A Dissertation for the Degree of Doctor of Philosophy

Impaired Transient Receptor Potential Vanilloid Type-1 Signaling Promotes Obesity and Leptin/Insulin Resistance In Mice

TRPV1 의 비만 및 렙틴/인슐린 저항성에의 역할과 그 작용기작 규명

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이 논문을 박사학위 논문으로 제출함

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Abstract

According to prevalence of obesity and its-associated metabolic disorders worldwide, these diseases become severe and global health problem. Obesity defined as a condition accumulated excess fat mass bodily is a hall marker and major cause of metabolic diseases such as cardiovascular disease, stroke, and type 2 diabetes (T2D). Because obesity and T2D patients have dramatically higher risks of cardiovascular disease, the most common cause of death in Western countries, an increase in the prevalence of obesity and diabetes in the population is one of the most serious problems of modern society. Thus, the prevention and treatment of obesity and T2D become more and more important. Insulin resistance, an attenuated or lack of response of the insulin receptor (IR) and its downstream signaling pathway to insulin stimulation even at high doses of insulin, is a representative characteristic of T2D. Although insulin resistance is caused by inflammation, oxidative stress, ER stress, and mitochondrial dysfunction, the specific mechanisms which lead from obesity to T2D is still unclear.

Recent evidences have been clearly showed that capsaicin, a pungent component of chili peppers, play a crucial role in obesity and metabolic disorders. Administration of capsaicin prevents obesity and improves glucose homeostasis and insulin secretion in small rodents and humans. Several previous studies have reported supportive clinical evidence that consumption of red peppers or capsaicin was shown to decrease appetite, cause weight loss and stimulate thermogenesis caused by substrate oxidation from carbohydrate to fat oxidation. However, the role of its receptor, transient receptor potential vanilloid subfamily type 1 (TRPV1), in development of obesity and its-associated insulin resistance is controversial, which suggests that its specific function and mechanistic studies in metabolic disorders are poorly understood.

Here, I examined the effect of TRPV1, capsaicin receptor, on diet-induced obesity and insulin resistance in mice. TRPV1-deficient mice became more obese and get more fat accumulation on high-fat diet (HFD) feeding than wild-type (WT) mice. These results were caused by reduced locomotor activity in TRPV1 KO mice fed HFD for 5 weeks. In TRPV1 KO mice, plasma leptin levels were decreased.

Although leptin up-regulates locomotor activity as well as energy expenditure, TRPV1 KO mice showed decreased activity and no changes in energy expenditure compared to WT mice, suggesting severe leptin resistance in TRPV1 KO mice fed HFD. All of these results indicated that TRPV1 is a regulator of energy balance and development of leptin resistance in obese mice. In addition, TRPV1 deletion accelerates diet-induced insulin resistance. Insulin-stimulated glucose uptake in adipose tissues and heart was significantly diminished in HFD-fed TRPV1 KO mice. As one of the major causes of inflammation, oxidative stress and mitochondrial dysfunction, aging has been showed to induce obesity and insulin resistance. Deletion of TRPV1 in mice accelerated aging-induced weight gain and insulin resistance. Unlike the results fed HFD, aging promoted hepatic insulin resistance in TRPV1 KO mice compared to WT mice. Thus, these results provide new insight into the involvement of TRPV1 in development of obesity and insulin resistance and promising strategy against their pathogenesis.

Keyword : TRPV1, metabolic syndrome, locomotor activity, leptin resistance, insulin resistance, aging, high-fat diet

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Contents

Chapter 1.

Implications of capsaicin and its receptor, TRPV1 in metabolic diseases: A review

Abstract

The prevalence of obesity and a plethora of associated metabolic diseases to which it contributes is rapidly becoming a severe problem throughout the developed world. Recent evidence has shown that capsaicin, a pungent component of chilli peppers, and its receptor (transient receptor potential vanilloid type-1; TRPV1) exert significant effects on obesity and metabolic disorders. The administration of capsaicin has been found to prevent obesity and improve glucose homeostasis and insulin secretion in small rodents and humans. This review aims to summarize an overview of the obesity problem, as well as insulin/glucose metabolism and leptin/insulin resistance as contributors to obesity development and related metabolic disorders. I will also describe important effects of capsaicin and its receptor, TRPV1, in prevention of these diseases. Although the activation of TRPV1 by capsaicin improves metabolic disorders and prevents weight gain in small rodents and humans, its mechanistic role in obesity and metabolic disease prevention remains poorly understood. The objective is to provide new insights into the involvement of both capsaicin and

TRPV1 in the development of obesity and metabolic disease, and potential strategies to attenuate pathogenesis.

1.1. Introduction

While obesity in itself represents a serious disease, the condition can also markedly increase the likelihood of other common metabolic disorders including cardiovascular disease and diabetes (1). For those who suffer from obesity, the development of type 2 diabetes (T2D) is a common eventuality in many developed countries, of which many have an obesity problem of epidemic proportions. Although some studies have estimated that over 250 million people will be affected by T2D worldwide in the coming years, its primary causes and mechanism of development remain unknown (2). However, it has become clear that obesity-associated insulin resistance, a major characteristic of T2D, plays an important role in its development (3, 4). Since impaired insulin signaling associated with hyperinsulinemia is a critical feature of insulin resistance-induced T2D, the detection of insulin resistance itself is useful for the diagnosis of T2D.

The medical definition of obesity is a condition in which a person has excess body fat mass. From fatty tissues, cytokines that regulate insulin/glucose metabolism are released, which act in positive or negative feedback loops to promote the development of insulin

resistance. High levels of several cytokines including interleukin (IL)-1β, resistain, and TNF-α promote inflammatory responses, which can accelerate the onset of obesity and its related disorders (5, 6). In contrast, IL-10, an anti-inflammatory cytokine, as well as adiponectin and leptin can regulate catabolic metabolism to reduce adiposity (7, 8). In particular, leptin, as a master regulator of appetite and energy expenditure, plays a critical role in the hypothalamus to inhibit obesity and metabolic diseases. While obese animals and humans generally have higher levels of plasma leptin, its signaling is often impaired so that it cannot function normally. This failure to respond to the regulation of appetite and energy expenditure by leptin is referred to as leptin resistance. In this condition, rodents and human patients typically exhibit insulin resistance as well, supporting the idea that obesity, insulin resistance and leptin resistance develop through complex and inter-related mechanisms (9).

Here, I review several lines of evidence that the capsaicin receptor, transient receptor potential vanilloid type-1 (TRPV1), represents a novel target for a new strategy to prevent obesity and its associated disorders. The receptor is widely expressed in adipose tissues, the pancreas, muscle and heart, which are major organs that

regulate glucose/insulin metabolism. It also targets primary sensory neurons, suggesting that TRPV1 may be involved in the development of metabolic disorders (10, 11). This review will focus on the molecular mechanisms responsible for metabolic diseases, with a focus on obesity and T2D, summarizing evidence supporting the hypothesis that TRPV1 plays an important role in their development.

1.2. Development of metabolic diseases

1.2.1. Obesity

Obesity is a significant risk factor for the development of other diseases including diabetes, coronary heart disease, hypertension, atherosclerosis and certain forms of cancer. As a disease, it is defined by the WHO to entail a body mass index of over 30 kg/m^2. Over 30% of the American population is considered obese, and 7-8% of Koreans have an obese phenotype. Obesity arises from excessive energy intake through food consumption and insufficient energy expenditure in order

to maintain cellular and organ function, physical activity and adaptive thermogenesis.

Obesity is characterized as an excessive accumulation of fat, which increases the secretion of pro-inflammatory cytokines and chemokines including TNF- α and MCP-1 from adipose tissue, which acts to recruit macrophages that infiltrate into areas containing adipocytes (12). Infiltrated macrophages trigger a broad and chronic inflammatory response that strongly promotes obesity through a positive feedback mechanism. Obesity also develops through complex and chronic mechanisms involving several factors including genetics, as well as environmental factors such as culture, lifestyle and nutritional state (1). Although obesity and its related metabolic diseases are widespread and represent a growing challenge, the mechanistic understanding of their pathology and therapeutics remain unsatisfactory, underlining the critical need for further studies.

1.2.2. Hyperglycemia, insulin resistance and type 2 diabetes

Chronic adiposity resulting from excess food intake and insufficient energy expenditure can be thought of more simply as an

oversupply of energy in the body. A major energy source for the body is glucose; obese animals and human therefore frequently exhibit hyperglycemi,a which is a representative characteristic of diabetes mellitus and defined as having excessive plasma glucose (over 11.1 mmol/L) (13). To reduce plasma glucose levels, β cells in the pancreas secret insulin to promote glucose uptake into organs such as muscle, adipose tissue, and the heart, while reducing glucose production in the liver (hepatic glucose production, HGP).

Diabetes is defined as a condition involving abnormal glucose and insulin metabolism. There are two major types of diabetes; Type 1 diabetes (T1D) is caused by genetic mutations that lead to defects in the immune system and destruction of insulin producing cells (islets). Patients diagnosed with T1D, therefore, exhibit impaired insulin secretion. In contrast, T2D is characterized by insulin resistance, and an attenuated or lack of response of the insulin receptor (IR) and its downstream signaling pathway to insulin stimulation even at high doses of insulin. Insulin resistance triggers an overload of the pancreas to compensate for the perceived lack of insulin, resulting in defects in the beta cells of the pancreas. In Korea, the number of patients diagnosed with type 2 diabetes was estimated at 7.08% of the total population in

2010 and has been expected to dramatically increase to over 10% by 2030 (14). It is now known that obesity is a major cause of T2D with inflammation, mitochondrial dysfunction and endoplasmic reticulum (ER) stress (Fig. 1) involved, and statistical data shows that the incidence of T2D is strongly associated with obesity (1, 15). However, the specific mechanisms which lead from obesity to T2D are still unclear, requiring further studies into the molecular mechanisms involved in the development of T2D.

1.2.3. Hyperlipidemia, hyperleptinemia and leptin resistance

Abnormally elevated levels of body fat in obese animals and humans have been strongly implicated as a major factor in the development of hyperlipidemia and hyperleptinemia. Hyperlipidemia is a condition marked by greater than normal levels of plasma lipid or lipoproteins. It is a primary cause of metabolic disorders that are characterized by abnormal lipid metabolism including atherosclerosis, coronary heart disease, and cardiovascular disease (16). Abnormally elevated levels of free fatty acids (FFA) that accompany hyperlipidemia greatly exacerbates existing conditions like impaired glucose tolerance,

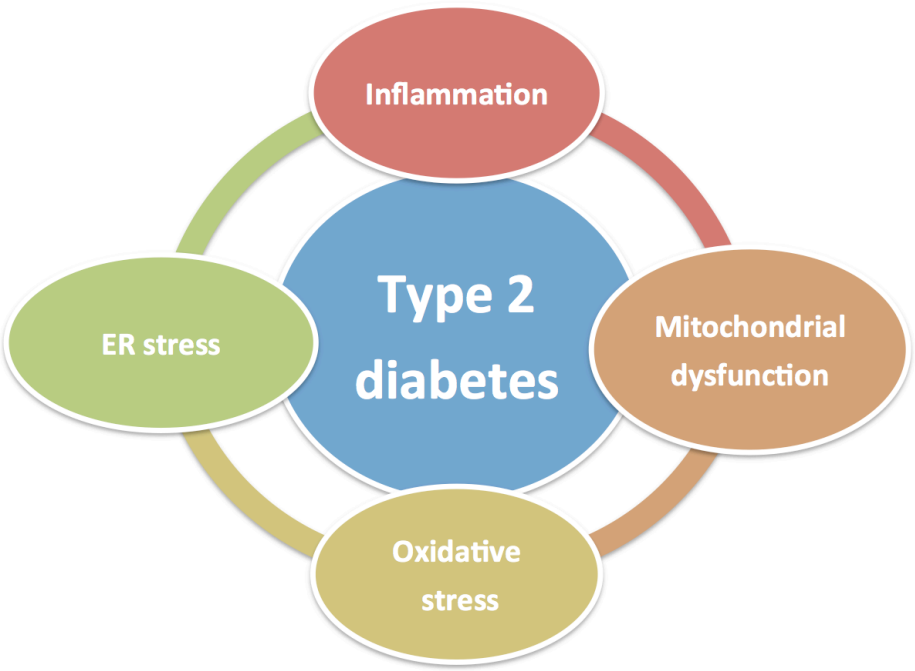

Figure 1. The causes of type 2 diabetes. Obesity induces chronic and systemic inflammation, oxidative stress, ER stress, and mitochondrial dysfunction. These are cellular events are strongly correlated to insulin resistance, which is characterized by impaired insulin signaling, and consequently promotes type 2 diabetes.

reduced glucose uptake in skeletal muscle, hepatic steatosis, increased secretion of pro-inflammatory cytokines, and consequently T2D [reviewed in (17)].

Adipocytes accumulate large numbers in obese animals and humans, and secrete high levels of pro-inflammatory cytokines. Examples include TNF-α, IL-1β, IL-6, and leptin. These signaling factors induce abnormal insulin action at high concentrations, whereas a lack of these cytokines can improve insulin sensitivity. This suggests that the overproduction of inflammatory cytokines in adipose tissue could play an important role in the development of insulin resistance. Leptin is a major hormone secreted from adipose tissue that regulates appetite and anabolic metabolic pathways as well as adaptive and innate immune responses (Fig. 2) (18, 19). Leptin was first characterized in 1950 in mutant obese mice that had deletions in the Ob gene that encodes leptin (20). The ob/ob mice, with point mutations in the leptin gene ob, or db/db mice, with homozygously deleted genes that encode the leptin receptor, are excessively obese, even when fed on chow diet. These mice also exhibit a decreased ability to control appetite, resulting in continuous food consumption. Leptin also acts on its receptor in the hypothalamus of the brain to suppress appetite.

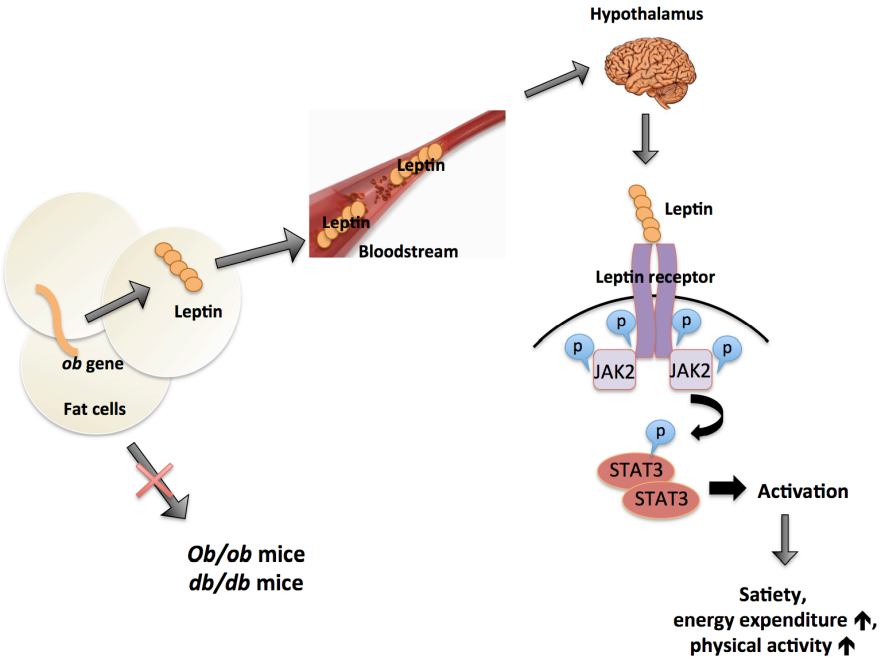

Figure 2. Regulatory mechanism of leptin feedback loop. Leptin produced from the ob gene in periphery cells including fatty tissues crosses the blood-brain barrier and activates the leptin receptor in hypothalamus. It triggers hypothalamic appetite suppression and elevated energy expenditure

Leptin administration experiments in rodent and humans clearly supports the notion that leptin stimulates the brain to feel satisfied, triggering the cessation of food consumption [reviewed in (21)]. In addition, recent studies have demonstrated that leptin regulates energy expenditure and physical activity as well as appetite, suggesting another novel route for preventing the development of obesity and diabetes. Infusions or acute injections of leptin induce an increase in energy expenditure and locomotor activity (physical activity) in ob/ob mice that normally show extremely low levels of energy expenditure and movement (22, 23).

