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Hepatic protein kinase Cbeta deficiency mitigates late-onset obesity

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Although aging is associated with progressive adiposity and a decline in liver function, the underlying molecular mechanisms and metabolic interplay are incompletely understood. Here, we demonstrate that aging induces hepatic protein kinase Cbeta (PKCβ) expression, while hepatocyte PKCβ deficiency ($PKC\beta^{Hep-/-}$) in mice significantly attenuates obesity in aged mice fed a high-fat diet. Compared with control $PKC\beta^{fl/fl}$ mice, $PKC\beta^{Hep-/-}$ mice showed elevated energy expenditure with augmentation of oxygen consumption and carbon dioxide production which was dependent on β3-adrenergic receptor signaling, thereby favoring negative energy balance. This effect was accompanied by induction of thermogenic genes in brown adipose tissue (BAT) and increased BAT respiratory capacity, as well as a shift to oxidative muscle fiber type with an improved mitochondrial function, thereby enhancing oxidative capacity of thermogenic tissues. Furthermore, in $PKC\beta^{Hep-/-}$ mice, we determined that PKCβ overexpression in the liver mitigated elevated expression of thermogenic genes in BAT. In conclusion, our study thus establishes hepatocyte PKCβ induction as a critical component of pathophysiological energy metabolism by promoting progressive hepatic and extrahepatic metabolic derangements in energy homeostasis, contributing to late-onset obesity. These findings have potential implications for augmenting thermogenesis as a means of combating aging-induced obesity.

Both obesity and aging have seen dramatic increases in prevalence throughout the world (1). In modern society, obesity is basically late onset, and its incidence apparently increases with age, likely due to a combination of genetic and environmental factors (https://www.who.int/en/news-room/fact-sheets/detail/obesity-and-overweight, Accessed 25 November 2022) (https://www.cdc.gov/obesity/data/adult.html, Accessed 25 November 2022). Although advanced age is a risk factor for obesity, a clear understanding of the changes that occur as we age that contribute to the development of obesity is currently lacking. Emerging evidence suggest that the thermoregulatory mechanisms associated with the regulation of body weight are complex because body weight changes considerably in the development phase and is affected by various environmental factors. Available data further indicates that dysregulation of the thermoregulatory mechanisms implicated in body weight regulation play a role in the etiology of obesity (2–4). Aging is accompanied by a loss of classical brown adipocytes as well as the brown-like adipocytes found in white adipose tissue (WAT), suggesting that loss of their energy expenditure capacity might contribute to an obesity-prone phenotype with increased age. Under obesogenic conditions, such as excessive and prolonged consumption of high-fat diet, energy intake exceeds expenditure, causing an accumulation of lipids in adipose tissues and ectopically in non-adipose tissues where excess lipids are stored as intramyocellular lipids (5). In extant research, several genes related to thermogenesis have been considered as candidate genes for human obesity and aging (6–9). Notably, age-related decline in β-adrenergic responsiveness and the virtual absence of uncoupling protein-1 (UCP-1) in adult humans also suggest a relationship between the reduced thermogenic ability and increased susceptibility to obesity with age (10, 11). Because obesity at a later age is a risk factor for life-threatening conditions such as insulin resistance, type 2 diabetes, and cardiovascular disease, understanding the causal molecular mechanisms of age-related obesity may provide a strategy for treating these disorders. Therefore, lack of effective therapies for obesity is a serious challenge and requires immediate attention.

The liver plays a key role in energy homeostasis and constantly adapts to meet the body's energy requirements of the whole body during the postprandial state, particularly during times of increased lipid burden (12, 13). Located at the nexus of the portal and various circulations, the hepatocyte is uniquely positioned to be the central hub of inter-tissue communication. There is also evidence that the liver plays a pivotal role in the aging of mammals *via* the modulation of a variety of metabolic pathways (14). Accordingly, interdicting hepatocyte signaling pathways are potential therapeutic targets for aging-induced metabolic diseases (15). Protein kinase Cbeta (PKCβ) has recently emerged as a critical regulator of hepatic lipid and glucose homeostasis, largely due to its

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downstream transcriptional regulation of critical genes to help the body's adaptation to energetic stress (16–18). Consequently, hepatocyte PKCβ inactivation is associated with protection from diet-induced hepatic steatosis and elevated glycogen accumulation (19, 20). Consistent with a central role of PKCβ in regulating energy metabolism, dietary fat intake induces hepatic PKCβ expression (21, 22), whereas exercise is shown to repress diet-induced hepatic expression (23). Furthermore, PKCβ is inhibited by beneficial omega fatty acids (24, 25). and activated by diacylglycerol and cholesterol, which were reported to be accumulated in obese liver (26, 27).

Guided by these findings, the present study aimed to examine the combinatory role of aging and high-fat diet on the development of obesity. Our findings indicate that aging is associated with hepatic PKCβ induction, whereas hepatocyte-specific PKCβ deficiency is related to increased BAT thermogenic gene expression and the resulting increase in energy expenditure. Conversely, hepatocyte-specific PKCβ overexpression was found to suppress the expression of thermogenic genes. These results establish a pivotal role for hepatic PKCβ induction in progressive obesity during aging and provide insight into the mechanisms by which aging becomes a risk factor for the development of obesity.

Results

Liver expression of PKCβ is elevated in aging

We analyzed and compared the expression of *Pkcβ* gene in the liver from young and old mice fed either chow or high-fat and high-cholesterol (HFHC; 45% calories derived from fat plus 1% cholesterol) diet and observed significant differences. Consistent with previous reports (21, 22), our present analysis indicated that HFHC diet-induced obesity increases the expression of *Pkcβ* in the liver and contributes to metabolic dysfunction during diet-induced obesity (Fig. 1). Furthermore, expression levels for *Pkcβ* was 1.8-fold higher in older livers compared to young livers from mice fed a chow diet. Moreover, an age-related increase in liver *Pkcβ* expression was even higher in livers of aged mice fed HFHC diet. Our results therefore indicate that aging-induced metabolic dysfunction under obesogenic conditions may be dependent on the hepatic PKCβ-mediated pathways.

Hepatocyte-specific deletion of PKCβ protects from late-onset obesity

To understand the role of hepatic PKCβ in aging-induced obesity, we subjected control $PKC\beta^{fl/fl}$ and hepatocyte-specific PKCβ-deficient ($PKC\beta^{Hep-/-}$) mice to HFHC diet feeding for 16 months and monitored their food intake and body weight gain over time. As shown in Figure 2*A*, body weights were identical between the two genotypes, which is consistent with previous results; however, their growth curves started to diverge at around 8 months of age. $PKC\beta^{Hep-/-}$ mice did not accumulate as much weight as $PKC\beta^{fl/fl}$ littermates with age and the average weight of these mice for the period between 8 to 12 months of age was 8% to 10% lower compared to $PKC\beta^{fl/fl}$ controls. The weights of the epididymal WAT (eWAT) and brown adipose tissue (BAT) were significantly reduced in aged $PKC\beta^{Hep-/-}$ mice compared with control mice (Fig. 2*B*). Consistently, eWAT (1.56 *versus* 2.29 × 10^4 μm^2) adipocyte size was significantly lower in aged $PKC\beta^{Hep-/-}$ mice than in control mice. The reduction in body weight and adiposity in $PKC\beta^{Hep-/-}$ mice was associated with reduced liver mass (Fig. 2*B*). Compared to control mice, aged $PKC\beta^{Hep-/-}$ mice had less fat accumulation in the liver (87

