Mutation of the RIIβ Subunit of Protein Kinase A Differentially Affects Lipolysis but Not Gene Induction in White Adipose Tissue*

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Targeted disruption of the RIIβ subunit of protein kinase A (PKA) produces lean mice that resist diet-induced obesity. In this report we examine the effects of the RIIβ knockout on white adipose tissue physiology. Loss of RIIβ is compensated by an increase in the RIA isoform, generating an isoform switch from a type II to a type I PKA. Type I holoenzyme binds cAMP more avidly and is more easily activated than the type II enzyme. These alterations are associated with increases in both basal kinase activity and the basal rate of lipolysis, possibly contributing to the lean phenotype. However, the ability of both β, selective and nonspecific β-adrenergic agonists to stimulate lipolysis is markedly compromised in mutant white adipose tissue. This defect was found in vitro and in vivo and does not result from reduced expression of β-adrenergic receptor or hormone-sensitive lipase genes. In contrast, β-adrenergic stimulated gene transcription remains intact, and the expression of key genes involved in lipid metabolism is normal under both fasted and fed conditions. We suggest that the R subunit isoform switch disrupts the subcellular localization of PKA that is required for efficient transduction of signals that modulate lipolysis but not for those that mediate gene expression.

Protein kinase A (PKA) transduces the cAMP-mediated signals from more than 30 different hormones and neurotransmitters, many of which may act simultaneously on a given cell to provoke discrete biological responses (1). The properties of PKA that modulate its signaling specificity are poorly understood, although it has been speculated that regulatory (R) and catalytic (C) subunit isoform diversity confers at least some of this specificity by assembling into discrete holoenzyme complexes that differ in subcellular targeting and sensitivity to activation by cAMP. Four R subunit isoforms (RIα, RIIα, RIIβ, and RIIγ) and two C subunit isoforms (Ca and Cβ) are transcribed in mice (2). Each is encoded by a separate gene and expressed in a tissue-specific pattern. Although much is understood regarding the physical properties of individual PKA isoforms, relatively little is known about the biological roles of each isoform in vivo. Knockout mice lacking individual PKA subunit genes represent powerful tools to elucidate these functions (3).

We have generated null mutant mice lacking the RIIβ isoform of PKA (4). Unlike some PKA subunit isoforms that are expressed ubiquitously, RIIβ demonstrates very restricted tissue distribution. It is most abundant in white and brown adipose tissue and brain, with very limited expression elsewhere. RIIβ knockout mice are lean on a standard diet and resist diet-induced obesity as well as some of its associated adverse consequences. On a standard diet they have a 50% reduction in adipose tissue mass throughout their bodies, despite normal food intake, lipid absorption, and adipocyte cellularity. These changes may arise at least in part from PKA perturbations in brown adipose tissue (BAT). PKA in mutant BAT is more sensitive to cAMP and shows increased basal enzyme activity, alterations that are associated with an induction of uncoupling protein 1. Elevated levels of this thermogenic molecule correlate with an increase in basal metabolic rate and body temperature and may contribute to the lean phenotype.

Here we report studies of PKA-mediated functions in the white adipose tissue (WAT) of RIIβ knockout mice. In WAT, PKA integrates several different hormonal signals to regulate the lipolytic catabolism of stored triglycerides into fatty acids and glycerol by hormone-sensitive lipase (HSL). Lipolysis is increased by β-adrenergic agonists, ACTH, and glucagon, all signaling via cAMP to stimulate PKA, which reversibly phosphorylates three serine residues on HSL to activate the enzyme (5–7) and promote translocation to lipid droplets (8, 9). Lipolysis is inhibited by insulin, which stimulates a phosphodiesterase (PDE3B) that lowers cAMP levels (10). PKA also regulates several lipogenic enzymes, generally inhibiting gene expression in opposition to insulin action. In addition, PKA mediates the induction of cAMP-response element–regulated genes in WAT, including the well studied PEPCK gene. We find that RIIβ mutant WAT has a blunted capacity for PKA-stimulated lipolysis, whereas PKA-mediated transcriptional regulation is relatively unaffected. Possible mechanisms underlying these changes are discussed.

