Journal of Oral Biosciences 61 (2019) 65–72

Contents lists available at ScienceDirect

Journal of Oral Biosciences

journal homepage: www.elsevier.com/locate/job

Review

Phospholipase C-related catalytically inactive protein: A novel signaling molecule for modulating fat metabolism and energy expenditure

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ARTICLE INFO

Article history:
Received 16 March 2019
Received in revised form
17 April 2019
Accepted 19 April 2019
Available online 15 May 2019

Keywords:
Adipose tissue
Energy metabolism
Obesity
Phospholipase C
Uncoupling protein 1

ABSTRACT

Background: Overweight and obesity are defined as excessive or abnormal fat accumulation in adipose tissues, and increase the risk of morbidity in many diseases, including hypertension, dyslipidemia, type 2 diabetes, coronary heart disease, and stroke, through pathophysiological mechanisms. There is strong evidence that weight loss reduces the risk of metabolic syndrome by limiting blood pressure and improving the levels of serum triglycerides, total cholesterol, low-density lipoprotein-cholesterol, and high-density lipoprotein-cholesterol. To date, several attempts have been made to develop effective anti-obesity medication or weight-loss drugs; however, satisfactory drugs for clinical use have not yet been developed. Therefore, elucidation of the molecular mechanisms driving fat metabolism (adipogenesis and lipolysis) represents the first step in developing clinically useful drugs and/or therapeutic treatments to control obesity.

Highlight: In our previous study on intracellular signaling of phospholipase C-related catalytically inactive protein (PRIP), we generated and analyzed *Prip*-double knockout (*Prip*-DKO) mice. *Prip*-DKO mice showed tolerance against insulin resistance and a lean phenotype with low fat mass. Here, we therefore reviewed the involvement of PRIP in fat metabolism and energy expenditure. We conclude that PRIP, a protein phosphatase-binding protein, can modulate fat metabolism via phosphoregulation of adipose lipolysis-related molecules, and regulates non-shivering heat generation in brown adipocytes.

Conclusion: We propose PRIP as a new therapeutic target for controlling obesity or developing novel anti-obesity drugs.

Abbreviations: ATGL, adipocyte triglyceride lipase; BAT, brown adipose tissue; CGI-58, comparative gene identification-58; CNS, central nervous system; DAG, diacylglycerol; GABARAP, $GABA_A$ receptor associated protein; GLP-1, glucagon-like peptide-1; HFD, high-fat diet; HSL, hormone-sensitive lipase; $Ins(1,4,5)P_3$, inositol 1,4,5-trisphosphate; LC3, microtubule-associated protein 1 light chain 3; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; PH, pleckstrin homology; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PKA, cyclic adenosine monophosphate-activated protein kinase (protein kinase A); PLC, phospholipase C; PLC-δ1, phospholipase C-δ1; PLC-L, phospholipase C-like protein; PP1, protein phosphatase 1; PP2A, protein phosphatase 2A; PRIP, PLC-related catalytically inactive protein; *Prip*-DKO, *Prip1*$^{-/-}$ and *Prip2*$^{-/-}$-double knockout; SNS, sympathetic nervous system; TAG, triacylglycerol; UCP1, uncoupling protein 1; WAT, white adipose tissue.

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https://doi.org/10.1016/j.job.2019.04.002
1349-0079/

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1. Introduction

The World Health Organization indicates that obesity is currently one of the most obvious, yet most neglected, public health problems [1]. If immediate action is not taken, millions of people will suffer from an array of obesity-linked serious health disorders such as type 2 diabetes mellitus, cardiovascular disease, and dyslipidemia, all of which decrease both the quality and length of life.

Obesity is characterized by excessive body fat accumulation in adipose tissue. During periods of energy excess, triacylglycerol (TAG) is synthesized and stored in adipose tissue. Under conditions of energy deprivation, stored TAG is sequentially broken down to diacylglycerol and monoacylglycerol as intermediates, and is eventually converted into three fatty acids and glycerol by a series of enzymes, including adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MGL) [2,3]. Ultimately, the fatty acids and glycerol are released from the adipocytes and transported to other tissues through the circulation.

There is convincing evidence that the central nervous system (CNS)—sympathetic nervous system (SNS) axis plays a role in modulating lipolysis in adipose tissues. Various hormones such as glucagon-like peptide-1 (GLP-1), leptin, and adrenaline, and the neurotransmitter noradrenaline, are secreted and regulate fat metabolism via the CNS—SNS axis. Adrenaline and noradrenaline secreted by SNS activation bind to a canonical G-coupled β-adrenergic receptor which is expressed on adipocytes. This activates ATGL and HSL through their phosphorylation by 5′-adenosine monophosphate-activated protein kinase (so called AMPK) and cyclic adenosine monophosphate-activated protein kinase (PKA) [4–6]. ATGL activation is further upregulated by the phosphorylation-dependent dissociation of comparative gene identification-58 (CGI-58) [2] and perilipin A, a lipid droplet-associated protein [7,8]. The lipolytic process is also regulated by dephosphorylation enzymes such as protein phosphatase 1 (PP1) [9] and protein phosphatase 2 A (PP2A) [10,11].

