ADP-ribosylation factor 6 modulates adrenergic stimulated lipolysis in adipocytes

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ELEVATED PLASMA free fatty acids (FFA) concentrations cause insulin resistance by inhibiting insulin-mediated glucose uptake by skeletal muscle and insulin-mediated suppression of glucose production by the liver (4, 18, 57). Therefore, alterations in adipose tissue lipolytic activity and the rate of FFA release into plasma are important factors in the pathogenesis of obesity-related metabolic abnormalities (36). Adipose tissue lipolytic activity is regulated by hormonal and biochemical signals (6, 16); catecholamines and insulin being the major physiological regulators that stimulate and inhibit adipose tissue lipolytic rates, respectively (6, 11). During energy restriction and endurance exercise, the two major physiological states that require the mobilization of endogenous triglycerides as fuel, lipolysis of adipose tissue triglycerides and release of FFA into plasma, are induced in part by catecholamine-stimulated β-adrenergic activation (13, 20). β-Adrenergic receptors (βARs) are G protein-coupled receptors (GPCR) that transmit a stimulatory signal to adenylyl cyclase to increase intracellular cAMP levels. Subsequent activation of protein kinase A (PKA) by cAMP causes phosphorylation-induced activation of hormone-sensitive lipase and its translocation from cytosol to the surface of the adipocyte lipid droplet. PKA also phosphorylates perilipin on the droplet, resulting in biochemical changes that enhance substrate accessibility and lipolysis by hormone-sensitive lipase. Additional signaling pathways including mitogen-activated protein kinase (MAPK), protein kinase C, and AMP-activated protein kinase have been identified to regulate the stimulation of lipolysis in adipocytes (17, 40, 49, 56). In addition to adrenergic stimulation, leptin and tumor necrosis factor (TNF)-α also contribute to enhancing lipolysis in adipocytes (14, 44). Recently, several more lipases [e.g., adipose triacylglycerol (TAG) lipase, TAG hydrolase, adiponutrin, and GS2] that cause lipolysis of TAG in adipocytes have been identified, suggesting that multiple regulatory pathways are involved in this process (11).

Membrane receptor internalization and trafficking might be important for determining some of the downstream events in the lipolytic pathway (39). After ligand binding, many GPCRs undergo internalization to early endosomes from which they are directed into recycling or degradative pathways in a highly regulated manner (12, 35). The β1- and β2AR subtypes undergo ligand-induced endocytosis via clathrin-coated vesicles but are then rapidly recycled back to the plasma membrane, restoring surface receptor numbers and function (42, 47). With prolonged agonist exposure, some receptors are redirected to lysosomes for degradation (35), which can result in tachyphylaxis (48). β3AR is less sensitive than the β1 and β2 to catecholamine-stimulated lipolysis and less prone to agonist-promoted internalization (32). Some recent studies demonstrated that distinct signaling pathways of adrenergic stimulation are differentially mediated by receptor trafficking. For example, inhibition of the endocytic machinery blocks β1AR-induced cardiac hypertrophy (37), suggesting receptor internalization is required for access to selected downstream effectors, which might not reside at the plasma membrane/cytosol interface. In contrast, Centaurin-α1-mediated inhibition of β2AR internalization enhances isoproterenol-stimulated cAMP accumulation (27).

The ADP-ribosylation factor 6 (Arf6), a member of the family of Ras-related, low molecular mass (∼20 kDa), GTP-binding proteins, is required for internalization of many membrane receptors, including βARs (22, 27). There are six mammalian Arfs that are grouped into three classes. Arf6 is the sole class III member, which is of interest with regard to GPCR trafficking because it is localized to both plasma membrane and endosomes (10). In this study, we asked whether receptor trafficking influences adrenergic stimulation of lipolysis in adipocytes. We evaluated the importance of Arf6-mediated...
GA receptor endocytosis in regulating adipocyte β-adrenergic stimulation of lipolysis and examined whether Arf6 levels may be subject to metabolic regulation.