Similar to conditions involving insulin resistance, obese animals and patients also exhibit leptin resistance. In the normal state, increased adipose tissue produces more leptin to lower food consumption and naturally regulate energy intake. Because obese animals and humans have dysregulated leptin signaling, they cannot recover from their abnormal state, exacerbating their obesity and diabetes. Recent clinical therapeutic studies to examine the positive effects of leptin for treatment of obesity and T2D have failed, possibly because obese patients with T2D have leptin resistance. However, the detailed

mechanisms responsible are not fully understood, strongly underlining the necessity of further studies for overcoming leptin resistance.

1.3. Molecular mechanisms of TRPV1 and capsaicin in metabolic disease

1.3.1. Capsaicin receptor: Transient receptor potential vanilloid type-1

Capsaicin, a major pungent component found in chili pepper, specifically stimulates transient receptor potential vanilloid type-1 (TRPV1), a non-selective cation channel. The consumption of capsaicin, which potently activates the TRPV1 channel, can arouse painful and thermogenic sensations that are commonly identified with as the spicy experience. The underlying importance of TRPV1 in neuroscience lies in its role as a major pain-sensing receptor, with its natural agonist, capsaicin, able to selectively react with TRPV1 and not with other TRP channels. There exist other agonists of TRPV1, for example, it can be activated by endovanilloid compounds, low pH (acidic condition) and high temperature (>43°C) (11). Since TRPV1

and capsaicin control thermogenesis, a crucial element in obesity and its related metabolic disorders, they could represent targets for novel therapeutic strategies to negate the onset of obesity and its associated metabolic disorders.

1.3.2. In obesity: adipogenesis and thermogenesis

Adipogenesis involves the production of fatty tissue through the conversion of preadipocytes to adipocytes via differentiation, and is the primary contributor to obesity. Several previous studies have reported clinical evidence that suggests the consumption of red peppers or capsaicin can decrease appetite, cause weight loss and stimulate thermogenesis via substrate oxidation from carbohydrate to the oxidation of fat (24-26). Epidemiological data also supports the notion that capsaicin consumption is related to a lower prevalence of obesity (27). Capsaicin treatment inhibits the differentiation of preadipocytes to adipocytes by regulating AMP-activated protein kinase (AMPK) and induces apoptosis and cell death in 3T3-L1 preadipocytes (28, 29). From the perspective of thermogenesis, capsaicin administration strongly increases whole-body energy expenditure, as well as brown

adipose tissue (BAT) temperature while increasing uncoupling protein 1 (UCP1), a key molecule for the regulation of BAT-related in small rodents and humans (30-32). Contrary to these reports, however, several studies have reported failures in inducing increased energy expenditure and thermogenesis by capsaicin or foods containing capsaicin (33, 34). These inconsistent results could be caused by differences in sensitivity to capsaicin, which may be affected by variable levels of endogenous TRPV1 expression. The repeated exposure to capsaicin (chronic) or exposure to high doses of capsaicin (acute) induces degradation of the TRPV1 protein triggering a process known as “desensitization” (35, 36). Consistent with this idea, levels of TRPV1 expression are significantly reduced and responsiveness to capsaicin can then be impaired in obese rodents and humans (37).

As a selective receptor of capsaicin, TRPV1 has been reported to be expressed in preadipocytes and visceral adipose tissue in both mice and humans (37), suggesting a possible role in the process of adipogenesis. Activation of TRPV1 in preadipocytes by capsaicin has been found to reduce adipogenesis by regulating peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein (C/EBPα), intracellular triglycerides, and glycerol-3-phosphate

dehydrogenase activity, while inducing apoptosis through the activation of caspase-3, Bas and Bak, cleavage of PARP, and decreased anti-apoptotic Bcl-2 expression (28, 37, 38). High-fat diet-containing capsaicin also reduces body weight gain and accumulation of visceral and subcutaneous fatty tissue in mice. All of these results indicate that the activation of TRPV1 and the consumption of capsaicin are beneficial for potential therapeutic intervention to prevent obesity and its related disorders.

In contrast, there have been contradictory reports using a TRPV1-null mouse model (39). TRPV1 knockout mice fed on a long term high-fat diet (HFD) for 25 weeks exhibited preventive effects against weight gain when compared to wild-type (WT) mice. This protective effect of TRPV1 deficiency against weight gain was found to be associated with a reduced accumulation of adipose tissue and hepatic steatosis by increased energy expenditure. These ambiguous results, strongly underline the need to reconsider the effects of TRPV1 and capsaicin as an agonist, for the treatment of obesity and its associated diseases.

1.3.3. In diabetes mellitus: insulin secretion and insulin resistance

Diabetes mellitus is categorized into 2 types. Type 1 diabetes (T1D) is caused by a lack of functional insulin secretion, while type 2 diabetes (T2D) arises from impaired insulin signaling as a result of various environmental factors. Previous studies have reported that TRPV1 activation by capsaicin affects insulin secretion in animals and humans. Highly elevated expression of TRPV1 in the sensory nerve fibres that innervate the pancreas implies that TRPV1 could play an important role in insulin secretion (40-42). The administration of capsaicin in diabetic rats increases insulin secretion and insulin responses, and consequently improves glucose homeostasiss (41, 43). These results indicate that TRPV1 and its agonist, capsaicin are involved in insulin secretion by regulating sensory nerve fibres which affect the pancreas, suggesting that TRPV1 and capsaicin could be potentially useful in new strategies for the treatment of T1D. In non-obese diabetic (NOD) mice, as a representative model for T1D, TRPV1 is mutated to hypofunctional form, and capsaicin treatment in these mice prevents islet beta cells stress and inflammation (44). Since the *in*

vivo data in small rodent models remains controversial (45), further studies using TRPV1-null mice are required.

1.5. Conclusions

Over the past few decades, the role and mechanism of capsaicin and TRPV1 in metabolic diseases has increasingly been demonstrated. Although capsaicin can influence the pathogenesis of obesity and metabolic diseases positively, the roles of TRPV1, a capsaicin receptor, in these diseases and its mechanism of action have not been clearly elucidated. TRPV1, as a non-selective cation channel, primarily regulates the influx of calcium ions involved in the pathogenesis of various human diseases. Various evidences exist that suggests the possibility that TRPV1 plays a crucial role in the development of metabolic diseases. Thus, a clear understanding of the role of TRPV1 in metabolic disease will facilitate the development of new therapeutic strategies for preventing and treating obesity and its associated disorders.

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Chapter 2.

TRPV1 deficiency aggravates diet-induced imbalance in energy homeostasis and leptin resistance

Abstract

Obesity is a hallmark of many metabolic disorders and in recent decades has become a severe global health problem. Although a considerable amount of previous studies have supported the claim that calcium channels (including transient receptor potential (TRP) channels) are involved in the development of obesity, the mechanistic aspects are not fully understood. Among the TRP channels, transient receptor potential vanilloid type-1 (TRPV1), a receptor for capsaicin (a pungent component of chilli peppers), has been reported to exhibit anti-obesity effects, although several studies have demostrated opposite results indicating pro-obesity effects for TRPV1-deficiency models. These controversial results indicate the necessity for further studies of TRPV1 and its involvement in the development of obesity. Here, I demonstrate that TRPV1 deletion promotes the accumulation of fat mass and obesity resulting from lowered locomotor activity. Moreover, TRPV1 deficiency impairs leptin signaling by inducing leptin resistance in mice. These results suggest that the activation of TRPV1 by the natural agonist capsaicin may represent a promising new therapeutic agent for the treatment and prevention of obesity.

Keywords: TRPV1, obesity, leptin resistance, high fat diet, locomotor activity.

2.1. Introduction

Abnormally elevated levels of fat accumulation in obese animals and subjects strongly promotes the induction of macrophage infiltration into the fatty tissue, which providing a major impetus for the secretion of inflammatory cytokines and hormones such as interleukin (IL)-1b, IL-6, monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-alpha (TNFα), adiponectin, and leptin (1, 2). Among these signaling factors, leptin is a critical regulator of energy balance by inducing satiety and energy expenditure. It binds with the leptin receptor and activates downstream signaling pathways in the hypothalamus, which work to reduce appetite. According to previous studies, leptin also regulates locomotor activity (or physical activity), and voluntary movement independent from food-anticipatory activity (3-5). In obese patients, leptin levels in the bloodstream are significantly lower, and leptin-replacement therapy can lead to weight loss (6, 7). Although there are many lines of evidence to support the notion that leptin remarkably improves obesity, associated hyperglycemia and insulin resistance in some animal models and humans (4, 8-12), several clinical leptin administration studies have failed to recover insulin resistance and weight gain in obese patients with type 2 diabetes (13-15). These ineffective leptin treatment studies may be the result of leptin resistance present in extremely obese or type 2 diabetic patients.

Leptin resistance is a representative characteristic of subjects with obesity and diabetes. Similar to insulin resistance, leptin resistance is define as a less or lack of response to leptin, even if administered with high doses of leptin. This outcome is a critical aspect for why leptin cannot be used as a therapeutic for the treatment of obesity and diabetes. Following a number of recent failures of leptin therapy due to leptin resistance, the demand for more insightful mechanistic studies to overcome leptin resistance has been growing. However, the underlying mechanisms and viable alternatives for the recovery of leptin resistance remain to be elucidated. In this study, I discovered that transient receptor potential (TRP) vanilloid type-1 (TRPV1) mediates the leptin signaling pathway, by demonstrating that a knockout of TRPV1 blocks leptin downstream pathway events such as STAT3 phosphorylation. This supports the idea that activation of TRPV1 by capsaicin or the other agonists might be a potential therapeutic strategy for the treatment of leptin resistance.

The TRPV1 channel is a member of the TRP protein superfamily, a large group of cation-permeable channels expressed in mammalian cells. TRPV1 is a nonselective cation channel that is highly permeable to Ca^{2+} and Na^{2+}, and is involved in thermogenesis and pain sensing (16-18). Capsaicin, a major component of chilli pepper, is a natural and specific agonist for the TRPV1 channel and induces the pain sensation and thermogenic reaction, colloquially known as the spicy sensation when eaten. Thermogenesis is a major component of energy expenditure and plays a crucial role in the energy

metabolism that regulates obesity (19). Therefore, chilli pepper and its major thermogenic component, capsaicin, could exhibit anti-obesity properties. A considerable number of previous studies have shown that chilli pepper or capsaicin-containing foods are associated with a reduced incidence of obesity, caused by reductions in heat generation and increases in energy expenditure (20-23). Epidemiological and clinical data shows that capsaicin consumption is correlated with a lower incidence of obesity in accordance with *in vitro* results indicating that capsaicin treatment reduces preadipocyte differentiation into mature adipocytes (20-22, 24-26). The activation of TRPV1 by capsaicin consumption has also been shown to significantly decrease the accumulation of body fat mass and body weight gain in mice (20). However, previous studies using a TRPV1 knockout mice model has revealed contradictory data indicating lower obesity and better thermogenic capacity in TRPV1 deficient mice compared to wild-type mice (27). Thus, I sought to determine the role of TRPV1 in high-fat diet (HFD)-induced obesity and energy imbalance using TRPV1-deficient mice. Here, I demonstrate that TRPV1 deletion aggravates HFD-induced fat accumulation and obesity, resulting from reduced leptin sensitivity.

2.2. Materials and Methods

2.2.1. Animals

TRPV1-deficient (TRPV1 KO) mice and WT littermates were purchased from Jackson Laboratory. For diet-induced obesity, mice were fed a high-fat diet (HFD) (55% fat by calories; Harlan Teklad TD93075) *ad libitum* for 5 or 9 weeks. The animal studies were approved by the Institutional Animal Care and Use Committee of the University Massachusetts Medical School. All studies at GSK were conducted after review by the GSK Institutional Animal Care and Use Committee and in accordance with the GSK Policy on the Care, Welfare, and Treatment of Laboratory Animals.

2.2.2. Body composition and energy balance measurements

Mice were housed under controlled temperature and lighting, with free access to food and water. Whole body fat and lean mass were noninvasively measured using ^{1}H-MRS (Echo Medical Systems, Houston, TX). The food/water intake, energy expenditure, respiratory exchange ratio, and physical activity were assessed for 3 days using metabolic cages (TSE Systems, Chesterfield, MO). I used the TSE Systems LabMaster platform with easy-to-use calorimetry featuring fully-automated monitoring for food,

water and XYZ activity. LabMaster cages were used, that are similar to normal cages used for pets, thereby allowing the use of bedding in the cage and minimizing animal anxiety during the experimental period. The system provides intuitive software with flexibility for experimental setup and data utilization.

2.2.3. Leptin/adiponectin measurement

The plasma levels of leptin and adiponectin were measured using ELISA kits (ALPCO Diagnostics, Salem, New Hampshire, USA). To collect plasma samples, mice were fasted for 5 h in the morning. Using heparin-treated capillary tubes, blood was collected carefully into heparin-treated eppen tubes, and centrifuged at 12,000 rpm for 3 mins in 4°C. For ELISA assays, the supernant of centrifuged blood was used, following the manufacturer's instructions.

2.2.4. Leptin infusion study

To infuse leptin continuously (20 μg/day), osmotic pumps (1007D, Alzet Corp., Palo Alto, CA) were used as described in the manufacturer's manual. Basal food intake was measured using metabolic cages for 3 days as described above. After survival surgery to insert the pumps, mice were housed in their original cages to recover from anesthesia and then placed in metablic

cages for 6 days to measure food intake during leptin infusion. To induce leptin resistance, mice were fed HFD for 9 weeks. The same procedures were then performed again.

2.2.5. Leptin signaling in primary cultured mouse embryonic fibloblasts (MEFs)

Primary cultured TRPV1 KO and WT MEFs were prepared from embryos at embryonic Day 13.5. The method for primary culture was modified from a previous study (28). Briefly, embryos were washed in DPBS and chopped with a blade and 23G needle. Embryos were trypsonized for 10 mins at 37°C and were centrifuged at 1,000 rpm for 3 mins at 4°C. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. TRPV1 KO and WT MEFs were treated with leptin (100 ng/ml) for 10 mins after serum deprivation for 18 h and then washed twice with cold DPBS and harvested.

2.2.6. Leptin stimulation study

To test leptin signaling in mice, mice were fasted overnight (~15 h) and injected with leptin intraperitoneally. After 2 h, mice were anesthestized with intraperitoneal ketamine (100 mg/ml) and xylazine (20 mg/ml) mixture.

The hypothalamus was taken from each mouse within 5 mins. After lysis of tissue samples, a total of 50 μg of protein was used for Western blotting.

2.2.7. Western blotting

Total cell and tissue lysates from mice were prepared and subjected to Western blot as described previously (29). After cell lysis, the protein concentration was determined using a dye-binding protein assay kit (Bio-Rad Laboratories, Hercules, CA) as described in the manufacturer's manual. Protein lysate (50 μg) was subjected to 10% SDS PAGE and electrophoretically transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). After blotting, the membrane was incubated with the p-STAT3 (Tyr705), STAT3, and β-actin primary antibodies at 4ºC overnight. Protein bands were visualized using a chemiluminescence detection kit after hybridization with a horseradish peroxidase-conjugated secondary antibody.