EXPERIMENTAL PROCEDURES

Mice—We have previously described the generation of RIIβ mutant mice (4, 11). Wild type and mutant mice used in all experiments were age- and gender-matched and maintained on the same mixed C57BL/6 × 129SvJ genetic background. For the experiments on the nutritional regulation of PKA-mediated gene expression, adult male mice were fasted for 24 h, after which half of the animals were sacrificed (fasted group) and the rest allowed to feed on standard mouse chow (Teklad Rodent Diet 8604) for 6 h (refed group) before being sacrificed. Epidid-
Lipolysis and Gene Expression in RIIβ Mutant WAT

yal fat pads were dissected, immediately frozen in liquid nitrogen, and stored at −70 °C. cAMP binding Capacity—Male wild type and mutant mice were sacrificed by cervical dislocation, and epididymal fat pads were immediately removed, weighed, and frozen at −70 °C. WAT was homogenized (10% w/v) in cold 6% trichloroacetic acid by Polytron treatment. Homogenates were centrifuged at 13,000 × g for 15 min at 4 °C. Internatants were harvested and extracted five times with 5-fold excess volumes of water-saturated ether (to remove trichloroacetic acid). Extracted aqueous samples were dried and resuspended in 300 μl of NEN assay buffer. The final cAMP concentration was determined by radioimmunoassay (NEN Life Science Products).

mRNA Quantitation—The steady-state amount of mRNA derived from specific genes was determined by solution hybridization as described previously (15). Briefly, total nucleic acid was isolated from individual tissues by proteinase K digestion and phenol/chloroform extraction (16). Samples were hybridized overnight with approximately 5000 cpm of [32P]CTP-labeled antisense RNA probe at 70 °C under paraffin oil. Free probe was then digested with RNase A and T1 at 1 h 37 °C. Samples were precipitated with 10% trichloroacetic acid and collected on Whatman GF/C glass microfiber filters (Whatman) to trap hybridized probe. The amount of RNAse-resistant probe was measured by liquid scintillation counting. Standard curves were generated from known amounts of appropriate sense strand RNA. The results were converted to molecules of mRNA per cell based on both the standard curves and the specific activity of the probe. cDNAs used as templates for the synthesis of RNA probes and standards were kindly provided by K.-H. Kim, Purdue University (ACC); M. D. Lane, The Johns Hopkins University (GLUT4); M. C. Schotz, UCLA (HSL and LPL); D. K. Graner, Vanderbilt University (PEPCk); D. S. Weigle, University of Washington (leptin); and S. Collins, Duke University (β-ARs).

RESULTS

Effects of RIIβ Knockout on PKA Holoenzyme Properties in WAT—We have previously published data showing that RIIβ is the predominant R subunit in WAT and that its loss in RIIβ knockouts is compensated for in this tissue by a 3–4-fold increase of the Rα isoform, arising by Rα protein stabilization (17). This is the only R subunit adjustment in mutant WAT, as neither Rβ nor RIIa is expressed at appreciable levels (data not shown). We have also demonstrated by high performance liquid chromatography/ion exchange chromatography that in WAT there is an isofrom switch from a nearly pure RII-containing holoenzyme in wild types to an entirely RI-containing holoenzyme in mutants (17).

In order to determine the cAMP affinities of PKA from wild type versus RIIβ mutant WAT, the cAMP-binding capacity of tissue homogenates was measured. As shown in Fig. 1A, the Rα-containing mutant holoenzyme binds cAMP more avidly than does the RIIβ-containing wild type holoenzyme, with Kd values of 170 and 400 nM, respectively. As a consequence, mutant PKA is more readily activated by cAMP than is wild type enzyme (Kc values of 80 and 220 nM, respectively, Fig. 1B). This increased cAMP sensitivity is reflected by a 4-fold elevation of basal PKA activity in mutant WAT (Fig. 1B, inset).

The overall complement of both R and C subunits is reduced in mutant WAT. The total number of R subunits (of any isoform) is reflected in the total cAMP-binding capacity at saturating cAMP concentrations. As shown in Fig. 1A, the maximal cAMP-binding capacity (and thus, total R subunit content) of RIIβ mutant WAT is reduced by 30% compared with wild type. Similarly, the amount of C subunit in mutant WAT is decreased by 55%, as judged by the reduction in total cAMP-stimulated PKA activity (Fig. 1B). We have previously published Western blot analysis showing a corresponding 43% decrease in the amount of C subunit protein in mutant WAT (17).

Lipolysis in Mutant WAT—It is well established that β-adrenergic stimulation of WAT leads to increased intracellular cAMP, activation of PKA, and stimulation of hormone-sensitive lipase by direct PKA phosphorylation (5, 6). Accordingly, to determine the functional impact of RIIβ deficiency on PKA-mediated signaling, the effects of various β-adrenergic agonists upon lipolysis were assessed. Lipolysis was assayed in vitro by glycerol release from cultured adipose tissue. The basal rate of lipolysis is mildly increased in mutant WAT, as might be pre-
in view of the increased basal PKA activity (Fig. 2A).

