Summer 2015

Potential Role of Protein Kinase Cbeta in High Fat Diet-Induced Adipose Dysfunction by Regulating Autophagy Levels

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Honors Research Project

Submitted to

The Honors College

ABSTRACT

Obesity is the most common nutritional disorder in the United States and worldwide. Public health efforts and other anti-obesity measures clearly have not controlled the obesity epidemic. There is a great need to understand the pathogenic mechanisms underlying fat accumulation. Research of the past decade supports a prominent role for diet-induced adipose tissue dysfunction in the development and/or progression of obesity and associated insulin resistance. Potential mechanisms for the development of adipose tissue dysfunction include ectopic (visceral) fat accumulation, genetic factors, and alterations in autophagy and inflammatory processes. However, the molecular mechanisms linking dietary fat intake with alterations in adipose tissue autophagy levels are not well understood. A critical role of protein kinase C beta (PKCβ) in obesity-associated adipose dysfunction is emerging. PKCβ deficiency protects from high fat diet-induced obesity and related complications. High fat diet is also shown to induce adipose PKCβ expression in mice, whereas PKCβ deficiency is reported to stimulate autophagy in a cell culture model. Based on the above results, we hypothesized that high fat diet-induced PKCβ activation in the adipose tissue inhibits autophagy and thereby promotes adipose dysfunction and fat accumulation. We report here that high fat diet-induced adipose PKCβ induction is accompanied by a simultaneous reduction in the ratio of autophagy markers LC3-II/LC3-I protein expression in mice. Importantly, high fat diet was unable to alter this ratio in the adipose tissue of PKCβ deficient mice. These results implicate PKCβ in mediating harmful effects of high fat diet on adipose tissue autophagy levels. It is hoped that understanding the PKCβ based pathogenic mechanisms leading to diet-induced adipose dysfunction will eventually lead to novel therapeutic approaches to combat the obesity epidemic.

ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Wei Huang for his support, constructive criticisms, and guidance. I am thankful for this opportunity and this project was a valuable experience. I want to thank my project readers: Dr. Carlson and Dr. Londraville for their valuable time and critiques. I am grateful to my Faculty Advisor Dr. Bagatto for his guidance.

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ABBREVIATIONS USED

HFD, high-fat diet; LC3, microtubule-associated protein 1 light chain 3; PKCβ, protein kinase C beta isoform; PKCβ$^{-/-}$, protein kinase Cβ-deficient mice; TG, triglycerides; WT, normal wild-type mice; WAT, white adipose tissue (visceral fat).

INTRODUCTION

A. Obesity rates are rising in USA and worldwide:

The population of obese (defined as a body mass index of 30 or higher) individuals has increased dramatically during recent decades (Fig. 1). In 1980, obese adults constituted roughly 15 percent of the US population. By 2010, that number had risen to 36%, with roughly 69% the US population considered overweight (defined as a body mass index between 25 and 29.9) or obese (2). Most disturbing, is the fact that obesity rates have been steadily rising in children too. Roughly 17% of children and teens are obese and 33% are considered overweight or obese (3). As long as these trends continue, it has been predicted that about half of the US population will be obese in 15 years (4).

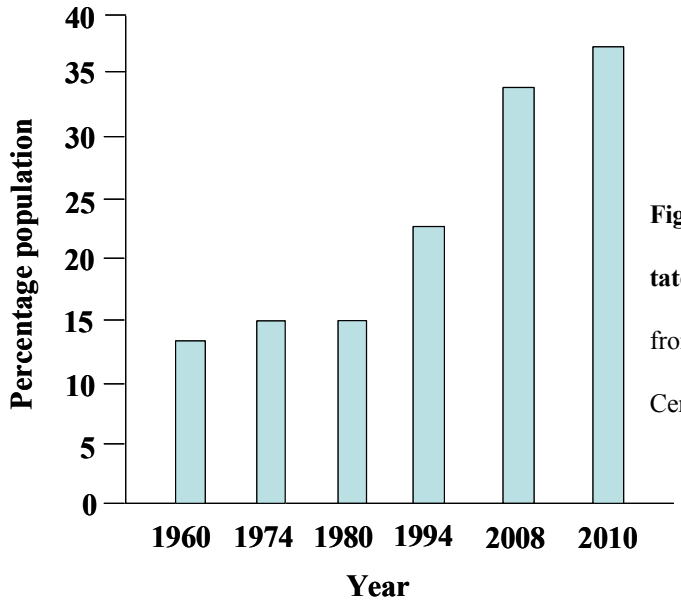


Fig. 1. Trends in obesity prevalence in the United tates in the indicated period. This figure is taken from National Center for Health Statistics, part of the Centers for Disease Control and Prevention.