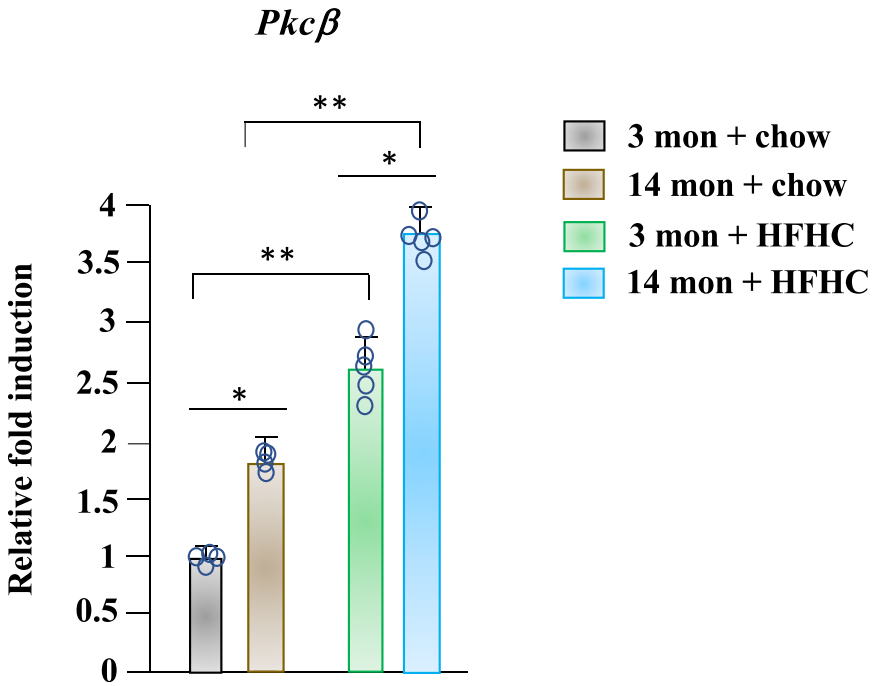

Figure 1. Effects of age and HFHC diet on hepatic *Pkcβ* expression in C57BL/6J mice fed either a chow or HFHC diet. The expression levels of liver *Pkcβ* in mice were examined by qPCR. Data are presented as mean ± SD, n = 8 to 10 mice per genotype. $^{**}p < 0.05$; $^{**}p < 0.01$.

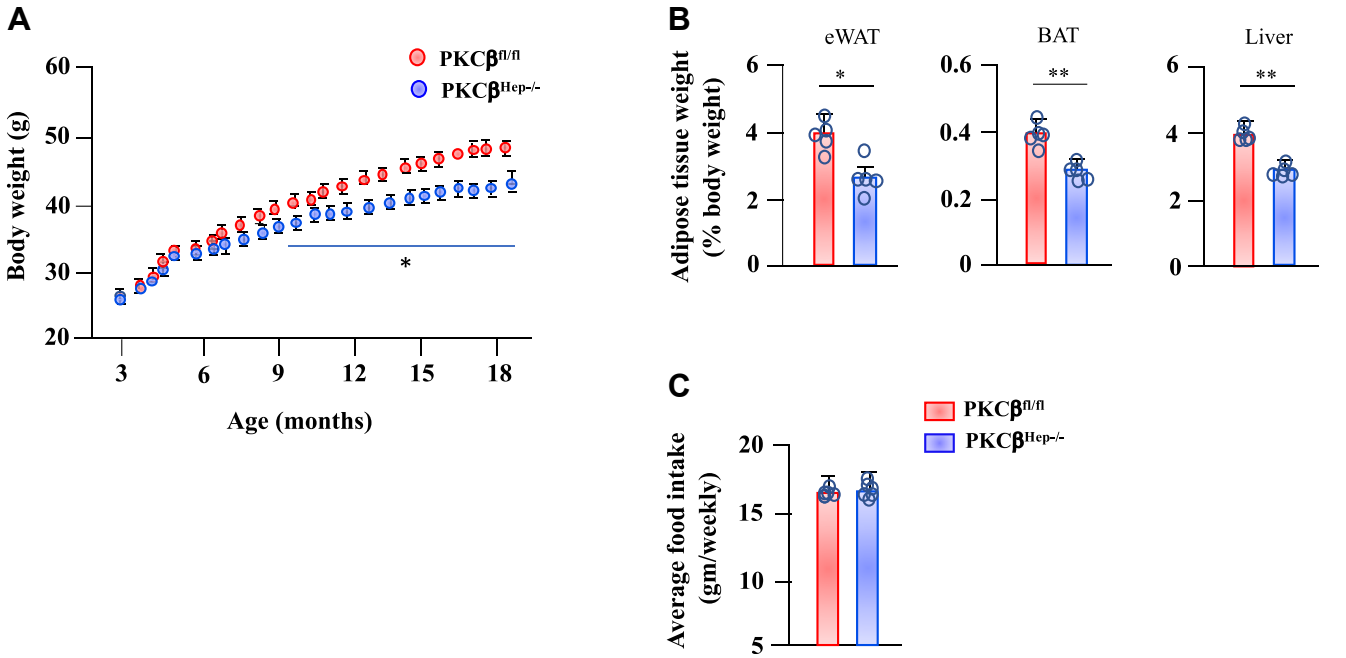

Figure 2. Attenuated aging-induced obesity in PKCβ$^{Hep-/-}$ mice under HFHC conditions. *A*, PKCβ$^{fl/fl}$ and PKCβ$^{Hep-/-}$ mice were fed HFHC diet starting at week 8 after birth. Weight gain of both genotypes over a time period of next 64 weeks were determined (n = 12/genotype). The average body weight of each group of mice between 35 and 75 weeks were compared by unpaired *t* test. *B*, epididymal WAT, interscapular BAT, and liver mass of 1.5-year-old male mice (n = 10 per genotype for WAT; n = 8 per genotype for BAT). *C*, food intake of approximately 1-year-old mice. Each data point represents an average measurement from a single cage. Data are presented as mean ± SD. Statistical analysis: unpaired two-tailed *t* test. *$p < 0.05$; ** < 0.01.

versus 46 mg/g). Despite reduced weight, the food intake of PKCβ$^{Hep-/-}$ mice was comparable to control PKCβ$^{fl/fl}$ littermates (Fig. 2*C*).

Hepatocyte PKCβ inactivation potentiates energy expenditure and thermogenesis in peripheral tissues

To determine whether there were differences in energy expenditure that would account for the differences in body weights and fat mass in HFHC-fed PKCβ$^{Hep-/-}$ mice, indirect calorimetry was measured. We measured whole body energy expenditure between PKCβ$^{Hep-/-}$ and PKCβ$^{fl/fl}$ mice by monitoring oxygen and carbon dioxide production, locomotor activity, and thermogenesis using indirect calorimetry at ambient temperature (22 °C) and during a cold challenge at 4 °C. Remarkably, PKCβ$^{Hep-/-}$ mice exhibited a significant increase in basal oxygen consumption and carbon dioxide production compared to their control counterparts in both light and dark cycles, particularly during the dark cycle, without affecting either locomotor activity or respiratory exchange ratios (Fig. 3, *A* and *B*). Heat production was significantly elevated in PKCβ$^{Hep-/-}$ mice under all conditions. The above data suggest that the changes in energy expenditure associated with hepatic PKCβ inactivation are significant, driving the resistance to diet-induced adiposity seen in PKCβ$^{Hep-/-}$ mice.

To determine the molecular underpinning of increased energy expenditure, we examined the expression of a signature group of genes involved in energy expenditure and fat metabolism. We found that PKCβ$^{Hep-/-}$ mice exhibit marked increase in FGF21 protein levels in liver and a milder increase in both BAT and iWAT (Fig. 4, *A*, *D*, and *E*). As expected, PKCβ$^{Hep-/-}$ mice exhibited increased hepatic expression of fibroblast growth factor 21 (*Fgf21*) mRNA and circulating amounts of FGF21 in the blood relative to control mice (Fig. 4, *B* and *C*). It is interesting to note in this regard that we recently reported that hepatic PKCβ deficiency reduces endogenous hepatic FoxO1 protein level (19) which is reported to negatively regulate hepatic *Fgf21* expression (28). Furthermore, PKCβ$^{Hep-/-}$ mice show elevated peroxisome proliferator-activated receptor-alpha (PGC-1α) and UCP-1 protein levels specifically in BAT and not in inguinal WAT (iWAT) (Fig. 4, *D* and *E*). There were no changes in expression of UCP-2 and UCP-3 in BAT, whereas the expression of these genes was slightly lower in the iWAT of PKCβ$^{Hep-/-}$ mice relative to the control (Fig. 4, *D* and *E*). In addition, P-ERK-1/2 levels were elevated in iWAT of PKCβ$^{Hep-/-}$ mice relative to control mice (Fig. 4*E*), suggesting enhanced lipolysis in WAT (29). In contrast, we did not observe any significant differences in expression levels of above proteins in PKCβ$^{Hep-/-}$ skeletal muscle (Fig. 4*F*).