For the *in vivo* Western blots, the hypothalamus was removed from each mouse, and extracted with T-PER tissue protein extraction reagent (Pierce, Rockford, IL) containing 1% phosphatase inhibitor cocktail 2 (Sigma Chemical, St. Louis, MO) and the protease inhibitor phenylmethylsulfonyl fluoride (Calbiochem, La Jolla, CA) to isolate the proteins. The samples were then homogenized using ultra turrax homogenizer (Next Advance Inc.). The lysates were centrifuged at 14,000 rpm for 15 min, and the protein

concentration was determined using a protein assay kit (Bio-Rad Laboratories). Tissue lysates were subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Buckhinghamshire, UK). Membranes were processed and proteins analyzed as for the *in vitro* Western blot assay.

2.2.8. Statistical analysis

Data are expressed as mean ± S.E. values, and ANOVA was used for multiple statistical comparisons. A probability value of $p<0.05$ was used as the criterion for statistical significance. All analyses were performed using Statistical Analysis Software (SAS, Inc., Cary, NC).

2.3. Results

2.3.1. Deletion of TRPV1 induces higher accumulation of fat and obesity during HFD feeding

To determine the effect of TRPV1 on HFD-induced obesity, TRPV1 knockout (KO) mice and wild-type (WT) littermates were fed HFD (55% fat by calories) *ad libitum* for 5 weeks and body composition was measured weekly. Body composition data showed that the body weight of TRPV1 KO mice was significantly higher than WT after 5 weeks of HFD (Figure 1A), but the lean mass of each group was not statistically different (Figure 1C). Additionally, TRPV1 KO mice had remarkably more fat mass than WT mice after HFD feeding, whereas there was no difference in the body weight of both TRPV1 and WT mice fed chow diet (Figure 1B). Unlike previous studies using TRPV1 KO mice fed 11% fat diet(27), TRPV1 KO mice were more obese, resulting from higher fat mass than WT mice on HFD feeding. This finding coincides with some previous reports demonstrating that TRPV1 activation by capsaicin consumption improves the prevention of obesity and fat accumulation (20).

2.3.2. TRPV1-deficient mice exhibit decreased locomoter activity

Figure 1

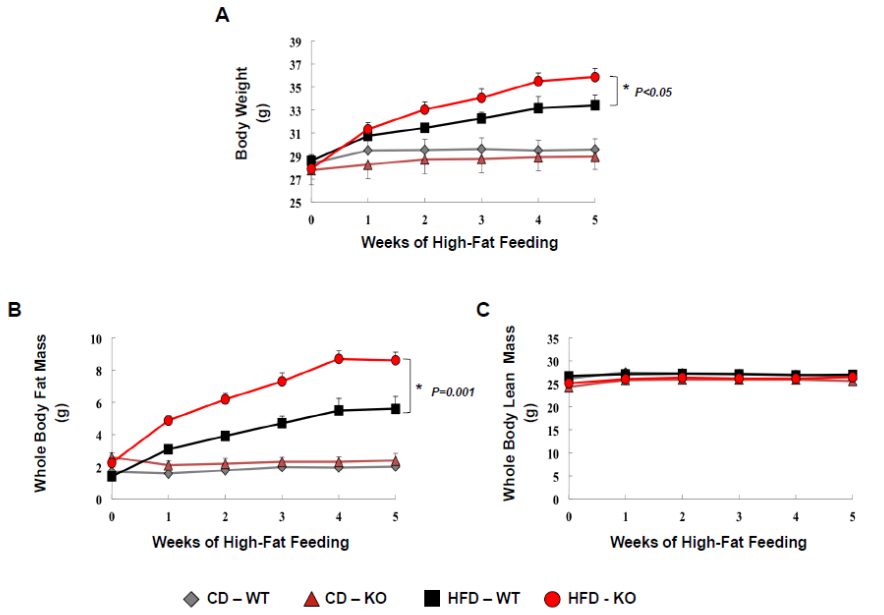

Figure 1. Effect of TRPV1 deletion on HFD-induced weight gain. (*A*) Body weight was measured during 5 weeks of HFD (n=10-12, *p<0.05 vs. WT mice fed HFD). (*B* and *C*) Whole body fat (*B*) and lean mass (*C*) were measured weekly using ^{1}H-MRS during the experiment. WT, wild type C57BL/6J mice; KO, TRPV1 KO mice. *p<0.05

Since TRPV1 KO mice fed HFD were more obese than WT mice fed HFD, we next examined the effect of TRPV1 deletion on energy balance using metabolic cages and TRPV1 KO and WT mice fed on a chow diet and after 4 weeks of HFD feeding. Food intake (Figure 2A), VO_2 consumption and VCO_2 production (Figure 2B), and energy expenditure (Figure 2C) normalized to whole body lean mass (measured using 1H-MRS) were not different between TRPV1 KO mice and WT mice on both fed chow diet and HFD. However, the TRPV1 deficiency caused a selective change in physical activity (35% of reduction) in TRPV1 KO mice during the night from 6pm to 6am for 3 days (Figure 2D and E), suggesting that TRPV1 deletion induces lower physical activity in mice.

2.3.3. Deletion of TRPV1 induces increased plasma leptin levels compared to WT mice after HFD

Since physical activity was decreased in TRPV1 KO mice, I sought to determine which factors were involved. Previous studies have suggested that leptin controls locomotor activity and energy expenditure as well as food intake (3, 5). The plasma leptin levels of TRPV1 KO mice were higher than in WT mice fed on both chow and HFD (Figure 3A). To confirm that the increased levels of plasma leptin in TRPV1 KO mice fed on HFD was caused

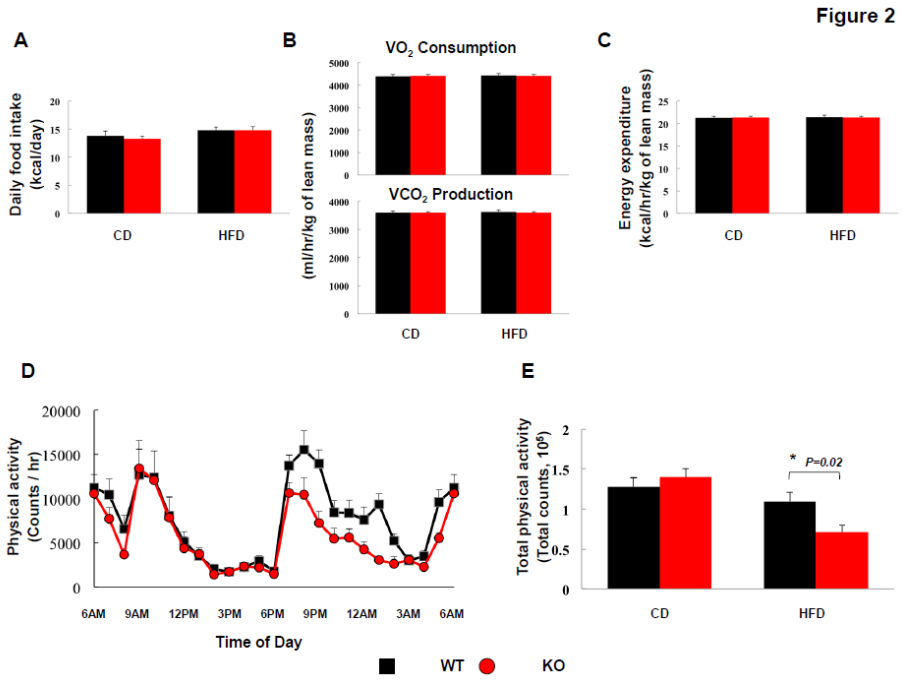

Figure 2. Reduced physical activity in TRPV1-deficient mice. Indirect calorimetry was performed using metabolic cages in male WT (n=6) and TRPV1 KO mice (n=6) before and after HFD feeding. (*A*) Daily food intake in WT and TRPV1 KO mice. All data are expressed per kg of whole body lean mass measured using ^{1}H-MRS. (*B*) Average VO_2 consumption (*upper*) and VCO_2 production (*bottom*) in each mouse. (*C*) Average energy expenditure rates. (*D*) 24-h physical activity in HFD-fed mice. (*E*) Total beam

break counts, used as a measure of physical activity during nighttime.

*$p<0.05$, p value denotes the statistical significance vs. WT mice fed HFD.

by higher fat mass, I normalized plasma leptin levels to fat mass for all mice (Figure 3B). Normalized leptin level in TRPV1 KO mice was also significantly higher than in WT mice. In addition, levels of the alternate adipokine secreted from fat tissues, adiponectin, were measured to confirm which leptin level was elevaed by increased fat mass in TRPV1-deficient mice (Figure 3C). Unlike leptin levels, plasma adiponectin levels of TRPV1 KO mice were not statistically different from levels in WT mice. TRPV1-deficient mice therefore had higher plasma leptin levels, suggesting that TRPV1 is involved in leptin secretion or related signaling pathways.

2.3.4. Negative correlation between leptin and physical activity/energy expenditure in TRPV1 KO mice fed HFD

Although TRPV1 KO mice had greater plasma leptin levels, their food intake and energy expenditure remained unchanged compared to WT mice after 5 weeks of HFD feeding (Figure 2A and C), and even physical activity during night time was significantly decreased in TRPV1 KO mice (Figure 2 D and E). Figure 4 reveals the opposite trends for physical activity and energy expenditure vs. plasma leptin levels for each mouse. WT mice had relatively positive correlations for physical activity (Pearson R = 0.939, *p* value=0.061) and energy expenditure (Pearson R = 0.847, *p* value=0.15) vs. concentrations

Figure 3

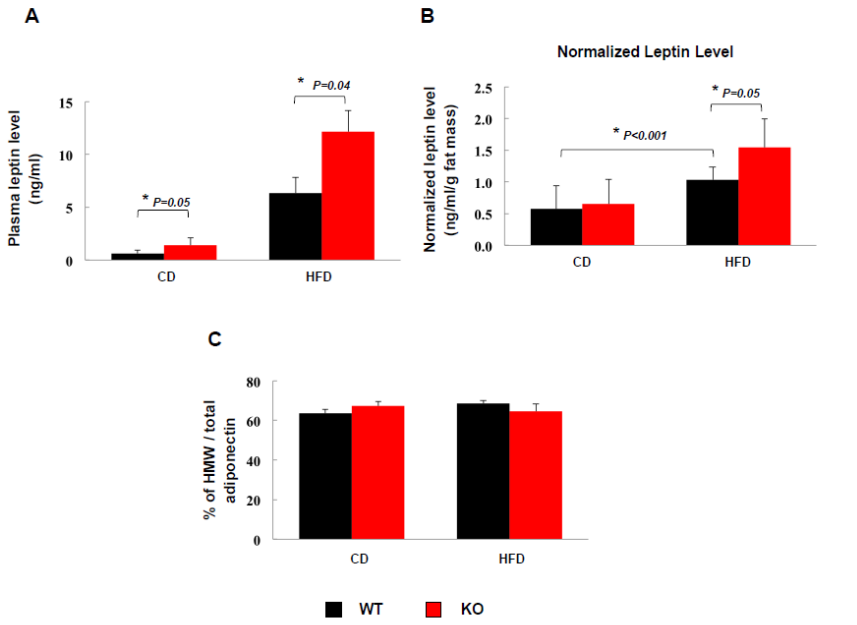

Figure 3. TRPV1 deficient mice have higher levels of leptin. (*A*) Plasma leptin levels were measured by ELISA assay according to the manufacturer's instructions in WT (n=12) and TRPV1 KO (n=10) mice before and after HFD feeding for 5 weeks. (*B*) Plasma leptin levels were normalized to whole body fat mass measured using ^{1}H-MRS. (*C*) Plasma high molecular weight (HMW) proteins and total adiponectin were measured using an ELISA kit. Average % of HMW adiponectin per total adiponectin is represented. *p<0.05

of leptin levels normalized to fat mass, whereas TRPV1 KO mice showed negative correlations (Pearson R = -0.929, *p* value = 0.022 for physical activity and Pearson R = -0.984, *p* value = 0.015 for energy expenditure) with statistical significance. All of these data indicate that there was impaired leptin sensitivity in TRPV1 KO mice fed HFD even with higher fat mass and plasma leptin levels, suggesting the existence of leptin resistance.

2.3.5. TRPV1 channel impairment promotes leptin resistance in mice fed HFD

To examine the effect of TRPV1 deletion on leptin resistance in mice, I performed leptin infusion study using an osmotic pump. To match the average plasma leptin levels of WT and TRPV1 KO mice fed HFD for 5 weeks (~10 ng/ml), leptin was infused at a concentration of 20 μg/day, for both groups of mice fed chow diet. After 6 days, the average plasma leptin levels in WT and TRPV1 KO mice were not significantly different (Figure 5A-b). Under leptin infusion conditions, food intake for WT mice was remarkably reduced by leptin stimulation at Day 1, this tendency remained until Day 6 (Figure 5A-a). However, in TRPV1 KO mice, reductions in food intake was less than for WT mice at Day 1 and disappeared shortly at Day 6 thereafter.

Figure 4

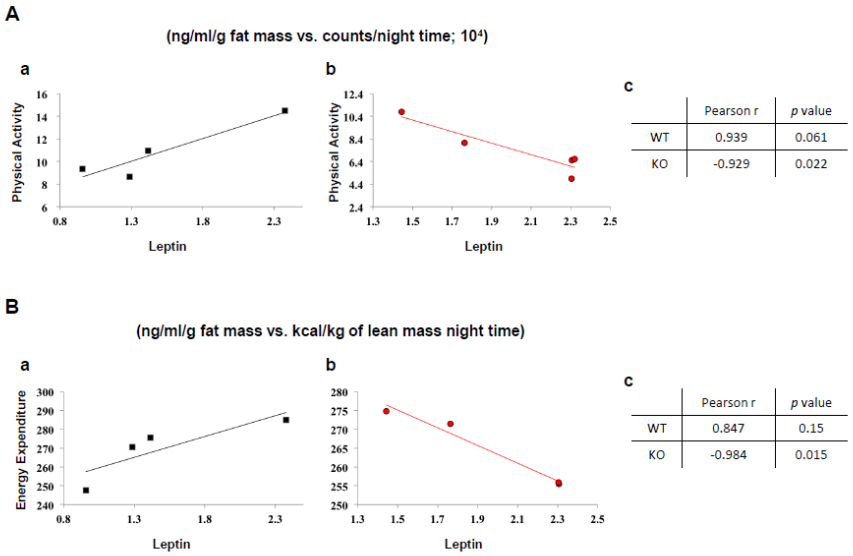

Figure 4. Correlation analysis between normalized leptin levels and physical activity or energy expenditure in HFD-fed TRPV1 KO. Plasma leptin levels were normalized to whole body fat mass measure using ^{1}H-MRS in each mouse. Energy expenditure is expressed per kg of whole body lean mass measured using ^{1}H-MRS. Correlation between physical activity (*A*) or energy expenditure (*B*) and normalized leptin levels were based on average values collected over 72 h. Each dot represents normalized plasma leptin vs. physical activity (*A*) or energy expenditure (*B*) in WT (n

= 4, *a*) and TRPV1 KO (n = 4-5, *b*) mice fed HFD. Pearson R value and p value for correlation coefficients of physical activity (*A*-*c*) or energy expenditure (*B*-*c*) vs. normalized leptin levels are shown.

To induce leptin resistance in both WT and TRPV1 KO mice, mice were fed HFD for 9 weeks. A leptin infusion study was then performed again in WT and TRPV1 KO mice (Figure 5B-a). I observed that after 9 weeks of HFD feeding, TRPV1 and WT mice exhibited leptin resistance at Day 6 with no changes in food intake. At Day 1, however, TRPV1 did not respond to leptin infusion, whereas WT mice on less food intake still responded to high doses of leptin. All of these results indicate that TRPV1 KO mice exhibited severe leptin resistance compared to WT mice. Consistent with these results from the leptin infusion study with TRPV1 KO mice, capsaicin oral administration for 12 weeks with HFD decreased food intake (Figure 6A), whereas plasma leptin levels were not altered in TRPV1 KO mice compared to WT mice (Figure 6B). Thus, these results suggest that the deletion of TRPV1 induces leptin resistance, and the activation of TRPV1 by capsaicin improves leptin sensitivity.