Obesity has become a worldwide phenomenon, now appearing in both developed and undeveloped countries. Globally, nearly 10 percent of men and 14 percent of women (roughly 500 million adults) are estimated to be obese. Nearly three times as many are considered overweight or obese (5, 6). There is less data available on global obesity rates in children, but a recent global estimate found 43 million preschool children to be overweight or obese (7). If these trends continue, over 1 billion adults are estimated to be obese in 15 years (8).

Obesity is a major contributor to the worldwide problem of chronic diseases. Abdominal obesity in particular is suggested to play a central role in the development of obesity-related illness, such as type 2 diabetes mellitus and cardiovascular diseases (9). Thus, it is unsurprising that the incidence of obesity-associated disorders is also dramatically increasing and body fat and weight gain over time are effective predictors of type 2 diabetes (11). While the relationship between obesity, insulin resistance, and cardiovascular disease is well established (12), the mechanisms underlying this relationship are still not well understood. It is estimated that the total cost of treating obesity-related diseases in the US is roughly $180 billion (13).

B. Energy imbalance and obesity:

Obesity is defined as an abnormal or excessive fat accumulation resulting from an imbalance between energy intake and energy expenditure. It is a complex polygenic disease that is caused by a number of lifestyle and environmental factors including: genetics, sedentary lifestyle, unhealthy diet, pregnancy, specific medications and sleep deficiency (15).

The combination of these factors results in a chronic positive energy imbalance, in which energy intake is greater than energy expenditure for an extended period of time. It is generally

believed that the development of obesity can be slowed down or avoided by modifying diet, exercise, and behavior habits. Even though it is estimated that 40-70% of the variation in body mass in a given population is a result of heritable factors (16, 17).

C. Adipose tissue dysfunction in obesity:

Adipose tissue is the most variable organ in mammals in terms of size. At the extremes in humans, it ranges from a few percent of body weight in the malnourished to over 50% of total weight in the morbidly obese (14). This excessive accumulation of fat in different depots is associated with different metabolic consequences. Visceral fat creates a much greater risk of developing diabetes than does subcutaneous or peripheral fat (18). In fact, fat accumulation in the intra-abdominal visceral depot has been demonstrated to be an independent risk factor for the development of cardiovascular diseases and type 2 diabetes (19). The insulin sensitivity of visceral fat is different from that of subcutaneous adipose tissue (20). Lipids and other metabolites from visceral adipose tissue can be drained directly into the liver via the portal vein. Additionally, adipose and subcutaneous fat differ in their metabolism, gene expression and in secretion of hormones called adipokines (16). They are cell signaling cytokines which communicate with other tissues to modulate inflammation. Visceral adipose tissue predominantly secretes many pro-inflammatory cytokines, whereas anti-inflammatory adiponectin is highly expressed in subcutaneous fat (21).

D. Abnormal regulation of adipose tissue autophagy in obesity and diabetes:

Autophagy is responsible for the removal of malfunctioning organelles and misfolded proteins via lysosomes (22). It is activated in response to stress and various physiological stimuli.

Autophagy involves the formation of an autophagosome, a double-membrane cytoplasmic vesicle that engulfs cellular components and fuses with lysosomes for degradation. The formation of double-vesicle membrane is a complex process and involves more than 16 proteins. A critical step in autophagy is the covalent lipidation of microtubule-associated protein 1 light chain 3 (LC3) in which cleavage of LC3 allows a carboxy-terminal glycine to conjugate with phospholipids. This process is required for the formation of autophagosomes. Migration of lipidated LC3-II on SDS-PAGE gel monitors levels of autophagy at the cellular level. LC3-II moves faster than LC3-I because of its association with the phosphatidylethanolamine group. Commercial antibodies are available to measure expression of both LC3-I and LC3-II simultaneously by immunoblotting. The above assay is generally used to measure cellular autophagy levels due to lack of a direct method to visualize autophagy in animal tissue.

Autophagy is considered a cellular degradation pathway essential for survival (23). As a protective mechanism, it safeguards organisms against normal and pathological conditions by regulating the turnover of dysfunctional organelles and proteins. Increasing evidence suggests that inhibition of autophagy greatly contributes to the accumulation of damaged organelles, leading to cell dysfunction or death. There are specific mechanisms for degradation of particular organelles; for example, mitochondria are degraded by autophagic mechanisms known as mitophagy (25). The efficiency of this process has been shown to decline with aging, and which can result in accumulation of damaged mitochondria (26, 27). The progressive decline of mitochondrial function is considered a major mechanism underlying obesity (25, 27-31). It is associated with less fatty acid oxidation and increased generation of reactive oxygen species, with detrimental structural and functional consequences.