Two different types of adipose tissue, white adipose tissue (WAT) and brown adipose tissue (BAT), exist in mammals. When energy intake is not equal to energy output, excessive energy is stored as TAG in WAT, whereas in a nutrient-starved state, TAG is hydrolyzed and supplied to the body through lipolysis. Since white adipocytes contain few mitochondria, their intrinsic metabolic rate contributes little to the whole-body energy expenditure. In contrast, brown adipocytes have the highest respiration capacity among mammalian cells because they contain high numbers of mitochondria [12]. Therefore, BAT dissipates the chemical energy contained in fatty acids as heat via mitochondrial uncoupling protein 1 (UCP1), which facilitates clearance of glucose, fatty acids, and TAG [13]. In a cold-acclimated rodent, thermogenesis in BAT can contribute to up to 50% of the total body heat production at rest despite the tissue wet weight only representing 5% of total body mass [14–16]. Inducible brown-like adipocytes, commonly referred to as brite adipocytes (brown-in-white adipocytes) or beige adipocytes, have been recently found in various WAT deposits and are especially prominent in subcutaneous inguinal WAT [15]. Upon cold exposure, SNS stimulates β-adrenergic receptor-mediated PKA signaling in brite adipocytes as well as in brown adipocytes, facilitating UCP1-dependent thermogenesis. Brite adipocytes also have high respiration capacity and contribute to whole-body thermogenesis [16].

Recently, we reported that phospholipase C (PLC)-related catalytically inactive protein (PRIP) downregulates lipolysis in white and brown adipocytes, and whole-body energy expenditure by modulating UCP1-mediated thermogenesis in brown and brite adipocytes [17–19]. We propose that PRIP is a new signaling molecule that negatively regulates energy balance and weight loss. Here, we review the role of PRIP roles in lipolysis and energy expenditure in adipose tissues.

2. Isolation of PRIP, an inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3]-binding protein

2.1. PRIP regulates intracellular inositol signaling and phospholipid signaling

PRIP was originally identified as an Ins(1,4,5)P_3 binding protein with a molecular mass of 130 kDa (Fig. 1) from both the cytosolic and membrane fractions of rat brain [20,21]. Subsequent gene cloning studies indicated that PRIP has considerable similarity to phospholipase C-δ1 (PLC-δ1), which is also identified as an