Skeletal muscles are a major contributor to basal metabolic rate, we therefore examined skeletal muscles from the above mice (30). Inactivation of PKCβ in hepatocytes resulted in a shift from glycolytic to oxidative in fast fiber-enriched gastrocnemius and quad muscles of PKCβ$^{Hep-/-}$ mice. An increase in the relative expression of transcript encoding slow myosin heavy chain 7 (*Myh7*) in G/P and quadriceps of PKCβ$^{Hep-/-}$ mice compared to control muscles was observed (Fig. 4*G*). We also found a decrease in the relative expression of transcript encoding fast *Myh2*, suggesting a muscle fiber type switching from fast to slow in more oxidative fibers in gastrocnemius and quadriceps of PKCβ$^{Hep-/-}$ mice (Fig. 4*G*).

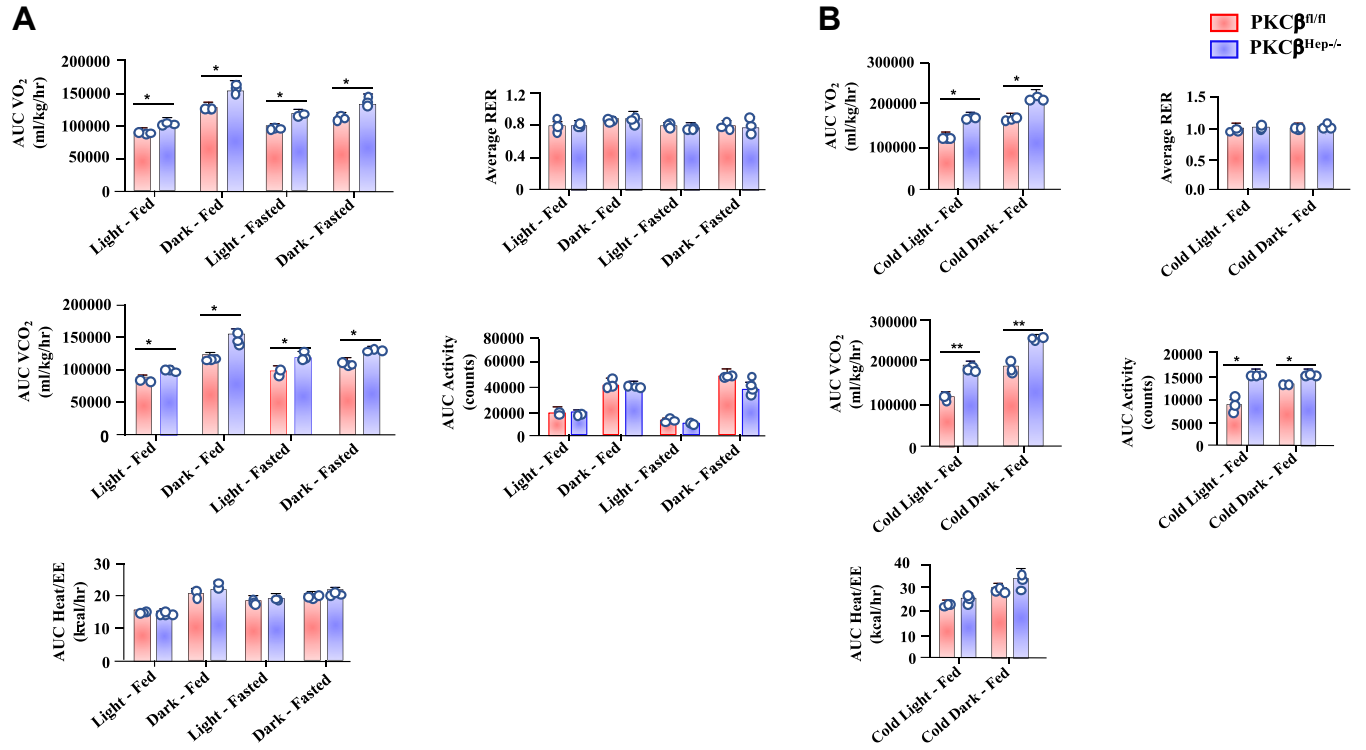

Figure 3. Hepatocyte-specific PKCβ deficiency promotes energy expenditure. *A*, HFHC-fed PKCβ$^{Hep-/-}$ mice show higher metabolic rates than HFHC-fed PKCβ$^{fl/fl}$ mice at room temperature. CLAMS measured O_2 consumption, CO_2 production, and heat generation; bar graphs indicate average O_2 consumption, CO_2 production or heat production during day and night. *B*, energy expenditure measured at 4 °C in the above sets of mice. Data are presented as mean ± SD. *$p < 0.05$ *versus* control mice. AUC, Area under the curve.

In agreement with this possibility, the quadricep muscle exhibited increase in the expression of mitochondrial markers succinate dehydrogenase (*Sdhb, Sdhc, Sdhd*) (Fig. 4*H*). Moreover, a decrease in slow *Myh3* and *Myh7* and a decrease in *Sdh*'s in soleus muscle of PKCβ$^{Hep-/-}$ mice was also observed (Fig. 4*H*). The age-related slowing of the muscle, due to a combination of an increased percentage of slow fibers as there is selective atrophy of type II fibers) and slowing of, in particular, type I fibers, will aggravate the loss of muscle function. These results suggested that the increase in energy expenditure of PKCβ$^{Hep-/-}$ mice during aging may also result from improved muscle function.

The above changes were observed in the absence of any significant changes in proinflammatory cytokines IL-1β and TNF-α between genotypes (Fig. 4*I*).

To determine further if the above changes in thermogenic gene expression altered the function of peripheral tissues, we performed high-resolution respiratory capacity of BAT and muscle. Mitochondrial respiration was sequentially measured with substrate present (basal respiration) and in the presence of ADP (complex V respiration) or FCCP (maximal respiration). Interestingly, basal mitochondrial OCR was significantly higher in PKCβ$^{Hep-/-}$ BAT (Fig. 5, *A* and *B*), and when provided with Rotenone, succinate, Antimycin A, and TMPD, mitochondrial OCR from PKCβ$^{Hep-/-}$ BAT were significantly lower (Fig. 5*A*). These results suggest that in PKCβ$^{Hep-/-}$ BAT, mitochondrial respiratory complex I is responsible for enhanced OCR, as substrates that activate other complexes do not increase OCR. Likewise, OCR under uncoupling and coupling conditions were also significantly increased in mitochondria from PKCβ$^{Hep-/-}$ gastrocnemius and plantaris muscle at baseline, in the presence of Antimycin A, Oligomycin, FCCP, and Antimycin/Rotenone, and insignificantly increased with Succinate and TMPD (Fig. 5, *C* and *D*). All provided substrates augmented OCR in mitochondria from PKCβ$^{Hep-/-}$ iWAT and GC, suggesting that all complexes within the electron transport chain within these tissues have significantly increased activity, which contributes to overall enhanced mitochondrial functions. Taken together, reduced obesity in PKCβ$^{Hep-/-}$ mice is linked to improvement in mitochondrial function in peripheral metabolic tissues, augmenting energy expenditure.

