EFFECT OF VOLUNTARY EXERCISE AND DIET ON THE UNFOLDED PROTEIN RESPONSE IN THE BRAIN OF MICE

by

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ABSTRACT

Effect of Voluntary Exercise and Diet on the Unfolded Protein Response in the Brain of Mice

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The Endoplasmic Reticulum (ER) is a net-like intracellular organelle where protein is folded, matures, and is transported. When cellular stressful circumstances affect the ER, unfolded proteins are stacked in the ER lumen. This cellular stress is called ER stress. To defeat ER stress, cells have a defensive mechanism called the Unfolded Protein Response (UPR). Many chronic diseases such as obesity and type 2 diabetes or neurodegenerative disease such as Alzheimer’s disease have recently been linked to ER stress. Exercise has a significant effect on ameliorating the development of these chronic diseases or neurodegenerative diseases. However, no studies have assessed the effect of exercise on UPR activity in the brain. So this study was mainly focused on identifying how voluntary running wheel exercise affects the UPR in the brain of C57BL/6 mice exposed to a variety of dietary conditions of differing levels of dietary fat and different periods of feeding. As an exercise protocol, access to a voluntary running wheel for 3 weeks was used and running mice were grouped depending on their level of running
activity. Using real-time PCR and western blotting, UPR-related gene/protein expression (XBP1, ATF6, eIF2α, and GRP78) was assessed in different brain regions. Exercise had a significant effect on up-regulating UPR activity in the brain of mice fed low fat diet (LFD) or high fat diet (HFD) for 3 weeks or 3 months. These effects were time and brain region dependent. However, the effect of exercise on up-regulating UPR disappeared in mice fed very high fat diet (VHFD) for 4 months. In addition to assessing UPR activity, the possibility that exercise-induced UPR activation was associated with activation of apoptosis was investigated. Apoptotic signaling was not affected by exercise. Trophic factors are activated by exercise and are known to be linked to UPR activity. The possibility that IGF-1, one such trophic factor, was responsible for exercise-induced UPR up-regulation without activating apoptosis was studied. The results showed that IGF-1 was not responsible for exercise-related activation of the UPR in the brain. The chemical chaperone 4-phenylbutyric acid (PBA) was given to mice to reduce ER stress and the effect of exercise on the UPR of the brain was studied. PBA had a tendency to lower ER stress in the hypothalamus. In this condition, exercise had a significant effect to decrease UPR activity. In conclusion, voluntary exercise activates the UPR in several brain regions of mice exposed to high-fat diet for up-to 3 months without activating apoptotic signaling. Only long-term exposure to dietary fat increased the brain UPR. It is possible that this exercise-induced UPR activation without apoptosis may contribute to the protective effect of exercise on brain health.
Public Abstract

Beneficial Effect of Exercise on Regulation of Cellular Stress in the Brain

The medical costs for many chronic diseases are increasing dramatically and placing a major financial burden on nations and individuals in both developed and developing countries. A number of chronic diseases, such as obesity, type 2 diabetes and some neurodegenerative disorders are all attenuated by a history of physical activity suggesting that they may be interconnected in some way. It has been suggested that cellular stress is a major factor promoting these chronic diseases.

Cellular stress occurs in a specific compartment within the cell, the endoplasmic reticulum, whose normal function is in the synthesis and folding of proteins into the correct 3 dimensional structure. Cells have a defensive mechanism to protect against this cellular stress that is known as the unfolded protein response (UPR). This involves the activation and/or inhibition of various genes that reduce protein synthesis and increase folding capacity.

With the support of USTAR (The Utah Science Technology and Research program), Yu Ho Kim, a Masters student in Dr. York’s research group in the Center for Advanced Nutrition & the Department of Biology at Utah State University, studied how exercise affects brain health. The hypothesis was that exercise increased the activity of the UPR to protect the brain from cellular stress. The experimental model used were mice allowed to have free access to running wheels for 3 weeks in their cages while fed with either low fat or high fat diets.

The results of this study confirmed the hypothesis that physical activity increased the activity of the unfolded protein response in multiple regions of the brain of mice suggesting that this mechanism may be, in part at least, responsible for the protective effects of exercise on some neurodegenerative diseases. Future work to identify the exercise-related signal that enhances the UPR mechanism in the brain may be helpful in the future treatment of neurodegenerative disorders such as Alzheimer’s disease.
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EXERCISE IN CHRONIC DISEASES

It has been well established that exercise has significant effects on ameliorating the development of a variety of chronic diseases such as obesity, type 2 diabetes, cardiovascular disease as well as in Alzheimer’s disease (AD). In regulating energy balance, exercise has an important effect by increasing energy expenditure and the resting metabolic rate (RMR) to maintain energy homeostasis.\(^1\)\(^-\)\(^3\) This improved metabolic rate, in turn, ameliorates the onset and/or development of obesity in humans and in rodents. In addition, many studies have shown that exercise has a positive effect on improving type 2 diabetic symptoms by improving insulin sensitivity.\(^4\)\(^-\)\(^10\) The risk of developing type 2 diabetes can be inferred by low physical fitness and VO\(_2\) max.\(^11\) In addition, exercise activates the expression of the GLUT 4 gene promoting the uptake of glucose into skeletal muscle.\(^12\)\(^-\)\(^13\)

Habitual physical activity, especially aerobic exercise, improves learning ability and the plasticity of the brain in humans, especially in the aged.\(^14\)\(^-\)\(^17\) Attention to the effects of exercise on cognitive function has recently increased and studies show that exercise slowed the progress of cognitive decline and decreased the risk of neurodegenerative disease such as AD.\(^18\)\(^-\)\(^20\) Though the mechanisms for the effect of exercise were not well elucidated, there have been a lot of studies showing that exercise has a strong effect on slowing the progress of neurodegenerative diseases. In recent studies using an AD transgenic model mouse, exercise not only increased cognitive function but also ameliorated the characteristics of AD (i.e., reduced expression of Aβ-42)
in the brain suggesting that exercise has the important potential of preventing the development of neurodegenerative disease such as AD. One interesting result suggested that exercise has the biggest effect on executive function rather than other tasks such as controlled, spatial, and speed works. Exercise training appeared to improve the function of learning and memory in rats tested in a water maze or 8-arm radial maze. It was also shown that physical activity had a significant effect on cognitive function along with academic achievement in young people.

Epidemiological studies of physical activity and neurodegenerative disease risk have been conducted with the purpose of showing the effect of exercise. One study showed that physical activity was more effective on AD (i.e., reduction of risk by 45%) than on dementia (28% reduction) and Parkinson’s disease (no effect). Many possible neuroprotective mechanisms could be induced by physical activity including improved vascular health, reduced obesity and type 2 diabetes, improved immunological status, and reduced hypercholesterolemia. High-technological neuroimaging such as functional magnetic resonance imaging (fMRI), positron emission tomography, and optical imaging are vigorously being developed and applied to identify the complex linkages between physical activity and human cognition.

Rodent models have been used to assess the comprehensive mechanisms of brain health following exercise. In such studies, it was shown that brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) are significantly elevated by physical activity in multiple brain regions of animals. Animals with high running activity have a significantly increased expression of BDNF in the hippocampus of brain. In the central nervous system, BDNF promotes neuronal plasticity by specific actions on
axon and dendritic remodeling.\textsuperscript{44-47} BDNF is processed from pro-BDNF and functions by binding to the trkB receptor, mediating synaptic transmission and cognitive control.\textsuperscript{48-49}

Taken together, these data show that exercise has a positive effect in that it improves or decelerates the development of chronic diseases such as obesity and type 2 diabetes and that it has a neuroprotective effect on neurodegenerative diseases including AD.

**ER STRESS AND UPR MECHANISM**

The endoplasmic reticulum (ER) is an intra-cellular organelle where many proteins destined to be secreted or to be membrane components are folded into proper tertiary structures.\textsuperscript{50} Once proteins are properly shaped, their hydrophobic amino acid residues are aggregated inside the entire protein structure. On the other hand, the presence of misfolded proteins generates the ubiquitin-proteasome pathway through which misfolded proteins are covalently bound with the ubiquitin, leading to degradation in the proteasome.\textsuperscript{51} Along with folding ability of the ER, cholesterol and many lipids are synthesized in this cellular organelle.\textsuperscript{52-53} However, cellular stressful environments such as glucose deprivation, perturbation of calcium homeostasis and viral infection, can hinder the role of ER leading to a build-up of unfolded proteins in the ER lumen. This pathological cellular stress is called ER stress and cells have a defensive mechanism defined as the unfolded protein response (UPR) in order to counteract to this cellular stress.\textsuperscript{54} The overall mechanism of the UPR is depicted in Figure 1-1. There are three major arms to the UPR mechanism and each is located in the ER lumen; Inositol-requiring protein 1 (IRE-1), Active Transcription Factor 6 (ATF-6) and PKR-like ER
Protein Kinase (PERK). In normal cellular status, these three arms are inactivated through binding with the ER chaperone immunoglobulin binding protein/glucose response protein 78 (Bip/GRP78). When unfolded proteins are stacked in the ER and ER stress is aroused, Bip/GRP78 is released from these three regulatory proteins, converting these UPR molecules into their active forms. Released Bip/GRP78 binds to and enhances folding of the unfolded proteins in ER lumen, decreasing the number of unfolded proteins. Once activated by dissociation of Bip/GRP78, IRE-1 forms a dimer, leading to autophosphorylation in the cytosolic kinase domain. This phosphorylated kinase acts as an endoribonuclease, a site-specific Ser/Thr protein kinase. Activated IRE-1 eliminates a 26-nucleotide intron of XBP1 mRNA, releasing spliced XBP1 (X-box binding protein 1; XBP1s). This frame-shift XBP1 is translocated into the nucleus where it binds to UPR elements (UPRE) and functions as a transcription factor which up-regulates genes for glycosylation proteins, disulphide bond proteins, and genes for elements of the ER associated degradation by which misfolded proteins are recognized and transferred to cytosol and degraded by the proteasome. Upon ER stress, ATF6, the second arm of UPR, is transported from the ER to the Golgi apparatus by unknown mechanism where its N-terminal cytoplasmic domain is cleaved by the Site 1 protease (S1P) and Site 2 protease (S2P). This cleaved ATF6 is translocated into the nucleus and functions as a transcription factor by binding to the ER stress response elements (ERSE), increasing the expression of UPR responsive genes including Bip/GRP78, CCAAT/enhancer-binding protein homologous protein (CHOP), and XBP1.
Figure 1-1 The Unfolded Protein Response (UPR) pathways. IRE-1, Inositol-requiring protein 1; ATF-6, Active transcription factor 6; PERK, PKR-like ER protein kinase; Bip/GRP78, immunoglobulin binding protein/glucose response protein 78; eIF2α, eukaryotic translation initiation factor 2α; S1P/S2P, Site 1 protease/Site 2 protease; UPRE, UPR elements; ERSE, ER stress response element; ERAD, ER-associated degradation.55-57
Along with these two UPR mechanisms, another UPR arm, PERK, affects the translation rate, reducing the synthesis of proteins and reducing the stress on the ER. PERK is protein kinase and ER stress activates PERK by phosphorylation. This activated form of PERK, in turn, phosphorylates eukaryotic translation initiation factor 2α (eIF2α). Because eIF2α is required in the process of binding the initiator methionyl-tRNA to the small ribosomal subunit, phosphorylated eIF2α no longer binds to the 80S ribosome, lowering the translational initiation events and eventual protein synthesis. It was shown that this cellular strategy of translational repression contributes to overall mRNA stabilization.

In summary, activated UPR increases the unfolded protein folding capacity along with decreasing the burden of new protein synthesis on the ER and these mechanisms eventually contribute to release the ER stress.

THE UNFOLDED PROTEIN RESPONSE (UPR) IN OBESITY

As societies have become westernized, the dietary habits of people all over the world have changed to the western diet, a high-calorie diet, and this dietary environment has resulted in an increased prevalence of obesity. Over the last several decades, the interest in obesity has increased. Many researchers have suggested the possibility that obesity could be induced by cellular stress signaling and inflammation, but have been unable to identify what is the exact origin for obesity.

Overnutrition leads to lipid accumulation in nonadipose tissues such as liver, pancreas, and muscles since fatty acids, triglycerides, and cholesterol are exogenously taken up. The buildup of intracellular lipids results in an increasing level of free fatty acids (FFA) and other lipids within the tissues which are harmful to intracellular
organelles such as ER and mitochondria due to the vulnerability of FFA to oxidative damage to produce reactive lipid peroxides.\(^{80-82}\) When the ER is overly exposed to FFA and lipid peroxides, this, in turn, causes structural changes in the ER and unfolded proteins are eventually accumulated in the ER, leading to the upregulation of UPR.\(^{83-84}\) Hotamisligil’s research group (2004) suggested that ER stress could be a main reason for obesity and type 2 diabetes. They showed that both obesity-induced by dietary conditions (High-Fat diet) and genetically obese mice (ob/ob mice) induced the activation of UPR in liver. In addition, in the UPR related gene depleted model (Xbp1\(^{+/-}\)), UPR responded to the ER stress inducer tunicamycin and this overexpression led to the impairment of glucose homeostasis and insulin signaling.\(^{85}\) His group also showed that increased ER stress in the hypothalamus of obese mice led to leptin resistance.\(^{86}\) They showed that brain tissue specific UPR gene knock-out led to both obesity and increased leptin levels after feeding a high-fat diet.

Recently, several researchers have suggested that ER stress could be the link between obesity and inflammation.\(^{87-90}\) Zhang et al. (2008) suggested that hypothalamic ER stress induced by high-fat diet was linked to inflammation (IkappaB kinase \(\beta/\)Nuclear Factor-KappaB [IKK\(\beta/\)NF-\(\kappa\)B]) which led to energy imbalance and obesity.\(^{89}\) IKK\(\beta/\)NF-\(\kappa\)B is the main switch for the control of intrinsic immune actions.\(^{91}\) In normal states NF-\(\kappa\)B is inactive through binding with the inhibitory protein I\(\kappa\)B, but when activated IKK\(\beta\) phosphorylates its substrate I\(\kappa\)B to produce an activated form. This activated I\(\kappa\)B releases NF-\(\kappa\)B which is translocated into nucleus where it acts as a transcription factor for other inflammatory actions.\(^{89}\) Viral vector mediated IKK\(\beta\) deletion in the mediobasal hypothalamus (MBH), a main region of sensing nutritional status, blocked the effect of
IKKβ and reduce the risk of obesity in high-fat diet conditions without increase of ER stress.\textsuperscript{89} On the other hand, there have been experimental trials to reduce the ER stress through applying a chemical chaperon, 4-phenyl butyric acid (PBA).\textsuperscript{86, 92-93} Oral treatment with PBA significantly decreased the level of both serum leptin and glucose in the high-fat dietary condition.\textsuperscript{86} PBA also reversed the hyperglycemia and improved the insulin sensitivity in ob/ob mice.\textsuperscript{93} These results revealed that increased ER stress could induce obesity as well as type 2 diabetes.

To summarize, overnutrition inducible obesity can be linked with ER stress when this cellular event leads to activation of the UPR as a defensive mechanism.

**THE UNFOLDED PROTEIN RESPONSE (UPR) IN ALZHEIMER’S DISEASE (AD)**

Alzheimer’s disease (AD) is one of the neurodegenerative diseases and is characterized by progressive decline of cognitive function. AD is also characterized by intracellular accumulation of tau protein into neurofibrillary tangles (NFT), and by extracellular aggregation of amyloid β (Aβ) protein, which forms senile plaques that are known to be neurotoxic.\textsuperscript{94} While in health brain β-amyloid precursor protein \textsuperscript{92} is processed by proteases such as α-, β-, and γ-secretases, mutations at the cleavage site of APP promote the accumulation of Aβ.\textsuperscript{95-97} Within the last decade, the UPR has been in the spotlight, as it was suggested that it might be involved in the underlying pathological causes of AD.\textsuperscript{98-100} One study showed that Bip/GRP78 chaperone in ER was bound to and enforced APP to be folded correctly, lowering the production of Aβ.\textsuperscript{101} In addition, when PERK activity was knock-downed by application of the PERK siRNA, it was shown that Aβ treatment increased neurotoxicity \textit{in vitro} \textsuperscript{102} due to the destruction of UPR mechanism. Mutations of the presenilin genes
(PS1 and PS2) appeared to be the main reason for causing an early onset AD and these proteins are usually found in ER. It was shown that PS1 takes part in UPR activation and the activation of IRE-1 is controlled by PS1. In cells expressing mutant PS1, mRNA expression level of Bip/GRP78 was significantly decreased. In addition, this mutation of PS1 also decreased the activities of all UPR related arms (PERK, IRE1, and ATF6). Loewen and Feany (2010) published their experimental results showing that upregulated UPR, especially XBP-1, ameliorated the neurotoxicity of tau using genetically-modified Drosophila.

