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Targeting IKK β in adipocyte lineage cells for treatment of obesity and metabolic dysfunctions

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Abstract

IKK kinase β (IKK β), a central coordinator of inflammation through activation of NF- κ B, has been identified as a potential therapeutic target for the treatment of obesity-associated metabolic dysfunctions. In this study, we evaluated an antisense oligonucleotide (ASO) inhibitor of IKK β and found that IKK β ASO ameliorated diet-induced metabolic dysfunctions in mice. Interestingly, IKK β ASO also inhibited adipocyte differentiation and reduced adiposity in high-fat (HF)-fed mice, indicating an important role of IKK β signaling in the regulation of adipocyte differentiation. Indeed, CRISPR/Cas9-mediated genomic deletion of IKK β in 3T3-L1 preadipocytes blocked these cells differentiating into adipocytes. To further elucidate the role of adipose progenitor IKK β signaling in diet-induced obesity, we generated mice that selectively lack IKK β in the white adipose lineage and confirmed the essential role of IKK β in mediating adipocyte differentiation *in vivo*. Deficiency of IKK β decreased HF-elicited adipogenesis in addition to reducing inflammation and protected mice from diet-induced obesity and insulin resistance. Further, pharmacological inhibition of IKK β also blocked human adipose stem cell differentiation. Our findings establish IKK β as a pivotal regulator of adipogenesis and suggest that overnutrition-mediated IKK β

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activation serves as an initial signal that triggers adipose progenitor cell differentiation in response to HF feeding. Inhibition of IKK β with antisense therapy may represent as a novel therapeutic approach to combat obesity and metabolic dysfunctions.

Keywords

Adipogenesis; Adipose Stem Cells; Antisense Oligonucleotide; IKK β ; Obesity

Introduction

Obesity is a rapidly growing epidemic representing a growing serious health threat in an increasing number of countries as the number of overweight and obese individuals are expected to increase to over half of the world's population by 2030 [1]. There is an urgent need to understand the mechanisms underlying obesity and obesity-related metabolic diseases. Obesity is associated with both increased adipocyte size (hypertrophy) and adipocyte number (hyperplasia). Adipocyte number is a major determinant of fat mass in adults [2, 3] and approximately 10% of the body's adipocytes are re-generated annually at all adult ages [3]. Obese individuals also have a significantly greater number of adipocytes added per year than lean individuals [2, 3], suggesting that regulation of new adipocyte production is a potential therapeutic target to treat obesity. However, the mechanisms underlying nutritionally induced hyperplasia remain largely unknown.

It is generally accepted that obesity is associated with a state of chronic low-grade inflammation that is a major contributor to type 2 diabetes and atherosclerosis [4, 5]. Many inflammatory pathways that contribute to the pathogenesis of insulin resistance and atherosclerosis are regulated by the transcriptional factor NF- κ B, a master regulator of the innate and adaptive immune responses [6]. I κ B kinase (IKK) β is the predominant catalytic subunit of the IKK complex and is required for activation of NF- κ B by inflammatory mediators in the canonical or classical activation pathway [6–9]. It has been well established that overnutrition can lead to IKK β activation *in vitro* and *in vivo* [10–12], and recent studies have implicated IKK β as a key molecular link between obesity, inflammation and metabolic disorders [5, 13, 14]. For example, diet-induced insulin resistance has been associated with the activation of IKK β /NF- κ B in multiple tissues including liver, adipose tissue, and brain [10, 15–20]. Deletion of IKK β in the liver improved diet-induced insulin resistance, and deficiency of IKK β in myeloid cells rendered global insulin sensitivity upon high-fat (HF) feeding [17]. By contrast, constitutive activation of IKK β in the liver caused systemic insulin resistance [18]. Activation of IKK β in the hypothalamus has also been linked to obesity and metabolic disease [19, 21].

We have recently demonstrated that IKK β functions in smooth muscle cells (SMCs) to regulate vascular inflammatory responses and atherosclerosis development [8]. Of particular interest is that many adipocyte precursor cells express SMC markers and ablation of IKK β blocked adipocyte differentiation *in vitro* and *in vivo*, suggesting that IKK β functions in adipocyte precursor cells to regulate adipose tissue development [8]. In the present study, we explored a novel and more efficient pharmacological approach to inhibit IKK β *in vivo* by

using antisense oligonucleotides (ASOs) and found that ASO-mediated IKK β knockdown ameliorated diet-induced obesity and metabolic disorders in mice. Interestingly, IKK β ASO also inhibited high-fat diet (HFD)-elicited adipocyte differentiation and reduced adipose tissue growth. As the functions of IKK β in regulating adipogenesis and adipose tissue development has not been fully understood, we then selectively deleted IKK β in the white adipose lineage in mice to further elucidate the role of adipose progenitor cell IKK β signaling in obesity and metabolic function. Deficiency of IKK β decreased adipogenesis and systemic inflammation elicited by HF feeding, leading to resistance to diet-induced obesity and insulin resistance. Lastly, inhibition of IKK β in human adipose stem cells also blocked adipogenesis in these cells. Our results establish IKK β as an important regulator of adipogenesis and adipose tissue development. Overnutrition-mediated IKK β activation may serve as an initial signal that triggers adipose progenitor cell differentiation in response to consumption of a HFD. Inhibition of IKK β with antisense therapy may present as a novel therapeutic approach to combat obesity and metabolic dysfunctions.