PKCβ$^{Hep-/-}$ mice exhibit increased energy expenditure in a β3-AR-dependent manner

The liver also has a nervous system containing both afferent and efferent neurons that are involved in energy homeostasis (31, 32). The afferent arm includes the sensation of metabolites which triggers the nervous system to make appropriate physiological changes. This prompted us to investigate the impact of hepatic PKCβ inactivation on the activity of adrenergic pathways in response to catecholamine stimulation. To parse out the potential contribution of β3-adrenergic receptor (β3-AR) signaling, we carried out energy expenditure studies by administering a specific β3-AR antagonist, L748,337. We found that L748,337 abolished this increase in energy expenditure (Fig. 6, *A* and *B*), without affecting either locomotor

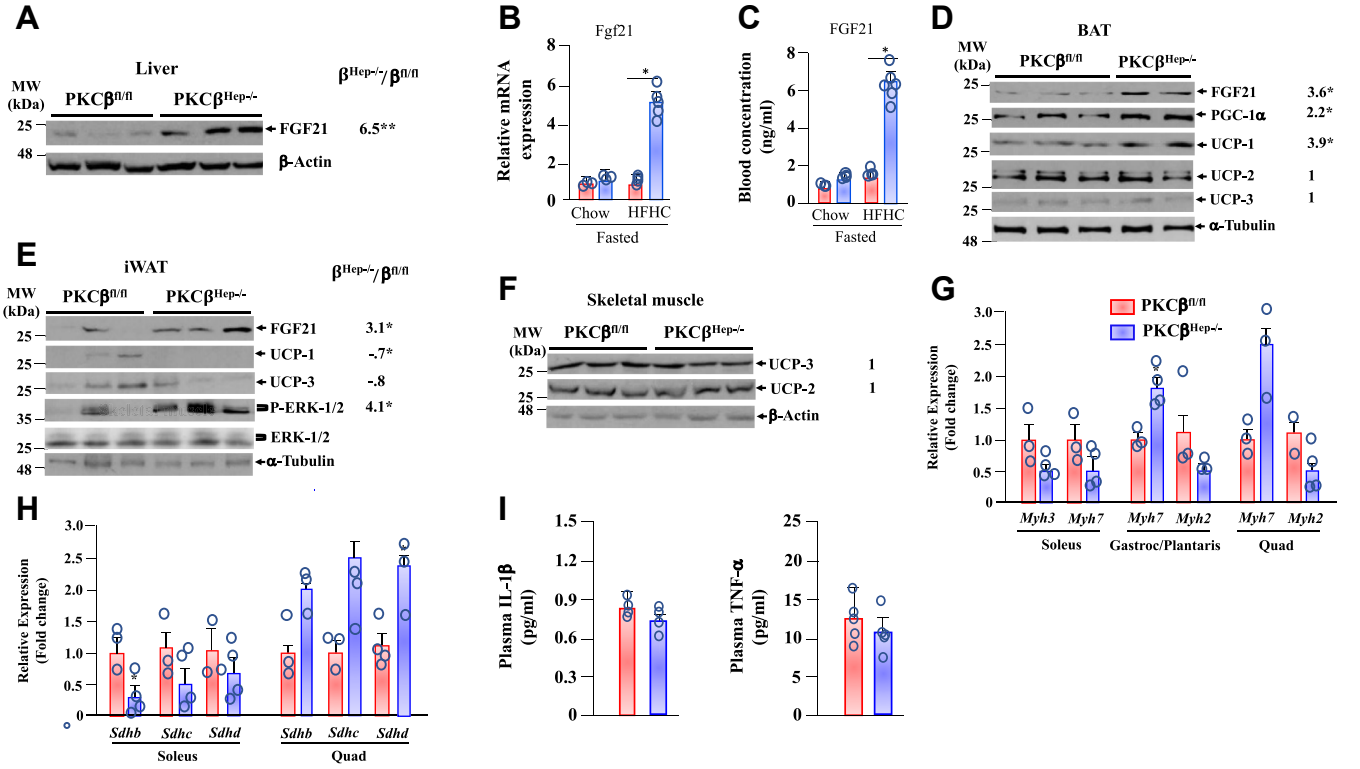

Figure 4. Effects of hepatic PKCβ deficiency on the protein expression in different tissues at the end of the study. *A*, overnight fasted PKCβ$^{Hep-/-}$ mice exhibit elevated FGF21 protein in the liver relative to control mice. Immunoblot analyses of indicated protein in the liver of HFHC-fed PKCβ$^{fl/fl}$ and PKCβ$^{Hep-/-}$ mice. Liver extracts were subjected to immunoblotting with FGF21 antibody. *B*, the hepatic expression of *Fgf21* mRNA in overnight fasted mice was examined by qPCR analysis. *C*, the concentration of FGF21 in the blood of chow-fed and HFHC-fed PKCβ$^{fl/fl}$ and PKCβ$^{fl/fl}$ mice was measured by ELISA. Blood was collected from mice fasted overnight. *D–F*, comparison of indicated protein expression in the BAT, skeletal muscle, and iWAT in the above mice. Western blots are representative of three separate experiments. Percentage relative decrease shows the band intensity ratio of PKCβ$^{fl/fl}$ over PKCβ$^{fl/fl}$ liver. *G* and *H*, hepatocyte-specific PKCβ deficiency upregulates expression of slow, oxidative major histocompatibility complex isoforms. Transcripts coding for *Myh2*, *Myh7*, and mitochondrial markers were determined in soleus, gastroc/plantaris and quadriceps muscles of PKCβ$^{fl/fl}$ and PKCβ$^{Hep-/-}$ mice by qPCR. mRNA is expressed relative to 18S mRNA, used as endogenous control. *I*, comparison of proinflammatory cytokines between genotypes. Data are presented as mean ± SD; n = 6; *$p < 0.05$; **$p < 0.01$.

activity or respiratory exchange ratios (results not shown) and attenuated elevated expression of BAT markers (Fig. 6*C*), suggesting a role of β3-AR signaling in increasing energy expenditure of PKCβ$^{Hep-/-}$ mice. Accordingly, no significant effects on either blood pressure or heart rate were observed (Fig. 6*D*), supporting that the inactivation of hepatic PKCβ potentiates sympathetic nervous system (SNS)/β3-adrenergic signaling *in vivo*. It is also now recognized that skeletal muscles, targeted by both β3-AR signaling and FGF21, also contribute to muscle hypertrophy and elevated thermogenic capacity (33, 34). It is tempting to speculate that the attenuation of aging-induced obesity *via* improving BAT and muscle functions in PKCβ$^{Hep-/-}$ mice appears to be caused at least in part by augmentation of β3-adrenergic signaling.

It is possible that a higher absolute SNS signal and a possible increase in sensitivity of BAT for the SNS stimulation may result in an increased ability to activate BAT in PKCβ$^{Hep-/-}$ mice. We sought to gain further insights into the molecular mechanism underlying the potentiation of adrenergic signaling by hepatic PKCβ inactivation. We administered CL316,243, a β3-AR-specific agonist, to PKCβ$^{Hep-/-}$ and PKCβ$^{fl/fl}$ mice daily for a period of 5 days. Administration of CL316,243 equally increased mRNA expression of Ucp-1 and *Pgc-1α*, markers of brown and beige adipocytes, in BAT of both PKCβ$^{Hep-/-}$ and PKCβ$^{fl/fl}$ mice (Results not shown). In line with these studies, explants from above mice responded equally to β3-AR agonist as assessed by measuring phosphorylation levels of hormone-sensitive lipase (Results not shown). It is tempting to speculate that the potential mechanism behind the loss of BAT function with age involves a reduction in sympathetic drive at least in part by hepatic PKCβ induction.

