Protein Kinase A Subunit Balance Regulates Lipid Metabolism in Caenorhabditis elegans and Mammalian Adipocytes

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Protein kinase A (PKA) is a cyclic AMP (cAMP)-dependent protein kinase composed of catalytic and regulatory subunits and involved in various physiological phenomena, including lipid metabolism. Here we demonstrated that the stoichiometric balance between catalytic and regulatory subunits is crucial for maintaining basal PKA activity and lipid homeostasis. To uncover the potential roles of each PKA subunit, Caenorhabditis elegans was used to investigate the effects of PKA subunit deficiency. In worms, suppression of PKA via RNAi resulted in severe phenotypes, including shortened life span, decreased egg laying, reduced locomotion, and altered lipid distribution. Similarly, in mammalian adipocytes, suppression of PKA regulatory subunits RIIα and RIIβ via siRNAs potently stimulated PKA activity, leading to potentiated lipolysis without increasing cAMP levels. Nevertheless, insulin exerted anti-lipolytic effects and restored lipid droplet integrity by antagonizing PKA action. Together, these data implicate the importance of subunit stoichiometry as another regulatory mechanism of PKA activity and lipid metabolism.

Protein kinase A (PKA) is a cyclic AMP (cAMP)-dependent serine/threonine kinase that mediates various cellular responses, including lipolysis. Since its discovery in the 1950s (1–4), the cAMP-PKA system has become one of the best understood signaling pathways in terms of biochemical properties. PKA is composed of catalytic subunits and cAMP-binding regulatory subunits (5). In the absence of cAMP stimulation, PKA forms an inactive tetramer composed of four subunits with two regulatory and two catalytic subunits. Upon nutritional deprivation or hormonal stimulation, activated adenyl cyclase produces cAMP from ATP. Then, the regulatory subunit of PKA binds cAMP, causing a conformational change that decreases its affinity for catalytic subunits by ~104-fold and leads to the release of active catalytic subunits to phosphorylate target proteins (6, 7).

PKA has a wide range of substrates that regulate a number of physiological processes. To date, several hundred PKA target proteins have been identified, and yet new PKA substrates are continually being reported (8, 9). In mammalian adipocytes, numerous studies over the last 40 years have revealed that the cAMP-PKA axis forms a critical node in the regulation of lipolysis. For instance, major components of lipolytic pathways, including hormone-sensitive lipase (Hsl)2 and perilipin 1 (Plin1), are direct targets of PKA (10, 11). Recently, adipose triglyceride lipase (Atg1) was also reported to be a target of PKA (12, 13). Upon receiving activation signals from molecules such as catecholamine and glucagon, activated PKA phosphorylates Plin1 and HSL to promote the recruitment of HSL to lipid droplets (14, 15). In addition, phosphorylated Plin1 releases competitive gene identification-58 (CGI-58: Abhd5), a coactivator of Atg1, to mediate lipolysis upon PKA activation (16–20). On the contrary, insulin can activate phosphodiesterase 3B (Pde3b) and reduce cAMP levels (10, 21, 22), thereby repressing PKA-mediated lipolysis. Receptors for other anti-lipolytic signals such as adenosine (23) and nicotinic acid (24) are coupled with inhibitory G proteins that inhibit adenyl cyclase. Thus, it is likely that most anabolic or catabolic signals affecting lipolysis are closely involved in the regulation of the cAMP-PKA axis.

Suppression of lipolysis in the basal state is crucial as excessive lipolysis would be detrimental and cause lipotoxicity. During the basal state, the pseudo-substrate domain of PKA regulatory subunits binds to the active site of catalytic subunits to inhibit kinase activity (25–27). Thus, regulatory subunits of PKA may contribute to maintain low levels of basal lipolysis by inhibiting the action of catalytic subunits. PKA has four regulatory subunit genes, RIIα, RIIβ, RIIα, and RIIβ, and two catalytic subunit genes, Ca and Cβ, in rodents (three in humans) (28). It has been reported that these subunits exhibit different tissue distributions and biochemical properties. To elucidate the role of these subunits, mouse models with genetically manipulated PKA subunits have been studied. In adipocytes, RIIβ is the predominant regulatory subunit isoform (29). RIIβ knock-out mice are morphologically normal and fertile despite reduced adiposity (29); they are also protected from diet-induced obesity and insulin resistance (30). Furthermore, it has been shown that the loss of RIIβ results in the up-regulation of RIIα subunit,

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2 The abbreviations used are: Hsl, hormone-sensitive lipase; ATGL, adipose triglyceride lipase; qRT-PCR, quantitative real time-PCR; hADSC, human adipose-derived stem cell.
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which leads to increased cAMP sensitivity and higher basal lipolysis in adipocytes (31, 32). Despite the abovementioned studies, deciphering fine-tuned regulatory mechanisms of PKA regulatory and catalytic subunits is limited in mouse models probably due to the compensatory effects of multiple subunit isoforms. Therefore, another approach is needed to reveal the regulatory processes of each PKA subunit, which would be important in decoding energy homeostasis.

