

Epac2a-knockout mice are resistant to dexamethasone-induced skeletal muscle atrophy and short-term cold stress

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Exchange protein directly activated by cAMP (Epac) 2a-knockout (KO) mice exhibit accelerated diet-induced obesity and are resistant to leptin-mediated adipostatic signaling from the hypothalamus to adipose tissue, with sustained food intake. However, the impact of Epac2a deficiency on hypothalamic regulation of sympathetic nervous activity (SNA) has not been elucidated. This study was performed to elucidate the response of Epac2a-KO mice to dexamethasone-induced muscle atrophy and acute cold stress. Compared to age-matched wild-type mice, Epac2a-KO mice showed higher energy expenditures and expression of myogenin and uncoupling protein-1 in skeletal muscle (SM) and brown adipose tissue (BAT), respectively. Epac2a-KO mice exhibited greater endurance to dexamethasone and cold stress. In wild-type mice, exogenous leptin mimicked the responses observed in Epac2a-KO mice. This suggests that leptin-mediated hypothalamic signaling toward SNA appears to be intact in these mice. Hence, the potentiated responses of SM and BAT may be due to their high plasma leptin levels. [BMB Reports 2018; 51(1): 39-44]

INTRODUCTION

Exchange protein directly activated by cAMP (Epac) is critical for cAMP signaling with cAMP-dependent protein kinases (1). Epac proteins are encoded by two distinct genes, yielding Epac1 and Epac2 (*Rapgef4*). Epac1 is widely expressed, whereas the three mRNA splice variants of Epac2 are differen-

tially expressed; Epac2a is mainly expressed in the brain, heart, and beta cells (2, 3). Epac2a-KO mice have normal body weight, glucose tolerance, and insulin sensitivity, at least until 12 weeks of age, but exhibit mild hyperleptinemia and have larger white adipocyte lipid droplets than those in age-matched wild-type (WT) mice (4). The arcuate nucleus (ARH) and ventromedial hypothalamus, which largely determine the degree of body adiposity, express Epac2a, and Epac2a-deletion exhibited leptin resistance (4). This suggests that hypothalamic Epac2a is vital for appropriate leptin signaling to regulate food satiety.

Leptin is a critical hormone that links energy intake and storage to energy consumption (5). Non-obese humans and animals are responsive to exogenous leptin (6-8), which increases food satiety and energy consumption. Plasma leptin levels increase in proportion to adipose tissue mass to facilitate its role in the hypothalamus (9-11). The ARH responds to leptin and regulates the release of orexigenic and anorexigenic hormones (5). Appetite control by anorexigenic hormones is mediated by melanocortin 4 receptors (MC4R) (12). However, at the high leptin levels that are observed in the obese state, the hypothalamus becomes less responsive to exogenous leptin.

Hypothalamic leptin signaling elevates sympathetic nervous activity (SNA) to decrease body fat mass by increasing energy expenditure (EE), which is mediated by both MC4R-dependent and independent pathways (13-15). Obesity is associated with high blood pressure and hyperleptinemia, implying that leptin resistance does not influence the leptin-mediated hypothalamic activation of SNA (16). In addition, under leptin resistance, the effects of leptin on sympathetic tone in brown adipose tissue (BAT) were maintained by signaling from the dorsomedial hypothalamus (13).

The distribution of Epac2a in the distinct nuclei of the hypothalamus and its role in the leptin-mediated hypothalamic regulation of SNA has not been fully clarified. Thus, this study aimed to evaluate the possible changes in SNA in Epac2a-KO mice, by observing the response of peripheral tissues to dexamethasone-induced muscle atrophy and cold stress. Because the skeletal muscle and BAT possess plasmalemmal