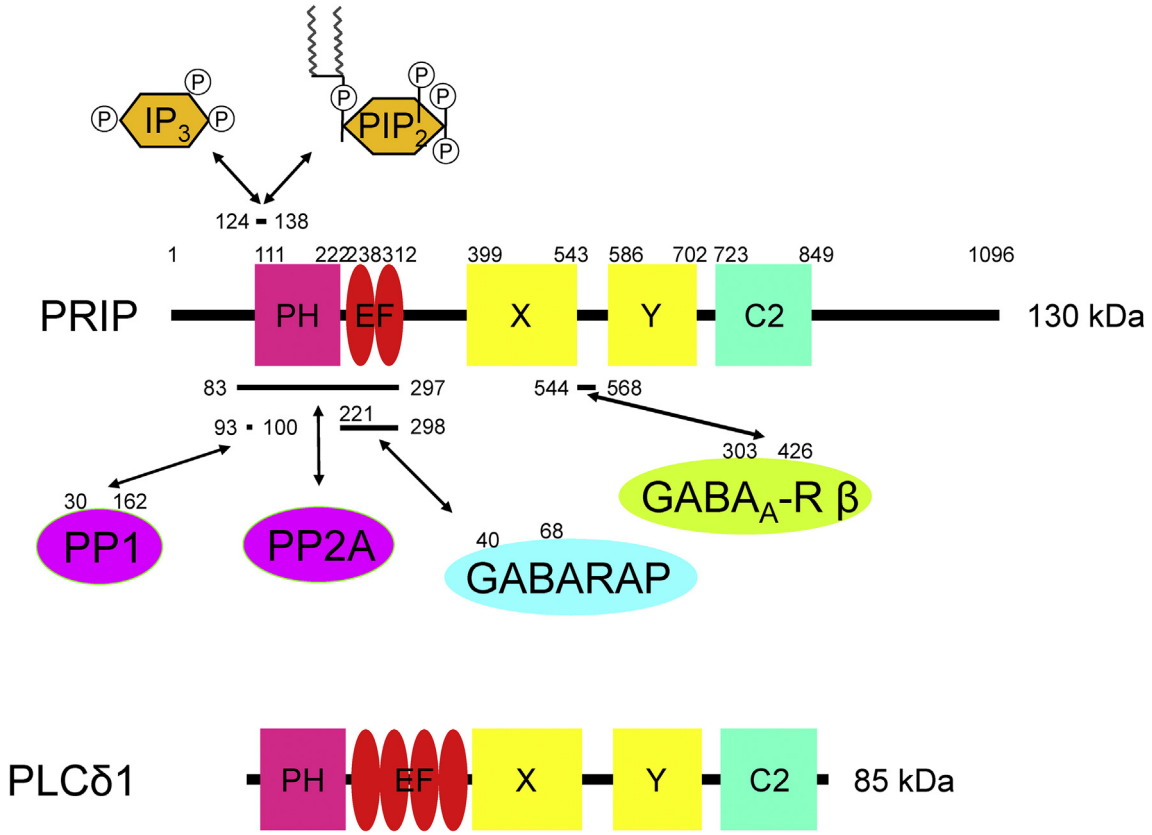

Fig. 1. Schematic for the structure of phospholipase C-related catalytically inactive protein (PRIP). PRIP, with a molecular mass of 130 kDa, has a domain organization similar to that of PLCδ1 with a molecular mass of 85 kDa. The binding partners of PRIP are shown in the upper panel. Numbers represent amino acid residues. C2, C2 domain; EF, EF hand domain; GABARAP, $GABA_A$ receptor-associated protein; $GABA_A$-R β, $GABA_A$ receptor β subunit; IP_3, inositol 1,4,5-trisphosphate; PH, pleckstrin homology domain; PLCδ1, phospholipase C (PLC) delta 1; PP1, protein phosphatase 1; PP2A, protein phosphatase 2 A; X and Y, catalytic subunit of PLC.

$Ins(1,4,5)P_3$ binding protein [20,22,23]. In addition, a crystal structure study of PLC-δ1 [24] and limited proteolysis experiments involving PRIP [25] predicted that PRIP has a domain organization similar to PLC-δ1, incorporating the pleckstrin homology (PH) domain, EF-hand domain, catalytic X and Y domains, and a C2 domain (Fig. 1). However, residues within the catalytic domain that are critical for PLC activity (Glu 341 and His 356) are not conserved in PRIP. Our mutagenesis study then determined that PRIP lacks PLC activity and was termed as a PLC-related but catalytically inactive protein [25].

The X-ray crystal structure of the PLC-δ1 PH domain showed that $Ins(1,4,5)P_3$ is bound to the positively charged surface of that domain [26]. The PRIP PH domain, which is similar to the PLC-δ1 PH, is important for binding to $Ins(1,4,5)P_3$ (Fig. 1) [27,28]. The ability of PRIP binding to $Ins(1,4,5)P_3$ could be regulated by $Ins(1,4,5)P_3$ receptor-mediated intracellular Ca^{2+} signaling. We analyzed PRIP overexpressing cells or primary culture cells prepared from *Prip* knockout mouse brain and demonstrated that PRIP is involved in the fine-tuning of $Ins(1,4,5)P_3/Ca^{2+}$ signaling in response to extracellular stimuli [29,30].

The PLC-δ1 PH domain is implicated in the stereospecific binding of both $Ins(1,4,5)P_3$ and phosphatidylinositol 4,5-bisphosphate [$PI(4,5)P_2$] [31,32]. It is proposed that the PLC-δ1 PH domain serves to anchor PLC-δ1 to $PI(4,5)P_2$-rich membranes, which facilitates hydrolysis of $PI(4,5)P_2$ [24]. We previously reported that the PRIP PH domain binds to $PI(4,5)P_2$ (Fig. 1) [33] and subsequently determined that PRIP, an enzymatically inactive protein, accumulates in $PI(4,5)P_2$-rich membranes and suppresses $PI(4,5)P_2$ metabolism, which negatively modulates PI3K-dependent production of phosphatidylinositol 3,4,5-bisphosphate from $PI(4,5)P_2$ followed by AKT signaling [34].