The PKA pathway is evolutionarily well conserved; PKA of lower organisms is simpler than that of vertebrates. Because the Caenorhabditis elegans genome encodes a single PKA catalytic (kin-1) and regulatory (kin-2) subunit (33, 34), compensation by other subunit isoforms is not possible. Thus, C. elegans would likely be a great genetic model organism to study the roles of regulatory and catalytic subunits of PKA. Previously, we reported that PKA signaling is activated upon fasting in worms and plays crucial roles in fasting-induced lipolysis with ATGL-1 (C05D11.7) (13). In fasted worms, ATGL-1 is phosphorylated and stabilized by PKA in intestinal cells. In addition, PKA augments the interaction between ATGL-1 and lipid droplet protein LID-1 (C25A1.12), leading to lipolysis in the intestinal cells of C. elegans.

In this study, we investigated the effects of PKA subunit depletion on lipid metabolism. To define the roles of each PKA subunit and to minimize complex compensatory regulation observed in mouse models, we used C. elegans and mammalian adipocytes. First, we tested whether the balance between regulatory and catalytic subunits might influence PKA activity and lipolysis. Second, we explored which specific isoforms may be functionally important in mammalian adipocytes. In C. elegans, modulation of PKA activity by kin-1 or kin-2 RNA interference (RNAi) showed pleiotropic effects, including altered lipid metabolism. In mammalian adipocytes, combinatorial small interfering RNA (siRNA) transfection revealed that among the four regulatory subunits of PKA, R1α and R1β were required for the inhibition of basal lipolysis. These data suggest that the balance between catalytic and regulatory PKA subunits is important for blocking unnecessary PKA activation and avoiding futile lipolysis in lipid-storing tissues, independent of cAMP signaling.

Results

PKA Subunit Stoichiometry Hypothesis—To prevent unwanted PKA activation in the basal state, the kinase activity of catalytic subunits is efficiently controlled by regulatory subunits. Regulatory subunits of PKA have been proposed to be sensitive inhibitors of catalytic subunits in the absence of cAMP (5). Biochemical studies using purified PKA subunits have revealed that the apparent dissociation constant would be 0.1–0.6 nM (35, 36). It has been reported that the addition of a purified cAMP-binding component of PKA, the so-called regulatory subunits, to the protein kinase fraction of PKA inhibits its kinase activity in the absence of cAMP (37–39). We hypothesized that a decrease in the regulatory subunit would result in stimulation of PKA activity (Fig. 1A). Because the opening of the active site is critical for PKA catalytic subunits to phosphorylate target substrates, it is likely that the decrease of regulatory subunits, which bind to the active site cleft, would lead to increased kinase activity. Before thoroughly investigating how PKA activity is modulated by different ratios of regulatory and catalytic subunits in the absence of cAMP changes, we decided to analyze a simple inhibitor model of PKA to test whether our hypothesis is plausible. Assuming there is no change in cAMP concentration and the dissociation constant between catalytic and regulatory subunits is constant (0.1 nM), our analysis showed that the amounts of free and active catalytic subunits would steeply increase upon reduction of regulatory subunits (Fig. 1, B and C). However, this model has potential limitations to explain the action of tetrameric PKA action in living cells with varying cAMP concentrations. Thus, these calculations may give some clues for the hypothesis that the balance between catalytic and regulatory subunits of PKA might be crucial to determine basal PKA activity independent of cAMP levels.

PKA Subunit RNAi Modulates PKA Activity in C. elegans—Compared with other organisms, C. elegans has only one gene for the PKA catalytic and regulatory subunits (supplemental Fig. S1, A and B). C. elegans KIN-1 and KIN-2 show high protein sequence homology with mammalian PKA catalytic (~80%) and regulatory (~60%) subunit proteins. Multiple alignment and phylogram analysis showed that the isoforms of vertebrate PKA subunits would be diverged from ancestral forms as suggested previously (40). Thus, it appears that C. elegans PKA genes may have preserved ancestral forms of PKA and may be representative of many PKA genes among eukaryotes.

In worms, we investigated tissue distribution of kin-1 and kin-2 using their own promoters with green fluorescent protein (GFP) reporters. As shown in Fig. 1D, neither kin-1 nor kin-2 showed tissue specificity in C. elegans. Although kin-1 was highly expressed in body wall muscles, head muscles, and gonads, kin-2 was highly expressed in muscles and hypodermis. Expression of PKA genes in the intestine was relatively low compared with that in the muscles or gonad. To test the hypothesis that changes in PKA subunit balance may alter PKA activity in vivo, we introduced kin-1 or kin-2 RNAi in C. elegans. Feeding RNAi of kin-1 and kin-2 selectively reduced their respective mRNA levels (Fig. 1E). Next, we examined PKA activity with total protein extracts and the PKA-specific substrate Kemptide. As shown in Fig. 1F, kin-1 RNAi considerably decreased PKA activity both in the absence and presence of cAMP. In contrast, kin-2 RNAi greatly increased PKA activity in the absence of cAMP. However, in the presence of dibutyryl-cAMP, the PKA activity of kin-2 RNAi in worms was not different from that of controls. We also observed that addition of a PKA inhibitor drastically decreased PKA activity. Consistent with previous reports (33, 34), these data suggest that kin-1 and kin-2 are well conserved PKA subunit orthologs, and suppression of PKA subunits by feeding RNAi can effectively modulate PKA activity in C. elegans.