Liver PKCβ overexpression suppressed thermogenic genes in BAT, independent of FGF21

To test the hypothesis that hepatic PKCβ regulates peripheral BAT function and thermogenesis, we next performed complementation assays to test whether restoration of PKCβ expression attenuated the effect of hepatic PKCβ deficiency on elevated expression of BAT thermogenic genes. PKCβ was predominantly expressed in the liver of mice injected with Ad-PKCβ (Fig. 7*A*). Indeed, restoring hepatic PKCβ prevented the effect of hepatic PKCβ deficiency on elevated hepatic FGF21 expression in PKCβ$^{Hep-/-}$ mice (Fig. 7, *A* and *B*). Moreover, PKCβ expression in the liver of PKCβ$^{Hep-/-}$ mice attenuated increase in expression of both *Pgc-1α* and *Ucp*-1 mRNAs in BAT of the mice (Fig. 7*C*) and was accompanied by reduction in oxygen consumption (Fig. 7*D*). In agreement with negative

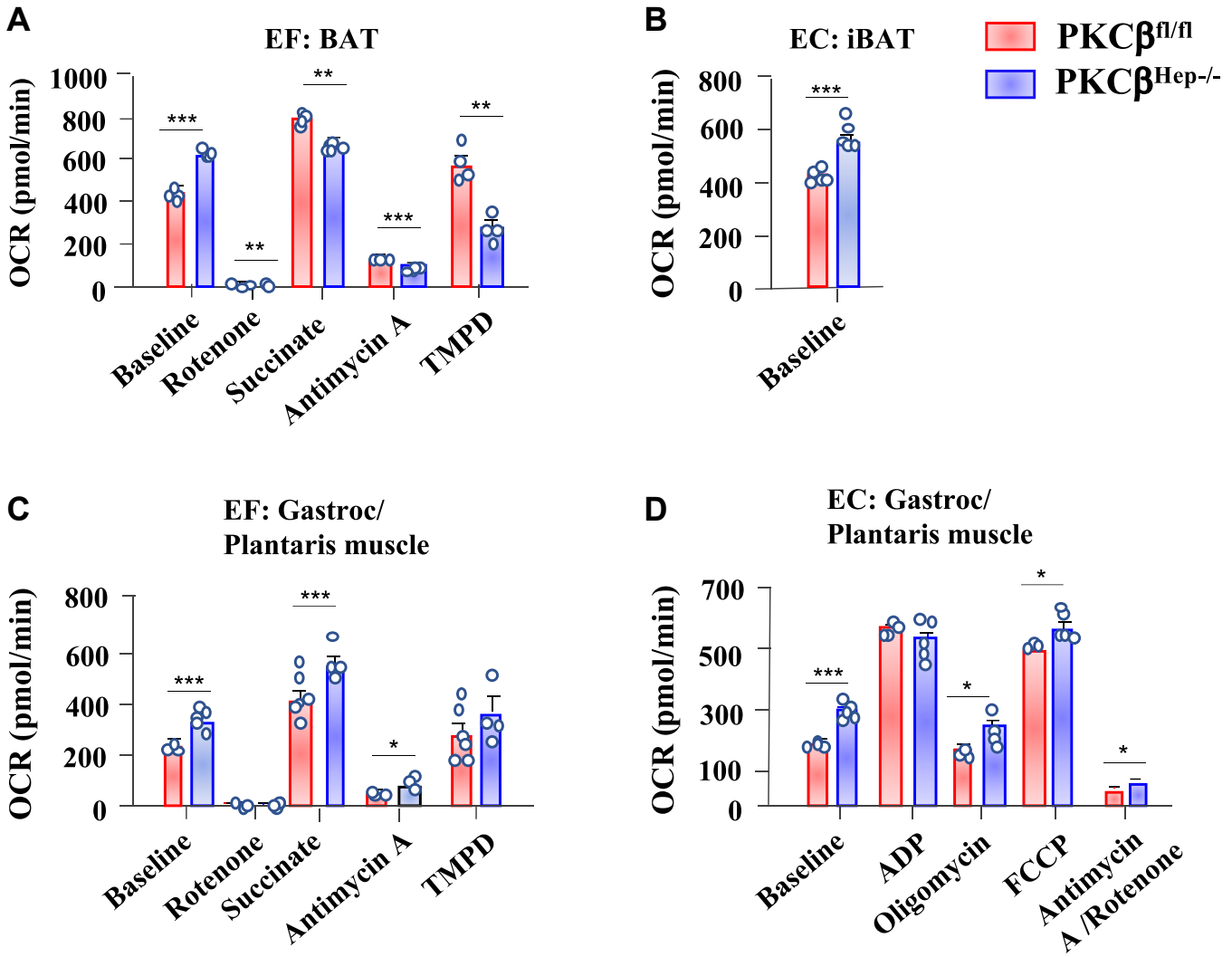

Figure 5. Hepatocyte-specific PKCβ deficiency improves mitochondrial function in peripheral tissues. Oxygen consumption rates were measured in freshly isolated isolated mitochondria from BAT (*A* and *B*) and gastric/plantaris muscle (*C* and *D*) of 1-year-old HFHC-fed PKCβ$^{fl/fl}$ and PKCβ$^{Hep-/-}$ mice fasted overnight. These measurements were performed on three separate isolations measured with at least six technical replicates in the absence or presence of indicated substrate, inhibitor, or modulator of electron transport chain. Data are presented as mean ± SD. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. EC, electron coupling assay; EF, electron flow assay.

regulation of hepatic FGF21 expression by hepatic PKCβ, treatment of mice with a specific inhibitor of PKCβ, INST3399 also increased hepatic FGF21 expression (Fig. 7*E*) (35). Together, these data support that PKCβ negatively regulates hepatic FGF21 and BAT markers.

FGF21 is reported to act centrally to induce sympathetic nerve activity and energy expenditure (36). It is thus possible that elevated hepatic FGF21 expression may contribute to the effects of hepatic PKCβ deficiency on energy metabolism. To test the potential role of FGF21 on BAT function, we used shRNA to knock down hepatic FGF21 expression in PKCβ$^{Hep-/-}$ mice. Control studies demonstrated that an AAV8-shFgf21 vector reduced hepatic *Fgf21* mRNA expression by 70% to 80%. (Fig. 7, *F* and *G*). Moreover, the amount of FGF21 in the blood was reduced in mice treated with the AAV8-shFgf21 vector compared with mice treated with the control AAV8-shLuc vector (Fig. 7*H*). We found that the shFgf21 vector did not prevent an increase in the expression of thermogenic genes and oxygen consumption caused by hepatic PKCβ deficiency (Fig. 7, *I* and *J*), thus ruling out potential contribution of PKCβ-FGF21-BAT axis in metabolic regulation by hepatic PKCβ.

Discussion

Our data provide novel insight into the mechanisms by which aging becomes a risk factor for the development of obesity. We demonstrate that hepatocyte PKCβ inactivation protects mice from diet-induced obesity as they age as well as mitigates hepatic steatosis, although the obesity protection was not apparent in early adulthood (19). It is intriguing that protection is linked to augmented energy expenditure due to potentiation of β3-AR signaling to adipose tissue, in particular BAT where it increased levels of PGC-1α and UCP-1, two known regulators of energy expenditure. Indeed, there is substantial evidence demonstrating that aging diminishes the

