Original Article

MOLECULAR METABOLISM

Rasal2 deficiency reduces adipogenesis and occurrence of obesity-related disorders

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ABSTRACT

Objective: Identification of additional regulatory factors involved in the onset of obesity is important to understand the mechanisms underlying this prevailing disease and its associated metabolic disorders and to develop therapeutic strategies. Through isolation and analysis of a mutant, we aimed to uncover the function of a Ras-GAP gene, Rasal2 (Ras protein activator like 2), in the development of obesity and related metabolic disorders and to obtain valuable insights regarding the mechanism underlying the function.

Methods: An obesity-based genetic screen was performed to identify an insertional mutation that disrupts the expression of Rasal2 (*Rasal2*$^{PB/PB}$ mice). Important metabolic parameters, such as fat mass and glucose tolerance, were measured in *Rasal2*$^{PB/PB}$ mice. The impact of Rasal2 on adipogenesis was evaluated in the mutant mice and in 3T3-L1 preadipocytes treated with Rasal2 siRNA. Ras and ERK activities were then evaluated in Rasal2-deficient preadipocytes or mice, and their functional relationships with Rasal2 on adipogenesis were investigated by employing Ras and MEK inhibitors.

Results: *Rasal2*$^{PB/PB}$ mice showed drastic decrease in Rasal2 expression and a lean phenotype. The mutant mice displayed decreased adiposity and resistance to high-fat diet induced metabolic disorders. Further analysis indicated that Rasal2 deficiency leads to impaired adipogenesis *in vivo* and *in vitro*. Moreover, while Rasal2 deficiency resulted in increased activity of both Ras and ERK in preadipocytes, reducing Ras, but not ERK, suppressed the impaired adipogenesis.

Conclusions: Rasal2 promotes adipogenesis, which may critically contribute to its role in the development of obesity and related metabolic disorders and may do so by repressing Ras activity in an ERK-independent manner.

Keywords Ras; ERK; Ras-GAP; Glucose tolerance; High-fat diet; Diabetes

1. INTRODUCTION

Obesity, which has grown to be a world-wide epidemic, has multiple metabolic consequences such as type II diabetes, hepatic steatosis, dyslipidemia, cardiovascular diseases, and certain types of cancer [1]. Identification of pathways that modulate the development of obesity will provide critical information regarding new therapeutic approaches to obesity and its associated diseases.

Ras proteins are known to play fundamental roles in cell growth and proliferation [2]. Potential important roles of Ras in regulating obesity-related cellular processes have been suggested by several studies, with certain caveats to be examined. First, transgenic mice over-expressing (8-fold) H-Ras specifically in adipose tissue displayed a remarkable decrease in fat mass [3], which suggested a potential role of Ras in repressing adipogenesis. However, since the study was performed under non-physiological conditions, the result is subjected to alternative explanations. Second, human and mouse genetic studies revealed that loss-of-function mutations in the Kinase suppressor of Ras 2 (*KSR2*) gene are associated with obesity in human and cause obesity in mice [4]. However, since KSR2 has been shown to bind to and regulate the activity of AMPK, in addition to its known role in promoting the Ras-Raf-MEK-ERK signaling pathway [5–7], further studies are required to determine if KSR2 acts through the Ras pathway to repress obesity. Finally, genetic ablation of docking protein 1 (Dok1), a protein known to repress Ras function by recruiting Ras-GAP to Ras [8,9], inhibits adipogenesis and causes mice to be lean [10]. This result also suggests a role of Ras in repressing adipogenesis. Therefore, additional studies are desirable to validate this role and fully understand this potentially important regulatory mechanism, which may lead to identification of potential new therapeutic targets.

Rasal2 was identified as a Ras-GAP through cancer-related studies [11–13]. A genome-wide association study has linked SNP rs10913469 (*Sec16B-Rasal2*) to increased body mass index in humans [14]. Two independent studies reproduced this association in Chinese and certain Mexican populations [15,16]. These data suggest potential roles of Sec16B or Rasal2 in the development of obesity. Genetic studies using mouse genetics should be effective and

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important to determine the role of Rasal2 in obesity-related cellular processes, which may elucidate the role of Ras in obesity.

In a screen for obesity-associated mutants based on systematic *Piggybac* (PB) transposon insertional mutagenesis, we identified a mutant strain with drastically reduced expression of *Rasal2*. This mutant strain provides an excellent mouse model to explore the roles of endogenous Ras proteins. The mutant mice display a lean phenotype with improvements in glucose tolerance, insulin sensitivity, and other metabolic parameters related to obesity. We carried out in-depth analysis to reveal the roles and mechanisms of Rasal2 in the development of obesity.

2. MATERIALS AND METHODS

2.1. Animals

FVB Mice were housed under 12/12 h light/dark cycles with free access to water and normal chow diet or 60 kcal% high-fat diet (Research Diets, Inc). All animal-related experiments were performed in accordance with guidelines from the Institute of Developmental Biology and Molecular Medicine Institutional Animal Care and Use Committee.

2.2. Indirect calorimetry

Mice were individually housed under room temperature (21 °C) with free access to food and water. Locomotor activity, O_2 consumption, and CO_2 production were measured using a Promethion Metabolic Measurement System.