However, mutant WAT shows a severely blunted capacity for lipolytic stimulation by isoproterenol, a non-selective agonist of $\beta_1$-, $\beta_2$-, and $\beta_3$-adrenergic receptors. All of these receptor subtypes are expressed in murine WAT (18) and normally couple via a $G_o$-adenylate cyclase mechanism to activate PKA and lipolysis. Isoproterenol increased the rate of lipolysis more than 5-fold in wild types but only approximately 50% in mutants (Fig. 2B).

The in vitro lipolysis experiments were repeated in the presence of adenosine deaminase (ADA), with and without the $A_1$-selective adenosine receptor agonist PIA. In differentiated adipocytes, adenosine and PIA both suppress lipolysis by interacting with $G_i$-coupled $A_1$-receptors that lower cAMP levels. Significant and variable amounts of endogenous adenosine can be released from cultured adipocytes, confounding lipolysis assays (19). ADA circumvents this problem by eliminating endogenous adenosine, thereby decreasing inter-assay variability (20) but also enhancing lipolysis (21, 22). As expected, ADA increased basal and stimulated rates of lipolysis in both wild type and mutant WAT (Fig. 2C). In contrast, PIA decreased these rates for both groups (Fig. 2D), presumably by decreasing intracellular cAMP concentration (23). However, the essential findings described above persisted in all conditions. Mutant WAT showed a slightly higher basal rate of lipolysis but a severely blunted capacity for hormone-mediated lipolytic stimulation. This defect was found equally with isoproterenol and the $\beta_3$-selective agonist, CL 316,243 (24), both of which were added at concentrations previously shown to stimulate lipolysis maximally (25).

In both wild type and mutant WAT the maximal lipolytic responses were equal for isoproterenol and CL. This might seem surprising, given that isoproterenol stimulates lipolysis via all three $\beta$-adrenergic receptor ($\beta$-AR) subtypes, all of which are expressed in WAT. However, our results agree with prior reports indicating that $\beta_3$-ARs predominate in regulating lipolysis. In murine WAT, $\beta_1$, $\beta_2$, and $\beta_3$-AR mRNA transcripts are expressed in a 3:1:150 ratio, respectively (18), and it has been estimated that $\beta_3$-ARs are responsible for at least 80% of the maximal isoproterenol-induced cAMP response (18, 26). Fur-
Lipolysis and Gene Expression in RIIβ Mutant WAT

In vivo assessment of lipolysis in RIIβ mutant and wild type mice. Serum glycerol was measured 20 min after intraperitoneal injection of normal saline, isoproterenol (0.3 mg/kg), or CL 316,243 (1.0 mg/kg). Results are means ± S.D. (n = 9/group for baseline, n = 7/group for isoproterenol, n = 9/group for CL 316,243). WT, wild type; KO, knockout.

Therefore, Northern blots probed with mRNAs were too low to be accurately quantitated by solution hybridization. Therefore, Northern blots probed with specific probes are shown in Fig. 4.

Expression of HSL and β-AR genes—To determine whether the perturbations in lipolysis seen in RII mutants result from alterations in the expression of either β-ARs or HSL, steady-state mRNA levels of these gene products were measured in WAT using solution hybridization and Northern blot. Because the expression of β-ARs and HSL could be affected by the state of feeding (28, 29), experiments were performed both in 24-h fasted and fed mice. In order to synchronize the feeding status of the latter group, mice were first fasted for 24 h and then refeed for 6 h before being sacrificed. As shown in Fig. 4, there were no significant differences between knockout and wild type WAT with regard to the expression of HSL regardless of nutritional status. Solution hybridizations with β-adrenergic receptor probes also detected no changes in β2-receptor mRNA levels, but levels of β1 and β3-receptor mRNAs were too low to be accurately quantitated by solution hybridization. Therefore, Northern blots probed with β-AR-specific probes are shown in Fig. 4B using RNA from fed animals.