MATERIALS AND METHODS

Materials. Fetal calf serum (FCS), calf serum (CS), Dulbecco’s modified Eagle’s medium (DMEM), and Lipofectamine RNAiMAX were from Invitrogen (Carlsbad, CA) and a small interfering RNA (siRNA) construction kit was from Ambion (Austin, TX). Mouse anti-Arf6 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-pAkt, anti-pMAPK, anti-total Akt and anti-total MAPK antibodies were from Cell Signaling Technology (Beverly, MA). Dynasore was from Scientific Exchange (Center Ossipee, NH). Other reagents were from Sigma (St. Louis, MO).

Cell culture of 3T3-L1 cells and differentiation into the adipocyte phenotype. 3T3-L1 cells were cultured to confluence in DMEM containing 20% calf serum with medium change every 2 days as previously described (52). Two days after cell confluence, differentiation was initiated by adding differentiation medium 1 (0.5 mM methylisobutylxanthine (IBMX), 0.25 mM dexamethasone, 1 μg/ml insulin in DMEM containing 10% fetal bovine serum). Two days later, methylisobutylxanthine and dexamethasone were removed and insulin (1 μg/ml) was maintained for 2 more days. Thereafter, cells were grown in DMEM containing 10% fetal bovine serum in the absence of differentiating reagents with media replacement every 2 days.

Whole cell protein extraction and immunoblotting. Whole cell lysates were prepared as previously described (50). Briefly, cell monolayers were washed twice with ice-cold phosphate-buffered saline and lysed at 4°C for 30 min with a lysis buffer containing 50 mM Tris-HCl, pH 7.6, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and 1% protease inhibitor cocktail solution (Sigma, St. Louis, MO). The cell lysates were clarified by centrifugation at 10,000 g for 10 min at 4°C before separation by SDS-PAGE and immunoblotting as described (50).

siRNA construction and transfection. The previously verified siRNA (30) directed against mouse Arf6 were constructed and purified employing the Silencer siRNA construction kit (Ambion) as described (50). Six to 8 days after initiation of differentiation, 3T3-L1 adipocytes were washed twice with PBS before treatment with trypsin-EDTA for 5 min at 37°C. Cells were collected into 10 ml collagenase (0.5 mg/ml) in PBS and spun at 400 g at 4°C in a table top centrifuge for 5 min. The collagenase solution was removed and the cells were resuspended in growth media. Cells were then plated in growth medium without antibiotics to obtain ~70% confluence at the time of transfection. Transfection of siRNA (10 nM final concentration) was performed using Lipofectamine RNAiMAX according to the manufacturer’s instructions. A scrambled siRNA (Ambion) was used as a negative control.

Glycerol release assay. Stimulation of adipocytes and measurement of glycerol release were performed as described previously (15). Before assaying glycerol release, adipocytes were starved in DMEM with 0.5% fatty acid free bovine serum albumin (BSA) for 4 h. The cells were then incubated with 2% BSA in DMEM and treated as indicated before lipolytic stimulation. Lipolysis was assessed from the release of glycerol in the culture medium (56) using free glycerol reagent (Sigma).

Thin layer chromatography. Adipocytes were incubated with DMEM containing 200 μM [3H]oleic acid/BSA (2:1, 1 μCi/ml) for 1 h. The cells were washed on ice three times with cold PBS containing 0.5% BSA and 500 μl of PBS. Lipids were extracted by the method of Bligh-Dyer (3) in presence of 50 μg dicylglycerol standard and separated on silica gel 60A plates (Whatman, Clifton, NJ) using a mobile phase of hexane/diethyl ether/acetatic acid, 70:30:1. Spots corresponding to major lipid species, identified by standards (trioleoylglycerol and 1-palmitoyl-2-oleoyl-sn-glycerol) run simultaneously and visualized by iodine vapors, were scraped and the radioactivity in each fraction was quantified by liquid scintillation spectrometry.