2.2. Identification of two isoforms of PRIP

We purified PRIP from rat brain [20] and identified a gene from a rat brain library [23]. The human *PRIP* gene was identified as a novel phospholipase C family gene on chromosome 2q33 that is homozygously deleted in human small cell lung carcinoma, and was named as phospholipase C-like protein (PLC-L) [35]. Thereafter, a cDNA that encodes a protein with 64% sequence identity to the full-length PLC-L from a mouse brain library was isolated [36]; a corresponding gene in humans has also been cloned [37]. We now propose to term the original PRIP (PLC-L in human) as PRIP1 (PLC-L1) and a gene highly homologous to PRIP1 as PRIP2 (PLC-L2). These PLC family member proteins have characteristic NH_2- and COOH-terminal extensions, which differ from those in PLC-δ1 (Fig. 1). Moreover, they have mutations involving critical amino acid residues of their catalytic domain; therefore, the products of these genes do not exhibit PLC activity [25,36]. The tissue distributions of PRIP1 and PRIP2 exhibit clear differences. PRIP1 is predominantly expressed in the CNS, including the cerebrum and cerebellum, whereas PRIP2 shows relatively ubiquitous expression [38]. However, the regional distributions of PRIP1 and PRIP2 in the brain are similar [38,39].

2.3. Identification of PRIP-binding partners and determination of possible physiological functions of PRIP

Cellular functions are regulated by a relay of molecular interactions; for example, binding of a protein to other proteins, nucleotides, or lipids. Therefore, we identified a protein that interacts with PRIP besides Ins(1,4,5)P_3 and PI(4,5)P_2, which bind to the PRIP-PH domain, in order to predict additional biological functions of PRIP. We isolated two possible binding partners of PRIP using yeast two-hybrid screening; i.e. the catalytic subunit of protein phosphatase 1α (PP1) (Fig. 1) [40] and $GABA_A$ receptor associated protein (GABARAP), which binds to the $GABA_A$ receptor γ2 subunit and tubulin (Fig. 1) [41,42]. We also determined (Fig. 1) that PRIP interacts with the $GABA_A$ receptor β subunit [43], PP2A [44], AKT, a serine/threonine kinase (also known as protein kinase B) [45], syntaxin 1, and synaptosomal-associated protein 25 [46].

To identify the physiological functions of PRIP based on associations between PRIP and its binding partners, we generated and analyzed *Prip1*$^{-/-}$-knockout (*Prip1*-KO) mice [42], followed by the generation of *Prip1*$^{-/-}$ and *Prip2*$^{-/-}$-double knockout (*Prip*-DKO) mice [44] by mating *Prip1*-KO mice [42] and *Prip2*-KO mice [47]. By binding to GABARAP, $GABA_A$ receptor β subunit, PP1, PP2A, and AKT, PRIP is able to regulate intracellular trafficking of $GABA_A$ receptors (i.e. transport of *de novo* synthesized or recycled $GABA_A$ receptors to Refs. [42,45,48,49] and endocytosis of $GABA_A$ receptors from Refs. [44,50] the plasma membrane) so as to modulate the channel functions of cell surface-expressed $GABA_A$ receptors [43,51,52]. PRIP is also implicated in regulating pain reactions [53,54] and anesthetic responses [55–57], which are modulated by $GABA_A$ receptors. In addition, PRIP can regulate the trafficking of insulin-containing vesicles [58] and insulin exocytosis [46,59,60]. By binding to microtubule-associated protein 1 light chain 3 (LC3), as a mammalian paralog of GABARAP and an initiator for autophagy progression, PRIP regulates the LC3-mediated autophagy pathway and is involved in autophagic elimination of bacteria from infected cells [61,62].

3. PRIP regulates fat metabolism in adipocytes and whole-body energy expenditure

3.1. Molecular mechanism of TAG metabolism in adipocytes

Although TAG synthesis occurs in multiple tissues, TAG lipolysis during periods of energy demand occurs predominantly in adipose tissues. The hydrolytic action of TAG by lipases is rapid in WAT; this process is regulated by several molecules, including CGI-58, perilipin, and lipolytic enzymes, ATGL, HSL, and MGL, resulting in the formation of free fatty acids (FFAs) and glycerol [2]. Under basal conditions (Fig. 2, left-side panel), perilipin, a master lipolysis regulator of stored TAG, sequesters CGI-58, a coactivator protein of ATGL at the surface of lipid droplets, which prevents sequential hydrolysis of stored TAG. Under starvation conditions (Fig. 2, right-side panel), SNS is activated, which initiates adipose lipolysis. Noradrenaline and adrenaline bind to β-adrenergic receptors located on the adipocyte plasma membrane and trigger G protein-mediated adenylate cyclase activation and subsequent PKA activation. Activated PKA phosphorylates perilipin at multiple sites, which mediates CGI-58 dissociation from perilipin. Subsequently, CGI-58 associates with and fully activates ATGL. The activated PKA also phosphorylates HSL, which