Recently, it was shown that calcium homeostasis could be linked to AD. The accumulation of Aβ hinders calcium influx at the plasma membrane or ER membrane, and when calcium homeostasis is impaired, UPR is activated. In humans, UPR activity is increased in the brain of AD patients. Using immunohistochemistry for localization of pPERK, peIF2α, and pIRE-1 in the hippocampus of AD patients, it was shown that these UPR related proteins were expressed in neurodegenerative disease of human and that the upregulation of PERK was accompanied with phosphorylation of tau.

To summarize, it is possible that the UPR is linked to the etiology of AD and we propose that controlling the homeostasis of UPR may lead to the neuroprotective effect of exercise.

**APOPTOSIS**

All multicellular animals retain a balance between cell division and cell death, maintaining the number and size of cells. This tightly controlled normal cell death is
called “programmed cell death” or Apoptosis, a term first used by Currie and colleagues in 1972. Overall apoptosis mechanism is depicted in Figure 1-2.

Apoptosis is characterized by its specific morphological changes which are usually induced by cysteine proteases, one of a protein family known as the caspases which cleave substrates specifically at Asp-Xxx bonds (i.e., aspartic acid residues). Several important substrates for caspase actions have been identified. The DNA nuclease was firstly identified and shown that it was cleaved and activated by caspase. Activated nuclease cleaves the DNA fragments into shortened DNA fragment of about 180 base pairs. This DNA ladder is used as a marker of apoptotic cell death. It was confirmed that this DNA ladder nuclease is a caspase-activated DNase, or CAD and it is inactivated in the normal living cells by binding with an inhibitory subunit (ICAD). Activated caspase-3 cuts the cleavage site of the inhibitory subunit, leading to its release from nuclease which, in turn, is activated.

Alteration of apoptotic cellular structures is usually followed by caspase action and it typically occurs at nuclear lamina which have a role as supportive structures for the nuclear membrane. Caspases cleave lamina into fragments, resulting in the destruction of lamina and possible damage to the chromatin structure.

Caspases are usually activated by three representative mechanisms; caspase cascade, proximal induction, and holoenzyme formation. In the caspase cascade, an activation of an initiator caspase delivers a proapoptotic signal which sequentially turns on effector caspases, causing apoptosis. Each initiator caspase has a distinct role for mediating a proapoptotic signal. For instance, caspase-8 leads to apoptosis related with death receptors while caspase-9 delivers the signal induced by cytotoxic agents.
In addition, it was also shown that specific cofactors are necessary for activation of initiator caspases. For example, the cofactor Fas-associated protein with death domain (FADD) is required for the procaspase-8 activation\textsuperscript{36, 130} and procaspase-9 is activated by a complex composed of cofactor called apoptotic protease activating factor-1 (Apaf-1) with the caspase recruitment domain.\textsuperscript{8, 131} For the case of induced proximity, when CD95 ligand binds to CD95 (the death receptor superfamily), CD95 forms a receptor cluster, developing the death-inducible signaling complex. This complex, in turn, combines with procaspase-8 molecules, resulting in the activation of procaspase-8. As mentioned, Apaf-1 could be not only an activator for caspase-9 but also a necessary subunit for a caspase-9 holoenzyme (also called apoptosisome).\textsuperscript{132} Taken together, initiator caspases are usually activated by protein-protein interactions while effector caspases are turned on by the action of an upstream caspase.

Mitochondria are affected by apoptotic death signals.\textsuperscript{133} It was suggested that the Bcl-2 family contributes to control the mitochondria homeostasis. Once mitochondria are damaged by apoptosis, they release cytochrome c, and this combines with Apaf-1 to form a complex known as apoptosisome.\textsuperscript{134} This complex appears to activate procaspase-9, which, in turn, activates caspase-3, the main effector caspase.\textsuperscript{126, 135} It was shown that Bcl-2 family takes part in this mechanism related to the cytochrome c activation. Bcl-2 proteins seem to be aggregated on the outer mitochondrial membrane where channels were formed.\textsuperscript{136}

In summary, apoptosis is delicately controlled both catalytically and structurally. By this complex mechanism, cellular death is controlled and programmed.
**Figure 1-2** ER stress activated apoptosis signaling pathway. TRAF2, TNF receptor associated factor-2; CAD, caspase-activated DNase; ICAD, inhibitor of CAD; FADD, Fas-Associated protein with Death Domain; JNK, Jun N-terminal inhibitory kinase; ASK, apoptosis signaling kinase; JNK, c-Jun N-terminal kinase; CHOP, CCAAT/enhancer binding protein (C/EBP); GADD 34, growth arrest and DNA damage gene 34. 56, 59, 118, 137-138

**ER STRESS-SPECIFIC APOPTOTIC MECHANISM**

If the activated UPR cannot resolve the continued accumulation of unfolded proteins, the affected cells become toxic and apoptotic signaling is aroused to lead to cell death. Activation of caspases is also linked to ER stress and caspase-12 is especially related to this cellular stress. When intracellular calcium homeostasis is impaired, the perturbed intracellular calcium concentration initiates ER stress and unresolved ER stress also activates caspase-12 by calpains, a family of Ca$^{2+}$-dependent cysteine...
proteases. ER stress induced capsase-12 activity was reduced by treatment with calpain inhibitors such as E64 and MDL28170. Genetic deletion of capsase-12 in vitro and in vivo inhibited apoptosis in the presence of ER stress inducers thapsigargin and tunicamycin. This ER-stress specific capsase-12 interacts with IRE-1 (one of UPR arm) and TRAF2, an adaptor protein. Upregulated IRE-1 leads to the disassembly of heterodimers between capsase-12 and TRAF2, inducing the activation of capsase-12. Upon ER stress, procaspase-9 is cleaved by capsase-12, resulting in an activated form of caspase-9 which activates caspase-3, a main effector caspase responsible for the destruction of cellular substrates.

Meanwhile, CCAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP) is known to be linked to the ER stress induced apoptosis by acting as a transcription factor. As a transcription factor, CHOP does not affect apoptosis directly. Instead, CHOP increases the expression of target genes (e.g., GADD34) which tend to exacerbate the status of ER stress. GADD 34 dephosphorylates eIF2α on serine, deactivating eIF2α and eventually increasing the burden of ER due to the increase of RNA translation rate. Activated CHOP also increases the expression of Ero-1α, a thiol oxidase, that contributes to disulfide bond formation and protein folding in the ER. However, Ero-1α also releases a derivative such as reactive oxygen species (ROS) which can induce apoptosis. CHOP is also known as growth arrest- and DNA damage inducible gene 153 (GADD 153). The GADD 153 gene is one of a group that can be induced by genotoxic stress and growth arrest signals. It was shown that sustained exposure to ER stress leads to the upregulation of CHOP expression. Recently it was shown that activity of both IRE-1 and PERK was accompanied by the expression of
CHOP and that down-regulation of IRE-1 and PERK led to the up-regulation of CHOP, eventually triggering cell death.\textsuperscript{102, 150}

The c-Jun-N-terminal kinase (JNK) is also known to be involved in apoptotic signaling and can be induced by uncontrolled UPR activity. Upon ER stress, activated IRE-1 combines with tumor-necrosis factor-α (TNF-α)-receptor-associated factor 2 (TRAF2) and this complex can interact with apoptosis-signal-regulating kinase (ASK1).\textsuperscript{151} Activated ASK1, in turn, may increase the activity of downstream kinase JNK and lead to cell death.\textsuperscript{152} Using ASK\textsuperscript{−} of mouse embryonic fibroblasts (MEFs), these cells were unable to activate JNK and apoptotic signaling after treatment with ER stress inducers.\textsuperscript{153} From this result, it was suggested that ASK is an important activator of JNK and apoptosis. Taken together, it is thought that prolonged ER stress may be linked to the development of apoptotic signaling by complex mechanisms and cell death can be induced.

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CHAPTER 2

THE EFFECT OF EXERCISE AND DIET ON THE UNFOLDED PROTEIN RESPONSE (UPR) IN THE BRAIN OF MICE

INTRODUCTION

The brain has an important role in regulating energy balance and peripheral glucose homeostasis and abnormal central regulation can contribute not only to the development of obesity but also to type 2 diabetes. The brain responds to circulating leptin which is released from adipose tissue\textsuperscript{1-2} and circulating insulin\textsuperscript{3-4} both of which are released relative to the degree of adiposity.

As one way of sensing energy status, the hypothalamus of the brain responds to leptin and other endocrine and nutrient signals to reduce food intake and increase energy expenditure, leading to control of body weight.\textsuperscript{5-7} Friedman’s research group showed that the obese (ob) gene has a main role in regulating energy balance in the mouse and that mutation of this gene (lep\textsuperscript{ob}/lep\textsuperscript{ob}) leads to obesity and type 2 diabetes.\textsuperscript{8} Furthermore, they showed that the arcuate nucleus of hypothalamus is the central site for regulating leptin signaling which, in turn, is dependent on the presence of the long form of the leptin receptor (Ob-Rb) which is known to be absent in db/db mice.\textsuperscript{9} In high-fat fed mice and obese humans, circulating leptin levels are chronically increased and their responsiveness to leptin is severely attenuated.\textsuperscript{10-12} This down-regulated leptin sensitivity is called “leptin resistance”. Stimulation of the inhibitory molecule Suppressor of Cytokine Signaling 3 (SOCS3) in the hypothalamus in response to leptin normally modifies its activity and may have a role in the development of leptin resistance.\textsuperscript{13-14}
In addition to leptin actions in the brain, insulin also takes part in the regulation of energy balance. Although it was initially believed that insulin could not cross the blood-brain barrier, it is now recognized that insulin can access into the brain across the blood-brain barrier and that insulin receptors are widely expressed in the brain including the arcuate nucleus.\textsuperscript{15} Secretion of insulin is also affected by the amount of stored fat so that the basal level of circulating insulin is up-regulated according to the individual fat mass.\textsuperscript{3-4} Increasing insulin levels with increased adiposity leads to the insulin resistance and the development of type 2 diabetes. Studies have confirmed that the brain is sensitive to insulin. Using the technique of intracerebroventricular (icv) infusion, direct insulin administration into the third ventricle of the brain was shown to decrease food intake along with body weight.\textsuperscript{16-18} Numerous studies have also shown that insulin actions in the brain contribute to glucose homeostasis. Obici et al. (2002) suggested that hypothalamic insulin receptors are necessary for glucose homeostasis.\textsuperscript{19} By application of an antisense oligodeoxynucleotide for the insulin receptor precursor protein, hyperphagia and insulin resistance are induced in rats indicating that insulin receptor activity is needed in the hypothalamus for proper regulation of food intake and insulin action.\textsuperscript{20} Similarly, icv infusion of antibodies specific to insulin into the hypothalamus increased glucose production indicating that insulin action in the brain regulates glucose homeostasis.\textsuperscript{19}

Within the last decade, many studies have shown that leptin resistance can result from ER stress and Ozcan et al. (2004) suggested that uncontrolled ER stress is the principal cause of obesity as well as type 2 diabetes.\textsuperscript{21} ER stress inducible chemicals tunicamycin and dithiothreitol (DTT) induce leptin resistance \textit{in vitro} by inhibiting leptin induced tyrosine phosphorylations of both leptin receptor (LepRb) and signal transducer
and activator of transcription 3 (STAT3), a down-stream component of the leptin signaling pathway. In addition, direct infusion of an ER stress inducer into the brain of lean mice increased leptin resistance and increased mRNA expression of neuropeptide Y (NPY) and agouti-related peptide (AgRP), known changes associated with leptin resistance. Furthermore, in neuron specific XBP-1 knock-out mice, not only plasma leptin level but also fat mass were significantly increased in the presence of high fat diet.

Recently, it was shown that ER stress is connected to impaired insulin signaling in the brain. Insulin activation of phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3) in the mediobasal hypothalamus (MBH), a downstream insulin signaling component, was severely reduced in neuron specific IkappaB kinase beta (IKKβ) deleted mice in which ER stress was increased. In addition, ER stress triggered by icv injection of the ER stress inducer thapsigargin down-regulated insulin signaling in the hypothalamus as detected by western blotting using antibody specific for phosphorylated Akt. In the mHypoE-44 hypothalamic cell line, palmitate induced lipotoxicity up-regulated the expression of phosphorylated eIF2α, a component of the UPR response, and caspase-3, an apoptosis marker. This palmitate treatment also decreased the expression of phosphorylated Akt and prevented insulin signaling.

Many epidemiological studies have revealed a connection between metabolic diseases such as obesity and type 2 diabetes, and neuronal diseases such as Alzheimer’s disease (AD). Obesity has been recognized as a main risk factor for AD and much supportive evidence has accumulated. Recent studies in vivo and in vitro have shown that impaired leptin signaling could also lead to the increased onset of AD. Leptin treatment
lowered Aβ levels in a dose- and time-dependent manner in the Neuro2a neuronal cell line.\textsuperscript{33} When leptin (daily 20 μg in PBS) was continuously administered into an AD model mice (Tg2576 mice) using Alzet osmotic minipumps for 8 weeks, brain Aβ levels were significantly decreased even on high-fat dietary feeding.\textsuperscript{33} Meanwhile, human studies have shown that obesity affects the onset of AD. Whitmer et al. (2007) showed that obesity/overweight, measured by body mass index (BMI), has a strong relationship with the onset of AD in middle-aged person.\textsuperscript{34} In another study a significant correlation between BMI/body fat mass and Aβ levels was identified.\textsuperscript{35}

Although exercise has a significant effect in reversing or slowing the progress of chronic diseases such as obesity,\textsuperscript{36-37} type 2 diabetes \textsuperscript{38-39} and AD,\textsuperscript{40-41} there have been few trials aimed at elucidating the effects of exercise on ER stress which is a possible main factor for the cause of these diseases. In 2008, Um et al. showed that 16 weeks of exercise training up-regulated the expression of the ER chaperone ‘GRP78’ proteins and down-regulated expression of the apoptotic proteins caspase-9 and -3 in the brain of APPsw transgenic mice, an AD disease model.\textsuperscript{42} In this study, a water maze test also showed the effect of exercise on UPR activation in the brain of AD mice along with the connection to the improvement of behavioral dysfunction.\textsuperscript{42} Recently, it was shown that 6 weeks of forced running increased UPR related gene expression (Grp78) in liver of rats fed high-fat diet \textsuperscript{43} along with decreasing body fat mass. This implies the effect of exercise on UPR also occurs in peripheral tissues. In 2011, Wu et al. revealed that one bout of exercise led to increased expression of UPR related genes in skeletal muscle of C57BL/6 mice such as quadriceps.\textsuperscript{44} They also showed that expression of the UPR gene Xbp1 in skeletal muscle reduced to normal level after longer (4 weeks) exercise
training. In contrast to these results, some reports have shown opposite results. Ropelle et al. (2010) showed that physical activity, composed of two 3-hour swimming exercise sessions along with 45 minutes rest time, lowered the UPR related gene expression (phosphorylated PERK) in the hypothalamus of rats injected with the ER stress inducer of thapsigargin. It was also shown that chronic exercise of 4 weeks swimming (1 hr/day, 5 day/week) increased phosphorylated PERK protein expression without statistical significance in hypothalamus of diet-induced obese rats. Most recently, it was shown that 8 weeks of swimming exercise lowered the UPR related phosphorylated PERK and eIF2α protein expressions in both adipose and liver tissue of rats adapted to high-fat diet for 2 months. Taken together, the effect of exercise on the UPR activity is unclear at this time. Further experiments are needed to elucidate the effect of exercise on the UPR mechanism in the brain as well as in other tissues.

In this chapter, we have focused on showing how voluntary running wheel exercise affects the activities of UPR related genes/proteins in the brain of C57BL/6 mice and how this response is affected by feeding HFD for differing time periods, 3 weeks (section 2), 3 months (section 3), and over 3 months (section 1). These experiments have shown how diet affects the response of the UPR in the brain, information that has implications for the prevention of chronic diseases.

**HYPOTHESES**

(1) The UPR mechanism in brain of mice will be up-regulated by voluntary running wheel exercise and this effect will be enhanced by dietary fat.

(2) Activated UPR in response to exercise will not be connected with apoptotic signaling up-regulation.
(3) Dietary fat will up-regulate the UPR activity and apoptosis will be induced.