Based on current knowledge, a model can be proposed to account for the development of obesity in response to complex interactions between genes, environment, and behavior (Fig. 2).

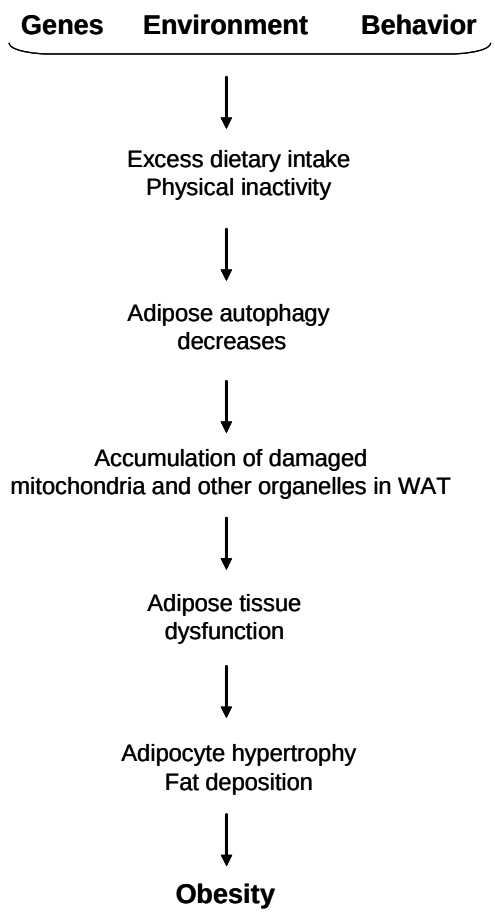


Fig. 2. Proposed model of the pathogenesis of diet-induced obesity. The cascade of pathogenic mechanism leads to adipose dysfunction via reducing autophagy and promoting accumulation of damaged mitochondria and other organelles. Compromised mitochondrial and cellular functions result in ectopic lipid deposition leading to the development of obesity and related diseases.

E. Established role of protein kinase Cbeta in diet-induced obesity and diabetes:

Protein kinase Cbeta (PKCβ), a member of the lipid-activated serine/threonine PKC family, has recently been implicated as the master regulator of triglyceride homeostasis, possibly via regulating mitochondrial function (32). PKCβ plays a critical role in mediating deleterious effects of high-fat diet (HFD) on adipose functions: (i) HFD feeding of normal mice for 12 weeks induces PKCβ protein expression in the white adipose tissue (WAT), but does

not affect expression in either liver or muscle (33), (ii) whole-body deficiency of PKCβ leads to leaner mice with significant reduction in body fat depots (32, 33) and these mice are resistant to diet-induced obesity and obesity-related metabolic complications such as type 2 diabetes (34), (iii) PKCβ$^{-/-}$ adipocytes exhibit increased numbers of healthy mitochondria and higher levels of fatty acid oxidation than WT (32); (iv) PKCβ levels are elevated in the adipose tissue of leptin-deficient *ob/ob* mice and PKCβ deficiency in these mice is also effective in reducing obesity and diabetes (34); (v) weight gain related to treatment with antipsychotic drugs in humans is linked to the activation of PKCβ expression in adipose tissue (35); and (vi) Polymorphism in the PKCβ gene is associated with development of Type 2 diabetes and related complications (36, 37). The molecular mechanism underlying PKCβ action in the development of obesity is not known but the potential role of autophagy is indicated by a recent study in which overexpression of PKCβ in cultured cells (mouse embryo fibroblasts and human embryonic kidney cells) negatively regulated autophagy by influencing mitochondrial membrane potential (38). In view of relationships among reduced autophagy, increased mitochondrial dysfunction and development of obesity (27-31, 39, 40), it will be interesting to determine the role of PKCβ in regulating autophagy in the adipose tissue.

HYPOTHESIS

Feeding mice a HFD for 12 weeks induces adipose PKCβ expression (32), whereas PKCβ activation has been shown to inhibit autophagy in a cell culture system (34). Based on these observations, **we hypothesize that HFD-induced PKCβ activation in the white adipose tissue (WAT) inhibits autophagy and thereby promotes adipose dysfunction and fat accumulation in mice leading to the development of obesity** (Fig. 3).