PKA Regulates Lipid Distribution in C. elegans—In living organisms, PKA participates in diverse cellular processes, which also seems to be the case in C. elegans. For instance, kin-2 RNAi caused dumpy morphology with aberrant intestinal structures, whereas kin-1 RNAi resulted in relatively normal morphology (Fig. 2A). Compared with control worms, both kin-1 and kin-2 RNAi worms had shortened life spans...
(Fig. 2B) and greatly reduced locomotor activity (Fig. 2C and supplemental Movies S1–S3). However, pharyngeal pumping rates were not significantly different (Fig. 2D). In addition, the number of eggs laid was markedly reduced in both kin-1 and kin-2 RNAi worms (Fig. 2E), which is consistent with a previous report that PKA signaling is important in oocyte maturation (41).

To investigate changes in lipid metabolism upon suppression of each PKA subunit, we biochemically measured triglyceride contents from whole worm extracts. As shown in Fig. 2F, kin-1 RNAi increased triglyceride contents compared with controls. However, kin-2 RNAi did not exhibit significant differences. These results were unexpected because PKA stimulated lipolysis in the intestine of C. elegans upon fasting (13). Therefore, we investigated lipid-storing organelles using several lipophilic dyes. It has been reported that intestinal organelles in C. elegans are highly heterogeneous, and different organelles stain differently depending on the kind of dye and fixation process (42, 43). When worms were stained with Nile Red and fatty acid-conjugated BODIPY in the live state, suppression of kin-2 by RNAi decreased the fluorescence intensity in intestinal cells (Fig. 2G). However, hypodermal signals stained by BODIPY fatty acids were not decreased by kin-2 RNAi. This result suggests that PKA activation by kin-2 RNAi would decrease Nile Red- and BODIPY fatty acid-positive intestinal granules known as lysosome-related organelles. On the contrary, Oil Red O staining,
FIGURE 2. PKA subunit alteration induces redistribution of lipids in C. elegans. A, differential interference contrast images of young adult worms grown on control, kin-1 RNAi, and kin-2 RNAi plates. B, life span assay (n = 60–90). C, bending rates of young adult worms in liquid media (n = 24). D, pumping rates of young adult worms in control, kin-1 RNAi, and kin-2 RNAi plates. E, number of eggs laid/h by young adult worms on control, kin-1 RNAi, and kin-2 RNAi plates (n = 10). F, triglyceride levels in young adult worms grown on control, kin-1 RNAi, and kin-2 RNAi plates. Triglyceride levels were normalized to total protein. G, images of C. elegans stained with BODIPY-conjugated fatty acid, Nile Red, and Oil Red O. BODIPY-conjugated fatty acid and Nile Red staining were performed on live worms, whereas Oil Red O stain was completed after fixation. H, high magnification observation of Oil Red O-stained images of control and kin-2 RNAi worms. White dashed lines indicate intestinal cells. I, confocal microscopic images of atgl-1(hj67) worms grown under control, kin-1 RNAi, and kin-2 RNAi conditions. J, mRNA levels of fil-1, cpt-3, and atgl-1 measured by qRT-PCR and normalized to act-1/3 mRNA. **, p < 0.01 versus control.
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which reportedly correlates well to biochemical triglyceride measurements (43), showed increased lipid content in kin-1 RNAi worms (Fig. 2G). However, in the kin-2 RNAi group, distribution of Oil Red O-stained granules exhibited an irregular pattern. More careful examination revealed that in kin-2 RNAi worms, intestinal lipid droplets were depleted, and stained lipids were mostly accumulated in extra-intestinal tissues (Fig. 2H). In accordance with a previous report (13), ATGL-1::GFP was found to be increased in the intestine upon kin-2 RNAi, which may explain the depletion of intestinal lipid storage (Fig. 2I). Quantitative real time-PCR (qRT-PCR) analysis showed that the expression levels of the fasting-induced gene fil-1 and fatty acid oxidation gene cpt-3 were enhanced by kin-2 RNAi (Fig. 2J). However, atgl-1 mRNA levels were not changed by kin-1 or kin-2 RNAi. Taken together, these data suggest that PKA activity would selectively regulate intestinal lipid metabolism in C. elegans.

Suppression of PKA Regulatory Subunit Increases Lipolysis in Mammalian Adipocytes—Compared with nematodes, PKA systems are complex in mammals. In mice, there are two catalytic and four regulatory subunit genes. Tissue distribution of each PKA subunit in mice showed different patterns (Fig. 3A); catalytic subunit Cα was ubiquitously expressed, whereas Cβ was highly expressed in the brain. Moreover, the expression pattern of the regulatory subunit RIα was ubiquitous, whereas that of other regulatory isoforms showed more tissue-specific patterns. For instance, RIIβ was abundant in the brain and low in other tissues, whereas RIα was enriched in the muscles, and RIIβ was predominantly expressed in adipose tissues and the brain. During adipocyte differentiation of 3T3-L1 cells, expression levels of catalytic subunit Cα and regulatory subunit RIIβ were increased (Fig. 3B). These data suggest that specific subunits of PKA might play certain roles in adipocytes.