2.3. Histology

For frozen section, tissues were dissected and fixed in 10% formalin solution overnight. The samples were then dehydrated in 30% sucrose and embedded in OCT. 10 μm sections were collected. For Oil-Red-O staining, cells or slides were fixed for 1 h at room temperature with 10% formalin before stained with Oil-Red-O as described previously [17]. After taking photos, the Oil-Red-O was extracted with isopropanol for spectrophotometric measurement at 510 nm.

2.4. Glucose tolerance test (GTT) and insulin tolerance test (ITT)

For GTT, 12-week old mice were fasted for 16—18 h, and injected with D-glucose (2 g/kg, intraperitoneally). Tail vein blood was sampled and analyzed with an Onetouch Ultra blood glucose monitoring system (LifeScan) at 0, 30, 60, 90, and 120 min after injection, respectively. For ITT, 12-week old mice were fasted for 6 h before being injected with insulin (Humulin, Lilly) (0.75 U/kg, intraperitoneally). 0, 15, 30, 45, 60 min after injection, tail vein blood was sampled for glucose determination.

2.5. siRNA transfection of 3T3-L1 preadipocytes and adipogenesis induction

Cells were maintained in expansion medium [High glucose DMEM (Gibco, 11965118) supplemented with 10% FCS (Gibco, 16170078), 1% Penicillin-Streptomycin (Gibco, 10378016)]. Transfection of siRNA against *Rasal2* was performed as previously described [18]. In brief, 3T3-L1 preadipocytes were trypsinized, resuspended in expansion medium, then seeded into 24-well plates and transfected with siRNA by Lipofectamine RNAiMAX (Life Technologies). Two days after reaching confluency, cells were induced with a defined adipogenic cocktail [High glucose DMEM (Sigma, 11965118) supplemented with 10% FBS (Gibco, 16000044), 1% Penicillin-Streptomycin (Gibco, 10378016), 5 μg/ml insulin (Sigma, I0259), Dexamethasone (Sigma, D1756), and 0.5 mM 3-Isobutyl-1-methylxanthine (IBMX) (Sigma, I7018)] for 2 days, then raised in the maintenance medium [High glucose DMEM (11965118) supplemented with 10% FBS (Gibco, 16000044), 1% Penicillin-Streptomycin (Gibco, 10378016), and 5 μg/ml insulin (Sigma, I0259)] for additional 4 days. The sequences of Rasal2 siRNA could be found in Table S2.

2.6. Assay of Ras activity

Cells were lysed on ice in the lysis buffer containing 25 mM HEPES (pH 7.5), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 10% glycerol, 25 mM NaF, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM sodium orthovanadate, protease inhibitor (Roche). 500 mg of cell lysate was incubated at 4 °C with 5 μg glutathione-Sepharose beads (Upstate biotechnology) coupled with a glutathione S-transferase fusion protein corresponding to Ras binding domain of human Raf-1. The bead-bound proteins were analyzed by Western blotting.

2.7. Farnesyl Thiosalicylic Acid (FTS) and U0126 treatment of 3T3-L1 preadipocytes

FTS was dissolved in ethanol (5 mM). Cells were treated with adipogenic cocktail added with 10 μM or 50 μM FTS for 2 days, then incubated with the maintenance medium containing 10 μM or 50 μM FTS for 2 days. After that, cells were cultured in maintenance medium for an additional 2 days.

U0126 was reconstituted in DMSO (10 mM). For U0126 treatment, cells were stimulated with adipogenic cocktail for 12 h, then switched to adipogenic cocktail supplemented with 10 nM, 50 nM, 250 nM, or 1250 nM U0126 for 36 h. The choice of applying the inhibitor at 12 h post-induction was based on a previous study that showed ERK acts to promote C/EBPα and PPARγ expression (which, in turn, enhances adipogenesis), and PPARγ expression was induced at 12 h post-induction [27]. Subsequently, cells were maintained in maintenance medium containing the same concentration of U0126 for 2 more days. The cells were then cultured in maintenance medium for an additional 2 days.

2.8. Real-time PCR

Tissues or cells were homogenized in Trizol (Invitrogen), and then total RNA was extracted following the standard protocol. One μg RNA was used for complementary DNA synthesis using a Reverse Transcription kit (Takara, RR047A). Real-time PCR was performed by using Brilliant II FAST SYBR QPCR Master Mix (Agilent) in LyghtCycler™ 480 system (Roche). 36B4 or 18s RNA was used as the internal control. Primers used in real-time PCR can be found in Table S2. Some primer sequences are from PrimerBank [19].

2.9. Western blotting

Protein was extracted from cells or tissues by using RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% SDS] supplemented with protease inhibitor (Roche) and phosphatase inhibitor (Roche). Protein was separated by SDS-PAGE and transferred to PVDF membranes. Antibodies used were: Phospho-ERK (Thr202/Tyr204) (Cell Signaling), ERK (Cell Signaling), Rasal2 (ProteinTech), beta-actin (Santa Cruz), beta-tubulin (Sigma), and GAPDH (Sigma).

2.10. Statistics

All data were analyzed with a two-tailed unpaired/paired Student's t-test and expressed as mean ± s.e.m. Statistical significance is represented by asterisks: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. All n values refer to biological repeats.

