 2003; Xu et al., 2003), the anti-inflammation effect of Rb1 in the peripheral tissues of obese mice may contributed to the improvement of glucose intolerance. Besides, Rb1 treatment elevated circulation adiponectin, which has been indicated to ameliorate insulin resistance and reduced fatty acids levels in rodent, may contribute to the improved glucose tolerance and reduced fat accumulation we observed in the obese mice treated with this compound. Last, but not the least, we observed that Rb1 ameliorated hyperleptinemia, reduced the hypothalamic inflammation, PTP1B and SOCS3, improved central leptin signaling and restored central leptin sensitivity.
Hypothalamic inflammation is considered a key pathology of obesity in rodents and humans (Cai and Liu, 2012), leading to central leptin resistance through activation of the negative regulators of leptin signaling, SOCS and PTP1B (Milanski et al., 2012; Zhang et al., 2008). Our results demonstrate that Rb1 decreased the upregulation of SOCS3 and PTP1B in the hypothalamus of HFD-induced obese mice. Therefore, the inhibition of SOCS3 and PTP1B and attenuation of hypothalamic inflammation, contributes to the therapeutic effect of Rb1 on central leptin resistance observed in our mouse model. The improvement of hyperleptinemia, results from the reduction of fat mass, also contributes to the improvement of central leptin sensitivity. Overall, this study demonstrated chronic Rb1 treatment inhibited inflammation in the adipose tissue, liver, and the mediobasal hypothalamus of HFD-induced obese mice. Rb1 treatment also resulted in the improvement of glucose tolerance, central leptin sensitivity and the hypothalamic leptin-pSTAT3 signaling. We also reported that Rb1 treatment increased circulating anorexigenic hormone PYY and regulated the melanocortin POMC/AgRP neuropeptides in the mediobasal hypothalamus, contributing to the negative energy balance. Therefore, ginsenoside Rb1 is a potential anti-obesity therapeutic agent by modulating obesity-associated inflammation, improving central leptin sensitivity in the HFD-induced obesity.

In chapter 3, we expended these findings to investigate the effect of chronic Rb1 treatment on leptin signaling and leptin-mediated BDNF regulation in the prefrontal cortex of high-saturated fat diet–induced obese mice. We found that Rb1 upregulated BDNF protein expression and corrected the impaired leptin-mediated BDNF regulation in the prefrontal cortex of obese mice. Furthermore, Rb1 improved hyperleptinemia and restored leptin sensitivity on the regulation of JAK2-STAT3 signaling in the prefrontal cortex of obese mice, by increasing these signal protein phosphorylation. Moreover, we used an in vitro cultural primary cortical neuron model, to evaluate the deleterious effect of saturated fatty acid, palmitic acid, on the leptin-mediated BDNF regulation, and the leptin-mediated neurite outgrowth and synaptogenesis; as well as investigate the treatment effect of Rb1 on the PA-pretreated cortical neurons. This study indicated that, in the cultured primary prefrontal cortical neurons, palmitic acid impaired the effect of leptin on BDNF, neurite outgrowth and synaptogenesis markers. Rb1 treatment
improved the leptin-mediated BDNF expression, leptin-mediated neurite outgrowth (branching and length) and synaptic proteins (SYN and PSD95) in palmitic acid pre-treated prefrontal cortical neurons.