Considering that PKA activity must be suppressed in the basal state, although its kinase activity is induced by hormonal stimulation, we hypothesized that the stoichiometry of regulatory and catalytic subunits of PKA is important to control basal PKA activity and, consequently, basal lipolytic activity in adipocytes. To test this, we transfected siRNAs that specifically targeted each PKA subunit into fully differentiated 3T3-L1 adipocytes. To verify whether PKA activity may be altered by knockdown, we examined phosphorylation of Plin1, a well known target of PKA in adipocytes, via Western blotting. Suppression of PKA activity would selectively regulate intestinal lipid metabolism in C. elegans.

PKA RIα and RIIβ Are Important for Lipolysis and Lipid Droplets in Adipocytes—To identify which PKA regulatory subunit isoforms are important for the regulation of basal lipolytic activity, we conducted combinatorial knockdown of each PKA subunit with siRNA in adipocytes. As shown in Fig. 4B, when RIIα and RIIβ subunits were not suppressed, increases in lipolysis discontinued, indicating that both are required to maintain basal lipolysis in adipocytes. We also noticed that knockdown of both RIIα and RIIβ subunits, but no other regulatory subunits, was sufficient to induce lipolysis (Fig. 4C), implying that these two subunits play key roles in PKA-mediated lipolysis in adipocytes. Furthermore, we observed that the knockdown of catalytic subunit Cα completely ablated the effect of regulatory subunit knockdown (Fig. 4C). Consistent with the results from glycerol release assays, siRNA transfection targeting PKA regulatory subunits led to a reduction in the size and number of lipid droplets in differentiated adipocytes (Fig. 4D). Moreover, the knockdown effect of regulatory subunits on lipid droplets was fully reversed by suppression of catalytic subunits of PKA.

To verify whether PKA activity may be altered by knockdown, we examined phosphorylation of Plin1, a well known target of PKA in adipocytes, via Western blotting. Suppression of RIIα and RIIβ markedly increased Plin1 phosphorylation,
whereas suppression of catalytic subunits reduced Plin1 phosphorylation (Fig. 4E). Similar to glycerol release assay data, Plin1 phosphorylation was not increased by suppression of either R1α or R1β alone. In Fig. 4E, the total amount of Plin1 was not changed by PKA activation, whereas the level of Plin1 mRNA was slightly decreased (data not shown). Interestingly, the increase in lipolysis and Plin1 phosphorylation was not accompanied by increased intracellular cAMP levels, proposing that regulation of PKA activity by suppressing regulatory subunits can be uncoupled from changes in intracellular cAMP levels (Fig. 4F). In the presence of the β-adrenergic receptor agonist isoproterenol, lipolysis was not further enhanced in R1α- and R1β-suppressed adipocytes (Fig. 4G), indicating that the maximal lipolytic rate could be achieved by suppression of R1α and R1β in adipocytes.

We also obtained similar results using differentiated human adipose-derived stem cells (hADSCs). The suppression of PKA regulatory subunits resulted in increased lipolysis and reduced lipid droplet size in hADSC (Fig. 4, H and I). Therefore, these data suggest the potential roles of PKA R1α and R1β subunits in cell-autonomous regulation of lipolysis and maintenance of lipid droplet morphology in adipocytes, which may be crucial for energy homeostasis.

**Atgl Is Required for PKA-mediated Lipolysis**—To elucidate the downstream effectors of PKA in lipolysis, we examined whether lipase might be involved in up-regulated lipolysis by suppression of PKA regulatory subunits in adipocytes. Cotransfection of siRNA against Atgl or Hsl with siRNA against PKA R1α and R1β showed that suppression of Atgl markedly decreased lipolysis, whereas suppression of HSL did not (Fig. 5A). Reduction of lipid droplets induced by PKA regulatory subunit suppression was also rescued by knockdown of ATGL (Fig. 5B). The level of Hsl phosphorylation by PKA activation was not changed by Atgl suppression, whereas Plin1 phosphorylation levels were slightly decreased, implying that there are some regulatory feedback mechanisms (Fig. 5C). To affirm these data, we tested the effects of the general lipase inhibitor orlistat and Atgl-selective inhibitor atglistatin on glycerol release. As shown in Fig. 5D, both orlistat and atglistatin down-regulated glycerol release induced by suppression of PKA reg-
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A, glycerol concentration in media from differentiated 3T3-L1 adipocytes 48 h after siRNA transfection. BODIPY 493/503 staining after fixation. C, Western blotting of lipolysis-related proteins in differentiated 3T3-L1 adipocytes 48 h after siRNA transfection. β-Actin protein was used as a loading control. Error bars represent standard deviations. D, measurement of glycerol concentrations in media from differentiated 3T3-L1 adipocytes in the presence or absence of lipase inhibitors. Glycerol concentrations were normalized to total protein and time. Scale bars, 10 μm; **, p < 0.01.