2.3.6. Deletion of TRPV1 attenuates leptin signaling in MEFs and mice

Leptin signaling was examined using Western blot in TRPV1 KO MEFs and WT MEFs. Leptin-stimulated phosphorylation of STAT3 at Tyr705 was elevated by ~3-fold in WT MEFs, but total STAT3 expression remained unchanged after leptin treatment (Figure 7A). However, basal levels of p-STAT3 in TRPV1 KO MEFs was considerably elevated in TRPV1 KO

Figure 5

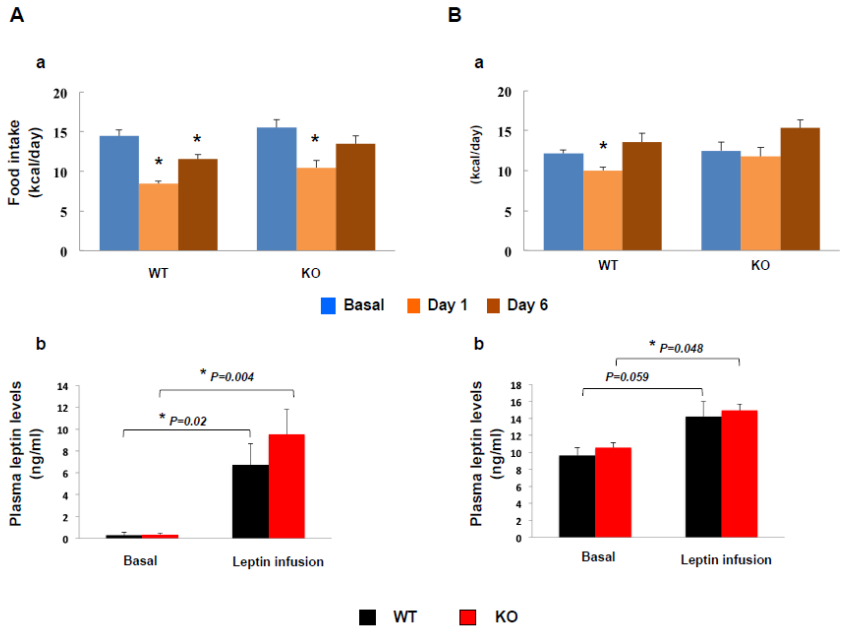

Figure 5. TRPV1-deficient mice are more leptin resistant compared to WT mice. A leptin infusion study was performed to test leptin resistance in WT (n=4) and TRPV1 KO mice (n=5) fed on chow diet and HFD for 9 weeks. After recovering from surgery for insertion of osmotic pumps, mice were acclimatized to metabolic cages for 24 h. Food intake was measured using the metabolic cage; basal levels in each mouse were obtained by measuring average food intake for 3 days. Food intake (*a*) and plasma leptin levels (*b*) of WT and TRPV1 KO mice fed chow diet (*A*) and HFD (*B*) during leptin infusion experiments. *p<0.05 vs. basal level of each mouse.

Figure 6

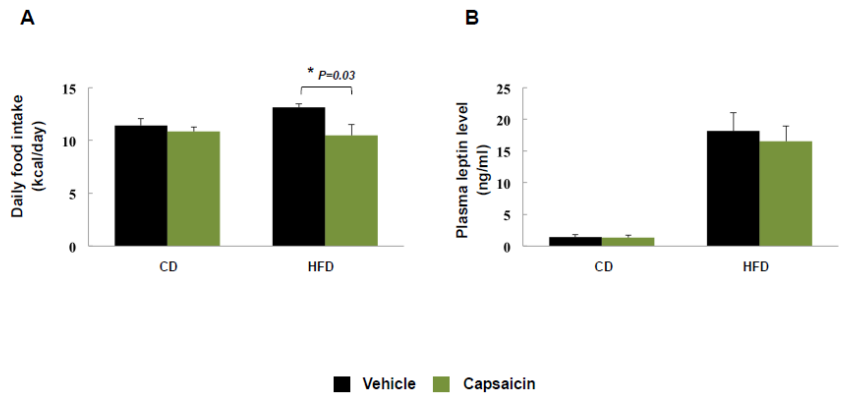

Figure 6. The inhibitory effect of capsaicin on energy intake during HFD feeding.

(*A*) Daily food intake was measured using metabolic cages for vehicle- and capsaicin-treated mice at the beginning and end of each experiment. (*B*) Plasma leptin levels in mice orally injected with vehicle and capsaicin were detected by ELISA. *$p<0.05$

MEFs in the absence of leptin stimulation compared to WT MEFS. In addition, TRPV1 KO MEFs did not respond to leptin stimulation, suggesting the existence of impaired leptin signaling in TRPV1 KO MEFs. To confirm the inhibitory effect of TRPV1 deletion on leptin-induced phosphorylation of STAT3, we injected leptin (2mg/kg body weight) intraperitoneally into WT and TRPV1 KO mice. After 2 h, phosphorylation of STAT3 at Tyr705 in the hypothalamus was increased in both WT and TRPV1 KO mice, but the increased levels of p-STAT3 in TRPV1 KO mice were reduced compared to levels in WT mice (Figure 7B). All of these results indicate that leptin signaling is defective in TRPV1-deficient mice.

Figure 7

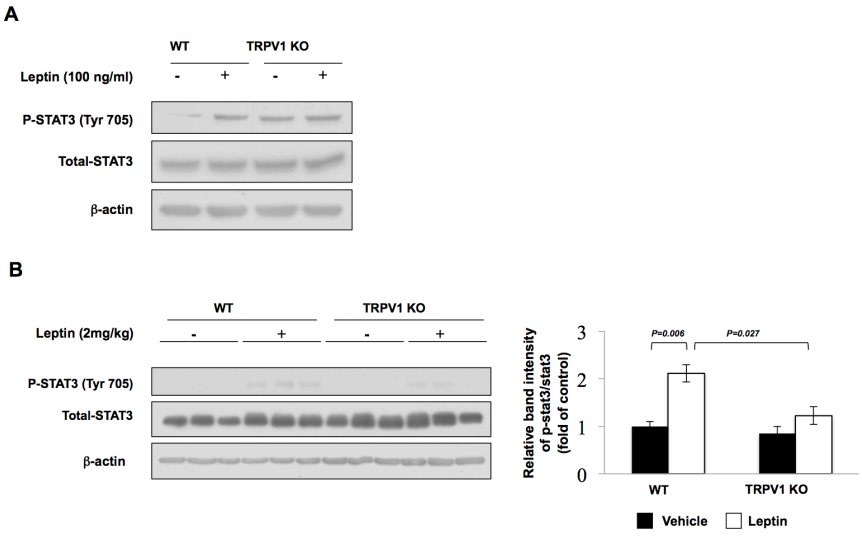

Figure 7. Phoshorylation of STAT3 at Tyr705 stimulated by leptin is reduced in TRPV1-deficient MEFs and mice. (*A*) Primary cultured MEFs from WT and TRPV1 KO embryos were seeded in 100 mm dishes and serum-deprived for ~18 h. After 10 mins treatment with leptin (100 ng/ml) or vehicle control, cells were harvested. Levels of protein phosphorylation and expression in lysates from WT and TRPV1 KO MEFs were determined by Western blot using specific antibodies against p-STAT3 (Tyr705), STAT3, and β-actin. (*B*) Phosphorylation of STAT3 at Tyr705 was examined in the hypothalamus of WT and TRPV1 KO mice intraperitoneally

injected with leptin.

2.4. Discussion

Previous evidence implicates dietary calcium consumption in the modulation of adipocyte metabolism and obesity (30-33), and recent studies have shown that calcium channel blockers attenuate obesity and related hypertension, suggesting that calcium channels are directly involved in energy metabolism and the development of metabolic syndromes (34, 35). Recently, TRP channels, as mediators of cation influx (including Ca^{2+}) induced by phycial and chemical stimuli, have been examined for their roles in metabolic diseases such as obesity, diabetes, hypertension, cardiovascular disease, and kidney disease [reviewed in (36, 37)]. In obesity studies, expression of TRPC4, TRPM8, TRPP2, TRPML, and TRPV6 were reported to correlate with obesity in genome scans (38). TRPM5 is a major component of taste sensory signaling and is highly expressed in the gastric mucosa of obese subjects, where it regulates energy consumption (39, 40). TRPV4 has been recently reported to control energy homeostasis and promote obesity (41, 42). However, the role of TRPV1 in obesity is controversial and considerable numbers of previous studies have reported anti-obesity effects due to TRPV1 activation (20, 21), while TRPV1-null mice are resistant to diet-induced obesity (27). In the present study, I demonstrated that TRPV1 deletion promotes HFD-induced obesity and leptin resistance by down-regulating leptin signaling. Even higher levels of plasma leptin were present in TRPV1-

deficient mice than WT mice, and leptin sensitivity of TRPV1 KO mice were remarkably attenuated through decreased locomotor activity.

Since leptin resistance is a primary cause for the failure of leptin therapy in recent obese patient studies, new strategies to overcome this condition are needed (14, 15). However, a detailed mechanistic understanding of the development and progression of leptin resistance is urgently needed. TRPV1-null mice have higher levels of plasma leptin than WT mice with impaired leptin signaling, which subsequently promotes leptin resistance, suggesting a new role for TRPV1 and its agonists as components of a novel strategy to address leptin resistance. Nevertheless, further studies into how TRPV1 regulates leptin signaling pathways are needed to elucidate the pathways responsible.

In the present study, I have shown that TRPV1 regulates energy balance, and its deletion promotes diet-induced obesity in mice. This detrimental effect of TRPV1 deficiency on obesity results from loss of sensitivity to leptin-controlled energy homeostasis. In this regard, the activation of TRPV1 by its agonists, such as capsaicin, may provide a solution for the maintenance of metabolic state and represent a novel therapeutic target for the treatment of metabolic syndromes.

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Chapter 3.

TRPV1 deficiency promotes diet-induced obesity and insulin resistance in mice

Abstract

Obesity-induced insulin resistance is a representative characteristic of type 2 diabetes (T2D). Impaired insulin signaling in insulin resistant states can destroy glucose homeostasis, causing various metabolic diseases such as hypertension, atherosclerosis and coronary disease. Although there have been reports that the activation of TRPV1 by capsaicin improves insulin sensitivity, the physiological role of TRPV1 is still unclear. In the current study, we used a hyperinsulinemic-euglycemic clamp assay to examine insulin sensitivity in live mice and found that TRPV1 deletion increased high-fat diet (HFD)-induced fat accumulation and whole body insulin resistance. The whole body insulin resistance observed in TRPV1-deficient mice fed with HFD was caused by impaired insulin sensitivity in insulin-responsive tissues including white adipose tissue, brown adipose tissue, and the heart. This study identifies a novel role for TRPV1 in the regulation of insulin resistance.

3.1. Introduction

Obesity, a hallmark of metabolic syndrome, is a major risk factor for the development of diabetes, hypertension and dyslipidemia. Obesity-associated insulin resistance strongly induces type 2 diabetes (T2D), which is a representative characteristic of T2D (1). Insulin resistance is commonly induced by mitochondrial dysfunction, endoplasmic recticulum (ER) stress, oxidative stress, and obesity-related inflammation (2-5). Insulin resistance is a condition in which cells fail to respond to insulin to reduce blood glucose levels, due to impaired insulin receptor (IR) activation. Inadequate responses to insulin signaling leads to an overproduction of insulin from the beta cells of the pancreas, which eventually damages them (6). To prevent T2D, controlling insulin resistance is a key strategy.

Insulin acts on multiple tissues including the pancreas, liver, muscle, adipose tissues and heart, to lower whole-body plasma glucose levels by stimulating glucose uptake. When blood glucose levels are high, beta cells in the pancreas generate high levels of insulin. Secreted insulin circulates through the body in the bloodstream to reduce glucose levels. Insulin stimulation in the liver ceases hepatic glucose

production to inhibit catabolism. In other insulin-responsive tissues such as the muscle, adipose tissues and heart, glucose is translocated and converted to glycogen or pyruvate and fat is stored as triglyceride (TG). Thus, sensitive responses to insulin are a crucial mechanism for maintaining energy homeostasis.

The development of insulin resistance is a complicated process with various signaling pathways involved. In normal conditions, insulin induces phosphorylation of the insulin receptor (IR), a tyrosine kinase, and phosphorylated IR in turn activates downstream effector molecules including insulin receptor substrate 1 and Akt, which promote translocation of GLUT4-containing storage vesicles to the plasma membrane to allow the entry of glucose into the cell. Translocated glucose into muscle cells is converted to glycogen or pyruvate through glycolysis. Insulin stimulates the liver to synthesize glycogen or TG. In adipose tissue, insulin increases TG levels and decreases lipolysis to reduce the production of fatty acids (FA). In insulin-resistant conditions, insulin-mediated glucose uptake in muscle, adipose tissue, and other tissues including the brain and heart is impaired. As a result, excess glucose is diverted to the liver, causing accumulation of liver lipids and impairment in controlling gluconeogenesis and glycogen synthesis. In

addition, insulin resistance in adipose tissue promotes re-esterification of lipids (FA) and further exacerbates insulin resistance by increasing lipolysis (7).

Transient receptor potential vanilloid subfamily type-1 (TRPV1) has been reported to be associated with obesity and insulin resistance. Previous studies have shown that chilli pepper or capsaicin-containing foods improve glucose homeostasis and insulin sensitivity (8, 9). However, the physiological role of TRPV1 in the development of insulin resistance remains poorly understood.

In the previous chapter, we demonstrated that TRPV1 deficient mice became more obese and accumulated more fat compared to wild-type (WT) mice in a high-fat diet (HFD) model. In addition, deletion of TRPV1 induced severe leptin resistance, which is consistent a result with prior capsaicin gavage data showing lowered food intake. Here, we examined the effect of TRPV1 deletion on HFD-induced insulin resistance to further investigate the function of TRPV1 in obesity and associated metabolic disorders. We found that loss of TRPV1 aggravated HFD-induced insulin resistance, which resulted from defects in insulin action in white adipose tissue (WAT), brown adipose tissue (BAT) and heart, but not in skeletal muscle and liver.

3.2. Materials and Methods

3.2.1. Animals and reagents

TRPV1-deficient (TRPV1 KO) mice (n = 10) and WT littermates (n = 12) were purchased from Jackson Laboratory. For the diet-induced obesity study, 11- to 14-week-old mice were fed a HFD (55% fat by calories; Harlan Teklad TD93075) *ad libitum* for 5 weeks. The animal studies were approved by the Institutional Animal Care and Use Committee of the University of Massachusetts Medical School.

3.2.2. Body composition

Mice were housed under controlled temperature and lighting, with free access to food and water. Whole body fat and lean mass were noninvasively measured using ^{1}H-MRS (Echo Medical Systems, Houston, TX).

3.2.3. Hyperinsulinemic-euglycemic clamp

Following chow or HFD, a survival surgery was performed at 4 –5 days before clamp experiments to establish an indwelling catheter in the jugular vein. On the day of the experiment, mice were fasted

overnight (~15 h), and a 2-h hyperinsulinemic euglycemic clamp was applied in conscious mice with a primed and continuous infusion of human insulin (150 mU/kg body wt priming followed by 2.5 $mU \cdot kg^{-1} \cdot min^{-1}$, Humulin; Eli Lilly). To maintain euglycemia, 20% glucose was infused at variable rates during clamps. Whole body glucose turnover was assessed with a continuous infusion of [3-^{3}H]glucose, and 2-deoxy-D-[1-^{14}C]glucose (PerkinElmer) was administered as a bolus (10 μCi) at 75 min after the start of the clamping to measure insulin-stimulated glucose uptake in individual organs. At the end of the clamping, mice were anesthetized, and tissues were extracted for biochemical analysis (10).