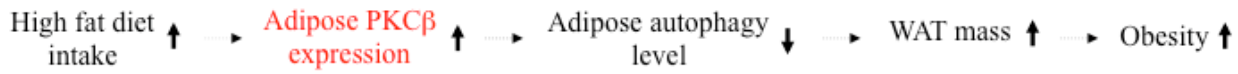


Fig. 3. Outline of proposed hypothesis and expected results. We propose that an increase in PKCβ expression mediates HFD-induced suppression of autophagy in adipose tissue and accounts for its obesity effects. We expect to see relationships among adipose PKCβ levels, suppression of autophagy and accumulation of triglyceride levels in wild-type mice on HFD. In contrast, HFD is not expected to suppress autophagy in the adipose tissue of PKCβ$^{-/-}$ mice.

To test the above hypothesis, we will examine the potential relationships among diet-induced changes in adipose PKCβ expression, autophagy and fat accumulation upon feeding a HFD to WT and PKCβ$^{-/-}$ mice.

MATERIALS AND METHODS

Animals and diet:

Generation of mice deficient in whole-body PKCβ expression ($PKC\beta^{-/-}$) has been previously published (32-34). Six-week-old WT and $PKC\beta^{-/-}$ male mice (n=6/genotype/diet group) were fed *ad libitum* either a HFD (60% of calories derived from fat; diet TD.06414 from Harlan Laboratories, IN) or a standard chow diet (17% of calories derived from fat; Harlan Tekland, WI) for 16 weeks (41, 42). Mice were housed under controlled temperature (23^oC) and lighting (12 hours light/dark) with free access to water at the Ohio State University Animal Care Facility located in the Biomedical Research Tower. All procedures on mice followed guidelines established by the Ohio State University College of Medicine Animal Care Committee. At the end of 16 weeks, mice were anesthetized and sacrificed. Body weight was measured for each mouse. WAT (visceral fat depot) from the above animals was dissected out, weighed, and immediately frozen in liquid nitrogen. The above procedures were carried out by lab personnel.

Immunoblotting:

Frozen WAT (200 milligrams) samples was homogenized in lysis buffer (40 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.5% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, phosphatase and protease inhibitors). Protein concentrations were determined using a Pierce BCA protein assay kit (Life Technologies, CA). After adjusting the protein concentration, equal amount of protein (30 μg/lane for β-Actin, 50 μg/lane for PKCβ, and 100 μg/lane for LC3s), along with colored molecular weight markers (BIO-RAD, CA), were

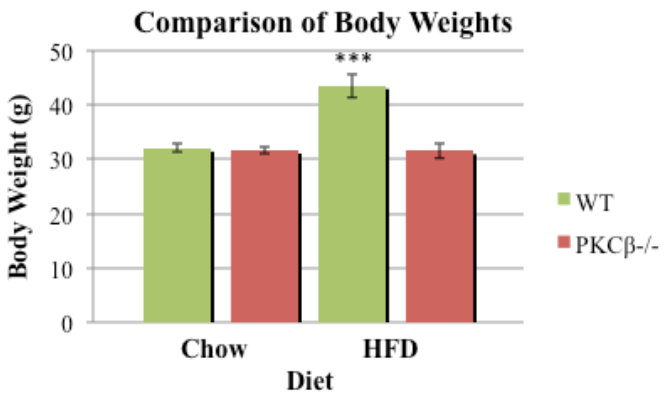
subjected to electrophoresis in SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose membranes (BIO-RAD, CA) using a standard transfer buffer containing 20% methanol. Membranes were blocked with Tris buffer containing 5% (w/v) non-fat dried milk for 1 hour. After washing with Tris buffer containg 500 mM NaCl and 0.05% (w/v) Tween 20, the membrane was probed with a primary PKCβ antibody (1:500 dilution; Santa Cruz Biotechnology, CA) to visualize the kinetics of PKCβ (77 kDa) induction in the adipose tissue of wild-type mice, or probed with LC3-1 and LC3-2 (16/18 kDa) antibody (1:250 dilution; EMD Millipore, MA) to analyze the expression levels of LC3 type 1 and 2 proteins (as markers for autophagy levels) in the adipose tissues of WT and PKCβ$^{-/-}$ mice, or using β-Actin (42 kDa) antibody (1:2000 dilution; Santa Cruz Biotechnology, CA). After washing with buffer, the membranes were incubated with a secondary antibody conjugated to horseradish peroxidase (1:5000 dilution) (BIO-RAD, CA) for 1 hour. Immunoreactive protein bands were detected by using a SuperSignal substrate kit (Thermo Scientific Inc., IL). Immunoreactive bands of predicted molecular mass were quantified with BioRad GS-800 calibrated densitometer using Quantify One analysis software (BIORAD, CA). Intensity of each band was corrected for background differences and normalized to β-actin loading control.