Quantitative gas chromatography analysis of TAG. Lipids were extracted by the method of Bligh-Dyer in presence of internal standard (T21:0 TAG, 10 nmol/mg protein) and separated on silica gel 60A plates. Spots corresponding to TAG were visualized with 0.01% rhodamine 6G and identified with TAG standard. The bands were scraped and extracted with chloroform:methanol 3:1. FA methyl esters of the TAG fractions were prepared by reaction with methanol:acetyl chloride 4:1 at 70°C for 1 h. Quantitative gas chromatography (GC) analysis was conducted (Hewlett-Packard 5890 GC, Palo Alto, CA) with a 30 m × 0.32 mm Omegawax 250 column (Sigma) and a flame ionization detector. The injector temperature was kept at 250°C. Helium was used as the carrier gas at constant flow rate of 1 ml/min. The initial temperature of the GC oven was 180°C, held for 2 min, ramped at 10 degree/min to 200°C, held for 2 min, ramped at 4 degree/min to 220°C, held for 15 min, and finally ramped at 70 degree/min to 240°C and kept for 2 min. Instrument response was calibrated using the Supelco 37 Component FAME Mix (Sigma) to relate relative peak areas of FAME peaks to molar ratios of a C21:0 internal standard.

Lipogenesis assay. Lipogenesis was assayed as previously described (28). Briefly, cells after the indicated treatment were incubated with 5 mM d-[U-14C]glucose (1 μCi per well) for 60 min at 37°C. Cells were then washed on ice three times with cold PBS, scraped into 1 ml of PBS, and shaken vigorously with 5 ml of Betafuor scintillant (National Diagnostics, Manville, NJ). The samples were settled overnight, and radioactivity in the organic phase was determined by liquid scintillation counting.

Protein extraction from mouse white adipose tissues. Mice were euthanized (CO2 overdose) and epidydimal fat pads (WAT) were removed and rapidly frozen in liquid nitrogen. About 50 mg tissue were homogenized with 500 μl lysis buffer by using a tissue homogenizer at maximum speed for 20 s. The homogenates were incubated on ice for 30 min and spun at 10,000 g at 4°C in a tabletop centrifuge for 10 min. The supernatant was transferred to a new tube and stored at −70°C until used for Western blot analysis.

RNA extraction and real-time quantitative PCR. RNA was extracted from adipose tissue samples by using TRIzol and reverse transcription was performed using the SuperScript III First-strand Synthesis System (Invitrogen). Real-time quantitative PCR assays were performed on an ABI 7500 Fast Real-time PCR System (Applied Biosystems) using SYBR Green PCR Master Mix. Primers used for mouse Arf6 analysis are 5’-GGAGCTGCACCGCATACATTCA-3’ and 5’-CTCATGGGGTTTTCTGGC4TAC-3’. The relative mRNA level of Arf6 was quantified and normalized to 36B4 mRNA.

Statistical analyses. The data are presented as means ± SE and Student’s t-test was used in the statistical analysis. A P value ≤0.05 was considered statistically significant.

RESULTS

Depletion of Arf6 in 3T3-L1 adipocytes impairs isoproteoneol-stimulated lipolysis. Arf6 mediates agonist-stimulated internalization of a broad array of GPCR including βARs (21). To understand whether receptor internalization plays a role in adrenergic stimulation of lipolysis in adipocytes, 3T3-L1 adipocytes were treated with a previously validated siRNA against Arf6 (30). The efficiency of the siRNA in downregulating Arf6 in differentiated adipocytes is shown in Fig. 1A. Two days after siRNA treatment, the rate of lipolysis was assessed by measuring glycerol release. As shown in Fig. 1B, depletion of Arf6 significantly decreased basal lipolysis (P < 0.05) suggesting a role of Arf6 in regulating nonadrenergic stimulated lipolysis.
The adrenergic signaling pathway acting via the βARs represents a prime regulator of triglyceride breakdown in adipocytes. Accordingly, cells were stimulated for 1 h with isoproterenol, a ligand for all three βARs, and a dramatic increase of lipolysis was observed as expected. In contrast, isoproterenol-stimulated lipolysis was inhibited in Arf6-depleted cells (Fig. 1B) \((P < 0.05)\). Similar inhibitory effects were observed when the cells were stimulated with isoproterenol for 10 or 30 min (Fig. 1C). These results suggest that internalization and/or trafficking of βARs may be important for its regulatory function on lipolysis in adipocytes.