Insulin Blocks Lipolysis in PKA-activated Adipocytes—Next, we addressed whether insulin, an anti-lipolytic hormone, could relieve lipolysis induced by knockdown of PKA regulatory subunits in adipocytes. As shown in Fig. 6A, insulin down-regulated glycerol release induced by PKA activation with RIα and RIβ siRNA. In accordance with glycerol release data, insulin treatment attenuated the reduction of lipid droplet size (Fig. 6, B and C) accompanied by a decrease in Atgl protein levels (Fig. 6D). Furthermore, insulin decreased phosphorylation levels of both Hsl and Plin1, implying that insulin antagonizes various effects of PKA on lipolysis in multiple ways.

Fat-specific protein 27 (Fsp27/Cidec), a lipid droplet-associated protein in adipocytes, is mainly localized at the contact point of lipid droplets and promotes atypical lipid droplet fusion and formation of unilocular lipid droplets in adipocytes (44). Upon examination, the levels of Fsp27 protein were increased by PKA activation (Fig. 6D), as reported previously (45). To verify whether Fsp27 activity might be changed with PKA activation, we analyzed lipid exchange rates, a hallmark of lipid droplet fusion activity. Next, to address the potential role of Fsp27 in suppression of lipolysis by insulin, we suppressed Fsp27 with siRNA in combination with PKA regulatory subunits and analyzed lipolysis rates with or without insulin. Similar to previous reports (46, 47), Fsp27 suppression increased basal lipolysis and reduced lipid droplet size (Fig. 6, E and F). However, in Fsp27 siRNA-transfected adipocytes, the effects of insulin on lipolysis and lipid droplet size were completely blunted, although insulin-mediated phosphorylation of Akt was normal (Fig. 6G). These data suggest that Fsp27 would be a key mediator of the anti-lipolytic action of insulin.

Discussion

In this study, we characterized the phenotypes of PKA subunit suppression in C. elegans and mammalian adipocytes. Our data propose that the stoichiometric balance between catalytic and regulatory subunits of PKA is critical to the regulation of its activity (Fig. 7). In both C. elegans and mammalian adipocytes, alterations in PKA subunit balance led to changes in PKA activity and consequently in lipid metabolism. Among four PKA regulatory subunits in mammals, we found that RIα and RIβ were necessary and sufficient for maintenance of basal lipolysis and lipid droplet integrity in adipocytes. Furthermore, it is likely that increased lipolysis induced by suppression of PKA regulatory subunits is a cell-autonomous event uncoupled from cAMP signaling. Nonetheless, the enhanced lipolysis induced by activated PKA was readily antagonized by insulin in adipocytes.

To understand the physiological roles of PKA, various genetic models have been investigated. For instance, mutants lacking PKA catalytic subunits are lethal in yeasts (48) and flies (49). It has also been shown that C. elegans kin-1(ok338) and kin-2(tm635) deletion mutants exhibit lethal and sterile phenotypes (41, 50). Furthermore, targeted disruption of Ca or RIα in mice results in severe phenotypes, ranging from embryonic lethality to growth defects and sterility (51, 52), implicating essential and/or evolutionarily well conserved roles of PKA. Moreover, PKA is involved in numerous processes, such as muscle contraction, germ cell development, and energy metabolism (8). In accordance with these findings, we observed that suppression of both catalytic and regulatory subunits conferred various phenotypic changes, including reproductive defects, reduced locomotion, premature death, and aberrant lipid metabolism, in C. elegans (Fig. 2, B–E). Therefore, it is plausible that suppression of PKA subunits would impair diverse aspects of C. elegans physiology, although the causes of such phenotypes have not yet been determined at the molecular level.

With respect to lipid metabolism in C. elegans, it is of interest to note that suppression of PKA activity increased lipid storage in intestinal cells and vice versa and were correlated with the level of ATGL-1 protein. Unexpectedly, kin-2 RNAi-induced PKA activation followed by increased ATGL-1 levels did not change whole-body triglyceride levels (Fig. 2F). These phenotypes appear to be different from that of PKA activation under fasting conditions, which stimulates intestinal lipolysis accompanied by increased oxygen consumption (13). Instead, kin-2 RNAi produced ectopic lipid accumulation outside intestinal cells as shown by Oil Red O staining (Fig. 2H). It seems that...
lipolysis induced by *kin-2* RNAi may not be accompanied by an increase in whole-body energy expenditure so that mobilized lipid metabolites may be redistributed to other tissues, unlike fasting. In mammals, ectopic lipid accumulation in liver or muscle is closely associated with insulin resistance and metabolic diseases (53). Thus, it is feasible that lipolysis induced by *kin-2* RNAi may mediate ectopic lipid accumulation. Besides, it has been demonstrated that certain aspects of lipid metabolism are linked to life span, development, or reproduction (54–56). For example, *lipl-4* is an intestinal triglyceride lipase required for longevity by germ line loss (57). It has also been shown that monomethyl branched-chain fatty acids produced by fatty acid elongation enzymes *elo-5* and *elo-6* are essential for development of *C. elegans* (58). Furthermore, *fat-6; fat-7* double mutant worms with defective unsaturated fatty acid production exhibit growth retardation and reduced fertility (59), implying that certain lipid metabolites are crucial in the development and longevity of *C. elegans*. Thus, it is likely that aberrant lipid metabolism by *kin-1* or *kin-2* RNAi may contribute to detrimental phenotypes in *C. elegans*.