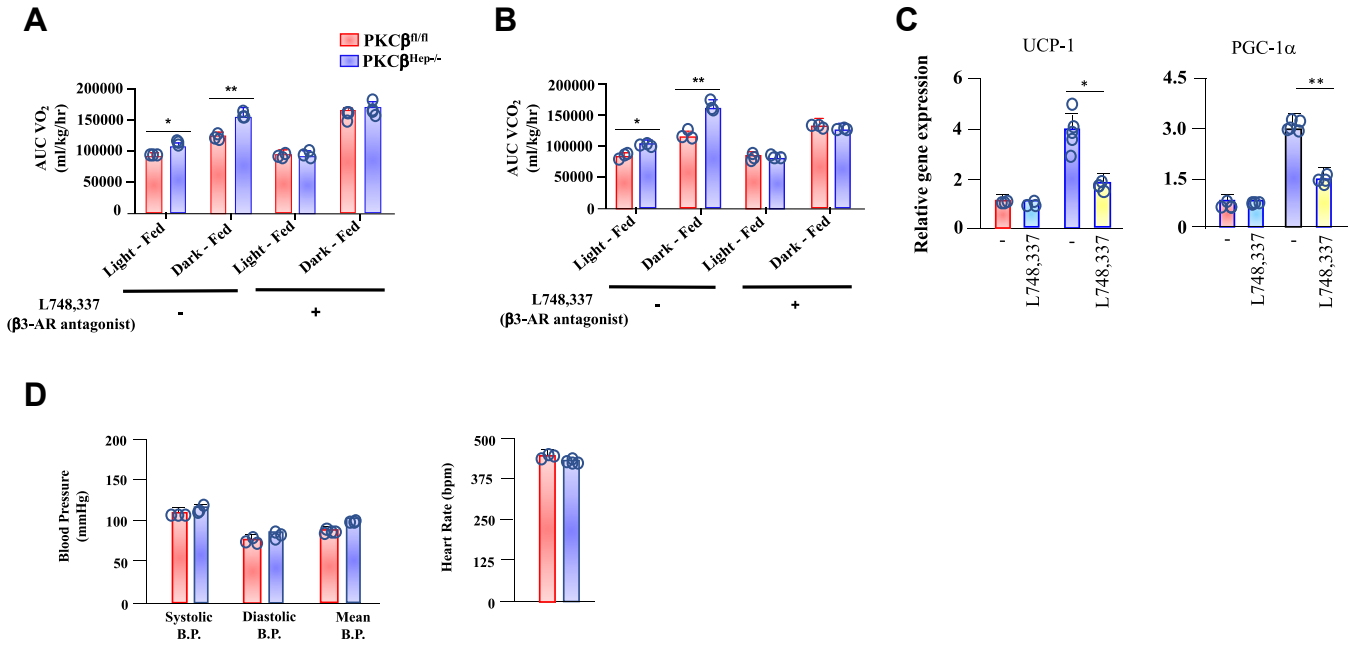

Figure 6. Specific β3-AR antagonist L748,337 blocks increase in energy expenditure of PKCβ$^{Hep-/-}$ mice. Effects of β-AR3 antagonist L748,337 on O_2 consumption (*A*) and CO_2 production (*B*) in HFHC-fed PKCβ$^{fl/fl}$ and PKCβ$^{Hep-/-}$ mice during day and night. *C*, analysis of UCP-1 and PGC-1α mRNA expression in the BAT of above mice. *D*, comparisons of blood pressure and heart rate in the above PKCβ$^{fl/fl}$ and PKCβ$^{Hep-/-}$ mice. Data are presented as mean ± SD. *$p < 0.05$; **$p < 0.01$.

sensitivity of adrenergic stimulation to reduce energy expenditure in humans as well as in rodents (37–39). The potentiating effect on adrenergic signaling appears to be modest and is thus likely insufficient to confer protection from weight gain in young mice fed high-fat diet. Aging is arguably a more pronounced physiological metabolic stress than high-fat feeding, demonstrating that even a modest effect of hepatic PKCβ deletion on adrenergic signaling is sufficient to confer beneficial effects under physiological conditions. Therefore, it is conceivable that a more profound induction of hepatic PKCβ expression by both diet and aging is required to trigger significant suppression of energy expenditure in order to

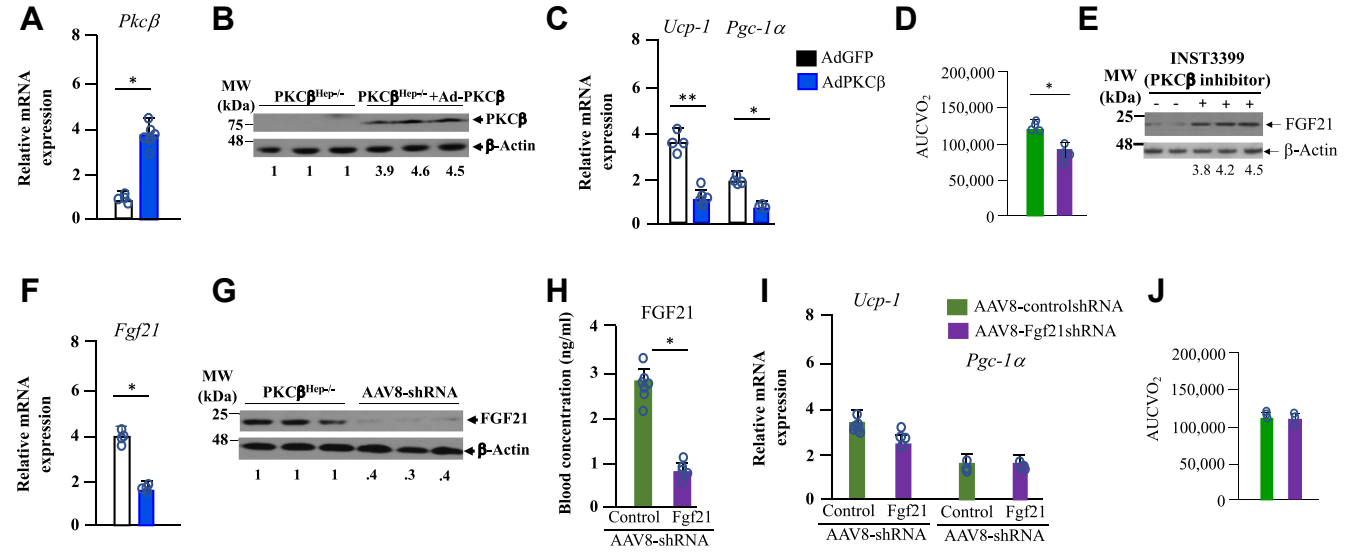

Figure 7. Hepatic PKCβ suppresses BAT thermogenic genes in an FGF21-independent manner. *A* and *B*, PKCβ or GFP (control) were expressed in the liver of HFD-fed of PKCβ$^{Hep-/-}$ mice using recombinant adenovirus vectors (12–16 days). The hepatic expression of PKCβ mRNA and protein were measured. *C*, comparison of expression of UCP-1 and PGC-1α in the BAT of the above mice. *D*, comparison of energy expenditure between PKCβ$^{Hep-/-}$ and PKCβ$^{Hep-/-}$+Ad-PKCβ mice fed HFHC diet. *E*, effect of a novel PKCβ inhibitor, INST3399 (4 mg/kg body weight for 6 weeks) on hepatic FGF21 expression was analyzed by immunoblotting (Mehta, 2022). *F–I*, FGF21 alone does not mediates metabolic effects of hepatic PKCβ deficiency. AAV8 vectors were employed to express control shRNA and Fgf21 shRNA in the liver of aged mice. Hepatic FGF21 mRNA and proteins were analyzed. FGF21 in the blood of mice fed ad-libitum or fasted overnight was measured by ELISA. Comparison of UCP-1 and PGC-1α expression in the BAT of the above mice. *J*, comparison of energy expenditure between control and AAV8-Fg21shRNA-treated mice under HFHC fed conditions. The data presented are the mean ± SD (n = 6; *$p < 0.05$; ***$p < 0.0001$).

promote energy storage. In line with this assertion, aging and obesity exert superimposable impact on mitochondria (40–42). It is thus reasonable to hypothesize that they could exert additive effects on mitochondrial dysfunction upon hepatic PKCβ induction. Our data support the premise that aging- and high-fat diet-induced PKCβ expression contributes to accentuating the decline in body capacity to metabolize fat, leading to a gradual accumulation of visceral adiposity. We therefore propose that hepatocyte PKCβ is an important component of energy balance under metabolic stress conditions, as it integrates dietary and metabolic signals to regulate energy balance by promoting hepatic and extrahepatic metabolic derangements in energy homeostasis. For most of human history, mankind has struggled with food insufficiency and short-life expectancy. Thus, the evolutionary process has led to the emergence of a so-called, thrifty genotype, which has driven the development of an efficient metabolism and a centrally regulated behavior designed to minimize energy expenditure at least in part by inducing hepatic PKCβ expression. As most people become obese slowly, we posit that inhibition of PKCβ may be useful for counteracting this gradual weight gain over time.