**Inhibition of dynamin impairs isoproterenol-stimulated lipolysis.** To determine whether Arf6-mediated lipolysis is due to impaired internalization of βARs, independent pharmaceutical inhibition of receptor internalization was employed. Dynamin is required for membrane budding at a late stage during the transition from a fully formed pit to a pinched-off vesicle and may also fulfill other roles during earlier stages of vesicle formation. Activation of dynamin is necessary for AR internalization (23). Dynasore has been recently identified to interfere in vitro with the GTPase activity of dynamin1, dynamin2, and Drp1, the mitochondrial dynamin, but not of other small GTPases (34). Accordingly, this membrane-permeable inhibitor has been widely used to disrupt endocytosis (1, 33). Adipocytes were treated with or without 80 μM dynasore for 20 min before stimulation with isoproterenol for 1 h. Similar to depletion of Arf6, inhibition of dynamin impaired both basal and stimulated lipolysis (Fig. 2A) \((P < 0.05)\). A time-course study showed that isoproterenol-stimulated lipolysis at 10 or 30 min was also inhibited by dynasore (Fig. 2B). These results suggest that receptor endocytosis following βAR activation is critical for its lipolytic effect. After agonist treatment, the βARs have been proposed to leave lipid rafts before internalization via clathrin-coated pits (43, 46). Since both clathrin-dependent and lipid rafts-dependent internalization pathways require dynamin activation, we specifically disrupted lipid rafts by cholesterol depletion as described (8). Adipocytes were treated with the cholesterol-depleting reagent methyl-β-cyclodextrin before stimulation. Disruption of lipid rafts enhanced basal lipolysis, which was suggested to be due to inhibitory effects on phosphodiesterase activity (41). Isoproterenol-stimulated lipolysis was not altered (Fig. 2C), suggesting lipid rafts are not involved in adrenergic-stimulated lipolysis. Collectively, the results using both genetic interference and pharmaceutical inhibition suggested a role of receptor endocytosis in maintaining basal lipolytic rates and in adrenergic stimulation of lipolysis in adipocytes.

**Depletion of Arf6 does not affect lipolysis stimulated by IBMX, forskolin, or bromo-cAMP.** To further confirm the important role of endocytosis in βAR-mediated lipolysis, lipolysis was stimulated by activating pathways downstream of receptor activation. Accordingly, 3T3-L1 adipocytes with or without depletion of Arf6 were stimulated with an adenylyl cyclase activator (forskolin, 10 μM), the phosphodiesterase inhibitor IBMX (0.5 mM), or a cell membrane-permeable cAMP analogue (8-bromo cAMP, 1 mM). Consistent with findings from a previous study, dramatic increases in lipolysis were observed in cells pretreated with negative control siRNA (Fig. 3) (38, 56). Significantly, lipolysis stimulated by forskolin, IBMX, or 8-bromo-cAMP was not affected by depletion of Arf6 (Fig. 3). This result confirms that Arf6 RNAi-mediated inhibition of lipolysis is due to impaired AR trafficking.