Statistical Analysis:

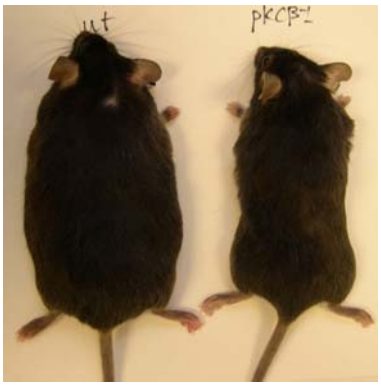
Experiments were performed in order to analyze all the groups of animals in parallel, n representing the number of different individuals used in each group. Results are presented as mean±SD of the number of samples indicated. Student's t-test was used when the values of two groups were analyzed. Data were considered statistically significant if $P < 0.05$.

RESULTS

1. PKCβ$^{-/-}$ mice gain less weight compared to WT mice in response to feeding a HFD. Both WT and PKCβ$^{-/-}$ mice were randomly divided into two groups. There were no significant differences in the initial body weights of these mice (Supplementary Data). One group was fed a chow diet and the other group was fed a HFD for 16 weeks, beginning at 6 weeks of age. At the end of the feeding period, body weight and WAT weight were examined for each mouse in both groups. It was observed that HFD feeding sharply increased body weights (43.58 ± 2.21 g WT versus 31.58 ± 1.4 g PKCβ$^{-/-}$, n=6, p<0.001) (Fig. 4) and WAT depot weights (2.43 ± 0.23 g WT versus 0.76 ± 0.15 g PKCβ$^{-/-}$, n=6, p<0.001) (Fig. 5 on next page) in WT mice compared to PKCβ$^{-/-}$ mice.

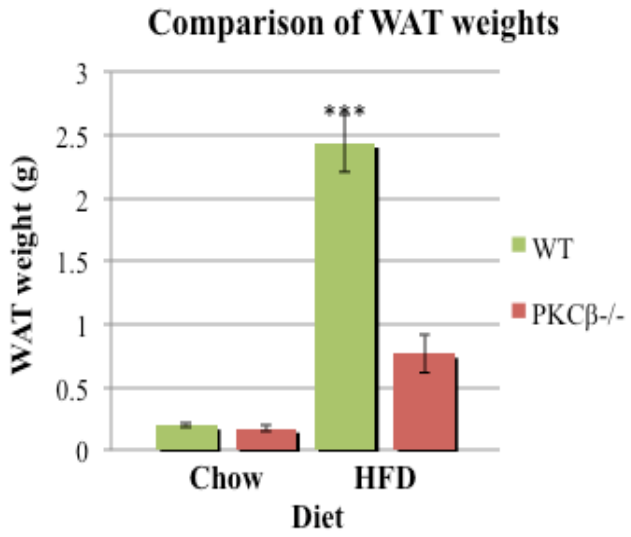


Comparison of body size between HFD-fed WT and PKCβ$^{-/-}$ mice



WT **PKCβ$^{-/-}$**

Fig. 4. PKCβ$^{-/-}$ mice are resistant to HFD-induced obesity compared to WT mice. Comparison of body weights for WT and PKCβ$^{-/-}$ mice fed either a chow or HFD for 16 weeks is shown in the left panel. Results are expressed as mean±SD. n=6, ***, p<0.001. A representative picture of WT and PKCβ$^{-/-}$ mice fed HFD for 16 weeks is shown in the right panel.



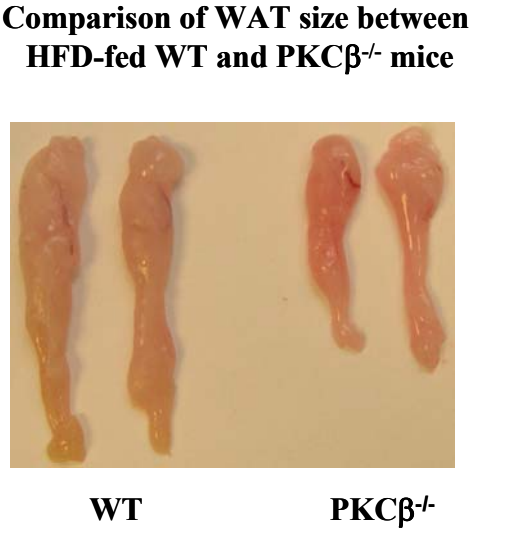


Fig. 5. HFD dramatically increases WAT depot weight in WT mice compared to PKCβ$^{-/-}$ mice. Comparison of WAT weights for WT and PKCβ$^{-/-}$ mice fed either a chow or HFD for 16 weeks is shown in the left panel. Results are expressed as mean±SD. n=6, ***, $p<0.001$. A representative picture of WATs of HFD-fed WT and PKCβ$^{-/-}$ mice is shown in the right panel.