METHODS

Animals and Diets

Male C57BL/6 mice (~ 6 weeks) were used in all of the experiments. All animal studies were approved by the Utah State University Institutional Animal Care and Use Committee. Once all animals arrived at the animal room, about one week was given for adaptation to the new environment. Each mouse was then moved to an individual cage (30.3 x 20.6 x 26 cm, Nalgene® plastic cage) which can be equipped with a running wheel and the type of diet was also changed as required by each experimental protocol. In this research, three types of diets were used: Low fat diet (LFD); 10 kcal% Fat (D12450B, Research Diets, New Brunswick, NJ), High fat diet (HFD); 45 kcal% Fat (D12451, Research Diets, New Brunswick, NJ) and Very High fat diet (VHFD); 60 kcal% Fat (TD 03661, Harlan Teklad, Madison, WI). Detailed dietary information is shown in Table 1. The food was given in feeding cups and both 45% HFD and 60% HFD were changed every second day due to the possibility of oxidation of the diet fatty acids. Before starting the 3 weeks running protocol, each diet was given for a different time (VHFD for 4 months, LFD/HFD for 3 weeks, or LFD/HFD for 3 months). The animal room was automatically maintained at 22-23 °C with a 12-hour light/dark cycle. All animals had access to food and water ad libitum.

Body Composition

An Echo nuclear magnetic resonance imaging (MRI)-700™ system (Echo Medical Systems, Houston, TX) was used to assay body composition of animals. Using this system, % fat composition could be obtained along with the results for lean mass and
body fluid. After setting up the zero point, each mouse was placed in a transparent plastic tube which is specifically designed for mice and body compositions were recorded.

**Voluntary Running Wheel Exercise**

For mice in the running group, mice were individually housed in plastic regular cages equipped with MiniMitter running wheels (740mm circumference, [MiniMitter, Bend, OR]). One week was allowed for all mice to become accustomed to the cages containing the aluminum running wheel and during this period the running wheels were locked with commercial plastic cables. After the adaptation period, the wheels were released and mice had free access to the running wheels for the duration of experiments (e.g., 3 weeks). Revolutions of the running wheel were automatically recorded by the VitalView program in turns per hour. It was programmed that running activity was recorded and saved every hour for the duration of the experiment.

**RNA Isolation and Purification**

Mice were sacrificed by cervical dislocation. The brain was removed and the hypothalamus, hippocampi and frontal cortex were dissected out on ice. For later analysis, liver, and white adipose tissues were also taken. All tissues were quickly frozen in liquid nitrogen and kept at -80 °C until processed. Total RNA from each tissue sample was isolated using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH). Using a Teflon pestle, tissues were gently homogenized in 1.5 mL tubes containing TRI Reagent and 100 µL of 1-bromo-3-chloropropane (BCP, Molecular Research Center, Inc., Cincinnati, OH) added for the phase separation. After samples were centrifuged (15 min, 13,000 rpm, 4 °C), the RNA-rich aqueous phase was transferred to a new 1.5 mL tube and 500 µL isopropanol added to precipitate RNA.
**Table 1** Compositions of experimental diet chows

<table>
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<tr>
<th>Ingredient</th>
<th>10kcal% LFD (Research Diet, D12450B)</th>
<th>45kcal% HFD (Research Diet, D12451)</th>
<th>60kcal% HFD (Harlan Teklad, TD 03661)</th>
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<td>Fat</td>
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<td>59.5%</td>
</tr>
<tr>
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<td>100%</td>
<td>100%</td>
</tr>
<tr>
<td>kcal/g</td>
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<td>4.73</td>
<td>5.3</td>
</tr>
<tr>
<td>Ingredient</td>
<td>g/kg</td>
<td>g/kg</td>
<td>g/kg</td>
</tr>
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<td>Casein</td>
<td>200</td>
<td>200</td>
<td>230</td>
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<tr>
<td>Sucrose</td>
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<td>172.8</td>
<td>150</td>
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<tr>
<td>Maltodextrin</td>
<td>35</td>
<td>100</td>
<td>170</td>
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<tr>
<td>Cellulose</td>
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<td>50</td>
<td>26.46</td>
</tr>
<tr>
<td>Soybean oil</td>
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<td>25</td>
<td>20</td>
</tr>
<tr>
<td>Lard</td>
<td>20</td>
<td>177.5</td>
<td>330</td>
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<tr>
<td>Mineral Mix</td>
<td>(S10026)</td>
<td>(S10026)</td>
<td>(AIN-93G-MX, 94046)</td>
</tr>
<tr>
<td>Calcium phosphate</td>
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<td>13</td>
<td>2.5</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>(V10001)</td>
<td>(V10001)</td>
<td>(AIN-93-VX, 94047)</td>
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<tr>
<td>Choline Bitartrate</td>
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<td>3.5</td>
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**Fatty acid**

<table>
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<th>Fatty acid</th>
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<th>% of total fat</th>
<th>% of total fat</th>
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<td>36.3</td>
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<tr>
<td>Monounsaturated</td>
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<td>48</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>40.2</td>
<td>18.5</td>
<td>14</td>
</tr>
</tbody>
</table>
After centrifugation at 10,000 rpm at 4 °C for 8 min, the RNA pellet was washed with 1mL 75 % (v/v) ethanol and then air-dried at room temperature for 5-10 min. The pellet was resuspended in RNase-free water. Using a TURBO DNA-free™ kit (Ambion, Inc., Austin, TX), carry-over DNA was removed. After the DNase treatment, the RNA concentration was measured by spectrophotometric analysis (A260/A280) and the quality was visually assessed using 1.5 % agarose gel electrophoresis.

Quantitative Real-Time PCR

Using the Superscript First-Strand Synthesis system for RT-PCR III (Invitrogen, Carlsbad, CA), 1 µg total RNA from individual tissue samples was used to synthesize cDNA. The reaction for making cDNA was conducted for 60 min at 50 °C and for 15 min at 70 °C. The expression level of each gene (Xbp1, Grp78, Eif2α and Atf6) was quantified by the use of SYBR Green (Quanta BioSciences, Inc., Gaithersburg, MD) and normalized to the expression of cyclophilin B (Ppib) gene. Following the manufacturer’s protocol, reaction cycles for real-time PCR were set: 1 cycle for 3 min at 95 °C and then 40 cycles with two steps of 10 seconds at 95 °C and 30 seconds at 55 °C. The following primers were used:

*Grp78*: Forward, 5´-CTG GAC TGA ATG TCA TGA GGA TCA-3´, Reverse, 5´-CTC TTA TCC AGG CCA TAT GCA ATA G-3´; *Xbp1*: Forward, 5´-GGA CTC TGA CAC TGT TGC CTC TT-3´, Reverse, 5´-AAC TTG TCC AGA ATG CCC AAA-3´; *Atf6*: Forward, 5´-TGG GCA GGA CTA TGA AGT AAT G-3´, Reverse, 5´-CAA CGA CTC AGG GAT GGT GCT G-3´; *Eif2α*: Forward, 5´-ATG GAA GCC AAAGCT GAA G-3´, Reverse, 5´-CTG ACA TGA AGG AGG GCA-3´; *Ppib* (Cyclophilin B): Forward, 5´-
GCT GGA TGG CAA GCA TGT G-3’, Reverse, 5’-TGT CTT GGT GCT CTC CAC CTT-3’

**Protein Extraction**

For the assay of protein expression, the hippocampus was used as its size provides sufficient protein for the assay and these are also two symmetrical tissues in each brain. By using a Teflon pestle, hippocampus was homogenized in the 1.5 mL tube containing whole cell lysis buffer (50 mM KCl, 1 % NP-40, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES, pH 7.8], 10 µg/ml Leupeptin, 20 µg/ml Aprotinin, 125 µM DTT, 1 mM Phenylmethanesulfonylfluoride [PMSF] and 1 mM sodium orthovanadate) including one tablet of a protease inhibitor cocktail (Complete Mini®, Roche, Indianapolis, IN) per 25 ml whole cell lysis buffer. Homogenized tissues were sonicated with two cycles of 10 strokes. After centrifugation at 13,000 rpm at 4 °C for 15 min, the clear supernatants containing the total protein were transferred to new 1.5 mL tubes. Using a Pierce Bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Inc., Rockford, IL), protein concentration was determined by the amount of reduction of the Cu²⁺ to Cu⁺⁺.

**Western Blotting**

Equal amount of protein from each sample were adjusted to a common volume with 1x whole cell lysis buffer. 6x Sodium dodecyl sulfate (SDS) loading buffer was added and samples were heated at 100 °C for 5 min to denature protein structures. Samples were separated on a 10–12 % SDS-polyacrylamide gel with 1x Tris-Glycine-SDS electrophoresis running buffer (0.025 M Tris, 0.192 M Glycine and 0.1 % SDS) at 85 ~ 95 V. After confirming that the 10 kD reference band reached the bottom, the
protein was transferred to Polyvinylidene fluoride (PVDF) membranes (Thermo Scientific Inc., Rockford, IL) by western blotting transfer kit of Bio-Rad’s product. Using transfer buffer (0.5 M Tris base, 3.9 M Glycine and 20 % methanol) which was kept cool with ice pack, the transfer step was conducted for 3 hours at 80 V. Then, the membrane was blocked for about 2 hours using 5 % (w/v) nonfat dry milk mixed in Tris Buffer Saline Tween-20 (TBS-T) buffer (1 M Tris [pH 7.4], 5 M NaCl and 0.1 % Tween 20). Specific primary antibodies diluted to 1:200-1:1000 with 5 % milk buffer were added to the buffer and membranes incubated overnight at 4 °C. The antibodies used were: Rabbit anti-XBP1 (SC-7160), rabbit anti-ATF6 (SC-22799), rabbit anti-phosphorylated PERK (SC-32577R), rabbit anti-PERK (SC-13073), goat anti-phosphorylated eIF2α (SC-12412), rabbit anti-CHOP(GADD 153) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit anti-eIF2α (Cell Signaling, Danvers, MA), rabbit anti-GRP78 (Stressgen, BC, Canada), β-Actin (Abcam, Cambridge, MA), rabbit anti-Caspase-12, rabbit anti-Caspase-3 (Cell signaling, Danvers, MA).

After incubation with the primary antibody, each membrane was washed with TBS-T buffer and incubated with horse radish peroxidase-conjugated secondary antibody for one hour at room temperature. After washing the membrane with TBS-T buffer, ECL Western Blotting Substrate (Thermo Scientific Inc., Rockford, IL) was added and protein expression was detected by exposure to a blue autoradiograph film (Bio Express, Kaysville, UT). The film was developed by an x-ray film processor (Konica Minolta, Ramsey, NJ) in the dark room. Image density was assessed on a Bio-Rad Imaging system using Bio-Rad Quantity One software.
Statistics

Data are shown as Means ± SEM and statistically analyzed by the program of Prism 5 for windows (GraphPad, La Jolla, CA). Two-way ANOVA (running level x diet) was used to study the interaction between diet and running ability on each physiological parameter. Individual groups were compared with either an unpaired t-test or a one-way ANOVA with Tukey’s multiple comparison tests. Values of $p<0.05$ were considered as statistically significant.

RESULTS

The Effect of Exercise on the UPR in the Brain of Mice Fed VHFD for 4 Months

Voluntary running wheel exercise

After feeding mice with 60% HFD for 4 months, mice were given free access to running wheels for 3 weeks except non-running sedentary (SED) groups. At the time of beginning running exercise, mice were 25 weeks old. Following their running activity, running animals were grouped into Low-runner (LR, n=6), Middle-runner (MR, n=8), and High-runner (HR, n=6). As shown in panel (A) of Figure 2-1, there were variations in running activity of individual animals. In panel (B), though there was no major difference in running activity between the running groups in the first weeks of running, MR and HR mice increased their activity reaching their highest level after 2 or 3 weeks, respectively. As shown in panel (C), when considering total average running activity during the 3 weeks running period, there were significant differences between running groups. HR and LR mice made $200.59 ± 20.18$ and $36.48 ± 4.94$ turns per hour, respectively, and there was a significant difference between running activity of each running group. In further experiments, only LR and HR were used for study of the UPR activity.
The effect of exercise on body weight

During the 3 weeks running period, both SED (n=5) and LR (n=6) mice slightly increased their body weights by 1.65 ± 0.43 g and 2.04 ± 0.78 g, respectively. However, HR mice showed a significantly decreased body weight after three weeks running wheel exercise relative to LR mice (Figure 2-2, A). As shown in panel (B), the reduction in body weight (-5.32 ± 0.85 g) of HR mice was significantly different from the change of body weight in the SED and LR mice.

The effect of exercise on food intake

Although HR mice seemed to ingest fewer daily calories compared to the other groups during the 3 weeks running period, there was no significant difference on any specific day between the daily caloric uptake of each group (Figure 2-3, panel A). However, the cumulative caloric intake during the three weeks running period was reduced in the HR mice (panel B).

The effect of exercise on UPR in the brain

Three weeks voluntary running exercise decreased the UPR related gene expression in the brain of mice adapted to very high fat diet (60% HFD) for 4 months (Figure 2-4). In the hypothalamus, LR mice had significantly decreased gene expression for Xbp1, Atf6 and Eif2α compared to SED mice. HR mice increased UPR-related gene expressions relative to the LR. These results in HR mice were still lower than that in the SED group although there was no significant difference between HR and SED values. In the hippocampus, UPR related gene expression was not significantly affected by exercise except for expression of Eif2α which was significantly decreased in the LR group. Western blotting (Figure 2-5) of hippocampus samples suggested that XBPI and ATF6
protein expression was decreased by running exercise, consistent with the changes in gene expression. But, contrary to the gene expression patterns, the protein levels of both eIF2α and GRP78 seemed to be increased by running exercise.

The Effect of Exercise on the UPR in the Brain of Mice fed LFD or HFD for 3 Weeks

Voluntary running wheel exercise

The 20 mice given LFD or HFD had free access to running wheels during the 3 weeks experimental period after which all running mice were sorted into three groups relative to their activities; low runner (LR, n=6), middle runner (MR, n=8), and high runner (HR, n=6). As shown in panel (A) of Figure 2-6, activity levels of mice were monitored during 3 weeks and there was a big individual variation in running ability between animals even though mice are of the same genetic background. As shown in panel (B), there was a significant difference in activity between running groups for 3 weeks running period. While HR mice in LFD and HFD had 573.00 ± 39.90 and 492.90 ± 18.80 turns per hour, respectively, LR groups in both diet groups showed less than 200 turns per hour (174.00 ± 55.80 vs. 117.00 ± 24.50, LFD vs. HFD). Comparing the running activity of each running group between LFD and HFD, HFD fed mice seemed to be less active than LFD fed mice and a significant difference was observed between MR mice of LFD and HFD. In panel (C), HR mice eating LFD showed significantly higher running activity during 3 weeks as compared to LR mice of same diet. In addition, MR mice showed significantly higher running activity than LR mice after 2 week. As shown in panel (D), both HR and MR mice fed HFD had significantly more running activity relative to the activity of LR mice during 3 weeks running period. After 2 weeks, there was also a significant difference between HR and MR. Interestingly, activity of mice fed
the HFD seemed to decrease after 2 weeks of running period. In the subsequent experimental analysis of the UPR, only tissues from LR and HR mice were used for comparison.

**The effect of exercise on body weight and body composition**

As shown in panels (A) and (B) of Figure 2-7, 3 weeks feeding of LFD and HFD increased the body weight of SED mice by 3.60 ± 0.30 g and 5.80 ± 0.70 g, respectively. Three weeks of high running activity had a significant effect on lowering the increase of body weight in mice fed LFD for 3 weeks (panel A). At the end of the running period, the body weight of HR mice was significantly less than the LR mice. As shown in panel (B), HR mice fed the HFD had significantly lower body weights during the 3 weeks running period relative to the SED mice. At 3 weeks, there was a significant difference between body weights of LR and HR mice.

In this experiment, the Echo MRI system was used for analyzing body composition change of mice fed with LFD or HFD for 3 weeks (Figure 2-7, C). 3 weeks HFD feeding significantly increased body fat almost two-fold in comparison to the LFD group (12.60 ± 0.60 vs. 23.30 ± 1.50). Voluntary running exercise had no effect on body fat composition of LFD fed mice but decreased the body fat composition in HFD fed mice. The decrease was significant in HR mice that decreased the body fat composition to the level of %fat observed in the LFD group. ANOVA also showed that there was a significant interaction between exercise and diet ($F_{2, 30} = 4.099, p=0.0267$). Lean body mass of animals was also measured by Echo MRI (panel D). Neither diet nor exercise had any effect on lean body mass of mice.
The effect of exercise on food intake

Figure 2-8 shows the daily caloric intake of all groups during the 3 weeks running period. Both LR and HR mice had a trend of higher caloric intakes compared to SED group during 3 weeks. However, there was no significant difference between groups on any specific day except on the first two in mice fed the HFD when LR mice ate significantly more calories than SED mice. When cumulative caloric intake of each diet group was compared over the 3-week period (panel C), ANOVA showed that both exercise ($F_{2, 30} = 12.35, p=0.0001$) and diet ($F_{1, 30} = 25.98, p<0.0001$) had a significant effect without interaction. Compared to the equivalent groups of LFD, all groups of HFD consumed more cumulative calories. In addition, HR mice of each diet group consumed significantly more calories during the three experimental weeks than did SED mice.