Here we demonstrated that an imbalance of PKA subunits led to changes in lipolytic activity and lipid droplet morphology in mammalian adipocytes. It has been proposed that suppression of basal lipolysis would be beneficial because futile lipolysis may cause lipotoxicity (60). For a tight regulation of basal PKA activity, it is plausible to speculate that stoichiometric balance is crucial for blocking unnecessary PKA activation. Our data show that the reduction of regulatory subunits alone is sufficient to induce PKA kinase activity and lipolytic activity, suggesting the importance of stoichiometric balance between regulatory and catalytic subunits. Considering that the molar ratios of regulatory and catalytic subunits are maintained within narrow ranges (nearly 1:1) in various mammalian tissues, it appears that abrupt changes in subunit stoichiometry may not occur with normal physiology (61). In adipose tissue, decreased expression of PKA regulatory subunits is observed in
obese humans (62) and mice fed high-fat diets over 17 weeks (data not shown), suggesting a potential relationship between PKA subunit imbalance and altered lipid metabolism. Stoichiometric regulatory modes are also found in other important cellular signaling cascades, such as class I phosphoinositide-3-kinase (PI3K) and nuclear factor κB (NF-κB) pathways. Both proteins have inhibitory/regulatory components that bind effector components (63–66). Similar to suppression of PKA regulatory subunits, it has been demonstrated that disruption of Pik3r1 and IkBα, regulatory genes of PI3K and NF-κB, results in enhanced signaling activity with severe phenotypes (67–69). Our data and these studies collectively suggest that stoichiometry of effector and inhibitory subunits could be an important strategy for efficiently responding to dynamic stimuli, as well as maintenance of the basal state.

Compared with lower eukaryotes, the mammalian PKA system is complex and cannot be fully explained by quantitative balance between subunits. During evolution, the PKA system probably increased the number of subunit isoforms to achieve selective signaling temporally, as well as spatially. This, in turn, could result in functional redundancy between different subunit isoforms. In mammalian adipocytes, suppression of one regulatory subunit alone, even the predominant RIIβ, did not cause any change in lipolysis. Instead, suppression of both RIIα and RIIβ subunits greatly potentiated basal lipolysis and reduced lipid droplets. These data may be explained by isoform compensation. For instance, increased RIIα has been reported in adipose tissue of RIIβ-deficient mice (29, 31), supporting the idea that these two genes are critical PKA regulatory subunit isoforms in adipocytes. The compensatory effect of multiple isoforms would be beneficial for maintaining constant basal PKA activity. Furthermore, we cannot exclude the possibility that certain isoforms may be more tightly linked to the lipolytic pathway, as each PKA subunit isoform may possess different functions or subcellular localization. Accordingly, protein kinase A anchoring proteins are able to bind to the regulatory subunit of PKA and lead to subcellular compartmentalization of cAMP-PKA signaling (70, 71). In adipocytes, it has been reported that optic atrophy 1, a regulator of mitochondrial dynamics, acts as a protein kinase A anchoring protein in lipid droplets and facilitates lipolysis upon stimulation (72). Thus, the regulatory mechanisms of PKA activity and lipolysis by subcellular distribution of RIIα and RIIβ would be important to pursue in further studies.