2. HFD induces adipose PKCβ expression in mice. To determine PKCβ expression in the above WT mice, we compared PKCβ protein levels in the WAT of these mice after feeding either a chow or a HFD. It was observed that HFD feeding dramatically increased PKCβ expression (>13-fold) in the WAT of WT mice (Fig. 6).

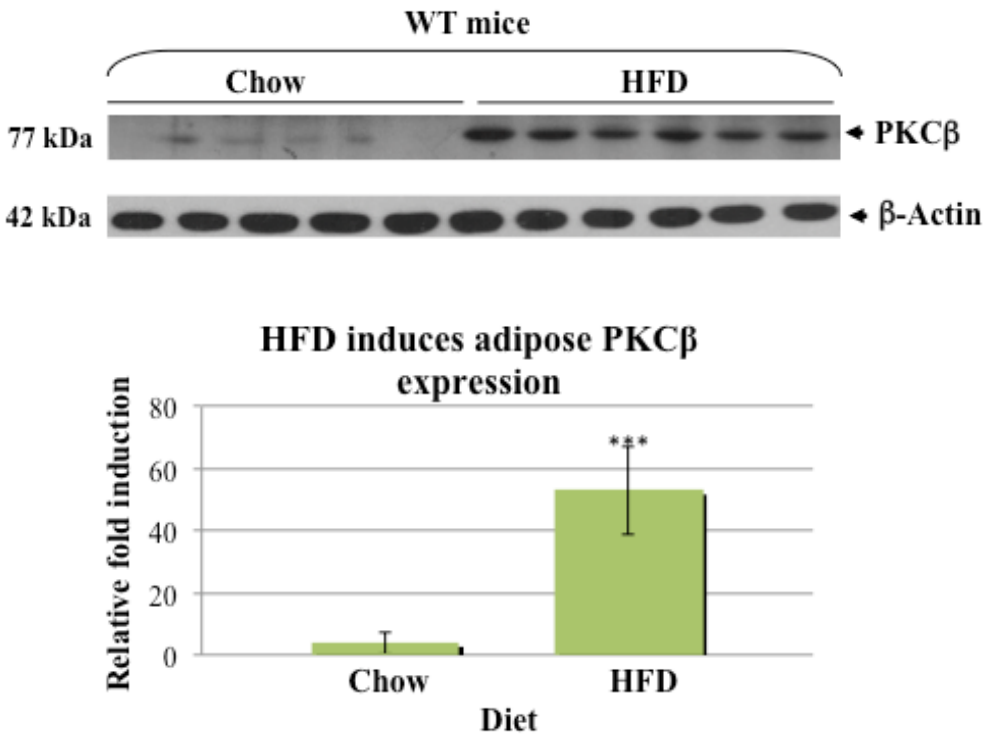


Fig. 6. Effect of HFD feeding on adipose PKCβ expression. WT mice fed a chow or a HFD were examined for proteins levels of PKCβ and β-Actin (loading control) in the adipose tissue by immunoblotting using an antibody to each protein. The quantitation of the immunoblot is shown in the bottom panel. Each value represents the mean±SD. n=6, ***, $p<0.001$.

3. HFD reduces autophagy in adipose tissue of WT, but not in PKCβ$^{-/-}$ mice. To determine if the change in adipose PKCβ expression was associated with an alteration in autophagy levels, we compared LC3 levels in the WATs obtained from WT mice fed a chow or a HFD. Autophagy level was determined as the change of the amount of the autophagosomal membrane marker LC3-II compared to LC3-I *in vivo*. This can serve as the indicator of autophagy intensity. The relative ratio of LC3-II/LC3-I protein expression is significantly reduced in the WAT of WT mice fed a HFD compared to WAT obtained from

mice fed a chow diet (Fig. 7). Quantitation of changes in LC3-II/LC3-I levels (normalized to β-actin levels) indicated that the autophagy levels have been reduced by almost 59%. To determine the effect of PKCβ deficiency on HFD-dependent suppression of autophagy levels, we also compared the relative protein expression of LC3-I and LC3-II in the adipose tissues of PKCβ$^{-/-}$ mice fed either a chow or HFD. Interestingly, feeding a HFD to PKCβ$^{-/-}$ mice had no significant effect on the ratio of LC3-II/LC3-I protein expression and was found to be comparable to mice fed a chow diet (Fig. 8).