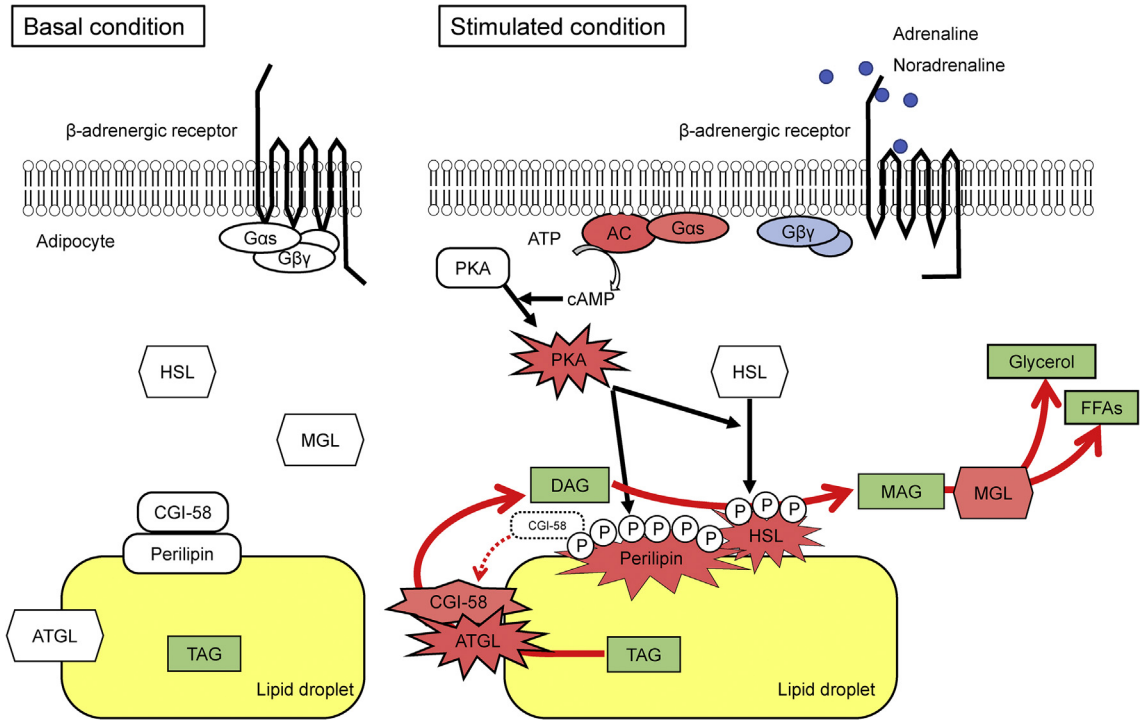

Fig. 2. Molecular model of the adipose lipolysis machinery. Catabolic hormone-mediated lipolysis in adipocytes is modulated by activation of a PKA-mediated pathway. The process is regulated by lipases (ATGL, HSL, and MGL) and other modulatory proteins including perilipin and CGI-58. Under basal conditions, these molecules are located as shown in the left panel. When adipocytes are stimulated with adrenaline and noradrenaline (right panel), PKA phosphorylates HSL and perilipin. Phosphorylation of perilipin releases CGI-58, resulting in activation of ATGL by association with CGI-58. Phosphorylated HSL is translocated to the lipid membrane and degrades DAG. AC, adenylate cyclase; ATGL, adipocyte triglyceride lipase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; CGI-58, comparative gene identification 58 (abhydrolase domain-containing protein 5); DAG, diacylglycerol; FFA, free fatty acid; Gαs, α subunit of Gs-protein; Gβγ, β and γ subunits of G-protein; HSL, hormone-sensitive lipase; MAG, monoacylglycerol; MGL, monoacylglycerol lipase; P, phosphate group; PKA, cyclic adenosine monophosphate-activated protein kinase (protein kinase A); TAG, triacylglycerol.