3.2.4. Biochemical analysis and calculation

The glucose concentration during the clamping was analyzed using 10 μl of plasma with a glucose oxidase method and a Beckman Glucose Analyzer 2 (Beckman, Fullerton, CA). Plasma concentrations of [3-^{3}H]glucose, 2-deoxy-D-[1-^{14}C]glucose, and $^{3}H_2O$ were determined after deproteinization of plasma samples as previously described (11). For the determination of tissue 2-deoxy-D-[1-^{14}C]glucose-6-phosphate content, tissue samples were homogenized,

and the supernatants were subjected to an ion-exchange column to separate 2-deoxy-D-[1-^{14}C]glucose-6-phosphate from 2-deoxy-D-[1-^{14}C]glucose.

Rates of basal and insulin-stimulated whole-body glucose turnover were determined as the ratio of the [^{3}H]glucose infusion rate (disintegrations per minute [dpm]) to the specific activity of plasma glucose (dpm/μmol) at the end of the basal period and during the final 30 min of the clamping, respectively. Hepatic glucose production (HGP) during the clamping was determined by subtracting the steady-state glucose infusion rate from the whole-body glucose turnover rate. Whole-body glycolysis and glycogen/lipid synthesis were calculated as previously described (11).

3.2.5. Plasma insulin measurement

The plasma levels of insulin were measured using ELISA kits (ALPCO Diagnostics, Salem, New Hampshire, USA). Plasma samples used for insulin ELISA were obtained from mice at the beginning and end of the hyperinsulinemic-euglycemic clamp. Using heparin-treated capillary tubes, blood was collected carefully into the heparin-treated eppen tube, and centrifuged at 10,000 rpm for 1 min. For the ELISA

assay, the supernatant of centrifuged blood was used following the manufacturer's instruction.

3.2.6. Glucose uptake assay

At the end of hyperinsulinemic-euglycemic clamping, gastrocnemius muscle, WAT, BAT, and heart were extracted from anesthetized mice. Glucose uptake in individual tissues was calculated from the plasma 2-deoxy-D-[1-^{14}C]glucose profile, which was fitted with a double exponential or linear curve using MLAB (Civilized Software, Bethesda, MD) and tissue 2-deoxy-D-[1-^{14}C]glucose-6-phosphate content.

2.2.7. Western blotting

WAT and heart tissue lysates were prepared from anesthetized mice at the end of the experiment and subjected to Western Blot. Tissues were extracted with T-PER tissue protein extraction reagent (Pierce, Rockford, IL) containing 1% phosphatase inhibitor cocktail 2 (Sigma Chemical, St. Louis, MO) and the protease inhibitor phenylmethylsulfonyl fluoride (Calbiochem, La Jolla, CA). The samples were then homogenized and centrifuged at 14,000 rpm for 15

min at 4°C. Tissue lysates (50 μg) were subjected to 10% SDS-PAGE and transferred to a PVDF membrane (Biorad Laboratories Inc.). After blotting, the membrane was incubated with p-Akt (Ser473), Akt, p-STAT3 (Tyr705), STAT3, or GAPDH primary antibodies at 4ºC overnight. Protein bands were visualized by a chemiluminescence detection kit after hybridization with a horseradish peroxidase-conjugated secondary antibody.

3.2.8. Statistical analysis

Data are expressed as mean ± S.E. values, and ANOVA was used for multiple statistical comparisons. A probability value of $p<0.05$ was used as the criterion for statistical significance. All analyses were performed using Statistical Analysis Software (SAS, Inc., Cary, NC).

3.3. Results

3.3.1. HFD-induced obesity and insulin resistance are increased in TRPV1 knockout mice

To determine the effect of TRPV1 on HFD-induced obesity, TRPV1 knockout (KO) mice and wild-type (WT) littermates were fed a HFD (55% fat by calories) *ad libitum* for 5 weeks and were measured for body composition at the beginning and end of the experiment. Whole body fat mass was significantly increased in TRPV1 KO mice compared to WT littermates after 5 weeks of HFD feeding (Figure 1). To further investigate the effect of TRPV1 deletion on HFD-induced insulin resistance, we performed a hyperinsulinemic-euglycemic clamp using WT and TRPV1 KO mice. It clearly exhibited defects in insulin sensitivity after 5 weeks of HFD feeding compared to chow diet feeding in WT mice (Figure 2A). Moreover, the glucose infusion rate of TRPV1 KO mice on HFD was significantly lower than that of WT mice on HFD (p = 0.038 vs. WT mice fed HFD). The whole body glucose turnover rate was reduced in TRPV1 KO mice after 5 weeks of HFD compared to HFD-fed WT mice (Figure 2B). The whole body

Figure 1

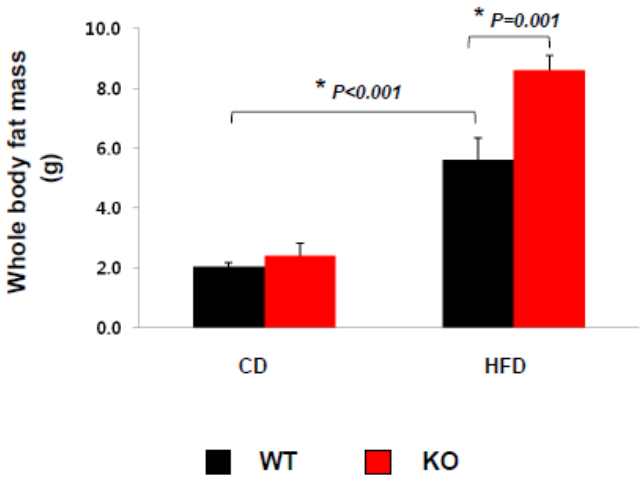

Figure 1. TRPV-deficient mice become more obese than WT mice fed a HFD. Whole body fat mass was measured using ^{1}H-MRS at the beginning and end of the experiment. WT, wild type C57BL/6J mice. KO, TRPV1 KO mice. *p<0.05

glycolysis of HFD-fed TRPV1 KO mice was slightly reduced compared to HFD-fed WT mice, but there was no statistical significance (p = 0.069, Figure 2C). There was no difference in plasma insulin level between WT and TRPV1 KO group (Figure 2D), and this result demonstrates that insulin resistance in TRPV1 KO mice is not caused by differences in the amount of whole body insulin. Collectively, these results indicate that TRPV1 deletion reduces whole body insulin sensitivity compared to WT mice after HFD feeding for 5 weeks.

3.3.2. Deletion of TRPV1 does not affect insulin action in liver and skeletal muscle

Since HFD-fed TRPV1 KO mice showed increased whole body insulin resistance compared to HFD-fed WT mice, we examined the effect of TRPV1 deletion on insulin action in insulin-responsive tissues. The liver is a representative organ that responds to insulin to reduce glucose production. There was no difference in basal levels of hepatic glucose production (HGP) between WT and TRPV1 KO mice in either chow diet or HFD groups (Figure 3A). After high doses of insulin stimulation during clamping, HGP was significantly reduced by HFD feeding in

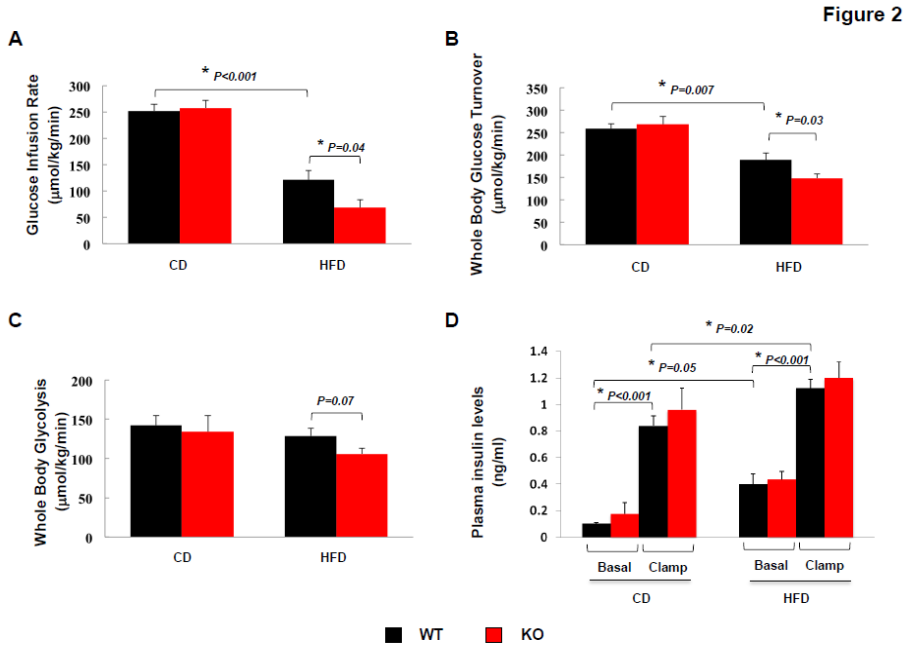

Figure 2. Deletion of TRPV1 promotes HFD-induced whole body insulin resistance in mice. (*A*) Steady-state glucose infusion rate during hyperinsulinemic-euglycemic clamping in conscious mice. (*B*) Insulin-stimulated whole-body glucose turnover was calculated using [^{3}H]glucose infusion during clamping. (C) Insulin-stimulated whole body glycolysis was estimated during clamping. (*D*) Plasma insulin levels were measured by ELISA. Plasma samples were taken from mice at the beginning and end of clamping. *p<0.05

both WT and TRPV1 KO mice (Figure 3B). Hepatic insulin action calculated using clamp/basal HGP was markedly reduced by HFD in WT mice (Figure 3C). However, the difference of insulin action in the liver between HFD-fed WT and HFD-fed TRPV1 KO mice was not detectable, suggesting that deletion of TRPV1 does not affect hepatic insulin action.

Skeletal muscle, which is another insuling-responsive tissue, was used to measure insulin action (Figure 3D). Muscle glucose uptake rates were not changed by TRPV1 deletion. These results suggest that TRPV1 is not involved in insulin action in the liver or the skeletal muscle.

3.3.3. Deletion of TRPV1 reduces glucose uptake into adipose tissues and heart

To further investigate which organ is involved in whole-body insulin resistance in TRPV1-deficient mice, we performed a glucose uptake assay using WAT, BAT, and heart tissue. In adipose tissue, insulin promotes storage of fats from glucose. By insulin stimulation during

Figure 3

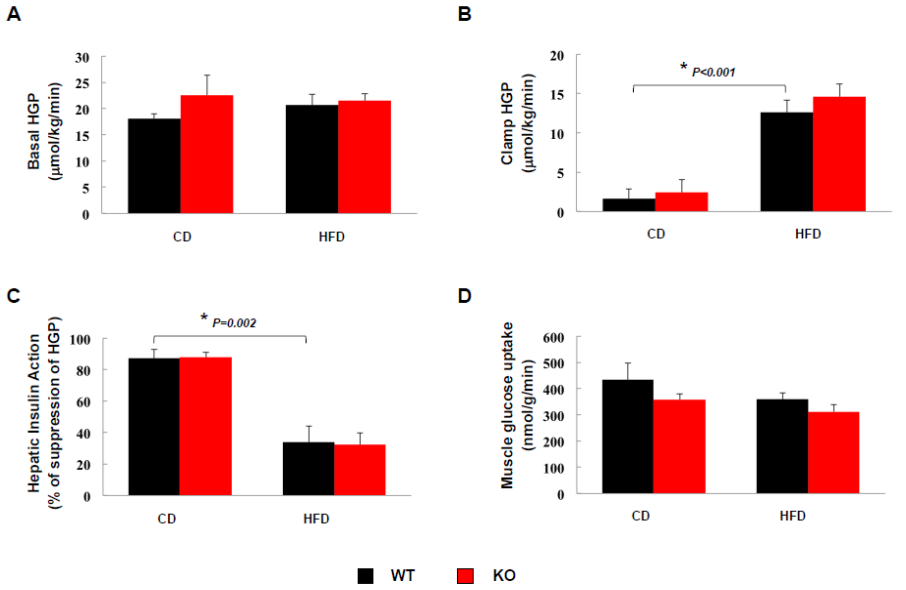

Figure 3. Deletion of TRPV1 does not affect HFD-induced insulin resistance in liver and skeletal muscle. (*A*, *B*, and *C*) Hepatic glucose production was estimated using [^{3}H]glucose during clamping. Using basal HGP (*A*) and clamp HGP (*B*), hepatic insulin action (*C*) was calculated. (D) Insulin-stimulated glucose uptake in skeletal muscle. *p<0.05

hyperinsulinemic-euglycemic clamping, glucose uptake was similarly accelerated in both WT and TRPV1 fed on chow diet (Figure 4A). After HFD feeding for 5 weeks, glucose uptake levels in adipose tissues including white and BAT tissues were significantly reduced in WT mice compared to chow diet-fed WT mice (Figure 4A and B). HFD-fed TRPV1 KO mice exhibited a stronger reduction in glucose uptake compared to that of HFD-fed WT mice in WAT (41% reduction) and BAT tissue (35% reduction). In addition, glucose uptake in the heart, a major organ participating in the uptake and use of glucose from fat sources, was decreased by 29% in HFD-fed TRPV1 KO mice compared to HFD-fed WT mice. Therefore, we have concluded that TRPV1 deletion induces impaired insulin sensitivity in adipose tissues and the heart, leading to whole-body insulin resistance during HFD feeding in mice.

3.3.4. TRPV1 deficiency induces impaired insulin signaling in WAT after HFD feeding in mice

Since, HFD-fed TRPV1 KO mice exhibited insulin resistance in adipose tissues and the heart, we further analyzed the effect of TRPV1

Figure 4

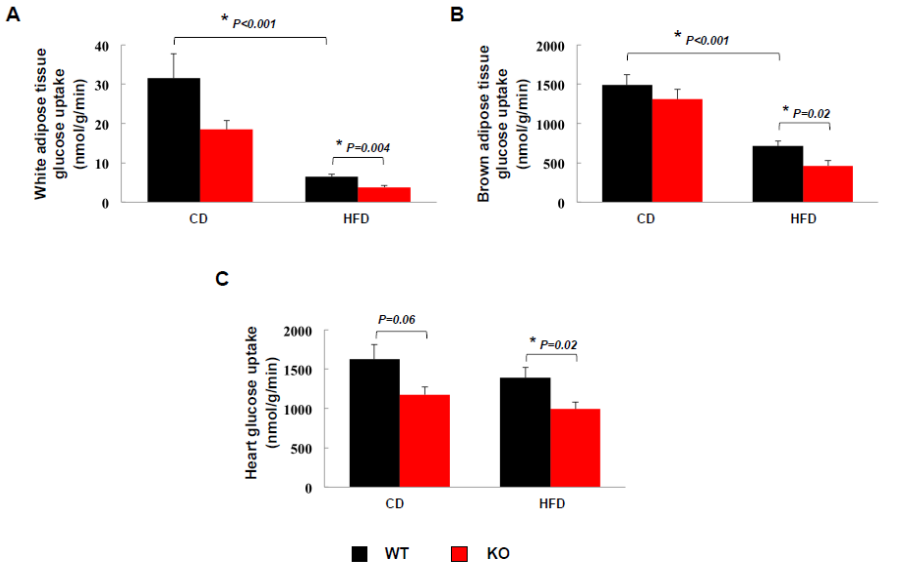

Figure 4. TRPV1-deficient mice become insulin resistant in adipose tissues and heart during HFD feeding. Insulin-stimulated glucose uptake in WAT (*A*), BAT (*B*), and heart (*C*) were measured using tissue 2-deoxy-D-[1-^{14}C]glucose-6-phosphate content during clamping. *p<0.05

deletion on HFD-induced insulin signaling pathways in WAT (Figure 5A).