Materials and Methods

Animals

For the IKK β ASO studies, 8-week-old male C57BL/6 mice (Jackson Laboratory) were fed a normal chow diet (ND) or a Western-type HFD (21.2% fat, 0.2% cholesterol; Harlan Teklad) and received biweekly intraperitoneal injections of either a nontargeting control ASO (5'-CCTTCCCTGAAGGTTCTCC-3') or an IKK β targeted ASO (5'-GCAGACTCTCATCTCCGTC-3') for 8 weeks at a dose of 25 mg/kg body weight/week. The 20-mer phosphorothioate ASOs were designed to contain 2'-O-methoxyethyl groups at positions 1 to 5 and 15 to 20, and were generated and purified by Ionis Pharmaceuticals (Carlsbad, CA). For cell lineage analysis, PDGFR β -Cre mice [22] were crossed with Rosa26^{lacZ} reporter mice [23] to generate PDGFR β -Cre/Rosa26^{lacZ} mice. To delete IKK β in adipocyte lineage cells, mice containing loxP-flanked IKK β alleles (IKK β ^{F/F}) [8, 24] was crossed with PDGFR β -Cre transgenic mice [22] to generate PDGFR β -Cre/IKK β ^{F/F} mice (termed as IKK β ^{PDGFR β}). For obesity studies, 4-week-old male IKK β ^{F/F} and IKK β ^{PDGFR β} littermates were fed a normal chow diet (ND) or a HFD for 16 weeks until euthanization at 20 weeks of age. For the BrdU studies, 4-week-old male IKK β ^{F/F} and IKK β ^{PDGFR β} littermates were intraperitoneal injected with 50 mg/kg body weight/day BrdU (TCI America) and mice were placed on a HFD for 7 days prior to euthanization. All animals were housed in a specific pathogen-free environment with a light-dark cycle, under a protocol approved by the University of Kentucky Institutional Animal Care and Use Committee.

Metabolic analyses

Body weight was measured weekly and body composition was measured by NMR spectroscopy (Echo MRI). Intraperitoneal glucose tolerance test (GTT) and insulin tolerance test (ITT) were performed, as previously described [8]. Plasma insulin was measured using a Rat/Mouse Insulin ELISA kit (Millipore). Plasma TNF α levels were measured by a mouse TNF α ELISA kit (BioLegend). Plasma cytokine levels were measured by a mouse cytokine multiplex assay kit and a BioPlex 200 system (Bio-Rad Laboratories). For insulin stimulation studies, 20-week-old male IKK β ^{F/F} and IKK β ^{PDGFR β} littermates or ASO-

treated C57BL/6 mice were injected with insulin (0.35 U/kg body weight) into the inferior vena cava [25]. After 5 minutes, mice were euthanized and tissues were collected for protein isolation and Western blot analysis.

Adipose SV cell isolation and differentiation

Adipose SV cells and mature adipocytes were isolated as previously described [8]. The SV cells were used for RNA and protein isolation or cultured in 12-well plates for differentiation. For differentiation assays, SV cells were induced by high-glucose DMEM containing dexamethasone (1 μ M), isobutylmethylxanthine (0.5 mM), insulin (10 μ g/ml), rosiglitazone (1 μ M), and 10% FBS until they were ready for analysis [8].

RNA isolation and QPCR analysis

Total RNA was isolated from mouse tissues or cells using TRIzol Reagent (Life Technologies) and QPCR was performed using gene-specific primers and the SYBR Green PCR kit (Life Technologies) as previously described [26, 27]. The sequences of primer sets used in this study are listed in Supporting Information Table 1.

Quantification of mitochondrial DNA

Genomic DNA was extracted from subcutaneous WAT by the DNAeasy Blood and Tissue kit (Qiagen). QPCR was performed on COX1 mitochondrial DNA and normalized to a nuclear 28S sequence [28, 29]. The sequences of primer sets used in this study are listed in Supporting Information Table 1.

Human subjects and adipose stem cell isolation

Human adipocytes were derived from the differentiation of adult derived human adipose stem cells (ADHASC) as described previously [30, 31]. The adipose tissue was from a collagenase digestion of the lipoaspirate of patients undergoing liposuction of subcutaneous fat. These patients were generally young, healthy women undergoing cosmetic procedures, and this method of collection was approved by the University of Kentucky Institutional Review Board. Differentiation was induced using differentiation media as previously described [30, 31]. At least 80 to 90% of the cells developed lipid droplets in 7–10 days.

Statistical analysis

All data are presented as the mean $\pm$ SEM. Individual pairwise comparisons were analyzed by two-sample, two-tailed Student's t-test unless otherwise noted, with $P < 0.05$ was regarded as significant. Two-way analysis of variance (ANOVA) was used when multiple comparisons were made followed by a Bonferroni multiple comparisons test. N numbers are listed in figure legends.

Results

ASO-mediated IKK β knockdown protects mice from diet-induced obesity

We recently demonstrated that IKK β inhibitors can inhibit adipocyte differentiation *in vitro* and ameliorate diet-induced adiposity in mice [8]. To explore novel and more efficient

pharmacology approaches for IKK β inhibition, we utilized second-generation ASO targeting technology [32] and investigated the impact IKK β ASO on diet-induced obesity and metabolic disorders. Intraperitoneal delivery of ASO has been shown to reach a variety of tissues including liver and adipose tissue [33, 34]. Indeed, we found that IKK β ASO can efficiently decrease IKK β gene expression in multiple tissues including liver, skeletal muscle, and white adipose tissue (WAT) at a dose of 25 mg/kg BW/week (Fig. 1A). Interestingly, IKK β mRNA levels were not significantly altered in brown adipose tissue (BAT) by IKK β ASO treatment at this dose. Western blot analysis also confirmed the specific and efficient IKK β knockdown in tissues including liver and WAT as IKK α protein levels were not affected by IKK β ASO treatment (Fig. 1B).