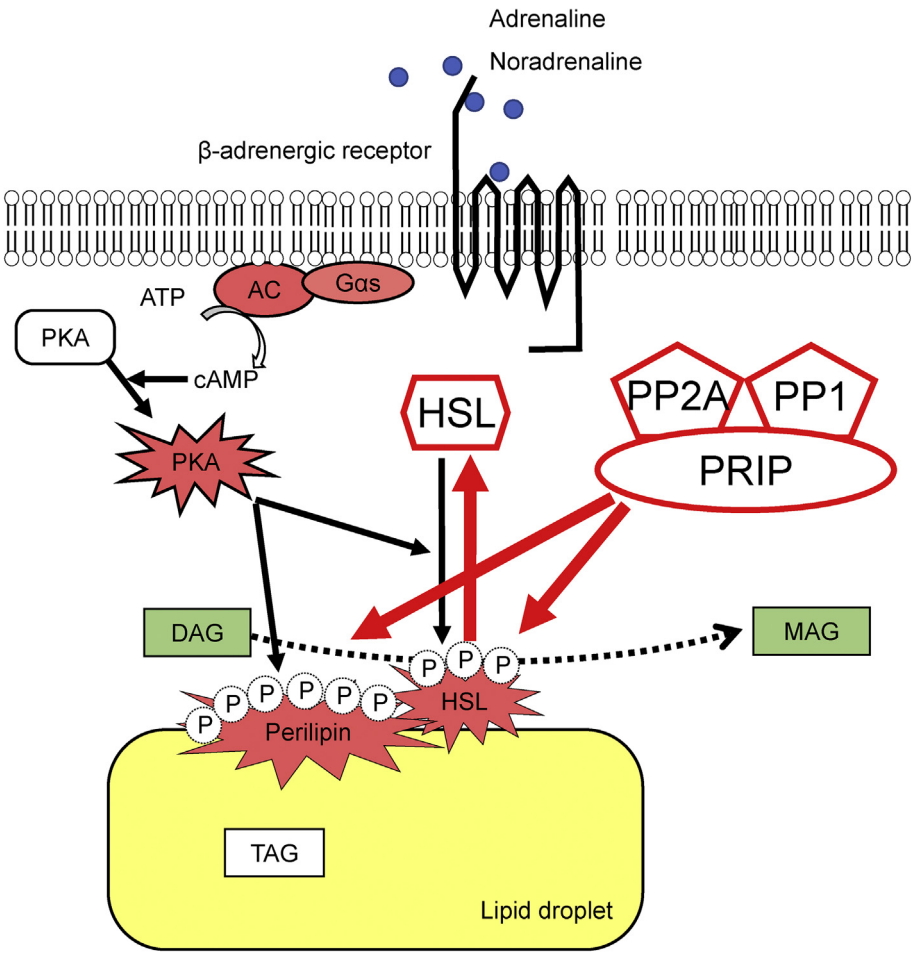

Fig. 3. Proposed model for PRIP-mediated phosphoregulation in adipocyte lipolysis. In response to catabolic hormone stimulation, PKA-mediated lipolysis is initiated. Presently, PRIP is translocated from the cytoplasm to lipid droplets together with PRIP-binding partners, protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A). These sequential events yield a sharp transient activation of lipolysis and subsequent attenuation of lipolysis. For abbreviations used, see the figure legend for Fig. 2.

facilitates the translocation of HSL from the cytosol to the surfaces of intracellular lipid droplets. HSL can be phosphorylated on at least five serine residues (amino acids 563, 565, 600, 659, and 660 of the rat sequence) *in vitro*. Ser-563, Ser-659, and Ser-660 are the major PKA phosphorylation sites responsible for activating HSL. Activated ATGL hydrolyzes TAG to diacylglycerol (DAG), and the phosphorylated HSL subsequently hydrolyzes DAG into monoacylglycerol (MAG), followed by degradation to FFA and glycerol by MGL in the final step of lipolysis. FFAs are released into the blood stream, where they bind to albumin for transport to surrounding tissues requiring energy. Glycerol is also transported into the bloodstream and is absorbed by the liver or kidneys, where it is converted to glycerol 3-phosphate by the enzyme glycerol kinase.

3.2. A new molecular machinery for attenuating adipose lipolysis

Dephosphorylation of HSL and perilipin by protein phosphatases is crucial for the attenuation of lipolysis. However, the molecular basis of such processes has not been clearly demonstrated. We have recently identified that PRIP suppresses phosphorylation levels of HSL and perilipin by binding to PP1 and PP2A and translocating them to the surface of lipid droplets [17–19], leading to attenuation of TAG breakdown (Fig. 3).

3.3. Prip-DKO mice present a lean phenotype

Studies in *Prip*-DKO mice have confirmed the importance of this molecule in TAG lipolysis [17,18]. Regular diet-fed *Prip*-DKO mice exhibited a lean phenotype with smaller WAT in size and weight, but showed slightly more food intake than wild-type control mice. In high-fat diet (HFD)-feeding experiments, *Prip*-DKO mice showed less body weight increase than wild-type controls. The *Prip*-DKO mice obviously displayed leanness with small-sized WAT. Interestingly, ectopic lipid accumulation was not observed in the livers of *Prip*-DKO mice, even though serum cholesterol and TAG levels were significantly higher in *Prip*-DKO mice than in wild-type mice. These results demonstrated an association involving the lean phenotype of *Prip*-DKO mice with altered fat metabolism in adipocytes.