Gene and protein expression in the brain

The effect of exercise in mice fed LFD for 3 weeks. In order to study how voluntary running wheel exercise affected the three arms (XBP1, ATF6, and PERK) of the UPR system in multiple brain regions (hypothalamus, hippocampus, and cortex), real-time PCR and western blotting were used to assay the expression of UPR related genes (Figure 2-9, panel A) and proteins (Figure 2-10, panel A), respectively.

Gene expressions of Xbp1, Atf6, Eif2a, and Grp78 were normalized to the expression of a housekeeping gene cyclophilin b. The results of LR and HR mice are shown as fold changes relative to those of SED mice. The expression of these UPR genes was generally increased by exercise in each brain region although there was significant variation in the magnitude of the response and in the level of activity that was required to induce a significant change. In the hypothalamus, HR mice up-regulated gene expression
of two arms, *Atf6* and *Eif2a* (down-stream of PERK signaling), as well as increasing *Grp78* gene expression. Specifically, *Atf6* gene expression was the most sensitive to exercise as this gene was activated even in LR mice. In contrast, LR mice did not show any change on *Eif2a* and *Grp78* expression although both were significantly increased in the HR group. However, running activity did not affect the *Xbp1* gene expression in this brain region. In the hippocampus, all genes of the UPR arms in addition to *Grp78* were activated by running exercise and a significant effect for *Atf6* gene was observed in LR mice. In the cortex, although all UPR related genes and *Grp78* gene were increased by exercise, only the changes in *Atf6* and *Eif2a* gene expressions were significant.

When UPR related protein expressions of hippocampus of mice fed LFD were assessed by western blotting experiments, the results were not consistent with the results of gene expression (Figure 2-10, panel A). Expression of XBP1 (spliced), phosphorylated PERK and total PERK were significantly upregulated by voluntary running wheel exercise. Though other arms and GRP78 had a tendency of being increased by running, these increases did not reach significance.

In addition to the brain regions, real-time PCR was also conducted on liver samples from the experimental groups (Figure 2-9, A). Running exercise increased the expressions of all UPR related genes in the liver, but there was considerable variability in all groups and only *Eif2a* gene expression was significantly increased.

**The effect of exercise in mice fed HFD for 3 weeks.** The same experimental procedures conducted in LFD animal samples were also applied to mice fed HFD for 3 weeks. UPR related gene expression (Figure 2-9, panel B) and protein expression (Figure 2-10, panel B) were analyzed in the multiple brain regions as well as in the liver.
In the hypothalamus the expression of genes in all three UPR arms and Grp78 were significantly increased by voluntary running wheel exercise and these maximal increases were also seen even in the LR groups. This effect of exercise was also detected in the hippocampus although it was not as pronounced. HR mice showed a significant increase in the expression of both Xbp1 and Atf6 genes as well as in Grp78 gene expression. However, exercise had no significant effect on Eif2α gene expression. Western blotting of UPR related protein expression in the hippocampus (Figure 2-10, panel B) identified an increase in XBP1 (spliced) protein expression, consistent with the increase in gene expression. However, neither ATF6 nor GRP78 protein expression were affected by exercise in comparison to the results of gene expression. In addition, the effect of exercise on the pPERK-peIF2α pathway also differed between mRNA level and protein level. While the Eif2α gene level was not changed by exercise, the protein expression levels of both pPERK and peIF2α were significantly increased in HR mice. In cortex of mice fed HFD, exercise only upregulated the Eif2α gene expression significantly. Although Xbp1 was also shown to be increased by exercise, the increase was not significant.

All the assessed UPR gene levels were increased in the liver in running mice fed HFD but only increases in Atf6 and Eif2α gene expression were significant in HR mice.

**The effect of exercise on apoptotic signaling in the brain**

In order to see if up-regulation of UPR gene expression induced by 3 weeks running exercise was associated with an increase in the apoptotic pathway, the protein expression levels of ER stress specific apoptotic components CHOP, caspase-12, and caspase-3 were evaluated using western blotting (Figure 2-11).
In the brain of mice fed LFD, 3 weeks running exercise had no effect on the level of apoptotic protein expression (panel A). Exercise had no effect on the level of CHOP, caspase-12 (full and cleaved) or caspase-3 (full and cleaved). Though caspase-12 (full) and caspase-12 (cleaved) was decreased by 3 weeks running exercise, these changes were not significant. In the HFD groups (panel B), exercise also had no effect on apoptotic signaling in the brain of mice fed HFD. There were trends of decreased expressions in caspase-12 (full and cleaved) and caspase-3 (full and cleaved) proteins, but these also did not reach significance.

**The effect of three weeks dietary (LFD/HFD) treatment on UPR in brain**

To identify the effects of high fat diets alone on UPR activity in the brain and liver, UPR related gene expressions were compared between sedentary groups of each diet (LFD or HFD) (Figure 2-12).

Compared to the LFD group, 3 weeks feeding of HFD had no significant effects on the expression of UPR genes except for Xbp1 gene expression in the hypothalamus and the Grp78 gene expression in the hippocampus. In the hypothalamus, Xbp1 gene expression was significantly decreased in mice after 3 weeks of HFD but other UPR genes were not changed by this dietary treatment. In the hippocampus, HFD feeding increased Grp78 gene expression significantly. In the cortex, even though no significant change was observed, 3 weeks HFD treatment showed a tendency to decrease the expression of UPR related genes.

The effect of diet on UPR in the liver was also studied (panel D). In this case only Eif2a gene expression was significantly increased. HFD feeding for 3 weeks had no effect on expressions of other UPR genes as compared to LFD.
The Effect of 3 Weeks Exercise on UPR in the Brain of Mice Adapted to Low or High Fat Diet for 3 Months

Voluntary running wheel exercise

After feeding mice LFD or HFD for three months, 20 mice of each diet group were allowed voluntary running wheel exercise for 3 weeks while maintained on these same diets. As in the previous experiments, running mice of each diet group were divided into three groups according to their running ability: Low-runner (LR, n=6), Middle-runner (MR, n=8), and High-runner (HR, n=6). As shown in panel (A) of Figure 2-13, there was a large variation in the individual exercise level. Diet and running ability had a significant effect on running activity and both MR and HR of HFD had significantly less activity than the same groups fed LFD (panel B). There was a significant difference within running groups of each dietary group. While HR groups of LFD and HFD ran 462.07 ± 13.68 and 339.97 ± 51.39 turns per hour, respectively (p<0.05), LR groups of LFD and HFD had 129.68 ± 18.55 and 61.63 ± 14.71 turns per hour (p<0.05). Though LR and HR mice fed LFD maintained their running level during the three weeks of the running period, running activity of MR mice appeared to decrease with time (panel C). From the result of mice fed HFD (panel D), MR and HR mice gradually increased their running activity while LR mice maintained similar low activity during the 3 weeks running period. There was a significant difference in running activity between running groups.

The effect of exercise on body weight

Figure 2-14 shows that body weights of mice adapted to LFD/HFD for 3 months were changed during the 3 weeks running period. At the beginning of the running period
(week 0), all LFD mice had a significantly lower body weight than all HFD mice. While LFD mice weighed 28.30 ± 0.60 g after 3 months feeding of LFD, mice fed HFD for 3 months weighed 36.00 ± 0.85 g (p<0.01). As shown in closed symbols, three weeks voluntary running activity decreased body weight of LFD fed mice relative to the SED mice fed LFD. The reduction was significant in the HR group. In mice fed HFD for 3 months (open symbols), the body weights of SED and LR mice increased during the 3 weeks running period while the body weights of HR mice were significantly reduced compared to both groups SED and LR groups. After 3 weeks of running exercise, HR mice of LFD and HFD reduced 2.40 ± 0.50 g and 3.40 ± 1.10 g, respectively while SED mice increased their body weight to 0.50 ± 0.30 g and 4.40 ± 0.90 g in mice fed LFD and HFD, respectively.

**The effect of exercise on food intake**

Each group of mice fed LFD had similar daily food intake during the 3 weeks running exercise period (Figure 2-15, panel A). HR mice of LFD tended to have more daily calories than other groups and this resulted in a significant cumulative increase over the 3 weeks of running period (panel C). In mice adapted to HFD (panel B), HR mice tended to have lower daily food intake and this resulted in a reduction in cumulative intake over the 3 weeks period (panel C). ANOVA showed (panel C) that there was a significant interaction between exercise and diet (F2, 30 = 15.31, p<0.0001) but neither factor had any independent effect on cumulative food intake during the 3 weeks of running period.
Gene and protein expression in the brain

The effect of exercise in mice fed LFD for 3 months. Using real-time PCR, UPR related gene expressions (Xbp1, Eif2a, and Grp78) were assayed in hypothalamus and hippocampus (Figure 2-16, panel A). In the hypothalamus, three weeks voluntary running exercise increased the expression of UPR genes and the increase in Grp78 gene of HR mice was significant relative to the level in SED mice. In the hippocampus though, HR mice only increased Eif2a gene expression with significance. There were no significant effects on either Xbp1 or Grp78. Only XBP1 (spliced) protein level was significantly increased by 3 weeks running in the hippocampus of mice adapted to LFD for 3 months (Figure 2-17, panel A). The other proteins levels were not changed by running exercise in this brain region.

The effect of exercise in mice fed HFD for 3 months. In the brain of mice fed HFD for 3 months, UPR related gene expressions (Xbp1, Atf6, Eif2a, and Grp78) were also increased by 3 weeks voluntary running wheel exercise (Figure 2-16, panel B). In the hypothalamus, it appeared that running exercise increased the expression of UPR genes but only the increase in Atf6 gene expression reached significance relative to SED mice. The hippocampus was more responsive to running exercise. Compared to the SED group, gene expression of Xbp1, Atf6, and Grp78 were all significantly increased in the hippocampus by 3 weeks running exercise. Western blotting for UPR related proteins showed that running exercise significantly increased the level of these proteins in the hippocampus of mice fed HFD for 3 months (Figure 2-17, panel B). The protein expressions of XBPI (spliced), ATF6, and peIF2α were all significantly increased by running exercise related to the results of SED group.
The effect of exercise on apoptotic signaling in the brain

Apoptotic related protein expressions (CHOP, caspase-12 and caspase-3) were assayed by western blotting (Figure 2-18). As shown in panel (A), 3 weeks running exercise had no effect on apoptotic protein expression in the hippocampus of mice fed LFD for 3 months. Although HR mice seemed to decrease the protein levels of CHOP and caspase-12 (full), neither change was statistically significant. Likewise, apoptotic protein expression in the hippocampus of mice adapted to HFD for 3 months was also not significantly changed compared to the LFD group (Panel B).

The effect of diet on apoptotic signaling in the brain

To assess the effect of diet, the apoptotic protein expression (CHOP and caspase-12) between SED mice of 3 weeks HFD and SED mice of 3 months HFD was compared (Figure 2-19). Long-term (3 months) HFD significantly increased apoptotic signaling in the hippocampus as compared to short-term (3 weeks) HFD.

The effects of prolonged feeding of high fat diet on UPR in the brain

The effects of long-term (3 months) HFD on UPR related gene expression in the brain of mice was also compared in the SED groups (Figure 2-20). Compared to the LFD group, gene expression of Eif2α and Grp78 were considerably increased in the hypothalamus of mice fed HFD for 3 months. In the hippocampus, 3 months treatment of HFD increased the expressions of both Eif2α and Grp78 but these changes did not reach statistical significance.
DISCUSSION

Recently, many studies have suggested a linkage between ER stress and chronic diseases. This was seen in the peripheral tissues such as liver and pancreatic β cells in obesity and type 2 diabetes. This possible linkage in the brain regions was also reported. Since exercise has beneficial effects in ameliorating metabolic disease (obesity and type 2 diabetes), it was hypothesized that exercise would show a positive effect on ameliorating the progression of these chronic diseases by up-regulating UPR and decreasing ER stress. In this study, I focused on the brain because exercise was shown to have an effect on UPR in the brain and it is known to reduce neurodegenerative diseases associated with aging. Since dietary fat is a risk factor for obesity, type 2 diabetes and neurodegenerative diseases, the studies reported in this chapter focused on the interaction between diet and exercise in regulating UPR in the brain.

Effect of Diet and Age on Voluntary Running Ability

Voluntary running wheel activity of male C57BL/6 mice was decreased in older mice and in mice eating higher fat diets (45% and 60% HFD). When converting the result of the running activity (turns per hour) into daily covered distance, HR mice in the 3 weeks diet experiments (LFD and HFD) ran about 10.76 km and 8.74 km a day on average, respectively. After feeding the diets for 3 months, HR mice ran about 8.20 km and 6.04 km a day during the 3 weeks of running period in the LFD and HFD groups, respectively. When mice were adapted to a more dense caloric diet (60% HFD) for a longer duration (4 months), HR mice on average covered only 3.56 km a day during the 3
weeks of running period. It is not clear if the further reduction was principally due to the older age or prolonged dietary fat intake.

There have been variable reports of the effects of diet on running ability. Animals adapted to a high-fat diet increased their running endurance capacity as assessed by the running time to exhaustion.\textsuperscript{57-58} This was assumed to result from an increased fat oxidation of animals fed the HFD that could give animals improved exercise ability. In contrast, another study showed that mice fed HFD ran significantly shorter distances as compared to mice fed regular chow.\textsuperscript{59} These different results may reflect the running protocols. The former study used one-time treadmill exercise to assess exhaustion time, while others used voluntary running wheel exercise during some period as used in the experiments in this thesis.

In the current studies, older mice showed less running activity compared to younger mice with similar dietary treatment. Since mice used in this study were not over one-year old, the decrease of running activity with age could be primarily induced by increased body weight, not by biochemical changes such as oxidant stress and inflammation.

In summary, the type of diet had a significant effect on voluntary running wheel activity and mice fed HFD showed lower running activity relative to LFD mice. Here, aging also had an effect on lowering running activity in animals on both diets.

\textbf{Effect of Exercise on Energy Balance}

As expected, this study confirmed that exercise has a significant effect to prevent weight gain or lower body weight independent of dietary type (LFD or HFD). Although HR mice did not decrease their body weight during 3 weeks feeding LFD/HFD, the
degree of increment was significantly lowered as compared to the SED mice. In the experiments in which mice were fed 45% HFD and 60% HFD for three months and four months, respectively, three weeks subsequent voluntary running exercise significantly decreased their body weight. This effect of exercise on lowering body weight was also detected in the mice adapted to LFD for three months. This effect of exercise on body weight has been well described in animals and human studies. Voluntary running wheel exercise has a positive effect on maintaining body weight and preventing or ameliorating obesity. The decrease in body weight of exercising mice fed high fat diet came from a reduced fat mass, confirming a previous report. This supports the suggestion that exercise increased fatty acid oxidation as an important regulatory response that contributes to the lower body weight.

Taken together, this study confirmed that exercise had a significant effect on maintaining body weight and contributed to the maintenance of energy balance by increasing energy expenditure.

**Effect of Diet and Exercise on Food Intake**

In the three weeks LFD/HFD experiments, HR mice in both diet groups consumed significantly more energy than LR and SED mice. However, the HR mice fed either 45% HFD or 60% HFD over three months significantly reduced their cumulative energy intake during the 3 weeks running period. This suggests that HR mice that become obese while chronically adapted to HFD get the energy to support activity from stored fat rather than from the direct energy source of food. It is also possible that three weeks running affects appetite and changes food intake depending on duration of fat diets. Other studies using 2~6 week exercise protocols found that exercise animals fed HFD
increased their cumulative caloric intake compared to control animals fed chow diet. Meanwhile, Patterson et al. (2009) showed that if selectively bred diet-induced obesity (DIO) rats had access to running wheels, running rats fed HFD had reduced food intake relative to sedentary rats 3 weeks after starting running but similar food intake after 10 weeks after starting running. It is likely that the effects of exercise on food intake are affected by the length of time on the diet, change in body fat, age, and intensity of exercise.