The present study demonstrated leptin elevates BDNF in the prefrontal cortex, as indicating by protein expression level in the *in vivo* study and immunochemistry and mRNA in the *in vitro* study. High-fat diet or palmitic acid altered the ability of leptin to elevates BDNF in the prefrontal cortex, suggesting that a high-saturated fat diet can affect leptin function in the prefrontal cortical neurons. These results are consistent with a previous report that demonstrated the impairment of central leptin-regulated BDNF in obese mice, which is associated with depressive behavior (Yamada et al., 2011). Moreover, mutation of the BDNF gene is associated with increasing early-onset obesity and cognitive impairment in BDNF-haploinsufficient patients (Gray et al., 2006). BDNF in the prefrontal cortex promotes neuronal plasticity and neurogenesis, which are important for learning and memory (Kanoski et al., 2007a; Sakata et al., 2013b). Thus, the impairment of leptin-regulated BDNF in the prefrontal cortex by saturated fat may play an important role in central nervous system dysfunction in obese subjects. The present study also demonstrated high-saturated diet substantially impaired leptin-JAK2-STAT3 signaling in the prefrontal cortex of obese mice, suggesting central leptin insensitivity in obese mice. Central leptin insensitivity also exists in obese individuals. The prevalence of dementia and other neurodegenerative diseases was higher in obese subjects with hyperleptinemia (Carpenter et al., 2000; Carter et al., 2000; Lieb et al., 2009a), which suggests that high plasma leptin levels unlikely activate signaling pathways in the brain of obese individuals. Therefore, central leptin resistance is a pathology of obesity (Myers et al., 2008b). Leptin was shown to regulate neurite outgrowth (branching and elongation) and synaptogenesis (PSD95 and SYN) in the primary prefrontal cortical neurons in the present *in vitro* study. However, this ability of leptin was blunt by palmitic acid, indicating saturated fat impaired leptin-mediated neurite outgrowth and synaptogenesis in cortical neurons. As prefrontal cortex is important for cognitive control (Cole et al., 2012b), and high-fat diet reduces synaptic plasticity in this region (Val-Laillet et al., 2011a) leading to learning and memory impairments (Laroche et al., 2000b).
Therefore, taken all together, the impairment of leptin-mediated BDNF expression and leptin-JAK2-STAT3 signaling by saturated fat in the prefrontal cortical neurons may attribute to the impairment of leptin-mediated neurite outgrowth and synaptogenesis, and thus accelerate neurodegeneration and cognitive decline. Importantly, Rb1 treatment improved hyperleptinemia, improved leptin-mediated BDNF expression, as well as leptin-JAK2-STAT3 signaling, which may facilitated to the improvements of leptin-mediated PSD95 and leptin-mediated SYN, and thus improved neurite outgrowth and synaptogenesis. These results suggest that these effects of Rb1 may partly contribute to the improvement of cognitive function of ginseng treatment in a clinic trial (Heo et al., 2011). Therefore, Rb1 may have beneficial effects on obesity-associated neurodegenerative disorders.

In chapter 4, we demonstrated chronic teasaponin treatment reduced obesity, hypothalamic inflammation, and central leptin resistance in high-fat diet-induced obese mice. Teasaponin treatment enhanced object recognition memory, and improved hypothalamic and cortical leptin-JAK2-STAT3 signaling and cortical leptin-induced BDNF expression of HFD fed obese mice, as well as improved hyperleptinemia, hyperinsulinemia and glucose tolerance in obese mice. Moreover, teasaponin treatment ameliorated the impairment on leptin-mediated BDNF, leptin-mediated neurite outgrowth (branching and elongation) caused by saturated-fat, palmitic acid. It is generally accepted that low-grade pro-inflammatory state is at the pathogenic core of obesity and type 2 diabetes. Therefore, teasaponin is a potential anti-obesity therapeutic agent that modulates obesity-induced inflammation and improves central leptin sensitivity in HFD-induced obesity. Previously, both clinical and animal studies have shown that tea improves cognitive function (Kuriyama et al., 2006; Ng et al., 2008) and prevents impairments of learning and memory (Morton et al., 2005). Furthermore, tea increases brain activity in the prefrontal cortex of healthy volunteers as assessed by functional MRI methods (Borgwardt et al., 2012). Therefore, the effect of teasaponin, an active gradient of tea extract, on enhancing object recognition memory in obese mice, may contribute to the ability of tea to improve cognitive function and memory, and increase brain activation in the prefrontal cortex, as described previously (Borgwardt et al., 2012; Kuriyama et al., 2006; Ng et al., 2008). Cortical leptin
resistance was observed in the obese mice, while teasaponin treatment restored leptin sensitivity, illustrating by the improving of leptin-JAK2-STAT3 signaling in the prefrontal cortex of teasaponin-treated obese mice. It has been reported that leptin replacement improves cognition in leptin-deficient patients with a delay or decline of cognitive function (Paz-Filho et al., 2008). Therefore, teasaponin may improve recognition memory via attenuate HFD induced impairments of leptin signaling in the prefrontal cortex.