Although PKA mediates a majority of cAMP signaling, there are other proteins that may control cAMP signals in cells, such as exchange proteins activated by cAMP (Epac1/2) (73, 74), cyclic GMP-dependent kinase (75), and cyclic nucleotide-gated channels (76). In this regard, PKA activation by suppression of its regulatory subunits may be useful for dissecting cAMP-independent regulation of lipolysis because our data suggest that suppression of PKA regulatory subunits activates PKA without exogenous hormonal stimulation. We also found out that insulin markedly alleviated lipolytic activity induced by PKA regulatory subunit suppression. Previously, it has been suggested that a key factor in insulin’s anti-lipolytic effect is Pde3b, which decreases intracellular cAMP levels (22, 77). Considering that activation of PKA by suppression of RIIα and RIIβ did not
increase cAMP levels (Fig. 4F), it is possible that insulin also attenuates lipolysis via pathways other than Pde5b activation. In accordance with this speculation, it has been proposed that the anti-lipolytic effect of insulin cannot be fully explained by a decrease in cAMP-PKA signaling by various lipolytic stimulato rs (78). More recently, it has been reported that Akt-mediated phosphorylation and activation are not required for insulin to down-regulate lipolytic activity in adipocytes (79). Our observation that insulin decreased phosphorylation of PKA target proteins implies that serine/threonine protein phosphatase may account, at least in part, for another anti-lipolytic activity of insulin. In addition, insulin is known to reduce Atg1 expression (80–82), which is a major lipase downstream of PKA signaling. Under these circumstances, it is conceivable that insulin activity would play key roles in preventing hyperlipidemia, lipodystrophy, or lipid wasting in cancer cachexia because insulin could exert anti-lipolytic effects even in autonomously PKA-activated adipocytes. On the contrary, lipodystrophy and cachexia are frequently associated with insulin resistance (83–85). In addition, there is a close correlation between insulin resistance and hyperlipidemia in metabolic diseases (53). Intriguingly, it has been reported that a human patient lacking Fsp27 showed partial lipodystrophy and insulin resistance (86). Accordingly, our data showed that PKA-mediated lipolysis was suppressed by insulin, and PKA activation was not accompanied by changes in Fsp27-mediated lipid droplet fusion (supplemental Fig. S3). These data indicate that the mechanism by which Fsp27 deficiency promotes lipolysis is different from that of PKA activation and that Fsp27 may be required for insulin-mediated anti-lipolysis (Fig. 6, E and F). The involvement of Fsp27 in the regulation of lipolysis seems to be further complicated by the observation that Fsp27 protein level was increased upon PKA activation (Fig. 6D). This result suggests that there might be a negative feedback regulatory mechanism to prevent excess lipolysis (45, 87). Although the molecular mechanisms involving Fsp27 in PKA and insulin signaling still remain elusive, Fsp27 likely plays certain roles in restraining excessive lipolysis and protecting lipid storage.

In summary, we characterized several phenotypes of PKA subunit deficiency induced by altered stoichiometric balance of catalytic and regulatory subunits. Imbalance of PKA subunits modulated PKA activity without a change in cAMP levels, eventually leading to the dysregulation of lipolysis in lipid-storing tissues. Together, these findings suggest that the balance between catalytic and regulatory subunits is an important factor determining PKA activity, which has not yet been explored in great detail.

Experimental Procedures

Nematode Culture and RNAi—The N2 Bristol strain of nematode was used as the wild type. All animals were raised at 20 °C in standard nematode growth medium. For feeding RNAi, kin-2 RNAi clones were obtained from the Ahringer RNAi library. kin-1 RNAi was generated by cloning the cDNA fragment of kin-1. Synchronized worms were cultured on RNAi plates until they reached the young adult stage. The efficiency of RNAi was confirmed by qRT-PCR using appropriate primers. Construction of Transgenic Strains—To generate kin-1 and kin-2 promoter reporter worms, the promoter regions ~2 kb upstream from transcription start sites were cloned into pH95.77 vectors to express GFP. Transgenic worms were generated by microinjection with co-injection of pRF4-dominant roller markers.

Cell Culture and Transfection—3T3-L1 preadipocytes were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum. 2 days after confluence, cells were induced to differentiate into adipocyte with DMEM containing 10% fetal bovine serum (FBS), 3-isobutyl-1-methylxanthine (520 μM), dexamethasone (1 μM), and insulin (167 nM) for 48 h. Then, the differentiation induction medium was replaced with DMEM with 10% FBS and insulin (167 nM). After 2 days, medium was changed with DMEM containing 10% FBS every 2 days for 5–7 days. hADSCs (Lonza; PT-5006) were cultured and differentiated into adipocyte according to the manufacturer’s protocol with slight modification. Briefly, hADSCs were maintained in preadipocyte basal medium (Lonza; PT-8002). After the cells reached confluence, hADSCs were incubated with DMEM containing 10% FBS, 3-isobutyl-1-methylxanthine (520 μM), dexamethasone (1 μM), insulin (167 nM), and troglitazone (1 μM) for 10–12 days with occasional changes. For transfection of siRNAs, differentiated 3T3-L1 adipocytes and hADSCs were extensively washed with phosphate-buffered saline (PBS) and treated with trypsin/EDTA. Then, cells were collected by centrifuging for 2 min at 1000 rpm. After washing with PBS, cells were mixed with siRNA and transfected with a single pulse of 1100 V for 30 ms using a Microporator MP-100 (Digital Bio).

Lipid Staining—Lipid staining with Nile Red and BODIPY-fatty acid in C. elegans was performed as reported previously (88). In brief, Nile Red (N3013; Sigma; 250 μg/ml in acetone) and BODIPY-fatty acid (D3823; Molecular Probes; 1 mg/ml in dimethyl sulfoxide) stock solutions were diluted in 1:1000 in PBS and added to the top of nematode growth medium plates with RNAi bacteria. Then, synchronized worms were grown until they reached the young adult stage and examined. Oil Red O staining in C. elegans was performed as reported previously (89). Briefly, worms were harvested and resuspended in 60 μl of 1× PBS (pH 7.4), 120 μl of 2X Modified Ruvkun’s Witches Brew buffer, and 60 μl of 4% paraformaldehyde. Worms were freeze-thawed three times and washed with PBS before being dehydrated in 60% isopropyl alcohol for 10 min at room temperature and stained with Oil Red O solution. To visualize lipid droplets in 3T3-L1 adipocytes, cells were fixed with 1% paraformaldehyde. BODIPY 493/503 stock solution (D-3922; Molecular Probes; 1 mg/ml in acetone) was diluted 1:1000 in PBS before being added and incubated with cells for 30 min. After washing with PBS, cells were observed using a confocal microscope.