**Depletion of Arf6 does not affect TAG formation.** We further studied whether TAG formation is affected by Arf6 depletion thus altering the intracellular pool available for lipolysis. Two days after siRNA transfection, cells were incubated with 200 μM \(^{3}H\)oleic acid, and the partitioning of oleic acid into polar lipid, diacylglycerol, and TAG was studied. The lipid synthesis from fatty acids was not altered by depletion of Arf6 (Fig. 4A). A previous study demonstrated the role of Arf6 on insulin-stimulated trafficking of glucose transporter type 4 (GLUT4) transporter to the plasma membrane and glucose uptake in
3T3-L1 adipocytes (30). We examined whether de novo lipogenesis was altered by Arf6. Cells treated with siRNAs were incubated with [14C]glucose, and lipid radioactivity was measured. Under basal condition, lipogenesis was not inhibited by treatment with Arf6 siRNA (Fig. 4B). However, insulin-stimulated lipogenesis was decreased around 30% (Fig. 4B) (P < 0.05) likely due to the decreased insulin-stimulated glucose uptake as previously demonstrated in the same cell system (30). To further validate that the effect of Arf6 depletion did not reflect a change in the TAG pool, we directly measured the contents and fatty acid compositions of TAG in adipocytes by quantitative GC. Total TAG amounts were not altered by Arf6 depletion (Fig. 4C). Moreover, compositions of the major fatty acids in TAG were also not significantly changed (supplemental Table S.1). These data indicate that the availability of TAG for lipolysis is not altered after Arf6 knockdown in our current experimental system, which is consistent with the observations that lipolysis stimulated by IBMX, forskolin, or bromo-cAMP was not altered in Arf6-depleted cells.

Depletion of Arf6 does not affect insulin signaling. Insulin signaling is a major physiological regulatory pathway that inhibits lipolysis in adipocytes. During fasting, lipolysis is initiated mainly by diminution of insulin signaling (24). Since both Arf6 depletion and insulin stimulation lead to inhibition of adrenergic-stimulated lipolysis, we explored whether these two pathways overlap. First, we confirmed the antilipolytic effect of insulin in our model system. As anticipated, insulin inhibits isoproterenol-stimulated lipolysis (Fig. 5A). We next studied the effects of Arf6 depletion on insulin signaling. 3T3-L1 adipocytes treated with negative control siRNA or siRNA-recognizing Arf6 were serum starved before stimulation with 0, 1, or 10 nM insulin for 10 min. Total cell lysates were prepared in the presence of protease and phosphatase inhibitors and analyzed by Western blot analysis. Knockdown of Arf6 had no significant effect on insulin-stimulated protein kinase B (PKB)/Akt activation (Fig. 5B). Moreover, insulin-stimulated MAPK activation was also not affected by siRNA against Arf6 (Fig. 5B). This result is consistent with our previous finding that inhibition of insulin receptor internalization by depletion of dynamin has no effect on insulin-stimulated PKB/Akt and MAPK activation (51). Since the inhibitory effect of insulin on lipolysis in adipocytes is mainly due to the activation of phosphodiesterase by PKB/Akt (25), this result suggests that Arf6 depletion-mediated inhibition of adrenergic-stimulated lipolysis is independent of that of insulin signaling.

Fig. 3. Arf6 depletion does not affect lipolysis stimulated by IBMX, forskolin, or cAMP analogue. 3T3-L1 adipocytes were treated with a negative control siRNA (NC) or an siRNA recognizing Arf6. Two days posttransfection, cells were starved and incubated with 1 μM 8-bromo-cAMP (8-Br-cAMP), 0.5 mM IBMX, or 10 μM forskolin for 1 h, and glycerol release was assayed and normalized to total protein. *P < 0.05. The data represent means ± SE of three independent experiments.
Regulation of white adipose tissue Arf6 levels in obesity-prone and -resistant mice. To gain insight into the potential physiological role of Arf6 in regulating the mobilization of endogenous adipose tissue triglycerides and release of FFA into plasma, we investigated regulation of white adipose tissue (WAT) Arf6 in several obesity-prone or -resistant mouse models. Arf6 levels were found to be significantly increased in ob/ob mice (Fig. 6A). Since aging is correlated with increased adipocity, we compared levels of Arf6 in WAT from 30- and 10-wk-old mice. Interestingly, Arf6 levels dramatically rose in the older mice (Fig. 6B). CD36 null mice have smaller adipose tissue mass and are protected from high-fat diet-induced obesity (19). In contrast to obese mice, Arf6 levels were dramatically decreased in CD36 null mice (Fig. 6C). Consistent with this, Arf6 mRNA levels were significantly increased in WAT from ob/ob mice and decreased in CD36 null mice, suggesting transcriptional regulation of Arf6 in mouse WAT (Fig. 6D). Collectively, these results demonstrate positive correlation of Arf6 protein and mRNA levels with adiposity. Considering the inhibitory effects of Arf6 depletion on lipolysis, regulation of Arf6 levels may represent a novel feedback mechanism to mobilize TAG in adipose tissues.