3.4. PRIP regulates catabolic hormone-induced lipolysis by translocating protein phosphatases to lipid droplets in adipocytes

We previously showed that phosphorylation of HSL and perilipin in *Prip*-DKO WAT is upregulated under both non-fasting and fasting conditions compared with that in wild-type mice [17]. In response to adrenaline stimulation, PRIP was translocated from the cytoplasm to lipid droplets in white adipocytes; meanwhile, PRIP-binding partners PP1 and PP2A were translocated to lipid

droplets (Fig. 3). Consistently, adrenaline stimulation-mediated time-dependent dephosphorylation changes in HSL were observed in wild-type adipocytes, but not in *Prip*-DKO adipocytes.

3.5. Proposed mechanism of PRIP-mediated phosphoregulation in adipose lipolysis

We propose a model of PRIP-mediated phosphoregulation of lipolysis in adipocytes (Fig. 3). Once emergent signals such as starvation or stress signals are activated, PKA is activated via stimulation of β-adrenergic receptors in adipocytes. Then, HSL and perilipin are phosphorylated, activating the TAG breakdown pathway. The signal also induces translocation of PRIP and protein phosphatase complexes to lipid droplets, which promotes HSL and perilipin dephosphorylation and attenuates lipolysis. These sequential events yield a sharp transient activation of lipolysis to provide fine-tuning of catabolic hormonal regulation in adipocytes.

3.6. PRIP regulates energy metabolism

Interestingly, HFD-fed *Prip*-DKO mice exhibited more moderate body weight increases, greater glucose tolerance, and higher insulin sensitivity than wild-type mice [18]. This underlined a protective mechanism against HFD-induced obesity in *Prip*-DKO mice. Histological analyses showed that ectopic lipid accumulation in the liver was strongly decreased in HFD-fed *Prip*-DKO mice. Consistently, energy expenditure and body temperature were higher in *Prip*-DKO mice than in wild-types.

Activation of UCP1-mediated thermogenesis in BAT prevents obesity and diabetes [13,63,64]. Brown adipocytes directly dissipate the chemical energy in fatty acids as heat through UCP1 activation (Fig. 4). In brown adipocytes as well as in white adipocytes, sympathetic hyperactivity causes β-adrenergic receptor-induced PKA activation-mediated lipolysis. FFAs are used not only as substrates for oxidative respiration, but also as allosteric activators of UCP1. In addition, PKA activation followed by elevated intracellular FFAs activates UCP1-mediated heat generation and regulates thermogenesis through transcriptional control in BAT [18,65–67]. Furthermore, in recent years, brite adipocytes have been identified as a third type of adipocyte [68], which are characterized by UCP1 expression and are localized in classical WAT deposits. However, the lineage of these beige fat cells and their contribution to whole-body energy expenditure is not fully understood. Cold temperature is a potent environmental signal that activates thermogenesis through the SNS and produces brite adipocytes. A *Prip*-DKO mouse study demonstrated that PRIP regulates β-adrenergic receptor signaling-induced UCP1-dependent thermogenesis in BAT through phosphoregulation of HSL and perilipin, and also regulates the