PKA-dependent Gene Expression in RIIβ Knockout WAT—In order to determine the functional consequences of RIIβ deficiency on PKA-mediated gene expression in WAT, steady-state levels of mRNA from PKA-regulated genes were examined by solution hybridization. It has been shown previously that several of the enzymes involved in lipogenesis are transcriptionally regulated by PKA. In WAT, PKA inhibits the expression of acetyl-CoA carboxylase (ACC) (30, 31), lipoprotein lipase (LPL) (32), and the insulin-responsive glucose transporter GLUT4 (33). In contrast, PKA activation is associated with enhanced expression of PEPCK (30). All of these genes are regulated by other factors, especially insulin, and their levels of expression vary with the state of feeding (34, 35). Accordingly, experiments were performed using mice subjected to 24-h fasting and 6-h refeeding protocols as described above.

Surprisingly, expression of some of these PKA-regulated genes was regulated normally in RIIβ mutant WAT (Fig. 5), despite the RIIβ-to-RIα isoform switch and consequent perturbations of PKA activity described above. As expected, expression of ACC and GLUT4 was low in fasted mice and increased with refeeding. However, there were no significant differences between wild type and mutant mice in the fasted state, and only a small but significant (p < 0.05) increase in GLUT4 mRNA comparing mutant with wild type in the refed group. LPL expression was completely unaffected by the knockout in either fasted or refeed animals. PEPCK expression showed the anticipated induction with fasting but was expressed similarly in wild types and mutants, regardless of feeding status.

To verify that PKA-mediated gene expression is unperturbed in mutant WAT even though PKA-mediated lipolytic stimulation is blunted, we examined PEPCK gene expression and lipolysis simultaneously in vitro. Lipolysis assays were performed on cultured adipose tissue as described above, with aliquots of media harvested every 15 min for 2 h to determine glycerol content. Incubations with or without isoproterenol continued for a total of 6 h, after which all cells were harvested and subjected to solution hybridization to measure PEPCK mRNA content. As shown in Fig. 6, there was a 2-fold increase in basal (unstimulated) lipolysis in mutant WAT compared with wild type, but isoproterenol stimulated lipolysis by about 6-fold in wild type samples, compared with only 1.4-fold in mutants. In contrast, PEPCK mRNA expression from these cells was induced approximately 5-fold in both mutant and wild type samples, and there was no change in basal PEPCK mRNA comparing mutant with wild type.

Leptin expression was inhibited dramatically by fasting in both wild type and mutant WAT (Fig. 5). There was no significant difference between the two fasted groups, although mRNA levels were near the minimal level of detection in our assay. Leptin was induced by refeeding in both groups; however, the mean level in refed mutants was nearly five times less...
than that in wild types, and although there were large animal to animal variations, the difference was statistically significant ($p, 0.05$).

**DISCUSSION**

RIIβ null mutant mice offer a model to study the specific functions of individual PKA isoforms. In both WAT and BAT, RIIβ is normally the prevailing R subunit, expressed far more abundantly than any other isoform. Its loss is compensated for solely by an increase in RIA protein, producing a switch from a predominantly type II to type I holoenzyme (4, 17). By studying adipose tissue in RIIβ mutants we can identify those PKA signaling functions that require an RII-type holoenzyme versus those that can also be subserved by an RIA-containing enzyme.

Because PKA signaling anomalies in RIIβ mutant mice could theoretically arise from changes in the number of R or C subunits, rather than from the isoform switch, we quantified these proteins in WAT. Whereas RIA compensation is virtually complete in mutant BAT (4), there is a 30% loss of R subunits overall in mutant WAT, as assessed by total cAMP-binding capacity. However, there is also an approximately 50% loss of C subunits in this tissue, as judged by Western blot analysis and total PKA activity. Since an R:C ratio of at least 1:1 is maintained, the increased basal PKA activity seen in mutant WAT cannot be accounted for by a gross disregrulation of C subunits due to an inadequate quantity of R subunits. Altered PKA activity also does not appear to be caused by any difference in overall cAMP concentration in mutant compared with wild type WAT (379 ± 177 versus 359 ± 64 nM, respectively). Instead, the increased basal PKA activity probably arises because the type I PKA in mutants has a greater affinity for cAMP and thus activates more easily than does the type II enzyme in wild types. In this regard, the PKA alterations seen in RIIβ mutant WAT are analogous to those previously described in BAT (4). Both tissues show a 4–5-fold increase in basal PKA activity but a decrease in total cAMP-stimulated activity. The only differences between mutant WAT and BAT with respect to changes in PKA appear to be quantitative. Compared with BAT, WAT shows a greater loss of both total R subunits (30% decrease versus 10%) and C subunits (50% decrease versus 30%).