**DISCUSSION**

The data from this study provide evidence that the ADP-ribosylation factor 6, Arf6, plays a role in regulating basal lipolysis and its β-adrenergic activation in adipocytes. First, depletion of Arf6 by RNAi-mediated gene knockdown in 3T3-L1 adipocytes decreased both basal and isoproterenol-stimulated lipolysis. Second, pharmaceutical inhibition of dynamin, which blocks receptor internalization, had similar effects. Third, the increase in lipolytic activity stimulated by reagents that bypass receptor activation was not affected by depletion of Arf6. Fourth, Arf6 depletion and insulin signaling are independent pathways leading to inhibition of β-adrenergic-stimulated lipolysis in adipocytes. Fifth, Arf6 levels are dramatically altered in adipose tissues from obese or obesity-resistant mice. Collectively, these data demonstrate that Arf6 regulation of βAR trafficking is a novel mechanism that modulates basal and stimulated adipocyte lipolytic activity. Arf6 depletion or pharmaceutical inhibition of dynamin impairs β-adrenergic-stimulated lipolysis in adipocytes (Fig. 1 and 2), suggesting that the effect of Arf6 depletion on adren-

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**Fig. 4.** Effects of Arf6 depletion on fatty acid (FA) partitioning, lipogenesis, and triacylglycerol (TAG) accumulation. 3T3-L1 adipocytes were treated with a negative control siRNA (NC) or an siRNA recognizing Arf6. A: 2 days posttransfection, cells were incubated with DMEM containing 200 μM [3H]oleic acid/BSA (2:1, 1 μCi/ml) for 1 h. FA partitioning into TAG, diacylglycerol (DAG), and polar lipids (PL) was measured and normalized to total protein. B: cells were starved and stimulated with 10 nM insulin for 15 min. Lipogenesis was assayed using 5 nM d-[U-14C]glucose (1 μCi per well) and normalized to total protein. *P < 0.05. Results are the means ± SE of three independent experiments. C: total lipids were extracted and TAG were measured by quantitative gas chromatography and normalized to total protein. Results are means ± SE of three independent experiments.

**Fig. 5.** Arf6 depletion does not alter insulin signaling. A: 3T3-L1 adipocytes were starved and treated with 10 nM insulin (Ins) for 15 min before stimulation with 10 μM Iso for 1 h, and the glycerol release was assayed and normalized to total protein. *P < 0.05. The data represent means ± SE of three independent experiments. B: 3T3-L1 adipocytes were treated with negative control siRNA (NC) or an siRNA recognizing Arf6. Cells were starved and stimulated with Ins at indicated concentrations for 10 min. The whole cell lysates were prepared and subjected to immunoblot (IB) analysis utilizing antibodies recognizing pAkt (Ser473), total Akt (tAkt), pMAPK, and total MAPK (tMAPK). Results are representative of three independent experiments.
ergic-stimulated lipolysis is due to impaired receptor internalization, which is an early event preceding receptor recycling. This is supported by our results that depletion of Arf6 or inhibition of dynamin decreases lipolysis after shorter term stimulation of isoproterenol (10 and 30 min) (Figs. 1C and 2B). Interestingly, depletion of Arf6 or inhibition of dynamin also inhibits basal lipolysis. Under basal condition, βARs can be constitutively internalized (45), and this could also be necessary for full lipolytic activity of the receptor. Alternatively, Arf6 and dynamin may mediate other βAR independent pathways that could play roles in lipolysis under basal condition without adrenergic stimulation.