It is known that BDNF-mediated synaptic plasticity is usually coordinated by the synaptogenic proteins, SYN and PSD-95 (Li and Keifer, 2012; Nelson et al., 2013; Robinet and Pellerin, 2011; Wolkowitz et al.). Consistent with this, along with increased BDNF levels, our findings demonstrated that leptin stimulated SYN and PSD95 mRNA expression in vitro (Yu et al., 2015). Furthermore, mRNA expression of the neurite outgrowth marker MAP2 increased in response to leptin in cultured prefrontal cortical neurons. It is well established that leptin has important effects on neurogenesis, synaptogenesis and dendritic morphology in the brain (Valerio et al., 2006; Yamada et al., 2011). Overall, these results suggest that leptin may promote neurogenesis and synaptic plasticity via increasing BDNF, SYN and PSD95 in cortical neurons. It has been reported that a diet high in saturated fatty acids induces neuronal degeneration and inactivates the leptin signaling molecule STAT3 in the hypothalamus of mice (McNay et al., 2012). This is consistent with our in vitro results, suggesting that saturated fatty acids also induce a leptin insensitivity effect on neurite outgrowth and synaptogenesis in the prefrontal cortical neurons. We also found that teasaponin reversed this adverse effect of palmitic acid on leptin-induced BDNF and neurite. It is known that the leptin signaling molecules, JAK2 and STAT3, are distributed around the postsynaptic sites in the cerebral cortex (Robinet and Pellerin, 2011). This suggests that teasaponin may modulate leptin signaling, BDNF expression and post-synaptic function in the prefrontal cortex, leading to improved recognition memory, suggesting the potential therapeutic effects on obesity-associated neurodegenerative diseases and cognition decline.

Chronic treatment with triterpene saponins, i.e. ginsenoside Rb1 and teasaponin, in obese mice is effective in the anti-obesity, anti-obesity-associated peripheral and central inflammation,
improving hyperleptinemia and glucose intolerance, and correcting central leptin resistance, as well as improving hypothalamic and cortical leptin sensitivity. Those results are consistent with some of the clinic and animal data that ginseng or tea may act on anti-inflammation, anti-obesity, and improve cognition (Borgwardt et al., 2012; Heo et al., 2011; Xiong et al., 2010). Ginsenoside Rb1 and teasaponin are triterpene saponin, belonging to steroidal saponins, which share structure features with steroid hormones (Attele et al., 1999a; Morikawa et al., 2006). Due to the steroid-like structure and the amphiphilic nature (Abid Ali Khan et al., 2012; Attele et al., 1999a; Morikawa et al., 2006), ginsenosides Rb1 or teasaponin can intercalate into the plasma membrane replacing membrane cholesterol, which increases membrane fluidity and changes the immediate environment of cell membrane proteins, such as the GABA receptor (Abid Ali Khan et al., 2012; Attele et al., 1999a). Furthermore, ginsenoside may traverse cell membranes freely and activate intracellular membrane receptors, such as steroid receptors, which may regulate the transcription of target genes (Attele et al., 1999a). However, it remains to be determined if Rb1 or teasaponin directly interacts with leptin receptor.

Finally, both ginsenoside Rb1 and teasaponin ameliorated the impairment caused by palmitic acid on leptin-mediated neurite outgrowth and synaptogenesis in the primary cortical neurons. Besides, oral teasaponin has been demonstrated to improve cognitive memory. Taken all together, triterpene saponins (both ginsenoside Rb1 and teasaponin) have neuroprotective effects that can be potential therapeutic agents on treating obesity-associated neurodegenerative diseases.