**Effect of Exercise on UPR**

The main focus of my thesis study was to identify how voluntary running wheel exercise affects the UPR in the brain of mice in a variety of dietary condition. Firstly, it was assessed how exercise has an effect on UPR in the brain of mice adapted to a highly calorically dense fat diet (60% HFD) for four months. It was shown that three weeks running exercise did not up-regulate the UPR in the brain regions. In the hypothalamus, LR mice had significantly lower UPR related genes expression compared to SED mice and although HR mice activated UPR, it was still low relative to the SED mice. In the hippocampus, it appeared that HR mice decreased the UPR gene expression except for *Eif2α*. From these results, it was thought that exercise could not increase but decreased UPR in the brain if mice were totally adapted to HFD and were obese. From these results, it was supposed that UPR had already reached to the maximum level by the long period of highly dense fat diet and three weeks voluntary running exercise could not activate the further UPR. These experiments suggested further studies to show the effect of diet and the effect of time of exposure to the diet on exercise-regulated UPR.
The second experiments showed that, in contrary to previous data of 60% HFD experiments, mice undertaking voluntary physical activity while feeding with LFD or HFD for three weeks up-regulated the gene and/or protein expression of a number of UPR components (XBP1, ATF6, eIF2α, and Bip/GRP78) in multiple brain regions (hypothalamus, hippocampus, and cortex) though there was frequently discrepancy between gene and protein expression. The inconsistency between gene and protein levels could be explained from the possibility that all UPR arms and GRP78 do not respond to ER stress at the same time. Interestingly, UPR in the hypothalamus of mice fed HFD for three weeks was more sensitively up-regulated by running exercise than in other brain regions. This may be important since the hypothalamus is the central brain region that regulates energy homeostasis and is responsive to physical activity. In this experiment, UPR activity in the liver was also assessed to know how peripheral tissue responds to the running activity. The results showed that exercise had similar effects in this peripheral tissue as in the brain. These results are in line with recently published data. Chapados and Lavoie (2010) showed that 6 weeks treadmill exercise activated the UPR, specifically Bip/GRP78, in the liver of rats fed with 45% HFD and this effect of exercise was more significant in the fatty liver where the expression of more UPR genes (Xbp1, Atf6, Eif2α, and Grp78) were activated by the exercise protocol.43

The effect of exercise on UPR in the brain of mice which were adapted to HFD for a longer time period (3 months) was also studied. After feeding mice with LFD or HFD for three months, the 3 weeks of voluntary running wheel exercise protocol was applied after which UPR related gene/protein expression were analyzed in different brain regions. In this experimental paradigm, voluntary running exercise activated the UPR
related gene/protein expression in the brain of both diet groups. However, in contrast to shorter exposure (3 weeks) to HFD, running activity did not significantly affect UPR in the hypothalamus of mice fed HFD for 3 months. It is possible that the hypothalamus could be desensitized by longer exposure to dietary fat. Meanwhile, the hippocampus of mice adapted to three months HFD sensitively responded to voluntary running wheel exercise and UPR gene/protein expression in this brain region were all activated by running activity as compared to the result of LFD mice. These results suggest that there is significant regional variation in the brain in response to exercise. As exercise seemed to have a significant effect on the hippocampus, which is important for cognitive function, the activation of UPR by exercise may have a possible effect against neurodegenerative disease.

The effect of diet itself on UPR activity in the brain was also assessed. Although 3 weeks of exposure to HFD appeared to have no effect on activating UPR as compared to LFD group, 3 months of HFD treatment was shown to up-regulate the UPR as compared to LFD mice. Ozcan et al. (2009, 2004) also identified the effect of HFD on activation of UPR in the hypothalamus22 and the liver,21 respectively. In these studies, they showed that 25 weeks HFD activated the expression of phosphorylated PERK and phosphorylated IRE1α (the upstream of XBP1) in the brain and 16 weeks HFD increased the expression of phosphorylated PERK and phosphorylated eIF2α in the liver. In addition, they indicated that these upregulated UPR by HFD could be linked to the insulin resistance in the liver.21 Considering the experimental results of our laboratory in which Boghossian et al. (2009) showed that 3 days HFD induced central insulin resistance,63 it is unlikely that UPR activation is responsible for insulin resistance in the
brain of mice fed the HFD for a short-term. It could be concluded that the dietary effect on activation of UPR depends on the length of time animals are exposed to the diet.

This study interestingly indicated that although short-term HFD by itself did not induce UPR activation, it did enhance the activating effect of exercise on UPR. The effect of exercise on UPR activity in the brain was greater in the HFD fed group than in LFD fed group. This result could be explained in a number of ways. Exercise could have an effect on reversing insulin resistance which was known to be induced in the brain by short-term HFD and that improved insulin signaling, in turn, could activate trophic factors such as BDNF and IGF-1, contributing to the UPR up-regulation. Many studies have supported the idea that exercise has a significant effect on reversing insulin resistance. Specifically, it was recently shown that HFD-induced insulin signaling impairment in the hypothalamus of rats was restored by exercise. In addition, it was indicated that improved insulin signaling was accompanied with UPR up-regulation and that BDNF action was linked to UPR (i.e., XBP1). It could be also assumed that exercise could reverse any HFD-induced reduction of BDNF, contributing to prompt UPR up-regulation in the brain. The Gomez-Pinilla research group (2004) showed a decrease of hippocampal BDNF in animals fed HFD for 2 months that was reversed by voluntary running wheel exercise. A further possibility is that exercise in animals fed HFD could increase fatty acid oxidation, possibly up-regulating UPR. It has been shown that increased fat metabolism can activate UPR in adipose tissue and in the liver. It is possible that activation of β-oxidation during exercise could produce ketone bodies which, in turn, could lead to activation of UPR. At this time, there is no supportive evidence showing a linkage between ketone bodies and UPR. However, if dietary fat is
high and exposure time to diet is prolonged, the effect of exercise on up-regulating UPR disappeared. UPR may have a maximal limit of activation as no additional activation in response to exercise was possible if UPR was activated chronically by HFD.

In summary, 3 weeks voluntary running wheel exercise up-regulated UPR in multiple brain regions of mice in various dietary conditions. Short-term HFD increased UPR in response to exercise. However, short-term HFD treatment alone had no effect on UPR while HFD feeding for longer duration up-regulated the UPR. This study suggested that short term HFD induced insulin resistance could not be related to UPR in the brain.

**Relationship of Exercise Induced Change in the UPR to Apoptosis**

Many studies have identified that untreated ER stress could sustain UPR up-regulation and that prolonged UPR activation enhances apoptotic signaling in mice with obesity or impaired insulin signaling.\(^{72-73}\) Apoptosis in the brain would not be conducive to the prevention of neurodegenerative diseases. So, in this study, we investigated if the upregulation of UPR induced by exercise was associated with apoptosis in the brain of mice fed with LFD or HFD. Apoptotic signaling was assessed using western blotting of specific ER stress related apoptotic signaling components; CHOP, caspase-12 and caspase-3. Three weeks voluntary running exercise had no effect on apoptotic signaling in the brain of mice fed with LFD and HFD for 3 weeks or 3 months. If anything, the changes that were observed indicated a decrease in the apoptotic response to running activity in both three weeks and three months experiments although the changes did not reach statistical significance. Hence, up-regulation of UPR by exercise is not associated with any increase in apoptosis.
The effect of diet on apoptotic signaling in the brain of mice exposed to LFD or HFD during the short or long term was also studied. In SED mice fed HFD for 3 months, there was increased expression of apoptotic related proteins (CHOP and cleaved Caspase-12) in the hippocampus as compared to SED of 3 weeks HFD mice. Hence, long-term treatment with dietary fat was shown to up-regulate apoptotic signaling in the brain. This is consistent with results of several other studies which showed that diet could have an effect on activating apoptosis. 60% HFD for 3 months was shown to increase the CHOP protein expression in the hypothalamus of rats.\textsuperscript{45} Another study also showed that 16 weeks HFD activated the expression of phosphorylated JNK in the liver.\textsuperscript{21}

Taken together, it can be concluded that exercise may prevent the normal activation of apoptotic signaling that is associated with increased UPR. This result, in turn, supports the idea that UPR up-regulation induced by exercise has beneficial effects in the brain of mice exposed to high fat diet, inducing the defensive mechanism of reducing ER stress without the damaging effect of apoptosis.

\textit{Exercise and Neurodegenerative Disease}

To our knowledge, this study is the first trial to show that voluntary running exercise increases UPR in the brain of animals independent of the diet composition. Exercise induced UPR up-regulation of the brain could be helpful to maintain brain health by clearing malformed proteins associated with neurodegenerative disease. This effect would be further supported by the absence of any increase in apoptotic signaling. Exercise is known to have a positive effect on neurodegenerative disease. The data described in this chapter suggest that the effect of exercise on UPR up-regulation and apoptotic down-regulation could contribute to the protective effect of exercise on
neuronal diseases such as AD. Most recently, Lowen and Feany (2010) suggested that activated UPR, such as XBP1, was neuroprotective in vivo by decreasing the toxicity of tau. Further, brain-derived neurotrophic factor (BDNF) which is induced by exercise and is needed to activate XBP1, may be responsible for the increase of UPR and neuronal health. In addition, our laboratory has shown (Park-York, unpublished observations) that nerve growth factor (NGF) increased XBP1 expression and inhibited ER stress inducer (thapsigargin) induced CHOP expression in the hippocampal HT-22 cells. Thus we hypothesize that exercise possibly activates UPR by up-regulating BDNF expression in the brain and that this could have a beneficial effect on brain health.

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Figure 2-1 Voluntary running wheel ability of mice adapted to 60% HFD for 4 months. (A) Running activity of individual mice. (B) The change of running activity of each running group during the 3 weeks running period. High running level group (HR) increased their activity while low runner group kept their activity low for 3 weeks. (C) After completing 3 weeks running exercise, running mice could be grouped relative to average wheel turns per hour; LR (n=6), MR (n=8), and HR (n=6). There were significant differences between running mice. All data is shown as Mean ± SEM. ++p<0.01 MR vs. LR; ###p<0.001 HR vs. MR; ***p<0.001, ****p<0.0001 HR vs. LR.
Figure 2-2 The effect of exercise on body weight. (A) During three weeks of running exercise, body weights of each group (SED [n=5], LR [n=6], and HR [n=6]) were measured every week. The results are shown as Mean ± SEM. (B) The change of body weight after three weeks of running exercise period. ##p<0.01, ###p<0.001 HR vs. LR; ***p<0.001 HR vs. SED.
Figure 2-3 The effect of exercise on food intake. (A) Daily food intake of each group of mice adapted to 60% HFD for 4 months during the 3 weeks running period. (B) The cumulative food intake during the 3 weeks of whole running period. All data is shown as Mean ± SEM. #p<0.05 HR vs. LR.
Figure 2-4 The effects of three weeks voluntary running wheel exercise on UPR related gene expressions in the brain of mice adapted to 60% HFD for 4 months. The results of real-time PCR for UPR related gene expressions (Xbp1, Atf6, Eif2α, and Grp78) were normalized to the expression of the reference gene cyclophilin b (Ppib) and the results of both LR (n=6) and HR (n=6) was shown relative to the results of SED (n=5). +p<0.05, ++p<0.01 LR vs. SED.
Figure 2-5 The effects of three weeks voluntary running wheel exercise on UPR related protein expressions in the hippocampus of mice adapted to 60% HFD for 4 months. Panel (A) is the image of western blotting for identifying UPR related protein expression in the hippocampus. Each lane was from pooled samples of three mice of each group. β-Actin was used as the control for protein expression. Panel (B) shows the result of densitometry of the western image. The results of LR and HR were shown relative to density of SED group.
Figure 2-6 Voluntary running wheel activity of mice fed LFD or HFD for 3 weeks. (A) The plotted data of running activity of individual mice. (B) Mean of running activity of mice in each diet for 3 weeks. (C) The result of voluntary running wheel activity in mice fed LFD for 3 weeks. Activity in each group (LR, n=6; MR, n=8; HR, n=6) is shown as an average of wheel turns per hour ± SEM during every week. (D) The result of running activity in mice fed with HFD for 3 weeks. All data is shown as Mean ± SEM. +p<0.05, ++p<0.01, ++++p<0.0001 MR vs. LR, ##p<0.01, ###p<0.001 HR vs. MR ****p<0.0001 HR vs. LR; a p<0.05 LR of HFD vs. LR of LFD.
Figure 2-7 Effect of diet and exercise on body weight and body composition. (A) The change of body weight of mice fed 10% LFD. HR mice had a smaller increase of body weight during 3 weeks. (B) The body weight of mice fed 45% HFD. Though body weights of all groups (SED, LR, and HR) were increased by 3 weeks HFD, HR had significantly lower increase of body weight. (C) The effect of 3 weeks diet and exercise on body fat composition assessed by MRI. Body fat was significantly increased by HFD as compared to same group of LFD (++p<0.01, +++p<0.001 HFD vs. LFD) and running exercise had a significant effect on decreasing body fat. (D) Neither exercise nor diet had any effect on the lean body mass. All data is shown as Mean ± SEM. *p<0.05, **p<0.01 HR vs. SED; #p<0.05 HR vs. LR; +p<0.05 LR vs. SED.
Figure 2-8 The daily caloric intake and cumulated caloric intake of LFD (A) and HFD (B) groups. Except for the first 2 days, there was no significant difference between groups. (C) The effect of diet and exercise on the cumulative food intake during the 3 weeks running period. During total running period, all of groups in HFD eat significantly more calories than LFD groups (\( ^{a}p<0.05 \)). HR mice in both diets had significantly more calories, comparing to SED. \( ^{+}p<0.05 \) LR vs. SED; \(*p<0.05, **p<0.01 \) HR vs. LR.
Figure 2.9 The effect of voluntary running wheel exercise on UPR related gene expressions in multiple brain regions and liver of mice fed with LFD and HFD for 3 weeks. All data is shown as Mean ± SEM and the results of running groups (LR and HR) are shown relative to the results of each SED group. Panel (A) shows the results from the brain and liver samples of mice fed LFD. Panel (B) shows the results for HFD groups. All groups had six mice. +p<0.05, ++p<0.01 LR vs. SED; *p<0.05, **p<0.01, ***p<0.001 HR vs. SED.
Figure 2-10 UPR related protein expression in hippocampus of mice fed LFD or HFD. (A) The UPR protein expressions in the hippocampus of mice fed LFD. Left panel shows the results of western blotting of two representative animals in each group. Right bar graph is the result of the densitometry of UPR protein expressions from four to six mice in each group. (B) The UPR protein expressions in HFD groups. Left panel shows the images of western blotting from two representative animals of each group. Right graph shows the results of densitometry from four to six mice fed with HFD. All data was shown as Mean ± SEM. +p<0.05, ++p<0.01 LR vs. SED; *p<0.05, **p<0.01, ***p<0.001 HR vs. SED.
Figure 2-11 The effect of running exercise on apoptotic protein expression in hippocampus of mice fed LFD or HFD. Exercise affects on expressions of CHOP, caspase-12, and caspase-3 in the hippocampus of mice fed with LFD (panel A) or HFD (panel B) for 3 weeks. Left panels show the result of western blotting images for protein expression from two representative animals. Right panels showed the results of apoptotic protein expression densitometry for samples from four to six mice per each group. All data are shown as Mean ± SEM.
**Figure 2-12** The effect of diet on UPR related gene expressions in multiple brain regions and liver. The results of UPR gene expressions from the SED mice of HFD group were shown in the fold change relative to the results from the SED group of LFD. All data is shown as Mean ± SEM and came from six mice of each diet group. *p<0.05, **p<0.01 HFD vs. LFD.
Figure 2-13 Voluntary running wheel activity of mice adapted to LFD/HFD for 3 months. (A) Running activity of all individual mice in each diet. (B) The effect of diet and running level or activity. Running mice were grouped into three groups; LR (n=6), MR (n=8), and HR (n=6). (C) The result of running activity (wheel turns per hour) of LFD running group during the 3 weeks of running period. (D) The running activity of running group of HFD during 3 weeks. All data is shown as Mean ± SEM. +p<0.05, +++p<0.001 MR vs. LR, #p<0.05, ##p<0.01, ###p<0.001, ####p<0.0001 HR vs. MR, and ***p<0.001, ****p<0.0001 HR vs. LR.
Figure 2-14 The effect of diet and exercise on body weight. These graphs show the changes of body weight for 3 weeks of running exercise in mice fed LFD (closed symbols) or HFD (open symbols) for 3 months. On both diets, HR (n=6) significantly decreased their body weight compared with SED animals (n=6). In HFD, there was significant difference even between LR and HR. SED and LR group in HFD mice had significantly increased body weight than same group in LFD mice during three weeks running periods. **p<0.01, ***p<0.001 HR vs. SED and #p<0.05 HR vs. LR; +p<0.05, ++p<0.01, +++p<0.001, ++++p<0.0001 SED of LFD vs. SED of HFD and ††p<0.01, †††p<0.001, ††††p<0.0001 LR of LFD vs. LR of HFD.
Figure 2-15 The effect of diet and exercise on daily and cumulative food intake. (A) Daily food intake of mice adapted to 3 months LFD during the 3 weeks running exercise period. (B) The result of daily food intake of mice fed HFD for 3 months. (C) The effect of diet and exercise on cumulative food intake during the 3 weeks running exercise. The data is shown as Mean ± SEM. ##p<0.01 HR vs. MR and *p<0.05, **p<0.01 HR vs. SED.
Figure 2-16 The effect of exercise on UPR related gene expression in the brain of mice fed LFD and HFD for 3 months. Panel (A) shows how 3 weeks running exercise affects UPR related gene expressions in the brain of mice fed LFD for 3 months. Panel (B) shows the results of UPR related gene expressions of mice fed HFD for 3 months. All results of both LR (n=6) and HR (n=6) were normalized to the results of SED (n=6) and were shown as Mean ± SEM. +p<0.05, ++p<0.01 MR vs. SED and *p<0.05, **p<0.01 HR vs. SED.
Figure 2-17 The effect of exercise on UPR related protein expressions in the hippocampus of mice fed LFD or HFD for 3 months. In the left side panels (A) and (B), the images show the results of UPR specific western blotting with two individual samples from group of mice fed LFD or HFD fed 3 months. The expressions of β-Actin were used for control protein expression. In the right section of both diet (A and B), UPR related protein expressions are shown in densitometry graph (shown in arbitrary unit) and each result of both LR and HR was normalized to the result of SED. For constructing densitometry, protein expressions from four to six mice of each group were used and all data is shown as Mean ± SEM. +p<0.05 MR vs. SED and *p<0.05 HR vs. SED.
Figure 2-18 The effect of exercise on apoptotic related protein expressions in the hippocampus of mice fed LFD or HFD for 3 months. The left sides of panel (A) and (B) show the apoptotic specific western blotting images of two individual animals of each group fed LFD or HFD for three months. On the right side of each panel (A and B), the bar graphs show the result of densitometry from four to six mice from each group. The results of LR and HR were normalized to the results of SED and were shown as Mean ± SEM.
Figure 2-19 The effects of diet on apoptotic related protein expression in the hippocampus. Each lane in the western image (A) comes from individual animals fed HFD for either 3 weeks or 3 months and the results are shown in densitometry (B). The protein expression from 3 months HFD mice were normalized to the expression from 3 weeks HFD mice. All data is shown as Mean ± SEM for three mice per group. *p<0.05 3 months vs. 3 weeks.
Figure 2-20 The effects of 3 months LFD/HFD on UPR-related gene expressions in the brain. These bar graphs show the results of comparing the UPR-related gene expression between SED groups of each diet group (3 months LFD and 3 months HFD). The results of HFD were normalized to the ones of LFD and were shown as Mean ± SEM. **p<0.01 HFD vs. LFD.
CHAPTER 3
THE EFFECT OF IGF-1 AND EXERCISE ON UPR ACTIVATED BY
VOLUNTARY RUNNING EXERCISE