We next determined whether IKK β ASO treatment can ameliorate diet-induced obesity. Groups of 8-week-old wild-type male mice were fed a ND or HFD and were treated with control ASO or IKK β ASO for 8 weeks (Fig. 1C). HF-feeding increased body weight in mice treated with both control and IKK β ASOs. However, IKK β ASO treatment significantly decreased HFD-induced body weight gain and adiposity. While lean mass was slightly but significantly increased in IKK β ASO-treated mice, fat mass was decreased by 45% in HFD-fed mice treated with IKK β ASO (Fig. 1D). IKK β ASO was able to decrease both subcutaneous (sub) WAT and visceral WAT including epididymal (epi) and retroperitoneal (retro) fat pads in HFD-fed mice as compared with littermate controls (Fig. 1E).

IKK β ASO improves insulin sensitivity and reverses hepatic steatosis in obese mice

Obesity is frequently associated metabolic disorders such as insulin resistance and hepatic steatosis. We next investigated whether IKK β ASO treatment can protect HFD-fed mice from these disorders. IKK β ASO-treated mice had decreased fasting plasma glucose and insulin concentrations as compared with control mice (Fig. 1F), suggesting improved diabetic phenotype. Upon glucose and insulin tolerance test, IKK β ASO-treated mice had improved glucose tolerance and showed an increased hypoglycemic response to the injected insulin (Fig. 1G). To further assess the impact of IKK β ASO treatment on insulin signaling, HFD-fed mice were injected with insulin prior to euthanization and phosphorylation of Akt was analyzed in multiple tissues. IKK β ASO was able to enhance phosphorylation of Akt in response to insulin in liver, skeletal muscle and WAT (Fig. 1H). While hepatic IKK β signaling has been demonstrated to contribute to obesity-associated insulin resistance [17, 18], the role of adipose IKK β signaling in the regulation of insulin sensitivity has not been well-defined. We then performed adipose glucose uptake assays. Interestingly, adipose tissue explants from IKK β ASO-treated mice had elevated glucose uptake in the absence of insulin and insulin stimulation further enhanced glucose uptake by adipose tissues (Fig. 1I). Therefore, the enhanced Akt phosphorylation and increased glucose uptake in adipose tissue likely contribute to the improved insulin sensitivity in IKK β ASO-treated mice. Further, HF feeding also caused lipid accumulation and hepatic steatosis in control mice. However, IKK β ASO-treated mice were protected from these detrimental effects (Fig. 1J, 1K). Consistently, hepatic triglyceride and cholesterol contents were significantly reduced in IKK β ASO-treated mice (Fig. 1L). Collectively, these results suggest that pharmacological inhibition of IKK β by ASO ameliorates obesity-associated metabolic disorders in mice.

IKK β regulates murine adipocyte differentiation

We next investigated whether IKK β ASO treatment can also affect IKK β expression in adipocyte precursor cells and affect adipocyte differentiation. Indeed, we found that IKK β expression was decreased in both adipose SV cells and mature adipocytes in IKK β ASO-treated mice (Fig. 2A). Consistent with our previous study [8], ASO-mediated IKK β knockdown diminished the ability of adipose SV cells to differentiate into adipocytes (Fig. 2B). As expected, the expression levels of adipogenic genes including PPAR γ , Zfp423, and C/EBPs were significantly decreased in epiWAT from IKK β ASO-treated mice (Fig. 2C). Further, IKK β ASO treatment also decreased the expression of a known NF- κ B target, Smad ubiquitination regulatory factor 2 (Smurf 2) (Fig. 2D). Smurf2 is an ubiquitin E3 ligase that regulates proteasome-mediated degradation of several proteins including β -catenin [8, 35, 36]. Consistently, ASO-mediated IKK β knockdown increased nuclear β -catenin protein levels in epiWAT (Fig. 2E). Since Wnt/ β -catenin signaling has been well defined to inhibit adipogenesis *in vitro* and *in vivo* [37, 38], the increased Wnt signaling likely contributes to the decreased adipogenesis in IKK β ASO-treated mice.

To further define the role of IKK β in adipogenesis, we used the CRISPR-Cas9 system to delete the IKK β gene in murine 3T3-L1 preadipocytes (Fig. 2F). We found that deletion of IKK β almost completely blocked 3T3-L1 cell differentiation (Fig. 2G). Gene expression analysis showed that mRNA levels of adipogenic genes and adipocyte markers including PPAR γ and adiponectin were significantly decreased by IKK β -deficiency (Fig. 2H). Consistently, deletion of IKK β decreased the expression of Smurf2 (Fig. 2I) and increased nuclear β -catenin accumulation (Fig. 2J), leading to increased β -catenin activity in these cells (Fig. 2K). We further confirmed that β -catenin ubiquitination was inhibited by IKK β deficiency in 3T3-L1 cells (Fig. 2L), suggesting the impact of reduced Smurf2 expression. Taken together, these results confirm the important role of IKK β in adipogenesis and indicate that pharmacological inhibition of IKK β by ASO can decrease adipogenesis and diet-induced adiposity.