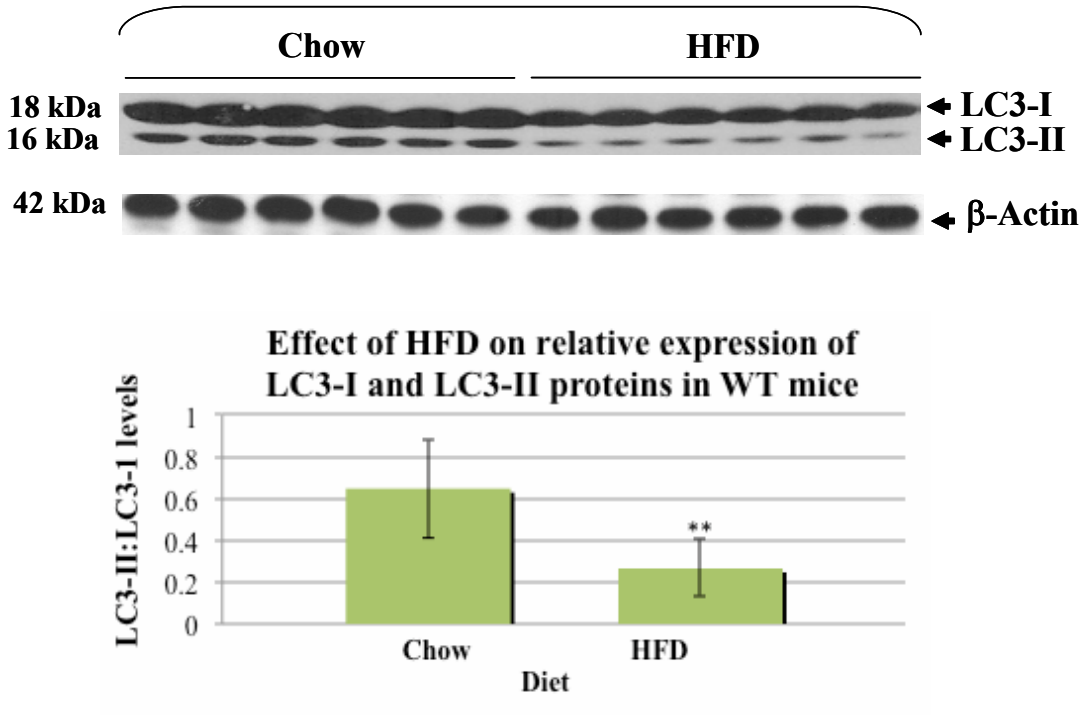


Fig. 7. Effect of HFD feeding on the relative protein expression of LC3-I and LC3-II in WT mice. WT mice fed a chow or HFD were examined for LC3-I, LC3-II, and β-Actin protein levels in the adipose tissue by immunoblottting using an antibody to each protein. The quantitation of the immunoblot is shown in the bottom panel. Each value represents the mean±SD. n=6, **, $p<0.01$.