5.1.2 Proposed Mechanisms of Triterpene saponins in Overall discussion of findings

Figure 5.1 proposed the possible mechanisms of triterpene saponins (ginsenoside Rb1 and teasaponin) on treating obesity, obesity-associated inflammation and leptin resistance, as well as mechanisms of triterpene saponins (ginsenoside Rb1 or Teasaponin) on treating leptin insensitivity in the prefrontal cortex impaired by high-fat diet or high saturated fat, palmitic acid.
1. Long term maintaining mice on a high-fat diet containing saturated fat enlarges the adipocytes size and increases the proportion of large adipocytes in adipose tissue, causing fat deposition; as well as increases food intake and body weight gain, and thus becomes obese. Fat deposition increases the circulating leptin, contributes to the hyperleptinemia. During the obesity development, central inflammation plays a pathologic role, that increasing negative leptin regulator PTP1B and SOCS3 protein expression, and thus contributes to central leptin resistance and the impairment of leptin signaling (including pSTAT3, and pFOXO1) in the hypothalamus. Hyperinsulinemia exists in the obese mice. High-fat diet-induced obesity results in peripheral inflammation in liver and adipose tissue, which together with hyperinsulinemia, causes glucose intolerance. Peripheral inflammation, together with hyperleptinemia, also contributes to the peripheral leptin resistance. Importantly, chronic treatment the obese mice with ginsenoside Rb1 or teasaponin for three weeks, reduced food intake via increasing circulating anorexigenic hormone PYY expression and upregulation of anorexigenic neuropeptide POMC, as well as downregulation of orexigenic neuropeptide AgRP in mediobasal hypothalamus. Rb1 treatment also reduced body weight gain and adipocyte size and fat deposition, thus ameliorated obesity. Rb1 or teasaponin treatment reduced central inflammation and negative regulators (PTP1B and/or SOCS3) of leptin signaling, then improved central leptin sensitivity. Rb1 treatment reduced peripheral inflammation in liver and adipose tissue, contributing to the improvement of glucose intolerance and peripheral leptin resistance.

2. High-fat diet-induced obese mice developed hyperleptinemia and central leptin resistance in prefrontal cortex, which induced the downregulation of leptin-mediated BDNF protein expression. As BDNF is essential for the neurite outgrowth and synaptogenesis, therefore, high-fat diet or high saturated fat caused the downregulation of leptin-mediated neurite outgrowth (MAP2) and synaptogenesis (PSD95 and SYN). Importantly, treatment with ginsenoside Rb1 or Teasaponin, ameliorated the impairment caused by high-fat diet or high saturated fat, improved leptin-pSTAT3 signaling, leptin-mediated BDNF, leptin-mediated neurite outgrowth (branching and elongation) and synaptogenesis in the prefrontal cortex. Thus, treatment with triterpene
Saponins (ginsenoside Rb1 or Teasaponin) will potentially improve cognition and neurodegenerative disorders.

Figure 5.1 Proposed mechanism of ginsenoside Rb1 treatment on obesity, obesity associated inflammation and leptin resistance; and proposed mechanism Rb1 or teasaponin improves cortical leptin sensitivity and synaptogenesis in high-fat diet-induced obese mice.

Abbreviation: HFD: high-fat diet; Rb1: ginsenoside Rb1; FI: food intake; BWG: body weight gain; PYY: peptide YY; POMC: Proopiomelanocortin; AgRP: Agouti gene-related peptide; PTP1B: Protein Tyrosine Phosphatase 1B; SOCS3: Suppressor of Cytokine Signaling 3; pSTAT3: Phosphorylated Signal Transducer and Activator of Transcription; pFOXO1: Phosphorylated Forkhead box protein O1; BDNF: brain-derived neurotrophic factor; MAP2: microtubule-associated protein 2; SYN: synaptophysin; PSD95: Protein Tyrosine Phosphatase 1B.

5.1.3 Conclusions
The results of present PhD thesis have been demonstrated the treatment effects of triterpene saponins (ginsenoside Rb1 and Teasaponin) on the high-fat diet-induced obese mice. All results are summarized in table 5.1, and proposed mechanisms are showed in Figure 5.1.