INTRODUCTION

In chapter 2, the preliminary data showed that three weeks voluntary running wheel exercise up-regulated UPR in multiple brain regions of C57BL/6 mice without activating any apoptotic signaling pathway such as CHOP, caspase-12, and caspase-3. Because uncontrolled UPR activity was shown to lead to programmed cell death, another question addressed in this thesis is to identify what is the mechanism that leads to down-regulation of apoptosis despite exercise-induced UPR activation. Exercise is known to activate many trophic factors such as Insulin-like growth factor-1 (IGF-1), Brain-derived neurotrophic factor (BDNF), Vascular endothelial growth factor (VEGF), and galanin. Hence, it is possible that UPR up-regulation or suppression of apoptosis induced by running wheel exercise could result from activation of these trophic factors. Specifically, in this thesis chapter, the role of IGF-1 was studied to identify if this trophic factor has an effect on exercise induced UPR up-regulation.

Recently, it has been shown that trophic factors regulate UPR. BDNF, one of the neurotrophic factors, is known to control UPR regulation in the brain, contributing to brain health. Using in situ hybridization, Hayashi et al. (2007) found that the mRNA level of XBP1 spliced form (XBP1s) was greatly up-regulated in developing mouse brain as compared to the developed mouse brain. Furthermore, they identified that treatment with BDNF prompted mRNA expression of both Xbp1 and Eif2a in cultured hippocampal neurons. Meanwhile, BDNF had a significant effect on inhibiting apoptotic signaling
(caspase-12) *in vitro* in the presence of an ER stress inducer tunicamycin. On the other hand, exercise appears to have a significant effect on activating *Bdnf* mRNA expression in the hippocampus which is the principal site for cognitive function. BDNF upregulation followed by exercise was shown to lead to the improvement of learning and memory. Gomez-Pinilla et al. (2008) also showed that exercise has a significant effect on cognitive function by using a strong BDNF receptor inhibitor, a recombinant TrkB-IgG chimera, to impair BDNF action. Wheel running exercise increased *Bdnf* mRNA expression in the hippocampus of Alzheimer’s disease mice model (Amyloid precursor protein [APP]-23 mice) without any changes in spatial learning and neurogenesis.

Another trophic factor IGF-1, a polypeptide of 7,500 kDa, is known to have common downstream signaling with BDNF. After binding to the TrkB receptor, a BDNF receptor, BDNF activates the phosphatidylinositol 3-kinase (PI3-K) via IRS-1 and -2 which are known to be a common component in intracellular signaling pathways of IGF-1. IGF-1 is produced in the liver and secreted into the circulation. It regulates the action of growth hormone for body development and tissue remodeling in the body. In high concentrations (150-400 ng per ml), it is transported in plasma while bound to IGF binding proteins (IGFBPs) and only a small amount (less than 1% of total) of free IGF-1 is available to bind to their receptors (IGF-1 receptors). IGF-1 activity, transport and half-life is regulated by the presence of IGFBPs. Recently, it has been shown that IGF-1 has a critical ability in the central nervous system, contributing to the differentiation and proliferation of neuronal cells as well as promoting their survival *in vitro* and *in vivo*. 
Circulating peripheral IGF-1 can cross the blood brain barrier (BBB) and enter into the brain where it binds to the IGF-1 receptor, a receptor of the tyrosine kinase family.

IGF-1 actions in the brain are important for brain health and this could mediate some of the effects of exercise. Carro et al. (2000) showed that 1 hour treadmill exercise increased the uptake of systemically applied IGF-1 into the rat brain. This was confirmed by staining digoxigenin-labeled IGF-1 in the brain. The role of peripherally produced IGF-1 in regulating the brain’s response to exercise was shown using anti-IGF-1 antibody to block the access of IGF-1 into the brain from the circulation. The data suggest that IGF-1 entering into the brain elicits the exercise-related neuronal activation that can be detected by changes in electrophysiological properties. In genetically modified mice whose liver IGF-1 gene was ablated and circulating IGF-1 level was significantly lowered, the hippocampal function for spatial learning in the water maze test was greatly reduced. IGF-1, systemically injected, rescued this impaired brain function.

IGF-1 acts in the hippocampus. Using autoradiography with [\(^{125}\)I]IGF-1, it was shown that IGF-1 receptors were located in the dentate gyrus of the hippocampus. Aberg et al. (2000) showed that systemically applied IGF-1 had a capacity to induce neurogenesis even in the adult rat hippocampus. They showed that animals treated with IGF-1 peripherally for either 6 or 20 days up-regulated the proliferation of neural cells in the adult dentate gyrus and increased neural differentiation selectively in comparison to astrocytes.

It has been suggested that IGFBP could be a possible mediator of the response to ER stress. The activity of the bZIP-activating transcription factor 4 (ATF4), which is
activated by phosphorylated eIF2α, affects the level of IGFBP which is secreted and regulates the activity of circulating free IGF-1. In this study, ER stress induction with tunicamycin increased IGFBP-1 mRNA expression. The role of ATF4 was confirmed by using Atf4 knockout mice. Recent studies have suggested that there is a linkage between UPR and IGF-1 signaling. There have been some trials showing that IGF-1 signaling works downstream of an upregulated UPR mechanism in mediating its effects. It was shown that IGF-1 treatment reversed the ER stress inducible effect of tunicamycin in vivo and that it also decreased the apoptotic signaling of caspase-3 activity in PC-12 neuronal cells.

In this chapter, the effect of voluntary running wheel exercise on UPR activity was studied in the brain of mice whose circulating IGF-1 action was inhibited by anti-IGF-1 antibody.

**HYPOTHESES**

1. IGF-1 will increase UPR activity in the brain.

2. IGF-1 will increase exercise-activated UPR in the brain.

**METHODS**

**Animals and Diets**

Thirty-two male C57BL/6 mice, 6 weeks of age, were used in this experiment. As in chapter 2, all mice had a 1 week adaptation period once they arrived at the animal room. During this period, all mice were randomly grouped into four groups: Saline/Sedentary (Sed), Saline/Run, anti-IGF-1/Sed, and anti-IGF-1/Run. Each group was composed of eight animals and an osmotic pump filled with saline or anti-IGF-1 antibody was implanted in each mouse of each treatment group. In this study, all mice were fed
with 10% low fat diet (D 12450B, Research Diets, New Brunswick, NJ). Unlike the experiment of chapter 2 in which food was given in feeding cups, mice had access to food on the cage lid in this experiment. The animal room was kept at 22-23 °C with a 12-hour light/dark cycle. This animal study was approved by the Utah State University Institutional Animal Care and Use Committee (USU-IACUC).

Voluntary Running Wheel Exercise

The detailed exercise protocol was described in the methods of chapter 2. In this study, only 14 days running period was applied because the working duration of the osmotic pumps used in this study was only 14 days. Briefly, running mice (n=8) in each treatment group (Saline or anti-IGF-1) were given free access to running wheels and their activity (turns per hour) was automatically recorded and stored in the computer (VitalView program, Mini Mitter, OR) every hour. After completing the 14 days running protocol, running mice were grouped into two groups depending on their running activity; Low Runner (LR, n=4) and High Runner (HR, n=4). In this study, only high running mice were used as running group for the future analysis of mRNA expression: Saline/RUN or anti-IGF-1/RUN. Saline/SED or anti-IGF-1/SED were used as control groups.

Osmotic Minipumps

Following the experimental protocol of Carro et al. (2000), a rabbit polyclonal anti-IGF-1 antibody (SC-9013, Santa Cruz Biotechnology, Santa Cruz, CA) was used to block the entrance of circulating IGF-1 into the brain. Osmotic minipumps (Alzet micro-osmotic pump, model 1002, Durect Corporation, Cupertino, CA) were used to deliver the anti-IGF-1 antibody or saline vehicle. The reservoir volume of this pump is 90 µL (±10
μL), pumping rate is 0.25 μL per hour, and pumping duration is 14 days. Using a small syringe (1 mL), pumps were filled with 200 μg of rabbit polyclonal anti-IGF-1 antibody (20% in saline) or saline under sterile conditions. After inhalational anesthesia with isoflurane, a small incision was made in the skin in the back neck region of each mouse and the subcutaneous connective tissue was spread to form a small pocket where each pump filled with anti-IGF-1 antibody or saline was implanted. After implantation, the incised skin was closed with an animal wound clip.

**RNA Isolation and Purification**

The detailed method for RNA isolation and purification was described in chapter 2. In this study, the hypothalamus and hippocampus were used and the liver was also assessed. Briefly, brain and liver samples were homogenized in TRI reagent (Molecular Research Center, Cincinnati, OH) using a motor driven Teflon pestle. After adding 100 uL 1-bromo-3-chloropropane (BCP), samples were centrifuged at 13,000 rpm at 4 °C for 15 min. The aqueous upper phase was transferred to a new 1.5 mL tube and 500 uL isopropanol added. After centrifugation, RNA was precipitated with 75 % (v/v) ethanol and dried out at room temperature for 5-10 min. The RNA pellets were solubilized in RNase-free water and heated at 65 °C for 5 min. Extracted RNA samples were cleaned-up by a TURBO DNA-free™ kit (Ambion, Inc., Austin, TX). The extracted RNA quantity was assessed by the A260 absorbance and by 1.5% agarose gel electrophoresis.

**Quantitative Real-time PCR**

The detailed procedure for real-time PCR was described in chapter 2. In this experiment, KAPA SYBR green (KAPA Biosystems, Woburn, MA) was used instead of Quanta SYBR green used in the experiments of chapter 2. Gene expression was
normalized to the expression of the reference gene cyclophilin b. PCR reaction cycles were set as 1 cycle for 20 seconds at 95 °C and then 40 cycles for 1 second at 95 °C and 30 second at 60 °C. Primers additionally used in this chapter were:

*Igf1*: Forward, 5’-CCTAACACTTGTATTTGTTGAATTG-3’,
Reverse, 5’-ACAGATGGAGTCAGGTACGTTAAA-3’;

*Bdnf*: Forward, 5’-AATGTTCCACCAGGTGAGAAGAG-3’,
Reverse, ‘5-TGCAACCGAAGTATGAAATAACCA-3’

**Statistics**

All data are shown as Mean ± SEM and analyzed using the Prism 5 for windows (GraphPad, La Jolla, CA). To assess the interaction between exercise and anti-IGF-1 on each parameter, two-way ANOVA was used. Individual groups were compared with t-test. Only values of *p*<0.05 were considered as statistically significant.

**RESULTS**

**Voluntary Running Wheel Exercise**

After 14 days of voluntary running wheel exercise, running mice of each treatment group (Saline or anti IGF-1) were separated into two groups depending on their running activity; Low Runner (LR, n=4) and High Runner (HR, n=4). For further analysis, only HR mice of each group were used as running mice. Panel A of Figure 3-1 shows the plotted result of running activity of individual animals for 14 days running period. As shown in panel B, blocking circulating IGF-1 with the anti-IGF-1 antibody had no effect on running activity for the 14 days running period and there was no significant difference between LR and HR of saline or anti-IGF-1 treated mice. As shown in panel C, HR mice of each treatment group seemed to increase their activity from the
second day and there was a significant difference between LR and HR mice treated with saline at the end of running period.

**The Effect of Exercise and Anti-IGF-1 on Change of Body Weight**

Figure 3-2 shows the effect of voluntary running exercise and anti-IGF-1 treatment on the change of body weight after the 14 days running period. There was no effect of anti-IGF-1 antibody on body weight. HR mice (HR, n=4) in both treatment groups (Saline or anti-IGF-1) had a reduced body weight gain and this decrease was significant in Saline mice. Here, ANOVA indicated that there was no significant interaction between anti-IGF-1 and exercise ($F_{1,20} = 0.5489$, $p=0.4674$). However, when considering all running animals (n=8), the exercise effect on the change of body weight disappeared.

**The Effect of Exercise and Anti-IGF-1 on Expression of UPR-related Genes in the Brain**

In the hypothalamus, both exercise and anti-IGF-1 antibody affected the expression of Xbp1 and Eif2α (Figure 3-3). Compared to saline treated SED mice, treatment with anti-IGF-1 antibody induced a big increase in the level of both genes in this brain region of SED mice and the increase in Eif2α gene expression level reached statistical significance. Running (HR) had no effect on either Eif2α or Xbp1 gene expression in the control mice. However, comparisons between HR group and SED group in mice treated with anti-IGF-1 showed that exercise significantly decreased the expression of both Xbp1 and Eif2α. In the hippocampus, only Xbp1 gene expression was affected by anti-IGF-1 and exercise. Anti-IGF-1 treatment increased Xbp1 gene expression compared to SED group of saline. Running exercise significantly lowered this
gene expression in the hippocampus of anti IGF-1 treated mice. However, the other UPR related gene expressions (Atf6, Eif2a, and Grp78) were not changed by anti IGF-1 treatment or exercise in either the hypothalamus or hippocampus.

The Effect of Exercise and Anti-IGF-1 on the Expression of IGF-1 and BDNF in the Brain

Using real-time PCR, gene expression of both Igf1 and Bdnf were assessed in the hypothalamus and hippocampus of running and sedentary mice treated with either saline or anti-IGF-1 antibody (Figure 3-4). Igf1 mRNA expression was reduced by running wheel exercise in the hypothalamus regardless of blocking circulating IGF-1 although the effect did not reach statistical significance probably due to the small group sizes. Gene expression of Igf1 was not changed by exercise in the hippocampus. Neither exercise nor blocking IGF-1 had any effect on Bdnf mRNA expression in either brain region.

The Effect of Exercise and Anti-IGF-1 on UPR and IGF-1 Gene Expression in the Liver

In addition to the brain, the effects of exercise and antibody for IGF-1 on UPR related gene expressions were assessed in the liver (Figure 3-5). Treatment with antibody for IGF-1 had no effect on expression of Xbp1, Eif2a, or Grp78 in the liver. In the saline treated mice, exercise appeared to decrease the expression of Xbp1, Grp78, and Eif2a but none of these changes reached statistical significance probably because of small group sizes. In addition, it was shown that neither exercise nor anti-IGF-1 antibody had any effect on Igf1 mRNA expression in the liver.
DISCUSSION

In chapter 2, the possibility that exercise had a neuroprotective effect through activation of UPR was suggested. Hence, we addressed the questions of what might mediate the effect and what was the signaling pathway linking exercise and UPR regulation. Exercise was identified to have a neuroprotective effect through increasing the entrance of circulating IGF-1 into the brain \(^{21}\) and administration of IGF-1 into sedentary animals having brain damage improved behavioral abnormalities. \(^{27-28}\) Since the inhibitory role of IGF-1 on ER stress induced apoptosis has been reported along with the effect of IGF-1 to up-regulate UPR, \(^{25-26,29}\) it was hypothesized that IGF-1 takes part in the exercise regulated UPR. This chapter focused on identifying the effect of exercise on UPR in the brain of mice while their uptake of circulating IGF-1 into the brain was blocked by treatment with anti-IGF-1 antibody.