Targeted deletion of IKK β in the white adipose lineage

Since ASO treatment affects many tissues and cell types, it is not clear how significantly the ASO-mediated IKK β knockdown in adipose progenitors contributes to the beneficial effects on obesity and metabolic disorder in mice. Compared with other tissues or cell types, the role of IKK β signaling in adipose progenitors has not been well-defined. To investigate the function of IKK β signaling in adipose progenitors, we sought to generate a mouse model that selectively lacks IKK β in white adipose lineage. Recent studies have identified a subset of perivascular cells as adipose progenitors which express multiple mural cell markers including platelet-derived growth factor receptor (PDGFR) β , α smooth muscle actin (SMA), and NG2 [39]. For example, both PDGFR β , a human mesenchymal stem cell (MSC) marker [40], and PDGFR α , another isoform of PDGFR, have been confirmed to be adipocyte progenitor markers by independent groups [41–45]. Consistently, we also found that the high expression levels of PDGFR β in adipose SV cells as compared with mature adipocytes (Supporting Information Fig. 1A). We then crossed PDGFR β promoter-driven Cre mice [22] with Rosa26^{lacZ} reporter mice that express β -galactosidase (lacZ) in target tissues upon Cre-mediated excision of a “Stop” sequence to generate PDGFR β -Cre/Rosa26^{lacZ} mice

(Supporting Information Fig. S1B). While none of adipose SV cells from control Rosa26^{lacZ} mice was lacZ⁺ cells, many of adipose SV cells from PDGFR β -Cre/Rosa26^{lacZ} mice were lacZ⁺ cells (Supporting Information Fig. 1C). Staining of the white adipose depots and tissue sections also indicated that PDGFR β -Cre is activated in adipose progenitors that give rise to white adipocytes as PDGFR β generated strong lacZ expression in adipocytes in addition to the vasculature (Supporting Information Fig. 1D, 1E). Taken together, our results are consistent with previous reports [39, 41] and confirm that PDGFR β is marker for adipocyte lineage cells.

We then deleted IKK β in adipocyte lineage cells by intercrossing PDGFR β -Cre mice with mice containing loxP-flanked IKK β alleles (IKK $\beta^{F/F}$) [8, 24]. The resulting PDGFR β -Cre/IKK $\beta^{F/F}$ (termed IKK $\beta^{PDGFR\beta}$) mice were viable and appeared indistinguishable from their control IKK $\beta^{F/F}$ littermates. Western blot analysis confirmed the deletion of IKK β in WAT including epiWAT and subWAT but not in BAT, liver, and skeletal muscle (Fig. 3A). As expected, PDGFR β -mediated IKK β deletion also caused reduced IKK β expression in adipose SV cells of IKK $\beta^{PDGFR\beta}$ mice (Fig. 3B). Further, IKK β deficiency reduces NF- κ B-mediated pro-inflammatory gene expression in response to LPS in adipose SV cells of IKK $\beta^{PDGFR\beta}$ mice (Fig. 3C–3G), suggesting impaired NF- κ B activation in these cells.

Deficiency of IKK β in adipocyte lineage cells renders mice resistant to diet-induced obesity

To determine the role of adipose progenitor IKK β signaling in obesity, IKK $\beta^{F/F}$ and IKK $\beta^{PDGFR\beta}$ littermates were fed a normal chow diet (ND) or a HFD for 16 weeks. There were no significant differences in body weight for ND-fed mice. HFD-feeding elicited increases in body weight in both IKK $\beta^{F/F}$ and IKK $\beta^{PDGFR\beta}$ mice. However, IKK β deficiency significantly decreased diet-induced bodyweight gain in IKK $\beta^{PDGFR\beta}$ mice as compared with IKK $\beta^{F/F}$ mice (Fig. 4A). While lean mass was not affected by IKK β deficiency, HFD-fed IKK $\beta^{PDGFR\beta}$ mice had significantly decreased fat mass as compared with control littermates (Fig. 4B).

HFD-fed IKK $\beta^{PDGFR\beta}$ mice also had decreased visceral adipose pads including epididymal and retroperitoneal fat pads. However, deficiency of IKK β did not affect the size or weight of subWAT and BAT (Fig. 4C, Supporting Information Fig. 2A, 2B). As subWAT often undergoes “browning” process which enhances oxidative metabolism and protects mice from metabolic dysfunctions [28, 46–48], we then examined whether deficiency of IKK β affects “browning” of subWAT in these mice. Interestingly, the expression levels of beige or brown adipocyte markers as well as mitochondrial function-related genes such as UCP-1 and PGC-1 α [28, 47, 48] were not affected in subWAT of IKK $\beta^{PDGFR\beta}$ mice (Supporting Information Fig. 3A). Consistently, IKK $\beta^{PDGFR\beta}$ mice also had comparable mitochondrial content in subWAT as that of control littermates (Supporting Information Fig. 3B). MRI analyses also confirmed that IKK $\beta^{PDGFR\beta}$ mice had a significantly decreased volume of visceral adipose tissue but a similar subcutaneous adipose tissue volume as compared with IKK $\beta^{F/F}$ mice under HF feeding conditions (Fig. 4D, 4E). We next analyzed the expression levels of IKK β and adipose progenitor markers in SV cells isolated from subWAT and epiWAT of control mice. Interestingly, IKK β expression levels were significantly higher in

visceral SV cells than subcutaneous SV cells. Further, the expression levels of adipose progenitor markers such as PDGFR β were much higher in visceral SV cells as compared with subcutaneous SV cells (Supporting Information Fig. 4), indicating a more abundant adipose progenitor cell population in visceral WAT. Collectively, these results demonstrate that PDGFR β -Cre-driven IKK β deficiency in the white adipose lineage decreases diet-induced obesity and visceral adiposity.