In conclusions, food intake, body weight gain, fat deposition, hyperleptinemia, as well as glucose intolerance in the high-fat diet-induced obesity can be improved by chronic ginsenoside
Rb1 treatment. Obesity-associated peripheral inflammation and hypothalamic inflammation can be reduced by Rb1 treatment, indicating by the decrease expression of TNFα, IL-6, IL-1β and pIKK. Rb1 treatment also decreased the protein expression of SOCS3 and PTP1B, the negative regulators of leptin signaling. The reduced central inflammation and downregulation of SOCS3 and PTP1B by Rb1 treatment, contributed to the improvement of central leptin resistance and restore leptin signaling in the mediobasal hypothalamus of obese mice treated with Rb1. Reduced peripheral inflammation contributed to the improvement of glucose intolerance. Besides, the increased expression of circulating PYY, and hypothalamic POMC upregulation and AgRP downregulation extend the mechanism on suppressing food intake by Rb1. Obesity impaired prefrontal cortex and induced leptin insensitivity in the cortical neuron in vivo and in vitro. Treated by the triterpene saponins, ginsenoside Rb1, leptin sensitivity in the prefrontal cortex of obese was improved, evidencing by the improvement of cortical leptin-JAK2-STAT3 signaling, leptin-mediated BDNF expression, leptin-mediated neurite outgrowth (MAP2 mRNA, branching and neurite length) and synaptogenesis (PSD95 immunoreactivity and mRNA). Teasaponin, the second triterpene saponin we tested, has been demonstrated that reducing food intake, body weight, improving hyperleptinemia and hyperinsulinemia, as well as improving glucose intolerance in the high-fat diet-induced obese mice. Chronic teasaponin treatment also decreased hypothalamic inflammation, improved central leptin resistance, and restored hypothalamic and prefrontal cortical leptin sensitivity via leptin-STAT3 signaling in obese mice. Using an in vitro prefrontal cortical model, we demonstrated that teasaponin treatment reversed the impairment by palmitic acid on the leptin-mediated BDNF expression and neurite outgrowth (branching and elongation).

Taken all together, triterpene saponins, including ginsenoside Rb1 and teasaponin, have beneficial effects on anti-obesity, on treating obesity-associated leptin resistance, obesity-associated inflammation, and obesity-associated neurodegenerative disorders.

5.1.4 Recommendation for Future Research

Based on the findings of the current series of studies, recommendation for future research are
listed as follows:

1. In the current study treatment of obese mice with Rb1 restored leptin induced activation of STAT3 phosphorylation, but not phosphorylation of FOXO1 in the mediobasal hypothalamus. Therefore, in the hypothalamus, Rb1 acted on the STAT3 signaling pathways rather than the FOXO1 pathway to restore leptin signaling and sensitivity. The precise mechanisms need further examination.

2. In the current study, we demonstrated Rb1 elevated circulating PYY in the treated obese mice. PYY is a gut-brain anorexigenic hormone that promotes negative energy balance by reducing appetite (Boey et al., 2008). The increased plasma PYY in Rb1 treated obese mice may have contributed to the negative energy balance, lower body weight gain and fat accumulations in these animals. The mechanism by which Rb1 treatment increased circulating PYY levels requires to be further determined.

3. This study demonstrated that Rb1 increased leptin sensitivity in the prefrontal cortex of obese mice, and in prefrontal cortical neurons treated with palmitic acid, as well as reversed the alterations of leptin downstream signaling molecules (pJAK2, STAT3, BDNF, and PSD95). However, further study needs to be carried out to determine if Rb1 directly interacts with the leptin receptor.

4. This study illustrated that Rb1 treatment ameliorated alteration of leptin-JAK2-STAT3 and leptin-BDNF in the prefrontal cortex of obese mice and improves hyperleptinemia. Rb1 treatment also promoted leptin’s effect on neurite branching and elongation, and synaptogenesis in prefrontal cortical neurons. As high-fat diet-induced obesity has been implicated in the progression of neurodegenerative diseases, such as vascular dementia. Rb1treatment may have therapeutic effects in attenuating the progression of cognitive declines in obese patients and reducing the risk of neurodegenerative diseases. This will be the next work to further validate using obese mice model.

5. This study presented teasaponin improved leptin sensitivity in the prefrontal cortex of obese mice and primary cortical neurons treated with palmitic acid, and reversed the alterations of leptin downstream signaling. According to the amphiphilic nature and able to intercalated into

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the plasma membrane, replacing membrane cholesterol and increasing membrane fluidity, and thus this molecule could change the immediate environment of cell membrane proteins. However, it is required to further investigate if teasaponin directly interacts with the leptin receptor.

6. This study present oral teasaponin improved the recognition memory in treated obese mice. Since high fat diet-induced obesity has been implicated in the progression of neurodegenerative diseases, such as vascular dementia, teasaponin supplementation may have beneficial effects in attenuating the progression of cognitive decline in obese patients and reducing the risk of neurodegenerative diseases. However, the eventual toxicity of long-lasting exposition to elevated concentrations of teasaponin should be examined before teasaponin supplementation to be recommended.
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# Appendices

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