Loss of RIIβ from mutant WAT markedly impairs PKA-mediated activation of a pre-formed enzyme (HSL), yet leaves PKA-regulated gene expression relatively unperturbed. The disruption of lipolytic stimulation is seen both in vitro and in vivo and occurs equally for signaling from the $\beta_2$-receptor-specific agonist CL 316,243 and a nonspecific $\beta$-agonist, isoproterenol. It is unlikely that the signaling defect in RIIβ mutant WAT is caused by decreased expression of either $\beta$-ARs or HSL, given that mRNA levels for these genes are unaffected. Conceivably, impairment of lipolytic hormonal response could be a consequence of the chronic stimulation of basal lipolysis seen in RIIβ knockouts. However, adipocytes from transgenic mice deficient in the G-protein subunit, $G_{\alpha}\omega$, show a 3-fold increase in basal cAMP levels and an elevated basal rate of lipolysis but retain normal maximal response to $\beta$-adrenergic agonists (36). Thus, continuous stimulation of basal lipolysis alone does not
appear to limit lipolytic response to hormones. Another possibility is that maximal lipolytic stimulation in mutant WAT is rate-limited by the 50% loss of total C subunit protein in this tissue, whereas levels of C subunit are not rate-limiting for gene induction.

We favor the hypothesis that at least some of the elements mediating lipolytic stimulation (e.g. β-ARs, adenylate cyclase, PKA, and HSL) may be co-localized within adipocytes to facilitate efficient signal transduction, and that lipolytic stimulation is impaired in RIIβ mutants because RIIβ participates specifically in the formation of this complex. The following observations from previous studies suggest the existence of a compartmentalized apparatus mediating lipolytic stimulation. (i) At any given intracellular cAMP concentration, the lipolytic response from catecholamines is greater than that from forskolin, a nonspecific adenylate cyclase activator (21, 22, 37). Hence, low concentrations of isoproterenol (10 nM) can stimulate lipolysis without measurably altering overall cAMP levels, whereas low concentrations of forskolin (0.1–1.0 μM) increase intracellular cAMP levels without affecting lipolysis (37). (ii) The concentration of isoproterenol or β2-specific agonists required for half-maximal activation of adenylate cyclase activity in adipocyte membranes is ~80× greater than the concentration required to activate lipolysis (27, 38, 39). (iii) Recently a signaling complex including β2-AR, PKA, and phosphatases has been isolated that appears to be assembled by the scaffold protein, gravin (40). In summary, catecholamines stimulate lipolysis more potently than they increase overall intracellular cAMP, suggesting a preferential association between receptor, PKA, and perhaps its substrate, HSL.

In numerous cell types, co-localization of key components involved in PKA signaling is accomplished by protein kinase A anchoring proteins (AKAPs), multivalent binding proteins that serve as platforms for the assembly of signal transduction modules (41, 42). These targeting proteins bind simultaneously to specific sites on the amino terminus of R subunits and to discrete subcellular structures. By tethering PKA at precise intracellular sites, AKAPs ensure that the kinase is exposed to localized changes in cAMP adjacent to appropriate substrates, thus preventing cross-talk between functionally unrelated PKA signaling units within the same cell. Although AKAPs have not yet been described in WAT, they have been found in virtually all other tissues investigated. We have preliminary data demonstrating AKAPs expressed in WAT, and it seems reasonable to speculate that they may facilitate phosphorylation of HSL by PKA.

Since most of the mammalian AKAPs identified to date are strongly RII-specific, PKA-AKAP binding might be disrupted in RIIβ mutant WAT due to the type II to type I isoform switch. This alteration could displace the PKA holoenzyme from local waves of cAMP generated by β-adrenergic agonists and/or from HSL, which would explain the blunted lipolytic response to adrenergic stimuli seen in mutant WAT. Indeed, disruption of RII-AKAP binding has been shown to have diverse functional consequences in other model systems (43–45). Our findings that PKA-mediated gene expression remains relatively intact in RIIβ mutant WAT suggest that high affinity anchoring is not required for this particular PKA function.

Leptin and to a lesser extent GLUT4 were the only gene products whose expression was markedly different in RIIβ mutant compared with wild type WAT, comparing the refed state. Leptin is a long term anorexigenic factor, and in most settings its level mirrors the amount of body adiposity (46–49). We found the same strong positive correlation of leptin expres-

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2 A. Sikorski and G. S. McKnight, unpublished observations.

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3 S. Schreyer, D. E. Cummings, G. S. McKnight, and R. LeBoeuf, unpublished data.
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