IKK β -deficient mice are protected from obesity-associated metabolic disorders

We next investigated whether deficiency of IKK β also protected mice from obesity-associated insulin resistance. IKK β ^{PDGFR β} mice had comparable glucose and insulin levels and similar glucose tolerance and insulin tolerance as that of IKK β ^{F/F} when fed a ND (Fig. 5A, 5B, Supporting Information Fig. 5). As expected, HF feeding increased plasma glucose and insulin levels in both IKK β ^{F/F} and IKK β ^{PDGFR β} mice (Fig. 5A). Although glucose concentrations were not affected by IKK β deficiency, HFD-fed IKK β ^{PDGFR β} mice had significantly decreased insulin concentrations as compared with HFD-fed control littermates (Fig. 5A). Consistently, IKK β ^{PDGFR β} mice also had improved glucose tolerance and insulin tolerance as compared with IKK β ^{F/F} mice under HF feeding conditions (Fig. 5B). PDGFR β -Cre-mediated IKK β deletion also enhanced phosphorylation of Akt in response to insulin in WAT but not in liver and skeletal muscle (Fig. 5C). Further, adipose tissue explants from IKK β ^{PDGFR β} mice had significantly increased insulin-stimulated glucose uptake compared with that of IKK β ^{F/F} mice (Fig. 5D).

Obesity-associated macrophage infiltration contributes to the development of systemic insulin resistance [49]. Although IKK β expression levels were not affected in macrophages of IKK β ^{PDGFR β} mice (Fig. 5E), macrophage infiltration was substantially decreased in WAT of IKK β ^{PDGFR β} mice (Fig. 5F, Supporting Information Fig. 6), suggesting that deficiency of IKK β in adipose lineage blocks the increased inflammatory infiltrates in WAT under obese conditions. Consistently, the mRNA levels of macrophage markers and several key pro-inflammatory cytokines including TNF α , MCP-1, and IL-1 β were significantly decreased in WAT of IKK β ^{PDGFR β} mice (Fig. 5G). We next measured plasma cytokine levels to determine whether IKK β -deficient mice also had decreased systemic inflammation. Deficiency of IKK β significantly decreased HFD-induced plasma pro-inflammatory cytokines including TNF α , MCP-1 and IL-6 in IKK β ^{PDGFR β} mice (Fig. 5H). Taken together, deficiency of IKK β improved insulin signaling in adipose tissue and protected mice from obesity-associated metabolic disorders.

Deficiency of IKK β inhibits adipogenesis in mice

We next investigated whether deficiency of IKK β can decrease HF feeding-elicited adipogenesis *in vivo*. For this experiment, IKK β ^{F/F} and IKK β ^{PDGFR β} mice were treated with BrdU during the first week of HF feeding. We then used BrdU as a marker to track newly differentiated adipocytes in these mice after 7d of HFD. Immunostaining of adipose tissue sections with BrdU antibodies revealed that IKK β ^{PDGFR β} mice had significantly decreased BrdU positive adipocytes in WAT (Fig. 6A, 6B), indicating decreased adipogenesis. We also assessed the adipogenic potential of adipose SV cells isolated from visceral adipose tissue of IKK β ^{F/F} and IKK β ^{PDGFR β} mice and confirmed that IKK β

deficiency impaired the adipogenic potential of SV cells from IKK β PDGFR β mice (Fig. 6C). Further, the expression levels of adipocyte markers and key adipogenic genes including PPAR γ , C/EBP α , and Zfp423, were significantly reduced by IKK β deficiency (Fig. 6D). Consistent with results from IKK β ASO treatment and 3T3-L1 experiments (Fig. 3), IKK β PDGFR β mice had decreased Smurf2 levels and increased nuclear β -catenin levels in epiWAT (Fig. 6E, 6F) as well as in primary adipose SV cells (Fig. 6G, 6H). Further, deficiency of IKK β substantially decreased β -catenin ubiquitination in adipose SV cells (Fig. 6I), which likely contributes to the accumulation of nuclear β -catenin in IKK β PDGFR β mice.

Inhibition of IKK β decreases adipogenesis in human adipose stem cells

In addition to murine 3T3-L1 preadipocytes and primary SV cells, we also determined whether IKK β regulates the differentiation of human adipose stem cells. Human adipose stem cells, also known as adult-derived human adipose stem cells (ADHASCs) [30, 31, 50], were isolated from healthy subjects. Consistent with previous reports [30, 31, 50], human adipose stem cells were able to efficiently differentiate into mature adipocytes *in vitro* and at least 80 to 90% of the cells developed lipid droplets in 7–10 days (Fig. 7A). Pharmacological inhibition of IKK β by BMS-345541 strongly inhibited human adipose stem cell differentiation and repressed human adipogenic gene expression (Fig. 7B). BMS-345541 treatment also led to reduced Smurf2 expression and decreased β -catenin ubiquitination, leading to increased nuclear β -catenin levels (Fig. 7C–7E), which likely contribute to the decreased adipogenesis in human adipose stem cells. Collectively, these results demonstrated IKK β as an important regulator of both human and murine adipocyte differentiation.

Discussion

As a central coordinator of inflammatory responses, IKK β signaling in multiple tissues including liver, pancreas, and brain have been associated with obesity and obesity-related metabolic dysfunctions [15, 17–19, 21, 51]. High doses of IKK β inhibitors such as salicylates have been used to treat inflammatory conditions including diabetes in humans for more than a century [52, 53]. Moreover, inhibition of IKK β activity by salicylates also protected mice against insulin resistance triggered by HFD or obesity [15, 16]. Interestingly, long-term anti-inflammatory therapy has also been associated with weight loss in human studies [54]. We recently demonstrated that salicylates and the potent IKK β inhibitor, BMS-345541, can also inhibit adipocyte differentiation in a dose-dependent manner [8]. In the current study, we demonstrated that IKK β ASO can efficiently decrease IKK β expression in various tissues including liver and WAT, and ameliorate diet-induced obesity and metabolic disorders in mice. To our knowledge, our study is the first to use ASOs targeting IKK β *in vivo* and investigate its metabolic impact. Consistent with previous reports demonstrating that hepatic IKK β signaling contribute to insulin resistance [17, 18], IKK β ASO-treated mice had improved insulin signaling in the liver. Nevertheless, knockdown of IKK β in WAT also resulted in enhanced Akt phosphorylation and increased glucose uptake. Therefore, the improved diabetic phenotype in IKK β ASO-treated mice was likely due to the repressed IKK β signaling in multiple tissues including liver and WAT. In addition to improved insulin signaling, IKK β ASO treatment also repressed IKK β expression in SV

cells, leading to decreased adipogenesis in WAT. Collectively, our studies demonstrate IKK β as a potential target for future anti-obesity drugs and provide evidence for the use of appropriate IKK β ASOs as a potential therapeutic strategy to treat obesity and metabolic disease (Supporting Information Fig. 7).