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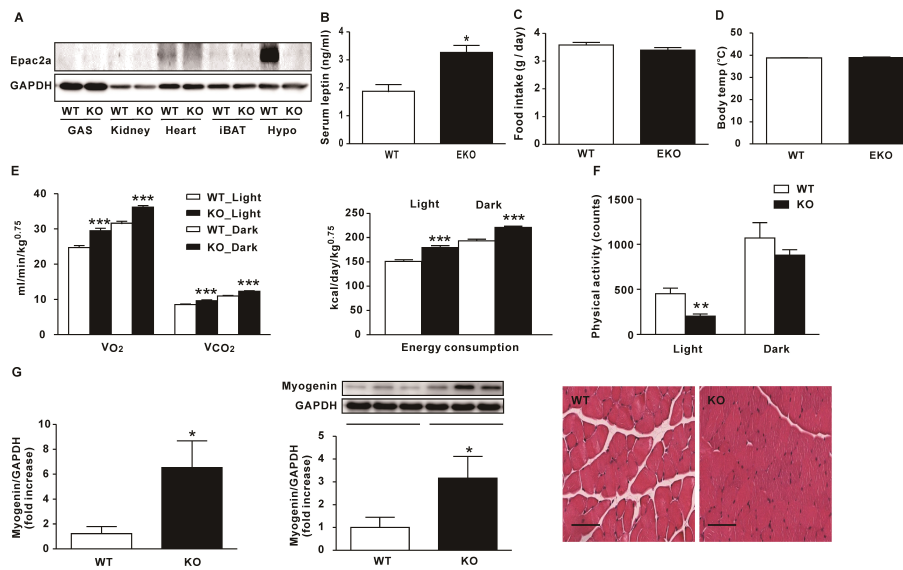


Fig. 1. Characteristics of Epac2a-KO mice. (A) Tissue distribution of Epac2a. Comparison of WT and Epac2a-KO mice by fasting serum leptin levels (B, $n = 4$), food intake (C, $n = 5$), rectal temperature (D, $n = 9$), energy consumption (E, $n = 6$), and physical activity (F, $n = 12$). Myogenin mRNA ($n = 15$) and protein ($n = 3$) expression and cross sectional appearance (G) of the gastrocnemius muscle in WT and KO mice. H & E staining. Scale bars: 100 μ m. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to WT. Data represent the mean \pm SEM. GAS: gastrocnemius muscle; Hypo: hypothalamus.

long-form leptin receptors (OB-Rb) (17) as well as sympathetic G-protein-coupled receptors, the results may be helpful for understanding whether leptin signaling in myocytes and brown adipocytes is affected under hypothalamic leptin resistance and hyperleptinemia.

RESULTS

Characteristics of Epac2a-KO mice

Epac2a-KO mice appeared to be normal when compared to WT mice. We checked the protein expression of Epac2a in WT mice. Consistent with previous reports (3), the hypothalamus and heart expressed Epac2a but interscapular BAT (iBAT) and gastrocnemius muscle did not express Epac2a (Fig. 1A). Remarkably, Epac2a-KO mice had elevated fasting serum leptin levels (Fig. 1B), but food intake was not different from that of WT mice (Fig. 1C). No difference in rectal temperature was detected between the two groups (Fig. 1D). Oxygen consumption (VO₂), carbon dioxide production (VCO₂), and EE in KO mice were higher than those in WT mice (Fig. 1E), although the physical activity of KO mice was lower compared to WT mice (Fig. 1F). Myogenin levels were increased in the muscle of Epac2a-KO mice compared to the expression in WT mice (Fig. 1G). In addition, the cross-section area (CSA) of the muscle fibers was greater in KO mice. The average CSA of muscle fiber was $1546.6 \pm 56.04 \mu\text{m}^2$ and $1808.9 \pm 75.50 \mu\text{m}^2$ in WT and Epac2a-KO mice, respectively ($n = 6$).

Epac2a-KO mice show greater endurance to acute cold stress

To observe the activity of BAT in Epac2a-KO mice, the mice were exposed to a cold environment (4°C) for 6 h. Epac2a-KO mice better-maintained body temperature in response to cold stress, and the decrease in body temperature induced by cold