Although the WAT of TRPV1 KO mice was more insulin resistant than that of WT mice, phosphorylation of Akt at Ser 473 was not altered in TRPV1 KO mice fed HFD. However, STAT3 phosphorylation levels stimulated by insulin during hyperinsulinemic-euglycemic clamping were significantly reduced in WAT taken from HFD-fed TRVP1 KO mice (Figures 5A and B). In the heart, phosphorylation of Akt was significantly reduced in both WT and TRPV1 KO mice after 5 weeks of HFD (Figure 6). However, we did not find any differences in phosphorylation levels of Akt and STAT3 between WT and TRPV1 KO mice. Thus, deletion of TRPV1 suppresses STAT3 dependent insulin signaling in mice.

Figure 5

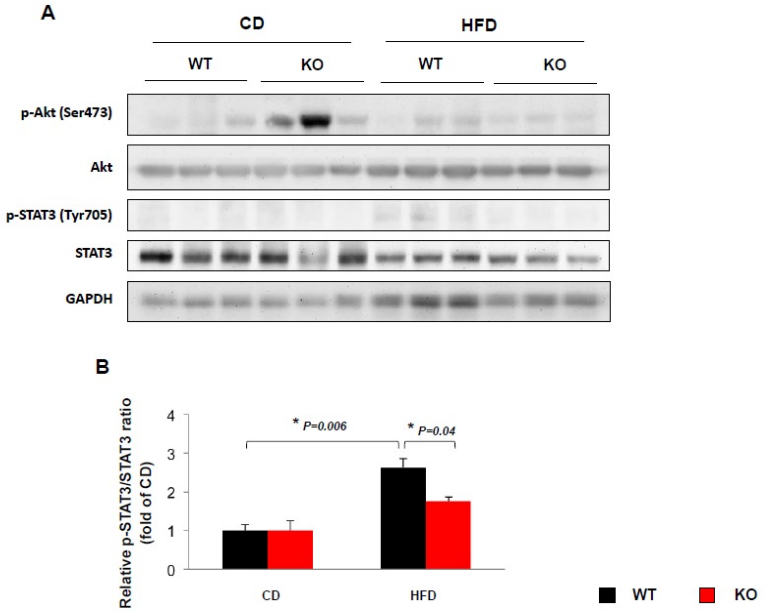

Figure 5. The effect of TRPV1 deletion on insulin-stimulated phosphorylation of Akt and STAT3 in WAT. (*A*) Insulin-stimulated phosphorylation of Akt and STAT3 levels were evaluated by Western blot. WAT were taken from WT and TRPV1 KO mice at the end of hyperinsulinemic-euglycemic clamping. (*B*) Quantitative analysis of p-STAT3 and STAT3 was performed using Image J software. **p*<0.05

Figure 6

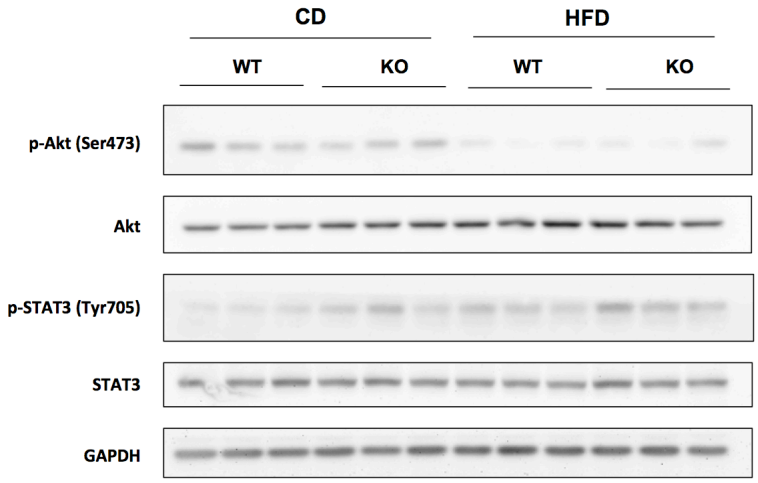

Figure 6. The effect of TRPV1 deficiency on phosphorylation of Akt and STAT3 induced by insulin in heart tissue. Heart tissues taken from WT and TRPV1 KO mice at the end of clamps were immunoblotted for detection of Akt and STAT3 phosphorylation levels.

3.4. Discussion

Insulin resistance is a representative characteristic of T2D and plays a major role in beta cell defects and the development of various metabolic syndromes. Insulin resistance is commonly identified in obese people, suggests its close association with obesity and T2D. Previous studies have reported the attenuation of IR-IRS-Akt and STAT3 signaling pathways as a mechanism for the development of insulin resistance. Although several strategies were suggested to overcome insulin resistance, the successful development of new agents to prevent and treat insulin resistance in obese or T2D patients is needed.

The majority of previous reports regarding TRPV1 and diabetes have focused on its effect on insulin secretion. TRPV1 channels in islet beta cells are involved in insulin secretion (12) and TRPV1+ fibers are associated with insulin release and energy expenditure (13-15). TRPV1 also innervates various tissues including pancreas and adipose tissues and regulates insulin response (13, 14, 16, 17). However, the role of TRPV1 *in vivo* is still controversial. A previous report has shown that sensory denervation of capsaicin-sensitive nerves by capsaicin, which

desensitizes TRPV1, increased glucose tolerance response to insulin (14), whereas another study suggested that glucose-stimulated insulin secretion is reduced in small rodents treated with capsaicin (17). TRPV1-null mice were protected against abnormal glucose and insulin resistance induced by HFD (15). In contrast, activation of TRPV1 by capsaicin enhanced glucose tolerance in mice fed HFD (18). Thus, it has been suggested in many studies that TRPV1 plays a critical role in obesity and diabetes however, whether TRPV1 functions as a repressor or promoter in the development of obesity and diabetes remains unclear.

In the present study, we have demonstrated that TRPV1 deletion exacerbates HFD-induced insulin resistance in WAT, BAT, and the heart. Indeed, insulin-stimulated phosphorylation of STAT3 in WAT was impaired in TRPV1 KO mice under HFD feeding for 5 weeks. However, we could not detect any difference in the phosphorylation levels of Akt and STAT3 in the heart between HFD-fed WT and TRPV1 KO mice, suggesting that further studies to elucidate the mechanisms of the pro-insulin resistance effects mediated by TRPV1 deletion is required. Taken together, our results suggest that TRPV1 may be a potential therapeutic target for obesity-related insulin resistance and T2D.

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Chapter 4.

Deficiency of TRPV1 accelerates aging-induced obesity and insulin resistance in mice

Abstract

Obesity as a major cause of insulin resistance promotes aging, which accerlerates obesity through a feedback response in various tissues including adipose tissues. Aging-induced obesity and insulin resistance have been clearly shown in animals and human. Although possible mechanism which involves in obesity-associated aging have been suggested by previous studies, it is still unclear. Transient receptor potential vanilloid subfamily type-1 (TRPV1) expression is remarkably decreased, which induces impaired pain sensing in aged mice and elderly human. With evidences indicating that TRPV1 involves in obesity and insulin resistance, I determined the effect of TRPV1 deletion on aging-induced obesity and insulin resistance *in vivo*. Deficiency of TRPV1 accelerates aging-induced weight and fat mass gain by decreasing energy expenditure. In hyperinsulinemic-euglycemic clamps, I found that TRPV1 deletion aggrevates aging-induced insulin resistance, especially regulating hepatic insulin action. These results suggest that TRPV1 may be a promising therapeutic target for aing-induced metabolic disorders.

Keyword : TRPV1, aging, obesity, insulin resistance, hepatic insulin action

4.1. Introduction

As changed lifestyle consuming more energy with less activity in these days, there has been a rise in aging-associated disorders such as cardiovascular disease, cancer, degenerative diseases, and metabolic diseases including obesity and diabetes(1). Obesity, a major cause of insulin resistance, and diabetes have been known to accerlarate aging, but the mechanisms connecting obesity, diabetes, and aging is not well studied. According to several reports, inflammation resulting from oxidative stress, DNA damage, and telomere dysfunction are associated in development metabolic diseases including obesity, diabetes, and atherosclerosis(2-4). Recently, the possible mechanisms supporting that aging accerlarates obesity and diabetes were supported by previous studies, suggesting involvement of mitochondria(5). Oxidative stress in mitochondria induces mitochondrial dysfunction, which promotes obesity and insulin resistance(6). In adipose tissue, especially, mitochondrial dysfunction which is a feature of normal aging triggers to high levels of oxidative stress and damaged cellular function involving in glucose metabolism and insulin sensitivity, promoting aging-associated diseases such as obesity and insulin resistance(5).

Besides, short telomere length strongly related to metabolic diseases such as cardiovascular disease and obesity has been known to lead abnormal fasting glucose/insulin, lipid and lipoprotein concentration, habitual physical activity, and other metabolic risk factors(7, 8). As a key regulator of aging, tumor suppressor protein 53 (p53) which prevent genome mutation during DNA damage induced by aging prevents obesity and insulin resistance by inhibiting pro-inflammatory response in the aging process of adipose tissue(9). However, some of them are found not to have no effect on aging-associated obesity in subjects according to sex or environmental factors(8). Thus, the specific mechanisms involving to aging-induced obesity and its-associated disorders are fully understood, further studies are still needed.

Transient receptor potential vanilloid subfamily type-1 (TRPV1) has been reported to show strong association with aging. TRPV1 expression is decreased in sensory neuron of aged mice leading to loss of thermal sensitivity(10). Decreased pain sensitivity during aging in human and animal models is associated in reduction of TRPV1 expression in peripheral nervous system (PNS) of aged mice(11). Therefore, these are supported that less or loss of TRPV1 activity

during aging may promotes aging-associated obesity and its-associated metabolic diseases. Previous report has been showed higher body weight in TRPV1-null mice with age (observed for up to 14 months), whereas the other study suggested protective effect of TRPV1 deficiency in mice against weigth gain by 44 weeks of age(12, 13). These controversial results are strongly suggesting necessity of further studies to elucidate the role of TRPV1 on obesity and its-related disorders during aging.

In present study, I found that TRPV1-deficient mice became spontaneously obese during aging by 9 months of age. This was caused by altered energy balance resulting from reduced VO_2 consumption and energy expenditure in TRPV1-null mice. Aging-induced insulin resistance was shown to be severe in TRPV1-null, and insulin-stimulated shutting down of hepatic glucose production (HGP) was completely impaired in TRPV1-deficient mice compared to WT mice. These results suggest that targeting TRPV1 could be a new strategy for the treatment obesity and insulin resistance induced by aging.

4.2. Materials and methods

4.2.1. Animals

TRPV1-deficient (TRPV1 KO) mice (n=4) and WT littermates (n=4) were purchased from Jackson Laboratory. 3-month-old mice were fed a chow deit *ad libitum* for 6 months. The animal studies were approved by the Institutional Animal Care and Use Committee of the University Massachusetts Medical School.

4.2.2. Body composition

Mice were housed under controlled temperature and lighting, with free access to food and water. Whole body fat and lean mass were noninvasively measured using ^{1}H-MRS (Echo Medical Systems, Houston, TX).

4.2.3. Energy balance measurement

The food/water intake, energy expenditure, respiratory exchange ratio, and physical activity were assessed for 3 days using metabolic cages (TSE Systems, Chesterfield, MO). We used the TSE Systems LabMaster platform with easy-to-use calorimetry featuring

fully automated mon- itoring for food and water and XYZ activity. LabMaster cages that are most similar to facility home cages were used, thereby allowing the use of bedding in the cage and minimizing any animal anxiety during the experimental period. The system provides intuitive software with flexibility for experimental setup and data utilization. Rectal body temperature was measured in aged WT and TRPV1 KO mice.

4.2.4. Hyperinsulinemic-euglycemic clamp

At the age of 9 month in WT and TRPV1 KO mice, a survival surgery was performed at 4–5 days before clamp experiments to establish an indwelling catheter in jugular vein. On the day of experiment, mice were fasted overnight (~15 hrs), and a 2-hrs hyperinsulinemic euglycemic clamp was conducted in conscious mice with a primed and continuous infusion of human insulin (150 mU/kg body wt priming followed by 2.5 $mU \cdot kg^{-1} \cdot min^{-1}$, Humulin; Eli Lilly). To maintain euglycemia, 20% glucose was infused at variable rates during clamps. Whole body glucose turnover was assessed with a continuous infusion of [3-^{3}H]glucose, and 2-deoxy-D-[1-^{14}C]glucose (PerkinElmer) was administered as a bolus (10 μCi) at 75 min after the

start of the clamps to measure insulin-stimulated glucose uptake in individual organs. At the end of the clamps, mice were anesthetized, and tissues were taken for biochemical analysis(14).

4.2.5. Biochemical analysis and calculation

The glucose concentration during the clamps was analyzed using 10 μl plasma by a glucose oxidase method on a Beckman Glucose Analyzer 2 (Beckman, Fullerton, CA). Plasma concentrations of [3-^{3}H]glucose, 2-deoxy-D-[1-^{14}C]glucose, and $^{3}H_2O$ were determined after deproteinization of plasma samples as previously described(15). For the determination of tissue 2-deoxy-D-[1-^{14}C]glucose-6-phosphate content, tissue samples were homogenized, and the supernatants were subjected to an ion-exchange column to separate 2-deoxy-D-[1-^{14}C]glucose-6-phosphate from 2-deoxy-D-[1-^{14}C]glucose.

Rates of basal and insulin-stimulated whole-body glucose turnover were determined as the ratio of the [^{3}H]glucose infusion rate (disintegrations per minute [dpm]) to the specific activity of plasma glucose (dpm/μmol) at the end of the basal period and during the final 30 min of the clamps, respectively. Hepatic glucose production (HGP)

during the clamps was determined by subtracting the steady-state glucose infusion rate from the whole-body glucose turnover rate. Whole-body glycolysis and glycogen/lipid synthesis were calculated as previously described(15).

4.2.6. Plasma leptin measurement

The plasma levels of leptin were measured using ELISA kits (ALPCO Diagnostics, Salem, New Hampshire, USA). To collect plasma samples, mice were fasted for 5 hours in the morning. Using heparin-treated capillary tube, blood were collected carefully into the heparin-treated eppen tube, and centrifuged at 12,000rpm for 3 mins in 4°C. For ELISA assay, supernant of centrifuged blood were used following the manufacturer's instruction.

4.2.7. Statistical analysis

Data were expressed as mean ± S.E. values, and ANOVA was used for multiple statistical comparisons. A probability value of $p < 0.05$ was used as the criterion for statistical significance. All analyses were performed using Statistical Analysis Software (SAS, Inc., Cary, NC).

4.3. Results

4.3.1. TRPV1 deficiency accelerates aging-induced obesity in mice

To examine the effect of TRPV1 on aging-induced obesity, TRPV1 knockout (KO) mice and wild-type (WT) littermates fed chow diet *ad libitum* for 6 months and measured body composition at the beginning and end of experiment. Body weight of aged WT mice was significantly higher than yound WT mice (3 months of age), and aged TRPV1 KO mice became heavier than WT mice at 9 months of age (Figure 1A). Elevated accumulation of whole body fats of WT mice was also detected at the 9 months of age (Figure1B). Whole body fat mass was also significantly increased in aged TRPV1 KO mice compared to aged WT mice (Figure 1B). Interestingly, I found that aged TRPV1 KO mice had more lean mass than aged WT mice, suggesting possible involvement of other factors such as bone and muscle growth by TRPV1 deletion (Figure 1C). All of these data indicate that TRPV1 deletion aging-induced weight gain and obesity.