C. elegans Assays—For life span assays, synchronized worms were grown and maintained on RNAi plates. The number of live and dead worms was counted every day until no live worms were left. For egg-laying assays, 1-day-old young adult worms were transferred to new plates (one worm per plate). After 2 h, the number of eggs laid was counted. To measure bending rates, worms were suspended in M9 buffer and their movements ana-
lyzed. Pumping rates were counted by measuring the number of contraction and relaxation cycles of pharyngeal muscles using a stereomicroscope.

Biochemical Triglyceride Measurement—Triglyceride contents were measured using a triglyceride assay kit (catalog no. TR22321; Thermo Scientific). Synchronized young adult worms were resuspended in 5% Triton X-100 solution and homogenized using glass beads and a Precellys® 24 homogenizer (Bertin Technologies). For complete lysis and triglyceride extraction, homogenates were sonicated and subjected to two cycles of heating (80 °C) and cooling (room temperature). Then, worm extracts obtained by centrifugation were used to measure total triglyceride amounts according to the manufacturer’s protocol.

cAMP Measurement—cAMP concentrations were measured using a direct cAMP ELISA kit (catalog no. 25-0114; Enzo Life Sciences) according to the manufacturer’s protocol. Cells were resuspended and lysed in 0.1 N HCl to inactive phosphodiesterase. After centrifugation, supernatants of cell extracts were used for ELISA. Results were analyzed using four parametric logistic curve fitting models. cAMP concentrations were normalized by the total protein.

PKA Activity Assay—Synchronized young adult worms were harvested and homogenized in protein extraction buffer (20 mM Tris-HCl, 10 mM dithiothreitol, 1 mM NaF, 1 mM NaVO₄, 10 mM EDTA, 10 mM EGTA, and a protease inhibitor mixture). Worm extracts were sonicated and centrifuged to obtain soluble proteins. PKA activity was measured according to a previously reported protocol (90). Briefly, 30 µg of protein was incubated with kinase assay buffer (50 mM Tris-HCl, 0.1 mM EGTA, and 10 mM magnesium acetate), Kemptide (PKA substrate peptide), and 1 mM [γ-³²P]ATP at 30 °C for 10 min. The reaction was terminated by spotting the reaction mixture onto P81 phosphocellulose paper. The paper was immediately immersed in 75 mM phosphoric acid. After washing three times with fresh phosphoric acid, the papers were briefly rinsed with acetone and air dried. Radioactivity was measured with a Cerenkov counter.

Immunoblot—Cells were lysed on ice with radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% (v/v) Nonidet P-40, 0.25% (w/v) sodium deoxycholate, and a protease inhibitor mixture) and subjected to Western blotting.

Glycerol Release Assay—The glycerol concentration in the medium of differentiated adipocytes was measured using Free Glycerol Reagent (F6428; Sigma) according to the manufacturer’s protocol. Briefly, cells were incubated with 2% fatty acid free-bovine serum albumin in serum-free high glucose Dulbecco’s modified Eagle’s medium with dimethyl sulfoxide and forskolin (Calbiochem) or isoprotenerol (Sigma). After 3 h, conditioned medium was collected and used for assay. Total protein was used for normalization.

qRT-PCR—Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase with random hexamer primers (Fermentas). qRT-PCR was performed on a CFX96 real time system (Bio-Rad) with SYBR Green (Invitrogen). For relative quantitative analysis of PKA subunit isoform genes, the PCR efficiencies of primer sets were normalized using known concentrations of templates.

Lipid Exchange Assay—FRAP-based lipid exchange assay was performed as reported previously (91). In siRNA-transfected 3T3-L1 adipocytes stained with BODIPY 558/568 C12 fatty acid (D-3835; Molecular Probes), select lipid droplet pairs were subjected to analysis. Select regions were bleached by 200 interactions at 100% laser power (543 diode laser), followed by time-lapse scanning with a 20-s interval. For FRAP analysis, mean optical intensity within lipid droplet core regions was measured using ImageJ. To obtain fluorescence recovery curves, mean optical intensity values were calculated as the percentage of the original fluorescent intensity and plotted using Prism 5.

Statistical Analysis—Values are shown as mean ± S.D. Unless otherwise mentioned, mean values were compared and evaluated with two-way analysis of variance with a Bonferroni post-test. A p value of <0.05 was considered significant.

Author Contributions—J. H. L. designed and performed the study and wrote the paper. J. S. H., J. K., Y. J., and X. L. performed and analyzed the experiments. J. L. and P. L. discussed the study and contributed to the writing of the manuscript. J. B. K. conceived and coordinated the study and wrote the paper. All authors reviewed the results and approved the final version of the manuscript.

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