Isoproterenol binds to all three βAR subtypes in adipocytes, and it is likely that the functional readout reflects their combined effects. However, the events that follow ligand receptor interaction have been best studied in the case of the β2AR. Ligand binding induces receptor internalization, which is followed by attenuation of signaling (desensitization) and receptor resensitization. (29). Desensitization is initiated at the plasma membrane with receptor phosphorylation by multiple protein kinases, including PKA and members of the GPCR kinase family, which ultimately results in receptor uncoupling from the stimulatory G protein (55) and in its trafficking to early endosomes via clathrin-coated vesicles (26). After dephosphorylation and resensitization on early endosomes, the receptor is then recycled back to the plasma membrane for renewed activation via Rab4-mediated rapid recycling (47) or Rab11-mediated slow recycling pathways (42). Internalization of the β2AR is dependent on activities of both Arf6 and dynamin. Further studies that disrupt different components of the endocytic and recycling pathways will be needed to determine the specific trafficking event(s) needed for β2AR lipolytic function.

The molecular details for the trafficking, signaling attenuation, and resensitization of β1AR and β3AR are less clear. Similar to β2AR, β1AR is rapidly internalized upon ligand binding, and its signaling capacity is attenuated (31). In contrast, β3AR seems less prone to these regulatory processes (32). The coexpression of all three subtypes of βAR in adipocytes suggests that coordination of the different receptors is required to finely regulate and achieve the full lipolytic action of catecholamine in adipocytes. This is supported by the presence of a hetero-oligomerization complex of β2AR and β3AR with unique signaling property (5). Signaling and lipolytic activity of the three βAR subtypes may be differentially regulated by Arf6 and dynamin, which will have to be determined. There are multiple signaling pathways leading to lipolysis in adipocytes, and it is possible that internalization and intracellular trafficking of the βARs will provide pathway selectivity. Therefore, the regulation of distinct signaling pathways resulting in the activation of different lipases (e.g., adipose TAG lipase, TAG hydrolase, Adiponutrin, and GS2) could involve vesicular trafficking.

The capacity of whole adipose tissues to release FFA after adrenergic stimulation integrates multiple factors, which include: 1) numbers of fat cells and total TAG accumulation in individual cells; 2) expression and functional activity of adrenergic receptors; and 3) other signaling pathways (e.g., insulin signaling). Ob/ob mice exhibit greater rates of basal and adrenergic-stimulated lipolysis in whole adipose tissues via an increase in fat cell number and intracellular TAG content and impaired insulin signaling (2, 53, 54). In contrast, adipocytes from other obesity models have impaired expression and activities of βARs (9) and higher levels of antilipolytic α2 adrenoceptors (7), indicating that net lipolytic capacity of adipose tissues is balanced by both stimulatory and inhibitory regulating factors. Our results suggest a role of Arf6 in basal and adrenergic-stimulated lipolysis in adipocytes. Moreover, WAT Arf6 levels are increased in ob/ob mice and aged mice but are decreased in lean CD36 null mice documenting a positive correlation with body fat mass (Fig. 6). These data suggest that Arf6 is likely to play an important role in chronic maintenance of lipolytic rates as well as in the acute regulation of these rates by adrenergic stimulation. In addition to its effects in the endocytic pathway, Arf6 also regulates a clathrin-coated complex for insulin-stimulated recycling of GLUT4 (30). Depletion of Arf6 or clathrin heavy chain impairs insulin-stimulated glucose uptake in adipocytes, suggesting a role of Arf6 in glucose homeostasis (30). Therefore, regulation of Arf6 could be a key factor balancing glucose and fatty acid metabolism.

Collectively, the results from the present study document that Arf6 is a novel regulator of adrenergic-stimulated and basal lipolysis via its influence on receptor trafficking. The
marked change of Arf6 levels as a function of adiposity suggests that it has an important function in regulating lipolysis in vivo. Additional studies are needed to determine whether targeting Arf6-mediated lipolysis can alter the rate of FFA release from adipocytes as a therapeutic approach to modulate lipolytic rates and manage obesity.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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