4.3.2. TRPV1 deletion reduces energy expenditure

Figure 1

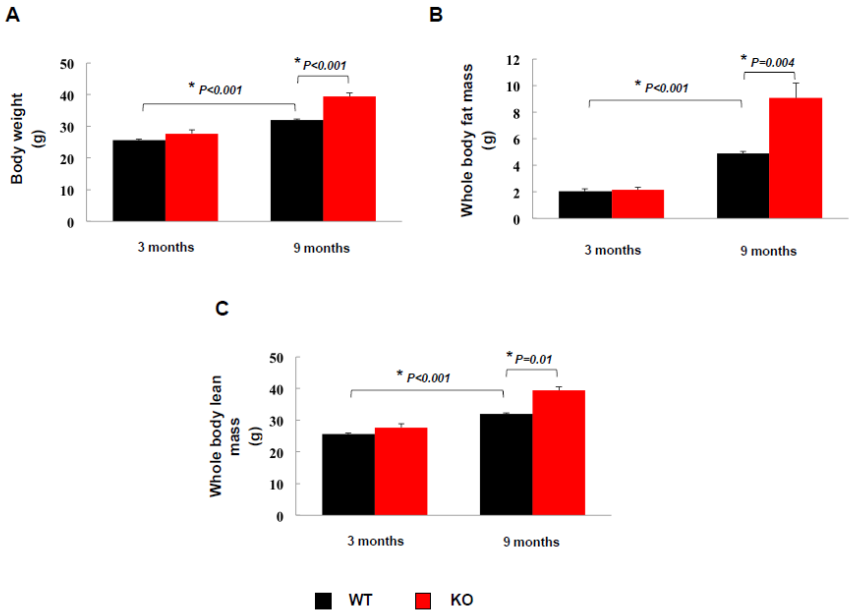

Figure 1. TRPV1 deletion promotes on aging-induced weight gain and obesity in mice. (*A*) body weight was measured at 3 and 9 months of age (n=4, **P*<0.05) in mice. (*B* and *C*) whole body fat and lean mass were measured weekly using ^{1}H-MRS during experiment. WT, wild type C57BL/6J mice. KO, TRPV1 KO mice. *P*<0.05

Since TRPV1 KO mice were more susceptible to aging-induced obesity than WT mice, I next examined the effect of TRPV1 deletion on energy balance using metabolic cage in TRPV1 KO and WT mice at the 3 months and 9 months of age. Food intake and physical activity were not altered in aged mice by TRPV1 deletion (Figure 2A and B). However, deficiency of TRPV1 changed indirect calorimetry such as VO_2, VCO_2, and energy expenditure normalized to whole body lean mass measured using ^{1}H-MRS. In aged mice, VO_2 consumption was increased by 11.2% and 6.7% compared to young WT and TRPV1 KO mice, respectively (Figure 3A). Aged TRPV1 mice showed remarkably decreased VO_2 consumption compared to aged WT mice (Figure 3A). In Figure 3B, VO_2 production were not different between young and aged WT mice (p=0.44) and young WT and TRPV1 KO mice (p=0.33). After 6 months, TRPV1 KO mice were significantly decreased VCO2 production, which leads to reduce energy expenditure (Figure 3C). All of these data suggest that TRPV1 deletion promotes aging-associated reduction of VCO_2 production, whereas VO2 consumption was increased in aged WT and TRPV1 KO mice, probably resulting from increased lean mass (Figure 1C), which trigger to reduce energy expenditure in aged TRPV1 KO mice. Also, I found that this reduced

Figure 2

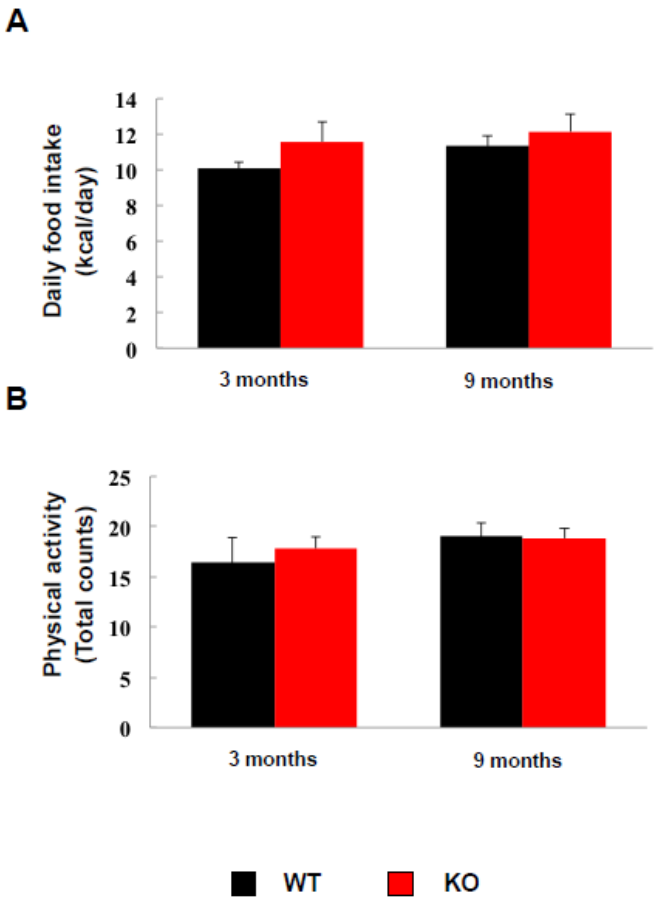

Figure 2. Deficiency of TRPV1 have no effect on food intake and physical activity during aging. (A) Daily food intake in WT and TRPV1 KO mice. (B) Total counts of beam break for measuring physical activity during nighttime.

energy expendutre in aged TRPV1 KO mice was not caused by decreased body temperature (Figure 3D).

4.3.3. Deletion of TRPV1 promotes aging-induced insulin resistance

Since aged TRPV1 KO mice showed obesity and less energy expenditure, I next examined the effect of TRPV1 deletion on aging-associated insulin resistance using hyperinsulinemic-euglycemic clamp. It was clearly showed the defects of insulin sensitivity in aged (9 months of age) WT mice compared to young (3 months of age) WT mice (Figure 4A). In addition, glucose infusion rate of aged TRPV1 KO mice considerably lower than aged WT mice. Whole body glucose turnover rate and glycolysis, however, were not changed by TRPV1 deletion in aged mice (Figure 4B and C). Because there are no difference in whole body glucose turnover rate, glycolysis, and consequently glycogen synthesis between aged WT and TRPV1 KO mice with showing impaired whole body insulin senstivity, I hypothesized that TRPV1 deficiency affects on hepatic glucose production and insulin action, which results in decreased glucose infusion rate in hyperinsulinemic-euglycemic clamp (Figure 3A).

Figure 3

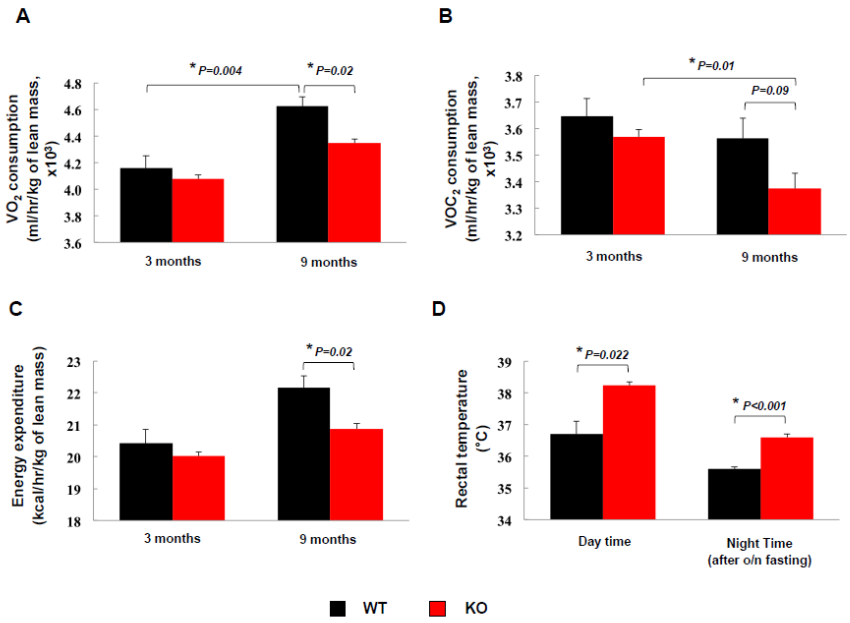

Figure 3. TRPV1 deletion induces reduction of energy expenditure during aging in mice. All data are expressed per kg of whole body lean mass measured using ^{1}H-MRS. (*A* and *B*) Average VO_2 consumption (*A*) and VCO_2 production (*B*). (*C*) Average energy expenditure rates. (*D*) Rectal body temperature measured at day time (6pm, *left*) and night time (6am, *right*). $P<0.05$

Figure 4

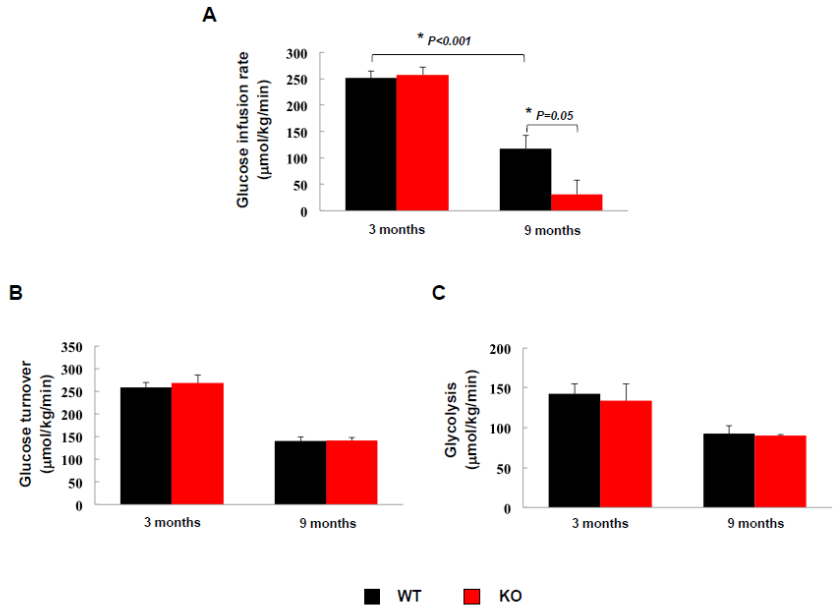

Figure 4. Deletion of TRPV1 promotes aging-induced whole body insulin resistance in mice. (*A*) Steady-state glucose infusion rate during hyperinsulinemic-euglycemic clamp in conscious mice. (*B*) Insulin-stimulated whole body glucose turnover was calculated using [^{3}H]glucose infusion during clamps. (*C*) Insulin-stimulated whole body glycolysis was estimated by during clamps. $P<0.05$

4.3.4. Deletion of TRPV1 aggrevates aging-induced impaired insulin sensitivity in liver

To further investigate how TRPV1 deletion exacerbates aging-induced whole body insulin resistance, I checked the levels of hepatic glucose production and insulin action measured [^{3}H]glucose from clamps. Insulin-stimulated reduction of hepatic glucose production was remarkably inhibited in TRPV1 KO mice compared to WT mice during clamps, whereas there is no difference in basal hepatic glucose production between WT and TRPV1 KO mice (Figure 5A). As a result, hepatic insulin action was significantly inhibited in aged TRPV1 mice compared to WT mice (Figure 5B), supporting decreased glucose infusion rate with no effect on whole body glucose turnover, glycogen synthesis, and glycolysis in aged TRPV1 KO mice.

4.3.5. Deletion of TRPV1 have no effect on glucose uptake levels in adipose tissues, muscle, and heart

To confirm the effect of TRPV1 deletion on aged-induced tissue specific insulin resistance, I performed glucose uptake assay by measured [^{3}H]glucose in skeletal muscle, white adipose tissue, and

Figure 5

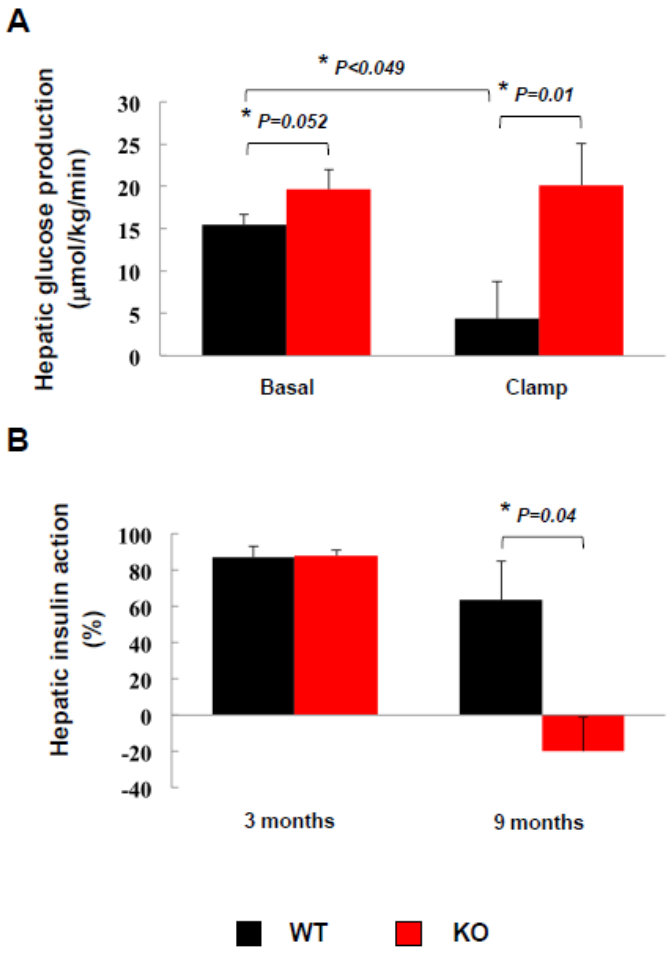

Figure 5. TRPV1 dificiency aggrevates aging-induced insulin resistance in liver. (*A*) Hepatic glucose production was estimated using [^{3}H]glucose during clamps in aged WT and TRPV1 KO mice. Using basal and clamp HGP (*A*), hepatic insulin action (*B*) was calculated. $P<0.05$

heart using samples obtained at the end of the clamps. Insulin-stimulated glucose uptake into white adipose tissue (Figure 6A) and muscle (Figre 6B). Grucose uptake rate were slightly reduced by TRPV1 deletion in brown adipose tissue (50% of reduction vs. aged WT mice, Figure 6C) and heart (31% of reduction vs. aged WT mice, Figure 6D), but there is no statistical significance (p=0.095 and 0.13, respectively).

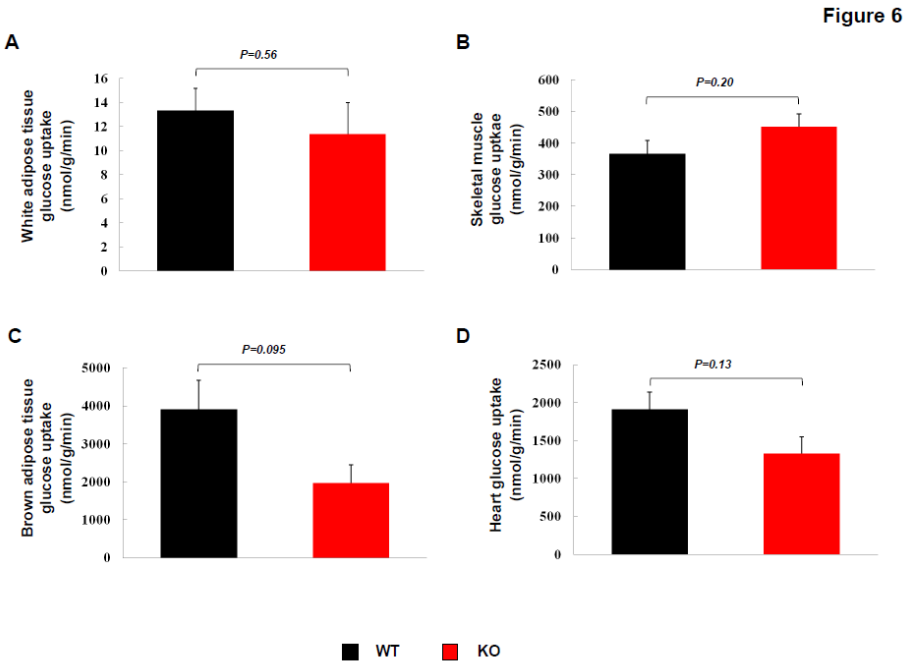

Figure 6. Deletion of TRPV1 dose not affect on insulin resistance in adipose tissues, skeletal muscle, and heart during aging. Insulin-stimulated glucose uptake in white adipose tissue (*A*), brown adipose tissue m(*B*), skeletal muscle (*C*), and heart (*D*) were measured using [^{14}C]glucose in aged WT and TRPV1 KO mice. *P* value means the statistical significance vs. aged WT mice.