exposure was smaller than that in WT mice (Fig. 2A). The mass of iBAT and white adipose tissue at the thermoneutral condition was less in KO mice than in WT mice (Fig. 2B), consistent with the greater EE in KO mice. Nevertheless, the magnitude of cold stress-induced fat-mass decrease was similar between the two groups. Serum creatine kinase concentrations in the two groups were not different (data not shown), indicating that the shivering activity of the skeletal muscle did not differ. Comparison of the expression of thermogenesis-related proteins in iBAT showed greater cold stress-mediated elevation in the expression of uncoupling protein-1 (UCP-1), peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC-1 α), CCAAT-enhancer-binding proteins beta (C/EBP β), carnitine palmitoyltransferase 1b (CPT1b), carnitine palmitoyltransferase 2 (CPT2), and peroxisome proliferator-activated receptor alpha (PPAR α) in Epac2a-KO mice (Fig. 2C). Likewise, mRNA expression levels exhibited similar results to proteins (Fig. 2D). Remarkably, the expression of the UCP-1 protein in Epac2a-KO mice was higher than that in WT mice even at ambient temperature, and protein levels further increased during low-temperature exposure. After cold stress, we observed that the mRNA expression of cluster of differentiation 36 (Cd36), cytochrome c oxidase subunit VIIIb (Cox8b), and cytochrome complex (CytoC), which are associated with fatty acid uptake and mitochondrial oxidative metabolism, was higher in Epac2a-KO mice than in WT mice (Fig. 2E). To determine whether the hyperleptinemia observed in Epac2a-KO mice caused the increased cold endurance, leptin (5 μ g/g) was administered intraperitoneally (i.p.) to WT mice, and then the mice were immediately exposed to a cold environment (4°C) for 6 h. Expectedly, the leptin-treated WT mice maintained a more stable body temperature than untreated WT mice (Fig. 2F).