Despite substantial progress in defining transcription factors such as PPAR γ and C/EBPs in the regulation of committed preadipocyte differentiation, the initial signals that trigger adipose progenitor cells commitment to adipocyte lineage in response to overnutrition remain unknown. In addition to its well-established role as a central mediator of inflammation and immune responses, IKK β /NF- κ B signaling plays key roles in the regulation of hematopoietic cell development and stem cell differentiation [36, 55–57]. It has also been reported that NF- κ B activity increases during adipocyte differentiation [58] and IKK β /NF- κ B activation is associated with increased adipogenesis and insulin resistance in maternal obesity [59]. Further, pro-inflammatory signals have been demonstrated to be important for adipogenesis *in vivo* and many IKK β /NF- κ B activators including LPS, IL-1 β , and MCP-1 can stimulate adipogenesis and promote adipocyte differentiation [60–62]. While the role of IKK β signaling in regulating HFD-elicited tissue inflammation and insulin resistance is well recognized [10, 13, 14], it remained elusive if activation of IKK β also mediates adipogenesis and adipose tissue growth in response to overnutrition. Since ASOs affects many cell types, we then selectively deleted IKK β in white adipose lineage in mice and confirmed that deficiency of IKK β in adipocyte lineage cells decreased adipogenesis and systemic inflammation elicited by HF feeding, leading to resistance to diet-induced obesity and insulin resistance.

Intriguingly, IKK β ^{PDGFR β} mice displayed no significant phenotype under standard laboratory conditions but were resistant to obesity when challenged with a HFD, indicating that HFD-induced IKK β activation in adipose progenitors is essential for HFD-induced adipose tissue growth. Consistent with our previous report [8], deficiency of IKK β significantly decreased Smurf 2 expression and substantially inhibited β -catenin ubiquitination in adipose SV cells, leading to accumulation of nuclear β -catenin and increased β -catenin activity. Wnt/ β -catenin signaling plays an important role in the regulation of MSC lineage and has been well defined to inhibit adipogenesis *in vitro* and *in vivo* [37, 38, 63]. Consistently, Cre-mediated IKK β deletion or ASO-mediated IKK β knockdown impaired the adipogenic potential of adipose SV cells in mice. We also demonstrated, for the first time, that inhibition of IKK β activity can also block human adipose stem cells differentiation, suggesting that IKK β plays an important role in regulation of both murine and human adipocyte differentiation.

It is worth noting that IKK β ^{PDGFR β} mice had decreased visceral adipose tissue but comparable subcutaneous adipose tissue as control mice when fed a HFD. There is a major ontogenetic difference between visceral and subcutaneous fat as they have different developmental origins [64]. Wang et al. [46] demonstrated that different fat depots have extensive differences in adipogenic potential. Visceral adipose tissue has a high capacity for adipogenesis *in vivo* but subcutaneous adipogenesis is limited [46]. A recent study also confirmed that HF feeding rapidly and specifically activates adipogenesis in visceral but not subcutaneous depots in mice [65]. Further, subcutaneous fat depot can also undergo

extensive “browning” process after cold exposure and the appearance brown-like cells or beige cells are mainly found in subcutaneous fat but not in visceral fat [46]. It has been recently demonstrated that the PDGFR β -positive adipocyte lineage cells contribute to beige adipogenesis after prolonged cold exposure [45]. While deficiency of IKK β did not affect the beige or brown adipocyte marker expression or mitochondrial content in subWAT of IKK β PDGFR β mice after HF feeding, it would be of interest to investigate whether IKK β PDGFR β mice have affected beige adipocyte formation or mitochondrial function after chronic cold exposure in the future. Consistent with previous studies demonstrating that human visceral adipose tissue expresses high levels of IKK β as compared with subcutaneous adipose tissue [66], we also found that IKK β expression levels were significantly higher in murine visceral SV cells than in murine subcutaneous SV cells. Moreover, the expression levels of adipose progenitor markers including PDGFR β were much higher in visceral SV cells than in subcutaneous SV cells. It is plausible that visceral adipose tissue has more abundant adipose progenitor population and activation of IKK β signaling is required for these cells differentiating into adipocytes in response to HF feeding. Deletion of IKK β in adipocyte lineage cells therefore has more impact on visceral adipose tissue growth than that of subcutaneous adipose tissue when challenged with a HFD. Further studies will be required to determine the detailed mechanisms that account for the different effects of IKK β deficiency on visceral vs. subcutaneous fat. While both subcutaneous and visceral fat are increased in obese mice and human, accumulation of visceral but not subcutaneous adipose tissue contributes to the increased risk of obesity-associated metabolic dysfunctions [67]. Our data suggest that targeting IKK β in adipocyte lineage cells may represent a novel therapeutic approach to reduce visceral adipose tissue mass in obesity.