Aging is accompanied by the loss of classical brown adipocytes as well as brown-like adipocytes found in WAT, suggesting that the loss of their energy expenditure capacity might contribute to an obesity-prone phenotype with increased age (43, 44). The underlying processes that govern reduced BAT function are largely unknown but could at least in part be due to changes in sympathetic activation. The possible molecular mechanism may include a reduction in the sympathetic nervous system contributing to impaired thermogenesis in brown adipocytes due to progressive hepatic PKCβ induction that increases with aging and visceral obesity. This phenomenon could be caused by a combination of several molecular factors. For example, PKCβ can sense and respond to nutrient overabundance by communicating the metabolic status of the liver and PKCβ-related decline in sympathetic activation, causing impaired thermogenesis in brown adipocytes. Alternatively, hepatic PKCβ may indirectly promote adipose inflammation, due to which inflammatory macrophages reduce adrenergic output by degrading catecholamines in the adipose tissue (45, 46). In view of lack of significant changes in proinflammatory cytokines by hepatocyte-specific PKCβ deficiency, the potential contribution of such a mechanism is less likely. One major pathway for sensing liver lipid content by self-regulated PKCβ is corroborated by lipidomic studies showing the accumulation of known PKCβ activators in obese livers, such as diacylglycerols (intermediates in TG biosynthesis and hydrolysis), free cholesterol, and saturated fatty acids (27). The mechanism underlying inter-tissue communication may therefore involve modulation of hepatic vagal afferent nerve by PKCβ by regulating receptor stability, transport, or interaction of the receptor with neighboring component(s). In line with these postulates, PKC has been reported to phosphorylate transient receptor channel protein 4 (Trpv4), as well as hepatic expression of this transporter is reduced in PKCβ$^{Hep-/-}$ liver relative to control liver, as revealed by RNA-seq analysis (20, 47–49). Notably, Trpv4 deletion is reported to confer resistance to diet-induced obesity (50). Therefore, the exact mechanism by which hepatic PKCβ disruption augments β3-AR signaling merits additional investigation. Nevertheless, our present study establishes novel aspect of aging-associated obesity and supports the premise that suppressing the hepatocyte-peripheral tissues axis restricts obesity and its underlying disease state during aging. To this end, we propose a model that features the hepatic PKCβ as a mandatory component for signal transduction from liver to BAT and concomitant suppression of energy expenditure (Fig. 8). An important implication of the present study is that modulation of hepatocyte signaling could be a promising therapeutic approach to promote thermogenic energy expenditure (51, 52). It is also tempting to speculate that concomitant administration of a PKCβ inhibitor could potentiate the effect of β3-adrenergic agonists, increasing their efficacy and their safety profile by reducing the effective doses, thus avoiding the side-effects of cross-activation of β3-AR in other organs (53). Furthermore, in addition of testing therapeutic efficacy of PKCβ inhibitors in isolation, it would be beneficial to test combinations of a PKCβ inhibitor with a food intake reducer GLP-1 agonist in the treatment of obesity is an avenue to be explored in future (54).

There is growing evidence that aging is a vital risk factor for the occurrence of nonalcoholic fatty liver disease (55). Our study provides a unique insight into the relationship between hepatic PKCβ and age-related increases in fatty liver disease. The impairment of liver function could be caused by a combination of several molecular mechanisms by persistently high levels of

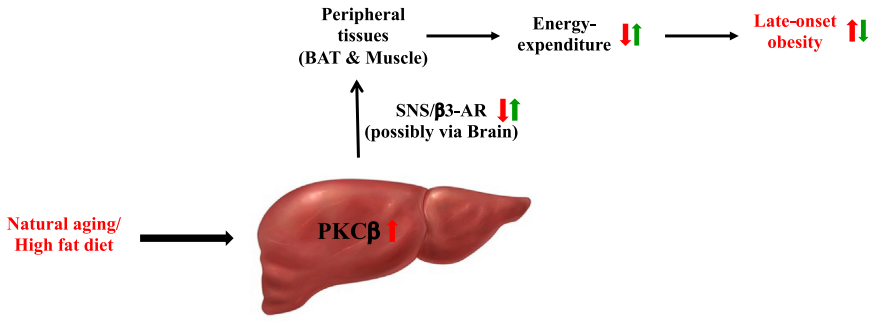

Figure 8. Our results are consistent with a model that aging- and diet-induced hepatic PKCβ induction suppresses BAT and muscle metabolism by acting as a brake on energy expenditure *via* SNS/β3AR signaling possibly *via* the brain. Hepatic PKCβ deficiency triggers activation of SNS/β3-AR signaling to peripheral tissues resulting in an increase in energy expenditure, thereby reducing obesity predisposition. Augmented stimulation of β3-AR signaling upregulates expression levels of FGF21, PGC-1α, and UCP-1 in BAT required for elevated thermogenesis. This may have important implications in the onset and pathophysiology of aging-induced obesity and therefore constitute a promising clinical target for obesity prevention and treatment.

hepatic PKCβ expression. First, hepatocyte-specific PKCβ deficiency attenuates age-related dysregulation of lipid metabolism and mitigates the development of hepatic steatosis and thereby progression toward nonalcoholic steatohepatitis and fibrosis (56). This protection can be attributed to a reduced capacity for biosynthesis due to reduced hepatic sterol response element-binding protein-1c activity (19). Second, an enhanced level of PKCβ expression promotes age-related increase in PKCβ/p66^{Shc} mitochondrial axis, thereby causing mitochondrial dysfunction and reduced autophagy (57), leading to hepatocellular stress (oxidative stress) that eventually predisposes affected individuals to steatosis, cirrhosis, and hepatocellular carcinoma. In obesity, significant mitochondrial dysfunction occurs in aged tissues, contributing to inflammation and insulin resistance and thus promoting fatty liver disease. Third, PKCβ can accentuate age-associated dysregulation of various transcription factors, specifically HNF4α, Nrf2, and NF-kB, and this can accelerate transcriptional networks resulting in an abnormal pattern similar to that observed in old mice. Intriguingly, the IKK/NF-kB signaling pathway has been shown to be one of the key mediators of aging (58). Finally, hepatic PKCβ induction appears to exacerbate fatty acid influx to the liver, thereby worsening the fatty liver disease (Fig. 4*E*). Thus, hepatic PKCβ deficiency improves mitochondrial function and causes reduced lipid content accumulation, thereby attenuating several mechanisms that provoke hepatocellular injury. According to this integrated view, combination of age-related changes in the liver and significant increase in chronic conditions such as obesity heightens the risk of liver disease in older individuals.

Aging is also characterized by a decline in biological functions, with a direct influence on lifestyle. The convergence of aging and obesity results in a unique pathology termed sarcopenic obesity that causes loss of muscle and physical strength as well as an increase in adiposity (59). The age-related decline in muscle function is aggravated by an HFD-induced increase in fat mass (60). The possible molecular mechanisms that lead to an age-related decline in muscle function may include the accumulation of intramyocellular lipids and an age-related slowing of the muscle due to a combination of an increased volume percentage of slow fibers and slowing of type 1 fibers. The disparity in response between control and PKCβ$^{Hep-/-}$ mice may in part be explained by PKCβ-related improvement in muscle fiber composition and attenuated intramyocellular lipid accumulation in muscle fibers resulting from a decline in the available lipid pool together with enrichment of slow fibers, thereby leading to resistance to muscle dysfunction. It is now recognized that both β3-AR and FGF21 contribute to muscle hypertrophy and elevated thermogenic capacity (60, 61). It is therefore conceivable that a more profound β3-AR signaling to muscle tissue due to hepatic PKCβ deficiency is required to trigger the aforementioned changes in order to attenuate intramyocellular lipid accumulation. Therefore, the degree of hepatic PKCβ induction may well be a factor underlying the earlier increase in body mass and intramyocellular lipid accumulation.

In conclusion, our study provides the first genetic evidence for the PKCβ in hepatocytes as a critical component in late-onset obesity. We show that hepatic PKCβ is the main driver for finely controlled BAT function suppression *via* the β3-AR signaling pathway, which may be more pronounced in older individuals, thereby putatively leading to aging-induced pathophysiology. This may have important implications for the development and pathophysiology of late-onset obesity and therefore constitutes a promising clinical target for obesity prevention and treatment. It will be interesting to see whether considerable inter-individual variations in weight gain in response to aging and caloric intake are due to variable expression and/or penetrance of PKCβ gene. A previous report describing an association between PKCβ polymorphism and insulin resistance in humans supports such a possibility (62).