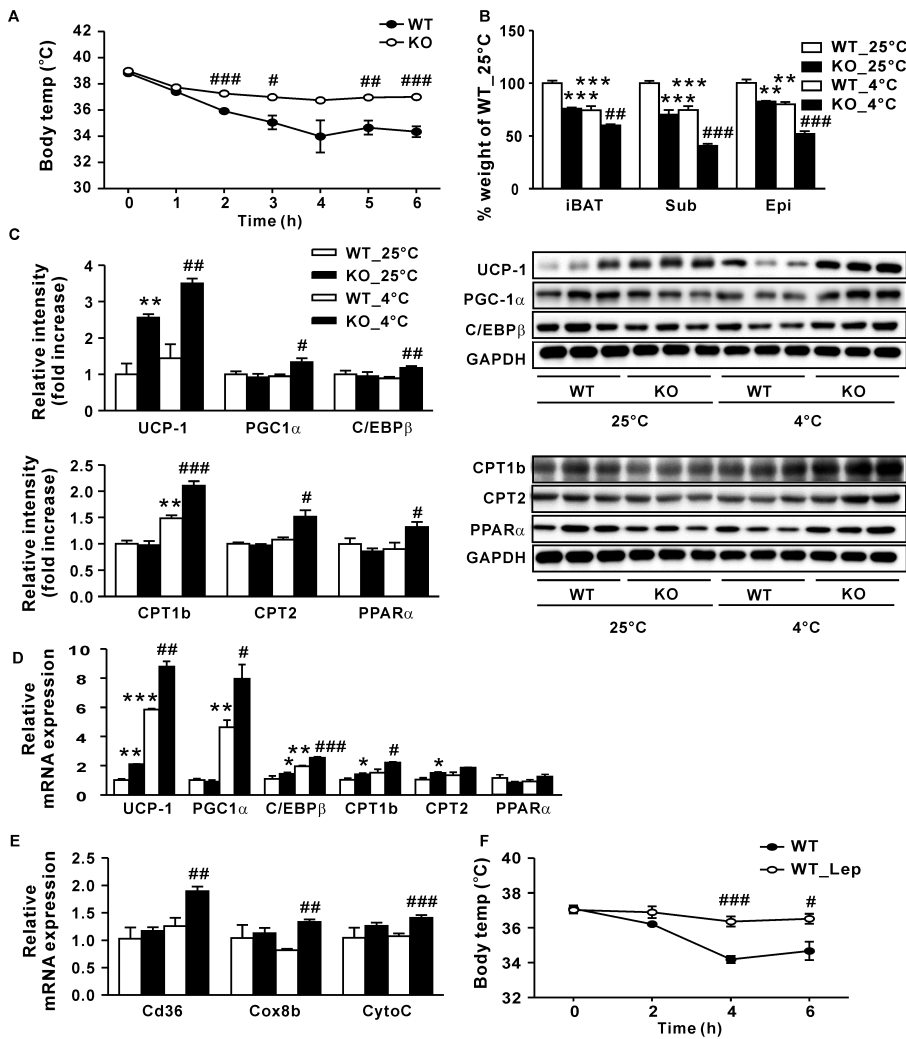


Fig. 2. Epac2a-KO mice display an enhanced response to the cold challenge. Changes in the body temperature (A, $n = 9$) and fat weight (B, $n = 8$) during a 6 h exposure to cold (4°C). Protein and mRNA expression of thermogenic markers (C and D, $n = 4$) and mRNA expression of fatty acid oxidation markers (E, $n = 8$) in the iBAT of WT and KO mice. (F) Changes in the body temperature of WT mice with or without leptin administration during a 6 h exposure to cold (4°C). WT mice in the leptin group were injected i.p. with leptin ($5 \mu\text{g/g}$) before cold exposure, whereas WT mice in the control group were injected i.p. with the same volume of PBS ($n = 8$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared to WT_25°C. # $P < 0.05$, ## $P < 0.01$, and ### $P < 0.001$ compared to WT_4°C without any treatment. Data represent the mean \pm SEM. Sub: subcutaneous fat; Epi: epididymal fat; Lep: leptin.

Greater endurance of Epac2a-KO mice to dexamethasone-induced muscle atrophy

The body weight of 7-week-old WT and Epac2a-KO mice did not differ (Fig. 3A). Dexamethasone treatment for 7 days (i.p.) significantly decreased the body weight of WT mice, but not that of Epac2a-KO mice. Accordingly, dexamethasone treatment reduced gastrocnemius muscle weight in WT mice, but not in Epac2a-KO mice. We then tested the functional performance of the gastrocnemius muscle with a treadmill exercise test. Treatment with dexamethasone significantly decreased the running time of the WT mice. Although dexamethasone treatment decreased the running time of Epac2a-KO mice, the decrease was less than that in WT mice (Fig. 3B). Consistent with the notion that dexamethasone damages skeletal muscle, serum creatine kinase levels in WT mice were elevated by dexamethasone treatment; however,

the effect of dexamethasone in Epac2a-KO mice was minimal (Fig. 3C). Next, we evaluated the expression of proteins associated with protein synthesis and degradation in the gastrocnemius muscle with or without dexamethasone treatment (Fig. 3D). In WT mice, dexamethasone treatment significantly decreased the expression of p-AKT and p-mammalian target of rapamycin (p-mTOR), but increased the expression of p-eukaryotic initiation factor 4E (p-eIF-4E), atrogin-1, and muscle RING-finger protein (MuRF), suggesting increased protein degradation and decreased protein synthesis. Conversely, in Epac2a-KO mice, dexamethasone treatment increased the protein expression of p-AKT and p-mTOR, while the dexamethasone-mediated increase in p-eIF-4E, atrogin-1, and MuRF expression observed in WT mice was attenuated. Dexamethasone-induced body weight reduction in WT mice shown in Fig. 3A was not due to the decrease in fat mass (Fig. 3E). To elucidate the increase in SNA and leptin signaling in