4.4. Discussion

Strong correlation between aging and obesity has been reported by a considerable amount of previous studies in a recent decade. Obesity accelerates aging of adipose tissue and skeletal muscle by inducing disruption of mitochondrial function, elevated DNA damage, and short telomere length(1, 9, 16, 17). Insulin resistance mainly caused by obesity is accelerated by inflammation, oxidative stress, and mitochondrial dysfunction(17-19). Since TRPV1 also involves in aging process by regulating pain and thermogenic sensitivity, it may play a crucial role in aging-associated obesity and insulin resistance.

Here, I demonstrated that the deletion of TRPV1 aggravates aging-induced weight gain and fat accumulation. Interestingly, both aged WT and TRPV1 KO mice showed increased fat mass compared to young (3 months of age) WT and TRPV1 KO mice, respectively, which results from the increased muscle or bone mass in aged mice during this experiment for 6 months (Figure 1C). Although old rodents or elderly human showed decreased lean mass and physical activity because of degeneration of muscle and bone(20), I did not find any decrease of lean mass and physical activity in age WT mice. This

probably means that 9 months of age cannot fully induce aging in C57BL/6J mice (WT) a background strain of TRPV1 KO mice, and their muscle and bone were still growing and working normally. It is also possible that distinct regulation of TRPV1 on energy balance during aging affects complexly in mice. Since the mechanisms associated with TRPV1 in aging-induced obesity is very complicated, further studies to demonstrate cause of hypometabolism in TRPV1 KO mice during aging should be conducted.

Aging-induced insulin resistance in WT mice was demonstrated in Figure 4A of the present study. Surprisingly, aged TRPV1-null mice showed almost completely stopped whole body insulin action during clamps, which suggests that TRPV1 plays an important role in development of insulin resistance during aging. As a primary target organ of TRPV1 deletion during aging-associated insulin resistance progression, liver totally did not respond to insulin stimulation in TRPV1 KO mice. This result is conflict with the data showing diet-induced insulin resistance in adipose tissues and heart, but not in liver in TRPV1 KO mice fed high-fat diet from Chapter 3, which indicates the different mechanisms are involved in aging-associated insulin resistance from diet-induced impaired insulin sensitivity in TRPV1 KO

mice. Besides, several factors such as VCO_2 production (p=0.09), brown adipose tissue and heart glucose uptake (p=0.095 and 0.13, respectively) between aged WT and TRPV1 KO mice did not have statistical significance, and 9 months of age was not enough to fully induce aging in this experiment, suggesting that further studies to increase number of mice used in experiment and sufficiently should be performed.

Here, I demonstrated that TRPV1 deficiency promotes aging-associated obesity and insulin resistance in mice. This is caused by reduced energy expenditure and inhibition of heptic insulin sensitivity by TRPV1 deletion. These results suggest that TRPV1 could be a promising therapeutic agent againt aging-associated obesity, insulin resistance, and type 2 diabetes.

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Chapter 5.

Conclusions

5.1. Deterioration of diet-induced obesity and leptin resistance in TRPV1 deficient mice

In the past few decades, the role and mechanism of capsaicin and transient receptor potential vanilloid type-1 (TRPV1) in metabolic diseases have been suggested. Consumption of red peppers or capsaicin was shown to decrease appetite, cause weight loss and stimulate thermogenesis caused by substrate oxidation from carbohydrate to fat oxidation(1-3). With these previous studies, deletion of TRPV1 exacerbates diet-induced obesity characterized by higher fat mass and weight gain than wild-type (WT) littermates fed high-fat diet (HFD). The elevated obesity in TRPV1 knockout (KO) mice were caused by reduced locomotor activity without any changes in food intake and energy expenditure. Because activity is regulated by hypothalamic leptin signaling, responsiveness to leptin is a critical mediator of energy balance to maintain homeostasis. TRPV1 deficient mice were impaired in the leptin/activity or energy expenditure axis of hypothalamus so that these mice showed opposite correlation pattern between activity/energy expenditure vs. leptin level. In leptin infusion study, also, TRPV1 KO mice were leptin resistance characterized by no response of food intake

reduction to leptin compared to WT mice. Together, these results strongly suggest that increased weight gain and fat mass upon HFD in TRPV1 KO mice were due to, at least in part, to an decreased activity with elevated plasma leptin levels, which indicates leptin resistance in these mice.

Capsaicin treatment inhibits differentiation of preadipocyte to adipocyte by regulating AMP-activated protein kinase (AMPK) and induces apoptosis and cell death in 3T3-L1 preadipocytes(4, 5). Repeated exposure to capsaicin (chronic) or exposure to high dose of capsaicin (acute) induces degradation of TRPV1 protein called as "desensitization"(6, 7). As a selective receptor of capsaicin, TRPV1 has been reported that it is expressed in preadipocytes and visceral adipose tissues both mice and humans(8), suggesting its possible role for biological process of adipocyte and adipogenesis. However, differentiation of primary cultured mouse embryonic fibroblasts (MEFs) from TRPV1 KO mice to adipocytes was markedly blocked compared to WT MEFs (data not shown), which is inconsistent with *in vivo* data showing increased fat accumulation in TRPV1 mice. This result suggests that deletion of TRPV1 affects on adipocyte differentiation, indicating that TRPV1 is required for normal

adipogenesis. Although TRPV1 deletion definitely promotes obesity and leptin resistance in mice, further studies to clarify mechanisms of TRPV1 involving obesity and leptin resistance were needed. In my study, I found that TRPV1 deletion influenced phosphorylation of STAT3 by leptin in primary cultured MEFs and hypothalamus of mice negatively, which induced blunt leptin signaling by TRPV1 deficiency. However, regulatory mechanism in TRPV1 deletion by leptin was still not fully understood.

5.2. Deterioration of diet- and aging-induced obesity and insulin resistance in TRPV1 deficient mice

Leptin resistance characterized by decreased sensitivity aginst leptin with hyperleptinemia is strongly correlated to obesity and its associated insulin resistance. TRPV1 deletion significantly reduced insulin sensitivity compared to WT littermates during HFD feeding. This result was also supported by decreased insulin-stimulated whole body glucose turnover in TRPV1 KO mice fed HFD for 5 weeks in Chapter 3. Among several insulin sensitive tissues, white and brown

adipose tissues and heart play critical roles in development of insulin resistance by TRPV1 deletion after HFD.

Indeed, It has been widely known that HFD mainly promotes obesity and insulin resistance. As another cuase of obesity and insulin resistance, aging affects on obesity and insulin resistance. In adipocyte, especially, aging plays a crucial role in the development of adipocyte inflammation and insulin resistance through the elevated accumulation of macrophage and oxidative stress by regulating p53, which is a representative survival and aging factors(9). However, the mechanism how aging accelerates obesity and insulin resistance is not fully understood. Here, TRPV1 KO mice became more obese under aging condition having higher fat accumulation and even higher lean mass than WT mice. The elevated obesity of TRPV1 KO mice was caused by imbalance of energy homeostasis shown reduced energy expenditure without any changes in energy intake. Similarly, insulin signaling in aged TRPV1 mice almost shut down compared to age-matched WT mice, which suggest severe insulin resistance in aged TRPV1 mice showing reduced hepatic insulin sensitivity. Together, deficiency of TRPV1 aggravates diet- and aging-induced obesity and insulin resistance *in vivo*, which supports that activation of TRPV1 could be

beneficial for treating or preventing obesity and its associated metabolic syndrome.

5.3. Discussion

Although the role of TRPV1 on diet- and aging-induced obesity and its associated metabolic abnormal condition such as leptin resistance and insulin resistance has been determined by present study, how and why TRPV1 regulates these metabolic diseases is still not fully understood. Also, the reason the results from this study are different from previous study reported anti-obesity effect in TRPV1 KO mice which is same strain that I used in the experiments(10). There are several explainable reasons; HFD used in my study has different fat source and calories. In previous report(10), they used HFD containing 11% of fat (25.8% of calories provided by fat), which is comparable to chow diet. I used HFD containing 55% fat by calories, and usually HFD of 45-65% fat by calories have been used by various researchers. Also, the fat source is cocoa butter having high proportion of saturated fat in previous study(10), whereas HFD used in my study contains corn oil with 28% of saturated fat, 30% of *trans*, 28% of monounsaturated (*cis*), and 14% of polyunsaturated (*cis*)(11). In

addition, the duration fed HFD is different from my study. I fed HFD for 5 weeks in mice, whereas they fed HFD by 6 months(10). Because they fed HFD for considerably long time, it can be suggested the involvement of aging in their experiments. To exclude the effect of aging in TRPV1 KO mice, I just fed only for 5 weeks and carried out separate experiment which examined the effect of TRPV1 deletion on aging-induced obesity and insulin resistance to explain the effect of aging. These differences of diet and duration can be critical reasons to induce opposite results with previous study(10).

As a down stream of insulin and leptin signaling, JAK/STAT3 pathway plays a multiple and critical role in the development of obesity and leptin/insulin resistance. Phosphorylation of STAT3 on Tyr 705 responds and activates transcription to various cytokines, which suggests it is a representative inflammatory marker. TRPV1 deletion was damaged in STAT3 pathway in response to leptin and insulin stimulation, which is caused by impaired suppressor of cytokine signaling 3 (SOCS3) expression in TRPV1 deleted MEFs and SOCS3 (data not shown). These data supports that impaired STAT3 phosphorylation was not directly altered by TRPV1 deletion,

suggesting the other molecular target mechanism of TRPV1 deletion by directly influencing these metabolic conditions.

In chapter 3 and 4, TRPV1 deficient mice showed abnormally obese and insulin resistance phenotype compared to WT mice by HFD and aging. However, the responsible mechanisms for obesity and insulin resistance were thought to be quite different between diet- and aging-induced models. HFD induced disability of insulin signaling in white/brown adipose tissue and heart, but aging induced impaired hepatic insulin action in TRPV1 KO mice. In addition, TRPV1 deletion mice had less energy expenditure by aging-induced obesity, whereas they became hypoactive during HFD feeding. These phenomena strongly suggest that mechanistic process induced by aging to develop obesity and insulin resistance is quite different between by diet.

Although failure of improvement of weight loss in Capsaicin treatment in mice (data not shown), Capsaicin induces leptin sensitivity characterized by reduced food intake with similar amount of plasma leptin levels to vehicle-treated group in chapter 2. Also, deficiency of TRPV1 promotes obesity and leptin/insulin resistance in mice (Figure 1), indicating that activation of TRPV1 by capsaicin should be a gold strategy for treating obesity and its associated metabolic diseases.

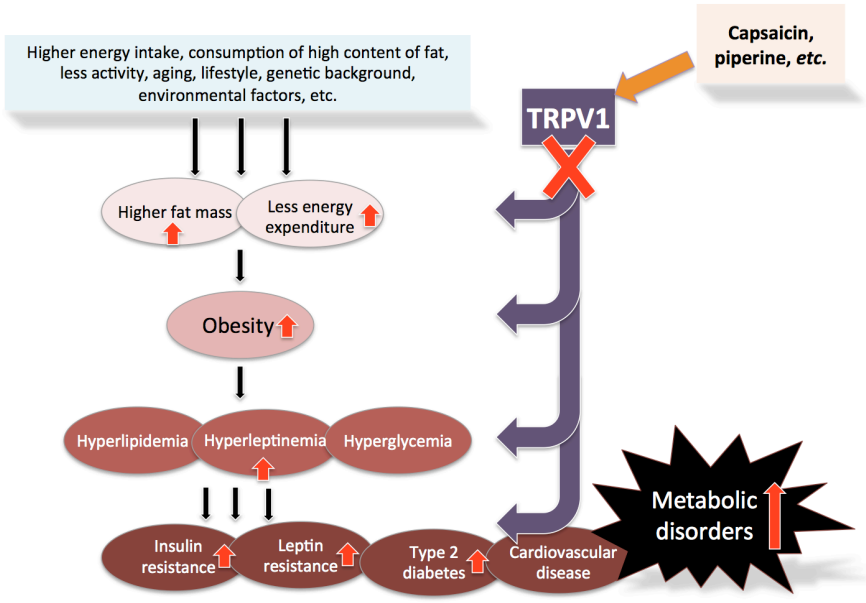

Figure 1. Possible mechanisms of TRPV1 on diet- and aging-induced obesity, leptin/insulin resistance, and subsequently metabolic disorders.

Moreover, these results provide new insight into the involvement of TRPV1 as a capsaicin receptor in the development of obesity and its-associated metabolic disorders and strategy against their pathogenesis as a therapeutic target.

5.4. References

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국문초록

비만이 전세계적으로 만연함에 따라 비만 및 그와 연관된 대사성 질환이 심각한 질병으로 떠오르고 있다. 본 연구에서는 캡사이신 수용체인 TRPV1 의 비만 및 대표적인 이형 당뇨의 지표로서 인슐린 저항성에 미치는 영향을 마우스 모델을 활용하여 밝히고자 하였다. 5 주간 고지방 식이를 투여함으로 인하여 유도되는 체중 증가와 지방 축적이 TRPV1 결핍 마우스에서 더욱 악화되어 나타나는 것을 확인하였으며, 이러한 비만의 악화는 TRPV1 결핍 마우스에서의 감소된 육체활동으로 인한 것임을 알 수 있었다. 육체활의 감소를 유도할 수 있는 상위 인자로 렙틴을 확인한 결과, 혈중 렙틴의 증가에 의하여 움직임과 에너지 소비량이 증가하고 에너지 섭취량이 감소해야 함에도 불구하고 TRPV1 결핍 마우스에서는 이러한 조절이 정 반대의, 비정상적인 패턴을 보이는 렙틴 저항성이 나타남을 확인하였다. 따라서 본 연구에서는 TRPV1 이 결핍되어 있는 경우에 고지방 식이로

유도되는 비만 및 렙틴 저항성이 악화됨을 통하여 TRPV1 이 고지방 식이에 의한 비만 및 렙틴 저항성 치료에 효과적인 목표가 될 수 있음을 보여주었다. 또한 TRPV1 결핍은 고지방 식이로 유도되는 인슐린 저항성을 더욱 악화시키며 이러한 것이 지방세포와 심장에서의 감소한 인슐린 민감성에 의한 것임을 증명하였다.

노화는 염증과 산화적 스트레스, 미토콘드리아의 기능 장애의 주요 원인 중 하나로 선행연구에 따르면 비만과 인슐린 저항성을 유발함으로서 당뇨 및 대사성 질환으로의 진행을 유도한다고 보고되어 있다. TRPV1 이 결핍된 마우스는 노화로 유도되는 체중 증가 및 지방, 제지방량 증가가 더욱 큰 폭으로 나타나며 인슐린 저항성 또한 악화됨을 확인하였다. 고지방식이로 유도되는 모델과는 달리, 노화로 유도되는 인슐린 저항성에 있어서 TRPV1 의 결핍은 간에서의 인슐린 활성을 현저히 감소시킴으로서 전체적인 인슐린 저항성을 유도함을 확인하였다. 따라서 본 연구결과를 통하여 TRPV1 이 비만 및 에너지 밸런스, 렙틴 저항성 및 인슐린 저항성의 발생

과정에 관여하는 중요한 조절자로서, 비만과 이형 당뇨를 목표로 하는 치료법에 대한 새로운 시각을 제시하였다.

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