Conclusion

Our study revealed a pivotal role of IKK β in the regulation of adipocyte differentiation and adipose tissue growth in obesity. ASO-mediated IKK β knockdown protected mice from diet-induced obesity and metabolic dysfunctions. Deficiency of IKK β in adipocyte lineage cells also inhibited HF feeding-elicited adipocyte differentiation and adipose tissue growth, leading to resistance to obesity and insulin resistance. Our findings suggest that overnutrition-induced IKK β activation in adipose progenitors is an important trigger for adipocyte differentiation and inhibition of IKK β with antisense therapy may represent as a novel therapeutic approach to combat obesity and related metabolic dysfunctions.

Supplementary Material

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Figure 1.

Pharmacological inhibition of IKKβ with ASOs protects mice from diet-induced obesity, improves insulin sensitivity, and reverses hepatic steatosis in obese mice. (A): IKKβ mRNA expression in liver, kidney, spleen, skeletal muscle (Sk.M.), brown adipose tissue (BAT), subcutaneous white adipose tissue (subWAT), retroperitoneal WAT (retroWAT), and epididymal WAT (epiWAT) from mice treated with control ASO or IKKβ ASO for 8 weeks (n=6–10; *P<0.05, ***P<0.001, assessed by Student's *t*-test). (B): Western blot analysis of IKKβ and IKKα expression in liver and epiWAT from mice treated with control or IKKβ

ASO for 4 weeks. **(C, D)**: Growth curves (C), and fat mass and lean mass (D) of ND and HFD-fed mice treated with control ASO or IKK β ASO (n=10 for ND and 30 for HFD; * P <0.05, ** P <0.01, and *** P <0.001, assessed by two-way ANOVA). **(E)**: Representative images of adipose depots (top) and weight of adipose depots (bottom) from mice treated with control or IKK β ASO for 8 weeks (n=20; *** P <0.001, assessed by Student's t -test). Results are presented as means $\pm$ SEM. **(F)**: Fasting plasma glucose and insulin levels of HFD-fed mice treated with control or IKK β ASO (n=29–30; *** P <0.001, assessed by Student's t -test). **(G)**: Intraperitoneal glucose tolerance test (IPGTT), intraperitoneal insulin tolerance tests (IPITT), and area of curve (AUC) of IPGTT and IPITT of HFD-fed mice treated with control or IKK β ASO (n=8–10; ** P <0.01, *** P <0.001, assessed by Student's t -test). **(H)**: Western blot analysis of phosphorylated Akt and total Akt levels in liver, epiWAT, and skeletal muscle of control or IKK β ASO-treated mice injected with saline or 0.35U/kg body weight. **(I)**: Glucose uptake was measured in primary adipose tissues from mice treated with control or IKK β ASO (n=9; ** P <0.01, *** P <0.001, assessed by two-way ANOVA). **(J, K)**: Representative appearance (J) and hematoxylin and eosin (top) and Oil-red-O (bottom) stained sections (K) of livers from mice treated with control or IKK β ASO (scale bar =100 μ m). **(L)**: Hepatic cholesterol and triglyceride levels of mice treated with control or IKK β ASO (n=10; *** P <0.001, assessed by Student's t -test). Results are presented as means $\pm$ SEM.

Figure 2.

IKK β regulates murine adipocyte differentiation. (A): IKK β mRNA expression in adipose SVF and mature adipocytes isolated from epiWAT of mice treated with control or IKK β ASO (n=6; ** P <0.01, assessed by Student's t -test). (B): Oil-red-O staining of adipose SV cells isolated from epiWAT of mice treated with control and IKK β ASO induced by differentiation media (scale bar=100 μ m, bottom panels). (C): Expression of adipogenic genes in epiWAT of control and IKK β ASO treated mice was measure by QPCR (n=6; ** P <0.01, *** P <0.001, assessed by Student's t -test). (D): Western blot analysis of Smurf2 protein levels in epiWAT. (E): Western blot analysis of nuclear β -catenin levels in epiWAT.

(F): Western blot analysis of IKK β and IKK α protein levels in control 3T3-L1 cells or CRISPR-mediated IKK β -deficient 3T3-L1 cells. (G): Oil-red-O staining of control and IKK β -deficient 3T3-L1 cells induced by differentiation media (scale bar=100 μ m, bottom panels). (H): Expression of adipogenic genes and adipocyte markers in control or IKK β -deficient 3T3-L1 cells (n=6; ** P <0.01, *** P <0.001, assessed by Student's t -test). (I): Western blot analysis of Smurf2 protein levels in control or IKK β -deficient 3T3-L1 cells. (J): Western blot analysis of nuclear β -catenin protein levels in control or IKK β -deficient 3T3-L1 cells. (K): β -catenin reporter (TOP-flash reporter) activity in control or IKK β -deficient 3T3-L1 cells (n=6; *** P <0.001, assessed by Student's t -test). (L): Control or IKK β -deficient 3T3-L1 cells were treated with vehicle control or 100 nM PS-341, as indicated, for 4 h. β -catenin was immunoprecipitated with anti- β -catenin antibodies and then probed with anti-ubiquitin monoclonal antibodies. The whole cell lysates were probed with anti- β -catenin antibodies as an internal control. Results are presented as means $\pm$ SEM.

Figure 3.

Generation of mice lacking IKKβ in the white adipose lineage. (A): Western blot analysis of IKKβ and IKKα protein levels in epiWAT, subWAT, liver, BAT, and skeletal muscle (Sk.M.) of IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice. (B): Western blot analysis of IKKβ and IKKα protein levels in adipose SV cells isolated from IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice. (C–G): Adipose SV cells isolated from IKKβ^{F/F} and IKKβ^{ΔPDGFRβ} mice were treated with LPS for 3 h. The expression levels for proinflammatory cytokines including TNFα (C), MCP-1 (D), IL-1α (E), IL-1β (F), and IL-6 (G) were examined by QPCR (n=5; ***P*<0.01, ****P*<0.001, assessed by two-way ANOVA). Results are presented as means ± SEM.