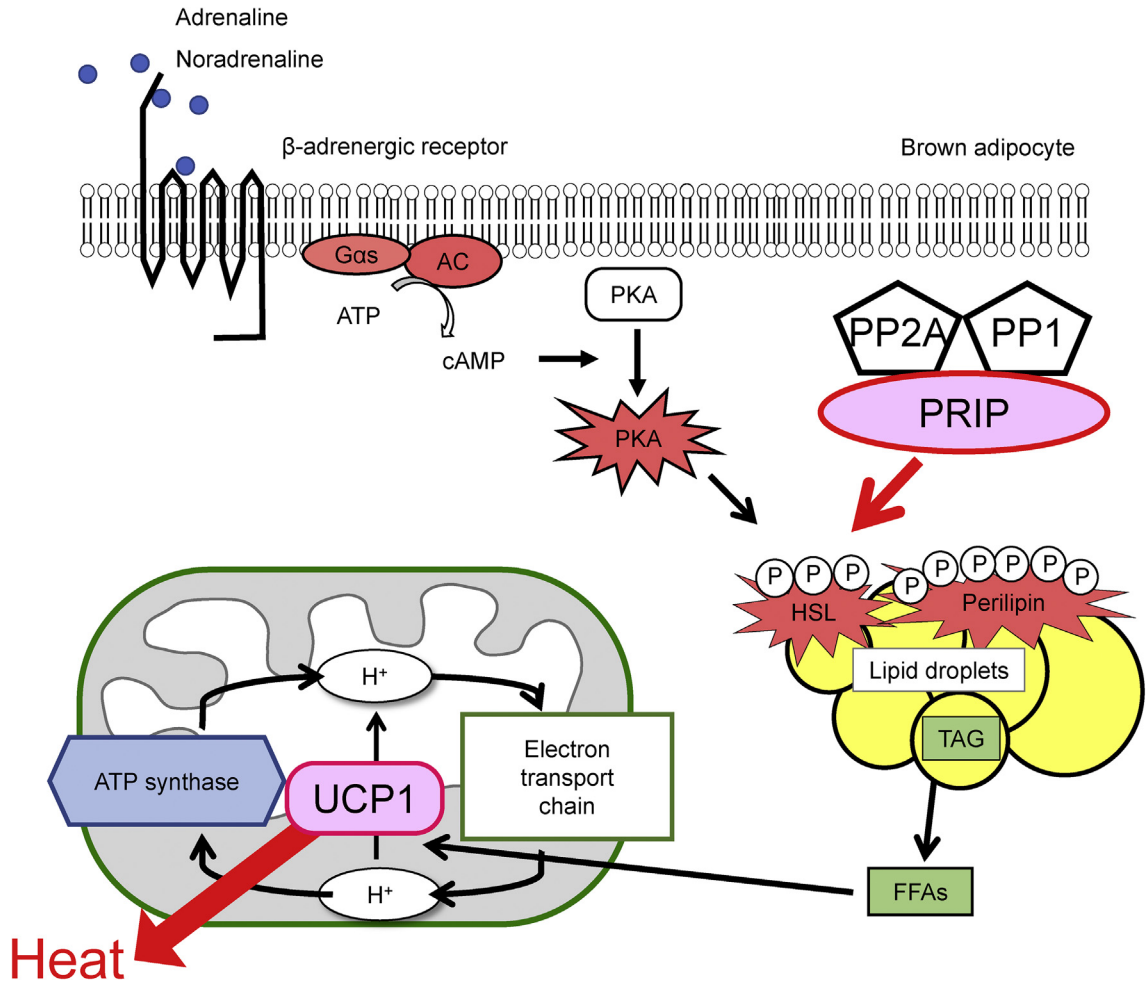

Fig. 4. PRIP regulates thermogenesis in brown adipocytes. Sympathetic nerve stimulation enhances receptor-mediated PKA activation in brown adipocytes. PKA facilitates the phosphorylation of HSL and perilipin, which promotes lipolysis. The resulting FFAs enhance UCP1 enzymatic activation. Translocation of PRIP and protein phosphatase complexes to lipid droplets facilitates the dephosphorylation of HSL and perilipin, which attenuates the PKA-mediated UCP activation-dependent heat generation. UCP1, uncoupling protein 1. For other abbreviations used, see the figure legend for Fig. 2.

development and/or activation of brite adipocytes in subcutaneous white adipocytes in response to cold exposure [18,19].

4. Conclusions

In this paper, we reviewed the molecular mechanisms underlying reduced fat mass observed in *Prip*-DKO mice and concluded that the lipolytic activity of adipocytes in mutant mice is upregulated. Importantly, our analyses showed that PRIP, together with PP1 and PP2A, regulates lipolysis by controlling phosphorylation-dependent HSL lipolytic activity. Furthermore, PRIP negatively regulates non-shivering thermogenesis through UCP1 activation to maintain energy homeostasis at an appropriate level for a given environmental condition. Elimination of PRIP or disruption of PRIP binding to protein phosphatases in adipocytes increased whole-body energy expenditure. Hence, PRIP is a newly identified TAG metabolism-regulating molecule and may serve as a potential therapeutic target to reduce obesity.

Ethical approval

All experiments were reviewed and approved by the Committee on the Guidelines for Animal Experimentation at Hiroshima University and Kyushu University.

Conflicts of interest

All authors state that they have no conflicts of interest. Takashi Kanematsu was awarded the Lion Dental Research Award from the Japanese Association for Dental Biosciences (JAOB), supported by Lion Corporation.

CRediT authorship contribution statement

Takashi Kanematsu: Conceptualization, Project administration, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing. **Kana Oue:** Investigation, Formal analysis, Resources, Funding acquisition. **Toshiya Okumura:** Investigation, Formal analysis. **Kae Harada:** Investigation, Methodology, Formal analysis, Data curation. **Yosuke Yamawaki:** Methodology, Resources. **Satoshi Asano:** Methodology. **Akiko Mizokami:** Investigation, Methodology, Resources. **Masahiro Irifune:** Resources, Funding acquisition. **Masato Hirata:** Resources, Funding acquisition.

Acknowledgements

This work was supported by grants from JSPS KAKENHI (grant numbers 16K11503 to T.K., 17K17254 to K.O., 18K09812 to M.I., and 17H01595 and 17K19766 to M.H.). T.K. thanks Lion Corporation for the Lion Dental Research Award.

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