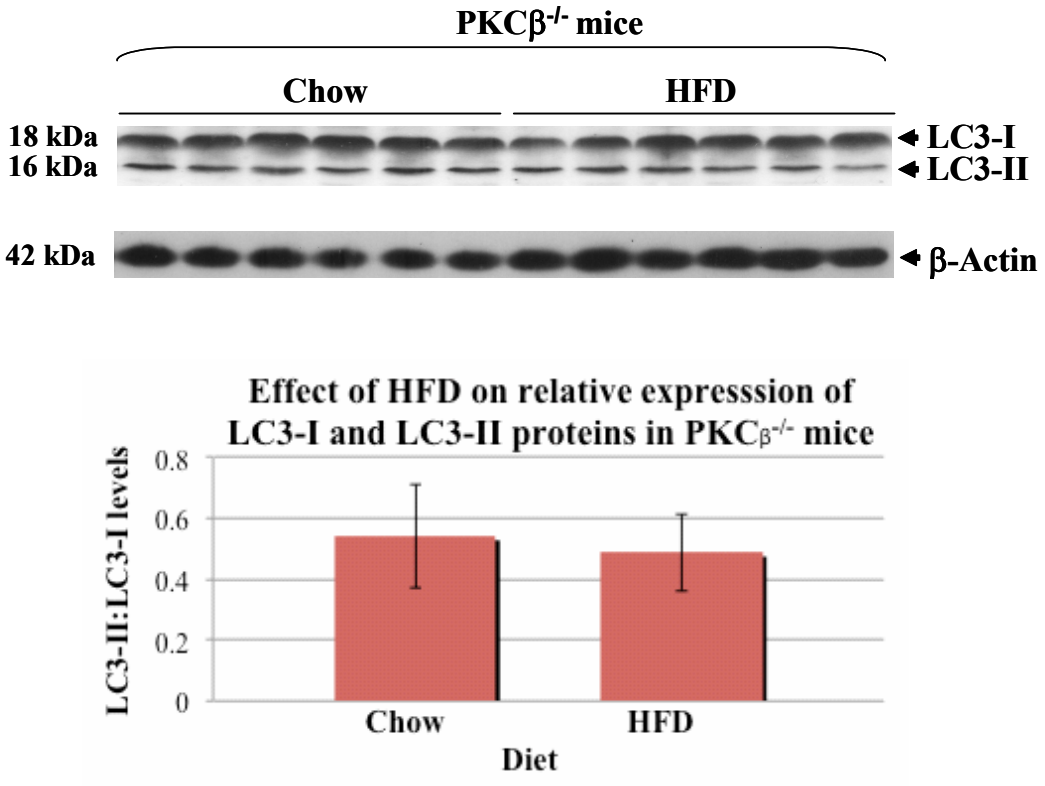


Fig. 8. Effect of HFD on relative protein expression of LC3-I and LC3-II in PKCβ$^{-/-}$ mice fed either a chow or a HFD for 16 weeks. PKCβ$^{-/-}$ mice fed either a chow diet or a HFD were examined for LC3-I, LC3-II, and β-Actin protein levels in the adipose tissue by immunoblottting using an antibody to each protein. Quantitation of immunoblot is shown in the bottom panel. Each value represents the mean±SD. n=6, (p=0.55).

DISCUSSION AND CONCLUSIONS

The results presented above highlight a novel role of PKCβ in regulating HFD-dependent suppression of adipose autophagy levels. We show that HFD-induced adipose PKCβ activation (Fig. 6) is accompanied by a simultaneous decrease in adipose autophagy levels in WT mice (Fig. 7). Most importantly, HFD was unable to significantly affect adipose autophagy levels in PKCβ$^{-/-}$ mice (Fig. 8), providing strong support for a novel role of PKCβ in regulating autophagy levels *in vivo*. Our observation that HFD reduces adipose autophagy levels is in agreement with recent reports that feeding HFD reduces autophagy and in WAT of WT mice, but the underlying mechanism was not investigated in their studies (43, 44). A defect in autophagic activity was also recently identified in liver tissues of insulin-resistant mice, and the correction of autophagy by genetic means was sufficient to restore the hepatic responsiveness to insulin, thus also supporting a strong link between defective autophagy and obesity-associated insulin resistance (45). Given that PKCβ deficiency protects from diet-induced obesity and diabetes (34), our results are also in agreement with the notion that defective regulation of autophagy could be one of the mechanisms linking caloric overload to the development of insulin resistance. It is important to note in this regard that common glucose-lowering medications, including metformin and thiazolidinediones, have also been shown to stimulate autophagy (46, 47). Lastly, an increase in autophagy has been recently shown to be required for the beneficial effects of exercise on glucose and lipid metabolism (48).

Several lines of evidence implicate mitochondrial dysfunction as a major contributor to obesity (27-31). It is likely that damaged mitochondria not only oxidize fat inefficiently, but

also generate large amounts of reactive oxygen species which are known to disrupt cellular function. Our results suggest that diet-dependent PKCβ activation contributes to a reduction in autophagic activity, which can exacerbate the accumulation of harmful cellular "garbage" including defective mitochondria. Failure to remove damaged mitochondria by a reduction in "mitophagy" is expected to make adipocytes store extra calories as triglyceride more efficiently (27-31, 39, 40).

Can a therapeutic strategy targeting adipose PKCβ be effective in treating dietary fat-induced obesity and diabetes? Given that whole body PKCβ deficiency is associated with protection from obesity possibly by increasing the percentage of healthy mitochondria in adipose tissue (25-27), it is possible that PKCβ deficiency exerts this effect through promoting mitophagy. Thus stimulating autophagy seems to be an attractive approach to improve adipose tissue adaptation to the caloric load. Before such a possibility is considered further, an important question to be answered is the specific contribution of adipose PKCβ deficiency to the lean phenotype of $PKC\beta^{-/-}$ mice. One approach to answering this question is by studying the sole contribution of adipose-specific PKCβ deficiency to the lean phenotype of $PKC\beta^{-/-}$ mice by generating tissue-specific mice using an inducible expression system (49, 50).

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