In this study, 14 days of running period was set due to the working duration of the osmotic minipumps. A previous study already confirmed that implantation of minipump does not affect exercise activity of animals. \(^{21}\) In addition, it was shown that blockage of circulating IGF-1 did not affect exercise activity in mice. Indeed increased physical activity normally has a positive correlation with circulating IGF-1 levels. \(^{30-31}\) Moreover, in experiments of this chapter, only HR mice in each treatment group were set as running mice in order to maximize the effect of exercise even though there was no significance between LR and HR.

In saline treated mice, 14 days wheel running exercise of HR mice significantly lowered weight gain compared to the sedentary mice. However, when considering all running animals, this significant effect was diminished. Here, the reason for not reaching
significance may be that the food given was low-fat diet and that the running duration was relatively short compared to the previous exercise protocol applied in chapter 2. It was indicated that the reduction of body weight in response to exercise was attenuated in anti-IGF-1 treated mice. Application of a polyclonal antibody against human IGF-1 strengthened the effect of IGF-1 on growth in vivo.\textsuperscript{32-33} In this study, the whole body weight of dwarf mice was greatly increased by the treatment of anti-IGF-1 antibody compared to the mice given solely exogenous IGF-1. They suggested that this growth promoting effect of anti-IGF-1 could result from the effect of antibody on protecting free IGF-1 from degradation, sustaining IGF-1 availability to promote anabolic activity.

The results presented in this thesis chapter showed that there was a trend of increase in $Xbp1$ signaling by blocking circulating IGF-1 level in both hypothalamus and hippocampus. The $Eif2\alpha$ mRNA expression was also significantly increased in the hypothalamus by blocking the circulating IGF-1 comparing between sedentary group of saline and anti-IGF mice. However, the expression of other UPR related genes were not changed by treatment of anti-IGF-1 antibody. Hu et al. (2007) showed that XBP-1 has a capacity to increase the IGF-1 transcription as a transcription regulatory factor.\textsuperscript{34} Considering their study results, it could be thought that the lowered level of IGF-1 that followed treatment with anti-IGF-1 antibody reversely activated the $Xbp1$ gene expression so as to increase the production of IGF-1 protein and maintain the level of IGF-1. In another study, addition of IGF-1 promoted the acute upregulation of XBP1 protein expression in MCF-7 cells in the presence of the ER stress inducer thapsigargin for 3 hours although the effect disappeared with longer incubation time.\textsuperscript{29} Consistent with the results reported here in which blocking systemic IGF-1 level increased $Eif2\alpha$ mRNA
expression in the hypothalamus, it was shown that IGF-1 treatment lowered the expression of phosphorylated eIF2α protein in the presence of the same ER stress inducer.\textsuperscript{29} Other studies showed that addition of IGF-1 increased the expression of ATF6 protein.\textsuperscript{29} No change in ATF6 expression was detected in response to IGF-1 antibody in the current study. This discrepancy could be explained by differences in tissues studied (brain tissue vs. brain cancer cell line).

Voluntary running wheel exercise for 14 days appeared to be effective on reducing over expression of Xbp1 and Eif2α mRNA induced by blocking IGF-1 level back to the level of saline treated running mice. However, running wheel exercise seemed to have no effect on other UPR related gene expression in the brain of mice whose IGF-1 level was blocked with antibody for IGF-1. In contrast to chapter 2, 14 days running wheel exercise in this experiment did not increase the UPR related gene expression. This inconsistent result could be a result of the change of feeding method. Unlike previous studies where food was provided in cups within the cage, food was given on the cage lid in this experiment. The physical activity of climbing and holding the lid while eating is very significant and completely alters energy balance. So, it is likely that all animals had significant exercise levels regardless of the presence of a running wheel (aerobic exercise or resistant exercise). One supportive result showed that the feeding method (cup feeding vs. lid feeding) affected voluntary running wheel activity.\textsuperscript{35} They showed that animals fed on the lid had significantly less running activity than ones fed in the cup. In our experiment, however, exercise seemed to reduce the possibly maximally activated UPR induced by blocking IGF-1.
IGF-1 is expressed in both the liver and brain. Running wheel exercise tended to
decrease the *Igf1* mRNA expression in the hypothalamus even in the presence of anti-
IGF-1 antibody. In contrast, in the hippocampus, running exercise had no effect on *Igf1*
mRNA expression. Only a few mice were involved in these studies. However, variable
effects of exercise in brain IGF-1 expression have been reported. While voluntary
running wheel exercise increased the *Igf1* mRNA expression in the cerebral cortex of
mice and hippocampus of rat, another study showed that exercise did not induce any change in *Igf1* mRNA expression in the rat hippocampus. One possible explanation for
lowered *Igf1* mRNA level in hypothalamus in response to exercise in my study is that
running wheel exercise could increase the level of free IGF-1 by exceeding the capacity
of IGF-1 antibody to bind IGF-1 which, in turn, could be sustained by binding with
IGFBP. This increased circulating IGF-1 level could inhibit *Igf1* gene transcription in
the brain. This would explain the decreased *Igf1* mRNA expression level in exercising
mice. Alternatively, an exercise-induced increased entrance rate of circulating IGF-1 into
the brain may also lead to decrease the *Igf1* mRNA level.

In the small number of mice used, I was unable to show the expected increase in
*Bdnf* mRNA expression with exercise. Again this probably is a result of the feeding lid
protocol that gave the control mice significant activity levels. Likewise in the brain, it
was shown that inhibition of IGF-1 action had no effect on UPR related genes expression.
Although experiments in the previous chapter showed that exercise increased UPR
related gene expression in the liver, this effect of exercise was not found in the current
experiment regardless of the presence of antibody for IGF-1. This difference could be
induced by feeding method, the small number of animals and different duration of
exercise. Blocking IGF-1 had no effect on \textit{Igf1} mRNA expression in the liver and exercise also did not give any effect on changing \textit{Igf1} mRNA expression in both treatments of saline and anti-IGF-1. Though one study identified an effect of exercise on increasing in \textit{Igf1} mRNA expression in the liver, this different result could be come from different exercise protocol applied, 4 weeks treadmill exercise using rats.

Taken together, the data suggest that circulating IGF-1 takes part in the regulation of XBP1 and eIF2α in the brain because blocking IGF-1 led to increase expression of these mRNAs. Voluntary running wheel exercise appeared to release the increased ER stress induced by the presence of anti-IGF-1, not up-regulating the UPR. This preliminary study has a number of limitations that include not assessing whether anti-IGF-1 antibody affects transport of IGF-1 into the brain or how circulating IGF-1 levels were changed. In conclusion, increased IGF-1 expression in response to exercise cannot be responsible for mediating normal increase in UPR in the brain. Instead, it could be speculated that IGF-1 could be responsible for reducing apoptosis.

**REFERENCES**


Figure 3-1 Voluntary running wheel activity in mice treated with IGF-1 antibody. (A) Average daily running activity of individual animals in either saline or anti-IGF-1 group for 14 days. (B) After 14 days running period, running mice (n=8) of each treatment group were separated into two groups depending on their running activity; Low Runner (LR, n=4) and High Runner (HR, n=4). Further analysis in this study only used HR mice as running group. (C) The running activity of saline or anti-IGF-1 treated running mice during 14 days running period. All data of activity is shown as Mean ± SEM. *p<0.05 HR vs. LR of saline treated mice.
Figure 3-2 The effect of exercise and anti IGF-1 on change of body weight. After 14 days of running period, it was assessed how body weights were changed by both exercise and antibody for IGF-1. (A) When choosing HR mice (n=4), there was a significant effect of exercise on the change of body weight. (B) There was no significant effect of exercise as considering all running animals (n=8). Treatment with antibody for IGF-1 did not affect the change of body weight comparing SED group of saline and anti IGF-1 mice.
Figure 3-3 The effect of exercise and anti IGF-1 on the UPR-related gene expressions in the brain. Using real-time PCR, UPR-related gene (*Xbp1, Atf6, Eif2a, and Grp78*) expressions were assessed in the hypothalamus and hippocampus of mice treated with saline or anti IGF-1. These groups were again divided into SED (n=4) and HR (n=4) group. All data is shown as Mean ± SEM. *p<0.05 anti IGF-1 vs. Saline; *p<0.05, ***p<0.001 HR vs. SED.
Figure 3-4 The effect of exercise and anti IGF-1 on Igf1 and Bdnf gene expressions in the brain. The gene levels of Igf1 and Bdnf in the hypothalamus (A) and hippocampus (B) were also analyzed using real-time PCR. These gene expressions were shown normalized to the expression of reference gene cyclophilin b. All data is shown as Mean ± SEM.
Figure 3-5 The effect of exercise and anti IGF-1 on UPR-related and IGF-1 gene expressions in the liver. Using liver samples of SED or HR mice treated with saline or anti IGF-1, the expressions of UPR related genes ($Xbp1$, $Eif2α$, and $Grp78$) and $Igf1$ gene were evaluated. Gene expression level is shown normalized to the reference gene cyclophilin b level. All data is shown as Mean ± SEM.
CHAPTER 4
THE EFFECT OF 4-PHENYL BUTYRIC ACID (PBA) ON UPR ACTIVATED BY VOLUNTARY RUNNING EXERCISE

INTRODUCTION

As mentioned in previous chapters, many pathological events can be linked to ER stress and cells activate a defensive mechanism called the UPR. However, if cellular stress is prolonged, the UPR mechanism might be insufficient and cells can be overridden with the accumulation of unfolded proteins, leading to a cellular death program. To gain supportive evidence for the role of UPR to promote the folding of unfolded proteins and release ER stress, the use of chemical chaperones, such as 4-phenyl butyric acid (PBA), trimethylamine N-oxide dehydrate (TMAO) and dimethyl sulfoxide has been suggested.

PBA was applied to promote the trafficking of cystic fibrosis transmembrane conductance regulator protein (CFTR) and was also approved as a drug for urea cycle disorder by the FDA. This chemical chaperone, a chemical of low molecular weight, was shown to cross the blood-brain barrier. Other studies have confirmed that this chemical can confer positive effects in the brain through releasing ER stress.

Ozcan et al. (2006) showed that PBA attenuates obesity induced ER stress along with improving glucose homeostasis and insulin signaling in peripheral tissues (muscle, liver, and adipose tissues) of ob/ob mice. They identified that expression of both phosphorylated PERK and phosphorylated IRE-1, which are up-regulated in ob/ob mice, were significantly decreased by PBA treatment. Additionally, tyrosine phosphorylation of both the insulin receptor (IR) and insulin receptor substrate-1 (IRS-1) were greatly up-regulated by PBA. Another study identified that adipocyte differentiation induced by
over-nutrition was decreased by treatment with PBA. In this study, it was shown that the expression of UPR related proteins GRP78 and phosphorylated eIF2α that is activated during adipocyte differentiation was significantly reduced by PBA treatment in vitro. Another study identified that PBA incubation reduced the expression of obesity-linked MC4R variants in the brain which is known to be linked to up-regulation of the expression of GRP78 and XBP1.

Many studies have been conducted to reveal the effect of PBA in the brain and have suggested the possibility of using PBA for treatment of neurodegenerative diseases such as Alzheimer’s disease (AD). Administration of PBA appeared to improve memory function as assessed by a conditioning paradigm in young- and old-AD model mice, Tg2576. Interestingly, this study showed that PBA treatment had an effect on up-regulating GRP78 level in old AD mice while eIF2α level was decreased by this chemical chaperone. Wiley et al. (2010) showed that PBA has the capacity to reverse the inhibition of β-amyloid precursor protein (APP) cleavage which is the pathological characteristic of AD and is induced by ER stress inducers such as thapsigargin and tunicamycin in neuronal cells. Most recently, they also identified that PBA application decreased the formation of amyloid plaque in the cortex and hippocampus by up-regulating APP proteolysis in vivo using AD mice model. Along with reduced size of plaques in these brain regions, cognitive function of AD mice appeared to be improved by this PBA treatment.

As shown in previous chapters, UPR in both the CNS and liver are affected by exercise. However, to our knowledge, there has been no trial to study if PBA would inhibit the exercise induced increase in the UPR. The purpose of the studies reported in
this chapter was to confirm the effect of the chemical chaperone PBA on the UPR in the brain of C57BL/6 mice. Furthermore, the other purpose of this study is to show if PBA could block the exercise induced increase in UPR in the brain of C57BL/6 mice.

**HYPOTHESES**

(1) PBA will down-regulate UPR in the brain.

(2) The effect of exercise on up-regulating UPR in the brain will be reduced in the presence of PBA.

**METHODS**

*Animals and Diets*

Total thirty two male C57BL/6 mice (~6 weeks old) were used and this animal study was approved by the Utah State University Institutional Animal Care and Use Committee (USU-IACUC). As in other chapters, 1 week was allowed for all animals to acclimate to their new environment after they arrived at the animal room. All mice were randomly separated into four groups: Water/Sedentary (Sed) (n=6), Water/Run (n=10), PBA/Sed (n=6), and PBA/Run (n=10). During the experimental period, a 10% low fat diet (D 12450B, Research Diets, New Brunswick, NJ) was given to animals that could freely access food on cage lid and water. The animal room was automatically controlled, being maintained at 22-23 °C with a 12-hour light/dark cycle.

*4-phenylbutyric acid (PBA)*

4-phenylbutyric acid sodium salt (Scandinavian Formulas, Inc., PA) was given to mice in solubilized in drinking water at a concentration of 2.5 mg/ml to provide a dose of 1g/kg/day. The control group received drinking water without PBA. All mice had access
to drinking bottles *ad libitum*. The amount of water intake was measured every second day and drinking bottles were replaced after 1 week.

**Voluntary Running Wheel Exercise**

The detailed exercise protocol was described in the methods of chapter 2. In this study, mice in the running groups receiving either Water or PBA were allowed to freely run on wheels for 14 days as in chapter 3. Running activity of mice (n=10) in each group (Water or PBA) was automatically recorded as turns per hour during 14 days of running using the VitalView program (Mini Mitter, OR). In addition, 6 mice in each group were used as control mice (Sedentary mice, SED) and were kept in locked wheel cages during the running period. Depending on their running activity during running period, running mice were grouped into Low Runner mice (LR, n=4) and High Runner mice (HR, n=4) and only HR mice were used for further experiments in this chapter.

**RNA Isolation and Purification**

The detailed method for RNA isolation and purification was described in the chapter 2. In this study, the hypothalamus and hippocampus were used for RNA analysis.

**Quantitative Real-time PCR**

The detailed procedure for real-time PCR was described in chapter 2. As in chapter 3, KAPA SYBR green (KAPA Biosystems, Woburn, MA) was used for assessing gene expression which was standardized to the expression of the reference gene cyclophilin b. PCR reaction cycles were shown in chapter 3.
Statistics

For statistical analysis, Prism 5 for windows (GraphPad, La Jolla, CA) was used and all data is shown as Mean ± SEM. To assess the interaction between exercise and PBA on each parameter, two-way ANOVA was used. Individual groups were compared with t-test. Only values of $p<0.05$ were considered as statistically significant.

RESULTS

Voluntary Running Wheel Exercise

Total 10 mice of each group (Water or PBA) had voluntary running wheel exercise for 14 days and were grouped into Low Runner (LR, n=4) and High Runner (HR, n=4) depending on their running activity during the running period (Figure 4-1, A). As shown in (B) of Figure 4-1, HR mice of both treatment group had significant more activity as compared to LR mice. As shown in (C), high running activity of HR mice in both groups appeared to be significantly increased 2 day after the start of running relative to LR mice and the increase was maintained during the continued running period. Comparing running activity within either LR or HR group of each group (Water or PBA), PBA did not affect running activity.

The Effect of Exercise and PBA on Change of Body Weight

As shown in Figure 4-2, 14 days treatment with PBA had no effect on the change of body weight of SED or RUN mice. HR mice had a tendency toward lower body weight gains in both water or PBA groups even though there was no statistical significance. When considering all 10 running animals (B of Figure 4-2), 14 days running exercise appeared to have no effect on decreasing body weight. The result of ANOVA indicated that neither PBA nor exercise had any significant effect on the change of body weight.
weight and that there was no significant interaction between these two factors (PBA × exercise) (F<sub>1, 15</sub> = 0.05852, p=0.8121).