Figure 4. Deficiency of $IKK\beta$ in adipocyte lineage cells renders mice resistant to diet-induced obesity. (A): Growth curves of ND or HFD-fed $IKK\beta^{F/F}$ and $IKK\beta^{\Delta PDGFR\beta}$ mice ($n=9-18$, *** $P < 0.001$ when comparing HFD-fed $IKK\beta^{F/F}$ mice to HFD-fed $IKK\beta^{\Delta PDGFR\beta}$ mice, assessed by Student's t -test). (B): Body weight, fat mass, percentage of fat, and lean mass of 10-week-old $IKK\beta^{F/F}$ and $IKK\beta^{\Delta PDGFR\beta}$ mice fed a ND or HFD for 16 weeks ($n=7-19$; ** $P < 0.01$, *** $P < 0.001$, assessed by two-way ANOVA). (C): Representative photographs of epiWAT, retroWAT, subWAT, and BAT from ND or HFD-fed $IKK\beta^{F/F}$ and $IKK\beta^{\Delta PDGFR\beta}$ mice. (D, E): Representative coronal section MRI images (D) and visceral and subcutaneous adipose tissue volume (E) of HFD-fed $IKK\beta^{F/F}$ and $IKK\beta^{\Delta PDGFR\beta}$ mice ($n=3$; *** $P < 0.001$, assessed by Student's t -test). Results are presented as means $\pm$ SEM.

Figure 5.

IKK β -deficient mice are protected from obesity-associated metabolic disorders. (A): Fasting plasma glucose and insulin levels in ND or HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice ($n=5-11$; $**P<0.01$, $***P<0.001$, assessed by two-way ANOVA). (B): IPGTT, IPITT, and area under the curve (AUC) of IPGTT and IPITT of HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice ($n=6-11$; $*P<0.05$, $**P<0.01$, $***P<0.001$, assessed by Student's t -test). (C): Western blot analysis of phosphorylated Akt and total Akt levels in epiWAT, liver, and skeletal muscle of IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice injected with saline or 0.35U/kg body weight. (D): Glucose uptake was measured in primary adipose tissues from HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice ($n=9$; $***P<0.001$, assessed by two-way ANOVA). (E): Western blot analysis of IKK β protein levels in peritoneal macrophages of IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice. (F): Representative immunohistochemistry for the macrophage marker, F4/80 in epiWAT from HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice (scale bar = 100 μ m). (G): The expression levels of pro-inflammatory genes and macrophage markers in epiWAT of HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice were measured by QPCR ($n=5$; $*P<0.05$, $**P<0.01$, $***P<0.001$, assessed by Student's t -test). (H): Plasma cytokine levels of ND or HFD-fed IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice ($n=5-7$; $**P<0.01$, $***P<0.001$, assessed by two-way ANOVA). Results are presented as means $\pm$ SEM.

Figure 6.

Deficiency of IKK β inhibits adipogenesis in mice. (A, B): Representative images (A) and quantification (B) of immunostaining for BrdU in epiWAT from BrdU-treated IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice fed a HFD for 7 days ($n=4-5$; $**P<0.01$, significance assessed by Student's t -test). The nuclei were stained with DAPI (blue) and the BrdU-positive cells are indicated by arrows. (C): Oil-red-O staining of adipose SV cells isolated from epiWAT of IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice induced by differentiation media. (scale bar=100 μ m, bottom panels). (D): The expression levels of adipogenic genes and adipocyte markers in adipose SV cells of IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice were measured by QPCR ($n=5-8$; $*P<0.05$, $**P<0.01$, $***P<0.001$, assessed by Student's t -test). (E, F): Western blot analysis of Smurf2 protein levels (E) and nuclear β -catenin protein levels (F) in epiWAT of IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice. (G, H): Western blot analysis of Smurf2 protein levels (G) and nuclear β -catenin protein levels (H) of adipose SV cells isolated from IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice. (I): Adipose SV cells isolated from IKK $\beta^{F/F}$ and IKK $\beta^{\Delta PDGFR\beta}$ mice were treated with vehicle control or 100 nM PS-341, as indicated, for 4 h. β -catenin was immunoprecipitated with anti- β -catenin antibodies and then probed with anti-ubiquitin monoclonal antibodies. The whole cell lysates were probed with anti- β -catenin antibodies as an internal control. Results are presented as means $\pm$ SEM.

Figure 7.

Inhibition of IKK β decreases adipogenesis in human adipose stem cells. **(A)**: Oil-redO staining of adult-derived human adipose stem cells induced by differentiation media or media containing 5 μ M IKK β inhibitor BMS-345541 (scale bar=100 μ m, bottom panels). **(B)**: The expression levels of adipogenic genes and adipocyte markers of human adipose stem cells treated with vehicle control or 5 μ M BMS-345541 were measured by QPCR (n=4; * P <0.05, ** P <0.01, *** P <0.001, assessed by Student's t -test). **(C, D)**: Western blot analysis of Smurf2 protein levels (C) and nuclear β -catenin protein levels (D) in control or BMS-345541-treated human adipose stem cells. **(E)**: Control or BMS-345541-treated human adipose stem cells were treated with vehicle control or 100 nM PS-341, as indicated,

for 4 h. β -catenin was immunoprecipitated with anti- β -catenin antibodies and then probed with anti-ubiquitin monoclonal antibodies. The whole cell lysates were probed with anti- β -catenin antibodies as an internal control. ** $p < 0.01$, *** $p < 0.001$. Results are presented as means $\pm$ SEM.

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