Experimental procedures

Mice

PKCβ$^{fl/fl}$ mice were generated through homologous recombination as described by us recently (19). As described earlier, to inactivate PKCβ in hepatocytes, we crossed PKCβ$^{fl/fl}$ mice with Albumin-Cre transgenic mice in the C57BL/6J genetic background. The littermates were screened by genotyping, and mice with two copies of loxP sites and Cre recombinase were characterized as PKCβ$^{Hep-/-}$ mice. Male mice were used in all experiments. PKCβ$^{fl/fl}$ and PKCβ$^{Hep-/-}$ mice were bred and maintained on a 12-hr-light and 12-hr-dark cycle with lights on from 7 AM to 7 PM. All mice were given standard food pellets (normal chow diet) and water ad libitum. Cohorts of age-matched male mice were used for the study. Body weight and food intake were measured weekly. For HFHC diet feeding experiments, mice were fed with HFHC diet (#D04102102, Rodent diet with 45% kcal fat and 1% added cholesterol, Research Diets) beginning at the age of 6 to 8 weeks. The institutional Animal Care and Use Committee at the Ohio State University Care Facility has approved all studies.

Histology

Liver, WAT, and BAT from *ad libitum*–fed mice were isolated and fixed in 4% paraformaldehyde and processed for H& E staining (16–18). For Oil Red O staining, liver tissues were fixed in 4% paraformaldehyde overnight and incubated in 12% sucrose for 12 h and then in 18% sucrose overnight before being cryoembedded and sectioned by HISTOWIZ.

Plasma and tissue chemistry

Blood was collected using a 1-ml syringe coated in 0.5 M EDTA, and serum was collected by centrifugation at 1000*g* for 20 min (16–20). Insulin levels were measured by ELISA. Serum and liver TG, cholesterol, and lipoprotein distribution were measured by the Mouse Metabolic Phenotyping Core Facility at University of Cincinnati College of Medicine and the Ohio State University.

Immunoblot analysis

Proteins were extracted from liver tissue of mice (16–19). Livers were homogenized in RIPA buffer, 10 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and protease inhibitor tablet (Roche

Diagnostic). Protein concentration was determined using a BCA protein assay kit (Thermo Scientific), and lysates were analyzed by SDS-polyacrylamide gel electrophoresis and Western blot analysis on a PVDF membrane. Antibody to UCP-1 (14670S), UCP-2 (89326S), UCP-3 (970000S), P-ERK-1/2$^{Thr202/Tyr204}$ (9101), and ERK-1/2 (#9102) were purchased from Cell Signaling Technology. Antibody to PKCβ (ab38279) and FGF21 (ab1711941) were purchased from Abcam, Cambridge, MA, and antibody to PGC-1α (A11971) was purchased from ABclonal. Goat anti-mouse and goat anti-rabbit HRP-conjugated secondary antibodies (Bio-Rad) were used.

Comprehensive lab animal monitoring system

The Comprehensive Lab Animal Monitoring System (Oxymax Opto-M3; Columbus Instruments) was used to measure activity level, volume of O_2 consumption, the volume of CO_2 production, and heat production. The total energy expenditure of mice was calculated as described previously (63).

Mitochondrial isolations and respiratory function analysis

Mitochondria were isolated as previously described (64). Four micrograms of isolated mitochondria from the liver, gastrocnemius/plantaris muscle, BAT, and iWAT were resuspended in respiratory assay buffer composed of 70 mM Sucrose, 220 mM mannitol, 10 mM K_2HPO_4, 5 mM $MgCl_2$, 2 mM HEPES, and 1 mM EGTA, pH 7.4). Electron coupling and electron flow assays were performed using the Seahorse Bioanalyzer. Briefly, mitochondria were incubated with the indicated substrates, and oxygen consumption rates (OCR) were determined as described previously. Mitochondria basal respiration in electron coupling assays was determined in a coupling state with 10 mM succinate initial substrate with 2 μM rotenone. State three respiration was initiated with the injection of ADP, State four respiration was initiated with the injection of oligomycin and maximal uncoupler-stimulated respiration was initiated with the injection of FCCP (Trifluoromethoxy carbonylcyanide phenylhydrazine). Mitochondrial basal respiration in electron flow assays was determined in an uncoupled state with initial substrates 10 mM pyruvate and 2 mM malate in the presence of FCCP. Sequential electron flow throughout the electron transport chain was determined by first injecting rotenone, followed by succinate, antimycin A, and ascorbate and TMPD (N,N,N′,N′-Tetramethyl-p-phenylenediamine).

Viral transduction studies

Complementation studies were performed using Ad-GFP (Control) and Ad-PKCβ adenovirus stocks described earlier by us (65). Male mice (52 weeks old) were fed HFHC diet (4 weeks) and then treated by intravenous injection (tail vein, 200 μl final volume) with 8×10^9 genomic copies/mouse of recombinant adenovirus. Studies were performed 72 h post-infection. We optimized expression conditions for assay of adenovirus-treated mice 48 h posttreatment. We also optimized conditions to achieve 2-3-fold hepatocyte PKCβ overexpression, which aligns with physiologic PKCβ upregulation in HFHC-fed aged mice. Recombinant Ad-GFP and Ad-PKCβ were prepared and amplified by the Ohio State University Children's Hospital.

The adeno-associated virus (AAV) eight vector that encodes an Fgf21 shRNA (5′-GATCAAAAAAGGGATTCAACACAG GAGAAACTTCGGTTTCTCCTGTGTT GAATCCC-3′) was used. Male mice (16 weeks old) were treated by intravenous injection (tail vein) with 3×10^{11} genomic copies/mouse of AAV8-shLuc (Control) or AAV8-(200 μl final volume). Studies were performed ∼30 to 90 days post-infection.

Measurements of blood pressure and heart rate

Blood pressure (BP) was measured by the noninvasive tail-cuff method in lightly sedated mice by using a CODA high-throughput BP acquisition system (Kent Scientific Inc). Briefly, mice were trained for 3 days by measuring BP daily before collecting actual data. Mice were initially anesthetized using 2.0% isoflurane in O2 at a rate of 0.8 l/min, and anesthesia was maintained with 1% isoflurane. Mice were then placed on a warming platform and allowed to acclimatize for 10 min before readings were obtained. Each BP measurement session consisted of five acclimatization cycles followed by 15 BP measurement cycles. On the data collection day, two sessions of 15 BP measurements were obtained, and the average of accepted readings from both sessions was used for systolic BP, diastolic BP, and mean BP in each individual mouse. The computer software of the CODA system measures the systolic and diastolic pressures with inflation of a pneumatic tail cuff while a transducer measures the BP waveform. The software determines signal-to-noise and accepted readings, discarding noisy inaccurate measurements.

Statistical analysis

All results are presented as means ± SD. When only two groups were analyzed, statistical significance was determined using an unpaired Student's *t* test. Two-way ANOVA was used to compare the effects of different diets on two genotypes, and when significant differences were observed individual means of column were compared by unpaired Student's *t* test as indicated. Repeated measurement-based parameters were analyzed using two-way ANOVA followed by Bonferroni's test. Data from multiple groups were compared with one-way analysis of variance followed by Tuckey's post hoc test. $p < 0.05$ was considered statistically significant.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Conflict of interest—The authors declare that they have no conflicts of interest.

Abbreviations—The abbreviations used are: AAV, adeno-associated virus; BAT, brown adipose tissue; eWAT, epididymal WAT; HFHC, high-fat and high-cholesterol; IWAT, inguinal WAT; PGC-1α, proliferator-activated receptor-alpha; PKCβ, protein kinase Cbeta; PKCβHep$^{-/-}$, hepatocyte-specific PKCβ deficiency; WAT, white adipose tissue.

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