**The Effect of Exercise and PBA on Food Intake**

PBA decreased food intake on day 1 after which food intake increased to levels similar to those in the water control groups (Figure 4-3, A). As shown in panel A, running mice (RUN) in each treatment group had higher daily food intake during the 14 days running period as compared to SED mice. The significant differences between RUN and SED mice were found on day 7 in Water mice and days 11 and 13 in PBA mice, separately. When comparing the cumulative food intake over the 14-day experimental period, RUN mice in both treatment groups (Water or PBA) had significantly increased intake of calories compared to the SED mice.

**The Effect of Exercise and PBA on Drinking**

As shown in (A) of Figure 4-4, there was no significant difference in fluid intake between SED and RUN mice in either water or PBA treated groups during 14 days of running period. However, ANOVA data indicated that PBA had an effect on increasing the amount of cumulative drinking during the experimental period (p<0.01) (Figure 4-4, B). This effect of PBA on drinking was shown to be statistically significant in SED mice.

**The Effect of Exercise and PBA on UPR-related Genes Expression in the Brain**

Even though the change did not reach statistical significance, PBA had a tendency to down-regulate the expression of both Eif2α and Grp78 in the hypothalamus (Figure 4-5, A). In this brain region, exercise also decreased the expression of both Eif2α and Grp78 genes in the control group (Water group) and the significant decrease was
observed in the *Eif2a* gene level. In the hippocampus (Figure 4-5, B), PBA treatment itself did not have any effect on the expression of UPR-related genes. However, exercise had an effect of reducing expression of these genes in the hippocampus of mice treated with PBA and expression of all UPR related genes (*Xbp1*, *Atf6*, *Eif2a*, and *Grp78*) were significantly down-regulated by 14 days running wheel exercise. However, from the result of two-way ANOVA (exercise × PBA), there was a significant effect of exercise in the expression of *Xbp1* and *Atf6* and there was no interaction between exercise and PBA.

**DISCUSSION**

In chapter 2, it was shown that 3 weeks voluntary running wheel exercise up-regulated the expression of UPR-related genes in the multiple brain regions of mice exposed to high-fat diet during a variety of periods. Along with these results, it was questioned how running wheel exercise affects the UPR in the brain of mice in the presence of a chemical chaperone such as PBA which has a capacity of reducing the accumulation of unfolded proteins and decreasing ER stress. So, in this chapter, treatment with PBA was used to reduce ER stress and we investigated its effects on the exercise induced UPR-related gene expression in the brain of mice.

PBA treatment for 14 days did not have any effect on running activity of mice. Neither did PBA treatment have any effect on body weight gain during the 14 days of our study. This is consistent with previous studies. In a study using *ob/ob* mice, 20 days PBA oral treatment did not reduce the body weight as compared to vehicle treated mouse even though blood glucose level and insulin level were significantly decreased during PBA treatment. In addition, 26 days treatment of PBA did not change the body weight in *ob/ob* mice as compared to vehicle treated mice.
In line with the absence of any change of body weight with PBA, PBA treatment did not affect food intake. However, 14 days running wheel exercise had a significant effect to increase the cumulative food uptake. Though there are variations in running periods, other studies have also shown that voluntary running wheel exercise increases food intake \(^{13-14}\) and they suggested that this increased food intake followed by running exercise could be one way to compensate the energy budget increased by high physical activity.

Contrary to another report,\(^6\) 14 days PBA treatment significantly increased the amount of drinking comparing SED groups given PBA with those in the Water group. It is possible that this reflects the osmolality of the 5 mM PBA, affecting the drinking behavioral patterns.

PBA treatment tended to lower the expression of both Eif2α and Grp78 in the hypothalamus. Exercise was shown to decrease these gene levels similar to the levels of PBA treated SED group. A study also showed that 26 days PBA treatment had no effect on UPR related phosphorylated PERK in the hypothalamus of lean mice while this chemical significantly lowered the expression of this gene in this brain region of ob/ob mice.\(^5\) To our knowledge, this thesis study is the first trial to study the effect of exercise in the brain of mice treated with PBA. Running wheel exercise had no additional effect on UPR related gene expressions in the hypothalamus of mice over treatment of PBA. However, the control water treated mice did not show the expected effect of exercise on the up-regulation of UPR. This different result probably reflected the differences in feeding way (cup vs. lid). Mice in the previous experiments were provided food in a feeding cup, but food was given on the cage lid in the current study. Harri et al. (1999)
showed that food location could affect behavior of mice that had access to running wheels.\textsuperscript{15} They showed that feeding from the cage lid decreased the access time to a running wheel as compared with feeding on floor. Accessing food on a cage lid appears to be a major form of exercise for mice. Control SED mice in this study were intact exercising mice.

When looking at the Figure 4-5 (B), 14 days PBA treatment had no effect on UPR related gene expressions ($Xbp1$, $Atf6$, $Eif2a$, and $Grp78$) in the hippocampus of SED mice. As shown in the hypothalamus, it is possible that 14 days treatment could be too short to induce the change in UPR and, since mice were not exposed to any stressful circumstance (i.e., high-fat diet), the effect of the chemical chaperone PBA could be limited. In contrast, running wheel exercise for 14 days had an effect of lowering all UPR genes expression in the hippocampus in mice treated with PBA. In this brain region, it is suggested that exercise may promote the action of PBA.

In summary, the chemical chaperone PBA lowered UPR in the brain. However, this chapter showed inconsistent results to the ones of previous chapter in that exercise did not increase the UPR related genes expression. This discrepancy could be induced by the shorter duration of running or to the altered food location used in this experiment. This complicates the interpretation of the data such that it is impossible to identify if PBA prevented the increase in UPR activity that was activated with running in the previous experiments. Running wheel exercise appeared to reduce the expression of UPR related genes in the hippocampus of mice treated with PBA for 14 days while it had no effect in the hypothalamus.
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Figure 4-1 The activity of voluntary running wheel exercise. (A) Running activity of individual mice in Water or PBA treated groups. (B) Total 10 mice in each treatment group (Water or PBA) were separated into Low Runner (LR, n=4) and High Runner (HR, n=4) relative to their activities for 14 days. (C) The running activity of mice given either water or PBA during running period of 14 days. All data was shown as Mean ± SEM of four mice in each group. *p<0.05, **p<0.01, ***p<0.001 HR vs. LR of Water mice; #p<0.05, ##p<0.01, ###p<0.001 HR vs. LR of PBA mice
Figure 4-2 The effect of exercise and PBA on change of body weight. (A) After 14 days, neither PBA nor exercise (HR mice, n=4) had any effect on the change of body weight although running exercise had a tendency of decreasing the body weight. (B) The results obtained for all running mice (n=10) and sedentary mice of each group show that neither PBA nor exercise had any effect on the change of body weight. All data was shown as Mean ± SEM.
Figure 4-3 The effect of exercise on the daily food intake in mice treated with PBA. (A) The change of food intake in either water or PBA treated mice during 14 days running period. RUN mice on both treatment groups have a tendency of having more food than SED mice and there were significant differences on day 7 in Water mice and on days 11 and 13 in PBA mice. At the first day, there was a significant difference in daily food intake between SED mice in Water and SED mice in PBA group (++++p<0.0001). (B) Exercise had a significant effect on cumulative food intake during 14 days running period and total food intake was increased by running activity (**p<0.01, ***p<0.001 RUN vs. SED). All data was shown as Mean ± SEM. *p<0.05, **p<0.01 RUN vs. SED
Figure 4-4 The effect of exercise and PBA on drinking behavior. (A) The change of daily water or PBA intake during 14 days. (B) PBA treatment had a significant effect on cumulative drinking (****p<0.0001 SED PBA vs. SED water). There was no significant interaction between exercise and PBA.
Figure 4-5 The effect of PBA and exercise on expression of UPR genes in the hypothalamus and hippocampus. In the hypothalamus (A), there was no significant effect of PBA on the expression of Xbp1 while two genes of both Eif2α and Grp78 had a tendency to be decreased by PBA treatment as comparing SED mice between Water and PBA mice. The effect of exercise was observed in expression of both Eif2α and Grp78 genes and Eif2α gene expression was significantly down-regulated by 14 days running exercise in Water mice (*p<0.05). In the hippocampus (B), PBA treatment had any effect on all UPR related genes expression (Xbp1, Atf6, Eif2α, and Grp78). In PBA group, the all UPR related gene expression was significantly decreased by 14 days running wheel exercise (*p<0.05, **p<0.01 HR vs. SED of PBA mice).
CHAPTER 5
OVERALL DISCUSSION AND FUTURE DIRECTIONS

A number of chronic diseases, such as obesity and type 2 diabetes \(^1\text{-}^3\) and neurodegenerative diseases such as Alzheimer’s disease (AD),\(^4\text{-}^6\) can be induced with ER stress. Meanwhile, many studies have shown that exercise has a significant effect on ameliorating the status/development of obesity \(^7\text{-}^8\) and type 2 diabetes \(^9\text{-}^{10}\) and neurodegenerative diseases.\(^11\text{-}^{13}\) In this thesis study, the focus was to understand the effect of voluntary running wheel exercise on the UPR, a defensive mechanism against ER stress, in the brain of mice exposed to two dietary circumstances (LFD or HFD) for short- or long-periods. In addition, the possibility that exercise regulated-UPR activity could be linked to exercise-responsive IGF-1 signaling in the brain of mice was studied. Lastly, how the effect of exercise on UPR was changed in the presence of chemical chaperones which are known to lower ER stress by releasing the accumulation of unfolded proteins was assessed. So, the overall theme of these studies was to elucidate the effect of exercise on brain health in terms of regulating UPR.

The regional response

The described results indicated that the UPR to diet and exercise was differentially regulated in each brain region. Although dietary treatment for 3 weeks did not activate UPR in the hypothalamus, running wheel exercise activated UPR in this brain region. One interesting result was that even low running activity up-regulated the UPR signaling in the hypothalamus of mice fed with HFD. Exercise has a significant effect on maintaining energy homeostasis by controlling the expression of hypothalamic neuropeptides such as neuropeptides Y (NPY) and pro-opiomelanocorin (POMC).\(^14\) In
addition, hypothalamic insulin and leptin signaling is improved by physical activity. Exercise induced hypothalamic changes were shown to be linked to the reduction of inflammation (IKKβ/NF-kB) along with reduction of ER stress. So, it is possible that exercise activated UPR activation in the hypothalamus of mice fed HFD for 3 weeks could be linked to alterations the expression of neuropeptides as well as to improved insulin and leptin signaling. When mice were exposed to HFD for 3 months, HFD itself increased the UPR in the hypothalamus more significantly than in the hippocampus. This result also suggested that the hypothalamus, the central part for sensing energy status and keeping energy homeostasis, activated UPR to defeat ER stress induced by a long-term HFD. However, the effect of exercise on UPR up-regulation was shown more significant in the hippocampus than in the hypothalamus. This result suggested the possibility that as the hippocampus is known to take part in cognitive function and memory, exercise could have a positive effect on improving cognitive function in the brain of mice exposed to high-fat diet for a long time.

When circulating IGF-1 was lowered by anti-IGF-1 antibody, UPR signaling in both the hypothalamus and hippocampus responded similarly. Blocking IGF-1 up-regulated Xbp1 mRNA expression and appeared to have similar effects in both regions. In addition, running wheel exercise for 14 days had similar effects in both brain regions, suggesting that IGF-1 normally down-regulate the UPR in the brain regions. Running wheel exercise appeared to decrease the UPR that was up-regulated by blocking IGF-1 level in both brain regions. These results suggested that both brain regions respond similarly to circulating IGF-1.
The effect of the chemical chaperone PBA differed depending on the brain region. While UPR (Eif2α and Grp78) in the hypothalamus seemed to be decreased by PBA, there was no change in UPR activity of the hippocampus. So, it appeared that the hypothalamus was more sensitive to PBA treatment than the hippocampus. The effect of exercise also differed in these two brain regions while in the presence of PBA. In the hypothalamus, running wheel exercise had no effect on regulating UPR related gene expressions, while in the hippocampus UPR related genes expression was significantly reduced by running wheel exercise. It is possible that co-treatment with exercise and PBA could have an effect on improving cognitive function and memory in the hippocampus by releasing ER stress.

Taken together, the UPR of each brain region was not responsive simultaneously to either diet or exercise. Although the responses to IGF-1 and exercise in the hypothalamus and hippocampus were similar, UPR in these two regions responded differently to PBA and exercise.

**The time-dependent effects of diet and exercise**

The effect of diet on the UPR was different depending on how long mice were exposed to the diet. Although short-term exposure (3 weeks) to HFD itself did not induce the activation of UPR in the brain, long-term exposure (3 months) to HFD had a significant effect of up-regulation of UPR. As even HFD feeding for 3 days induced insulin resistance in the brain, it is unlikely that the HFD increase in the UPR is linked to this change because it is not evident till 3 months. However, voluntary running exercise activated UPR signaling in the brain of mice fed HFD only for 3 weeks and this effect of exercise was also shown in the mice fed HFD for 3 months. This exercise
induced UPR up-regulation in the brain of mice exposed to short-term HFD could be linked to exercise-activated insulin sensitivity and this effect could be induced by up-regulated trophic factors such as BDNF and IGF-1 which, in turn, are activated by physical activity. However, when exposure time to VHFD was extended over 4 months, the effect of exercise on activating UPR signaling disappeared. It might be suggested that the UPR has a maximal limit and if UPR is continuously up-regulated by diet, this could be linked to cell death.

Although 3 weeks running wheel exercise had an effect on up-regulation of UPR signaling in the brain of animals fed either LFD or HFD, 14 days running wheel exercise which was applied in the experiments using anti-IGF-1 antibody and PBA failed to show an effect on the activation of UPR signaling in the brain. This different result could be induced by several factors such as the fact that food was placed on the cage lids rather than in cups in these experiments and that location is associated with a large increase in physical activity to obtain the food. In summary, UPR in the brain was responsive to diet and exercise in a time dependent manner.

**Possible implications in neurodegenerative disease**

This thesis study indicated that exercise induced UPR activation was not linked to the activation of apoptotic signaling in the brain of mice exposed to either LFD or HFD for 3 weeks or 3 months. Previous studies have shown that UPR up-regulation was accompanied with an activation of apoptotic signaling in the brain. Mayer et al. (2010) showed that treatment of hypothalamic cell with palmitate increased ER stress (eIF2α and XBP1) along with up-regulation of apoptotic signaling (phosphorylated JNK and cleaved caspase-3). Another study also identified that ER specific apoptosis (caspase-12) was
activated in response to disrupted calcium homeostasis in the ER along with ER stress *in vitro* and this ER stress induced apoptosis was confirmed using caspase-12 deficient mice.\(^{19}\) So, it was hypothesized that exercise may prevent UPR associated apoptosis by activating trophic factors that suppressed apoptotic activation. One such trophic factor, IGF-1, has an effect on exercise linked UPR activation in the brain. However, it was revealed that IGF-1 was not responsible for exercise activated UPR signaling, so other factors (e.g., BDNF) may be involved. On the other hand, it has been shown that UPR activity is required for preventing or slowing-down the progress of neurodegenerative disease.\(^{20,21}\) In summary, though the exact mechanisms still remains unknown, exercise induced UPR activation and suppression of apoptosis might contribute to the known beneficial effect of exercise on neurodegenerative disease.

**Future work**

Although this thesis study applied the exercise protocol for only 14 days or 3 weeks, studies of the effects of long-term exercise on UPR activity in the brain of animals will be needed to provide insight into the possible effects of habitual chronic physical activity on brain health. Previous studies identified that long term (16 weeks \(^{22}\) or 3 months \(^{23}\)) exercise ameliorated the pathological characteristics in neurodegenerative disease animal models.

An AD animal model could be used to reveal the interactions between running wheel exercise, UPR signaling, and AD development, identifying the direct effect of exercise on brain health. Using Alzheimer disease mouse NSE/APPsw-transgenic model, one research group has already indicated that forced-running has a significant effect on reducing the pathological phenotype (i.e., the decrease of deposition of Aβ peptides)
along with the up-regulation of GRP78.\textsuperscript{24} Instead of forced-running exercise, the future study will be able to apply voluntary running wheel exercise to minimize the possible stress, assessing the change of UPR in the brain of disease model which can be exposed to HFD for a short-term or a long-term. It will be worth to try to cross UPR gene knock-out mice with AD mice. It will be likely that the characteristic of AD would be not induced in this animal model. In addition, it will be possible that exercise protocol will be also shown to have no effect on up-regulating UPR signaling in this model.

Although IGF-1 in this thesis study was shown not to be responsible for exercise-induced UPR activation, other trophic factors should be considered to elucidate how exercise affects the UPR in the brain of mice. Since BDNF has been shown to have an effect on UPR,\textsuperscript{25,26} a study of the role of BDNF in exercise-activated UPR without activating apoptotic signaling is needed. In this future study, BDNF level could be able to be controlled by the application of the BDNF inhibitor TrkB antibody as used in Gomez-Pinilla’s research group.\textsuperscript{27}

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