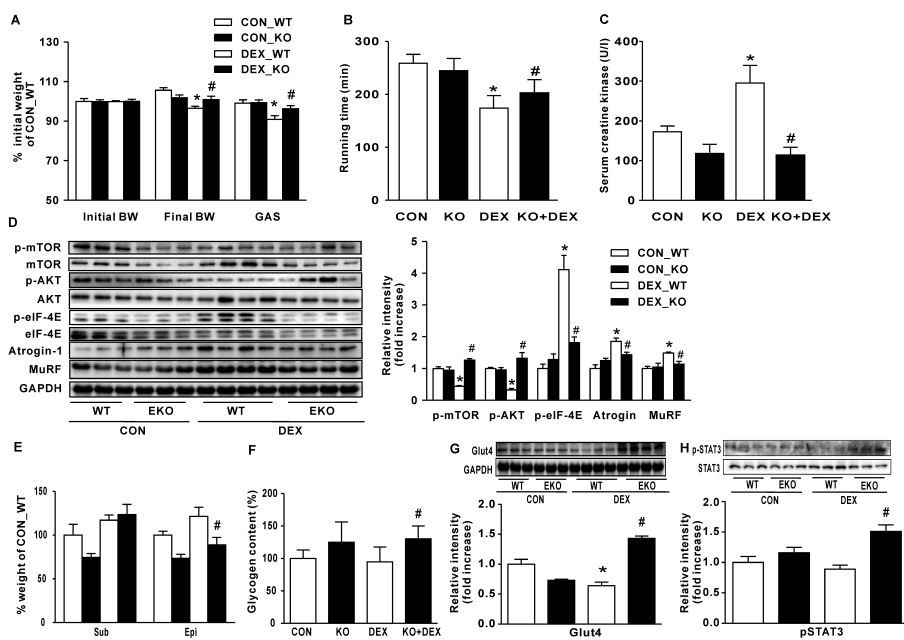


Fig. 3. Epac2a-KO mice better endure dexamethasone-induced muscle atrophy. (A) Changes in body weight and gastrocnemius muscle (GAS) weight following 7 days of dexamethasone (Dex) treatment (25 mg/kg/day; n = 15). (B) Total running time on a treadmill exercise test without or with a 7-day dexamethasone treatment (n = 11). (C) Serum creatine kinase activity without or with dexamethasone treatment. (D) The expression of proteins in the gastrocnemius muscle with or without dexamethasone treatment (n = 8). (E) Changes in fat weight following 7 days of dexamethasone treatment (25 mg/kg/day; n = 5). (F) Muscle glycogen content without or with dexamethasone treatment (n = 5). (G and H) The expression of Glut4 and phosphorylation of Stat3 in the gastrocnemius muscle with or without dexamethasone treatment (n = 4). *P < 0.05 compared to WT mice without dexamethasone treatment. #P < 0.05 compared to WT mice with dexamethasone treatment. Data represent the mean ± SEM.

skeletal muscle, we measured glycogen content, glucose transporter type 4 (GLUT4) expression, and pSTAT3 expression from gastrocnemius muscle. Consistent with previous findings (18), glycogen content and protein level of GLUT4 were greater in KO mice treated with dexamethasone compared to WT mice treated with dexamethasone. Additionally, STAT3 phosphorylation was also higher in KO mice treated with dexamethasone, which is a direct signaling molecule for leptin. Furthermore, we observed more potentiated pPKA expression in iBAT and gastrocnemius muscle in Epac2a-KO mice (Supplementary Fig. 1). Cold stress also decreased triglyceride content in iBAT much more in KO mice than in WT mice (Supplementary Fig. 2).

Effect of exogenous leptin treatment on dexamethasone-induced muscle atrophy

WT mice were administered leptin (i.p.). After 45 min, an *ex vivo* study of the gastrocnemius muscle was conducted. Following leptin treatment, the protein expression of p-AKT and p-mTOR was increased, which indicates a potentiation of protein anabolism induced by exogenous leptin (Fig. 4A). In addition, chronic leptin treatment for 7 days ameliorated the dexamethasone-mediated decrease in the weight of the gastrocnemius muscle and the dexamethasone-mediated change in the protein expression of p-AKT and MuRF (Fig. 4B and 4C).

DISCUSSION

The present study demonstrates that leptin-mediated activation of SNA through the hypothalamus is not impaired in

Epac2a-KO mice. We confirmed that Epac2a expression was not detected in gastrocnemius muscle and BAT in WT mice (Supplementary Fig. 3). In addition, we demonstrated that skeletal muscle and BAT in Epac2a-KO mice have normal leptin and adrenergic signaling through their own respective plasmalemmal receptors. They are known to express both leptin and the leptin receptor, which is the long-form (ObRb) for typical intracellular leptin signaling through Janus kinase (JAK) and signal transducer and activator of transcription (STAT) (19).

In BAT, leptin was shown to increase glucose utilization through ObRb and the JAK/STAT pathway. It demonstrates that leptin increases the expression of some metabolic enzymes and PPAR activity in preadipocytes and BAT (17, 20). However, direct leptin action on UCP-1 expression in BAT was not observed, suggesting that thermogenesis in BAT is not directly associated with leptin receptor signaling. In fact, UCP-1 gene expression in BAT was induced by leptin, but through sympathetic innervation (21). Diet-induced obese mice, which are resistant to the anorectic effect of leptin but are hyperleptinemic, exhibited a higher iBAT temperature than mice fed a regular diet, whereas *ob/ob* mice that do not have the leptin gene showed a lower iBAT temperature (22). Interestingly, exogenous leptin administration in diet-induced obese mice and *ob/ob* mice increased iBAT temperature through the β_3 receptors, and leptin receptor blockade in the dorsomedial hypothalamus prevented the thermogenic effects of leptin, suggesting a leptin-mediated hypothalamus-SNA-BAT axis (23). Unresponsiveness to leptin for appetite control in obesity is observed in the pro-opiomelanocortin (POMC)

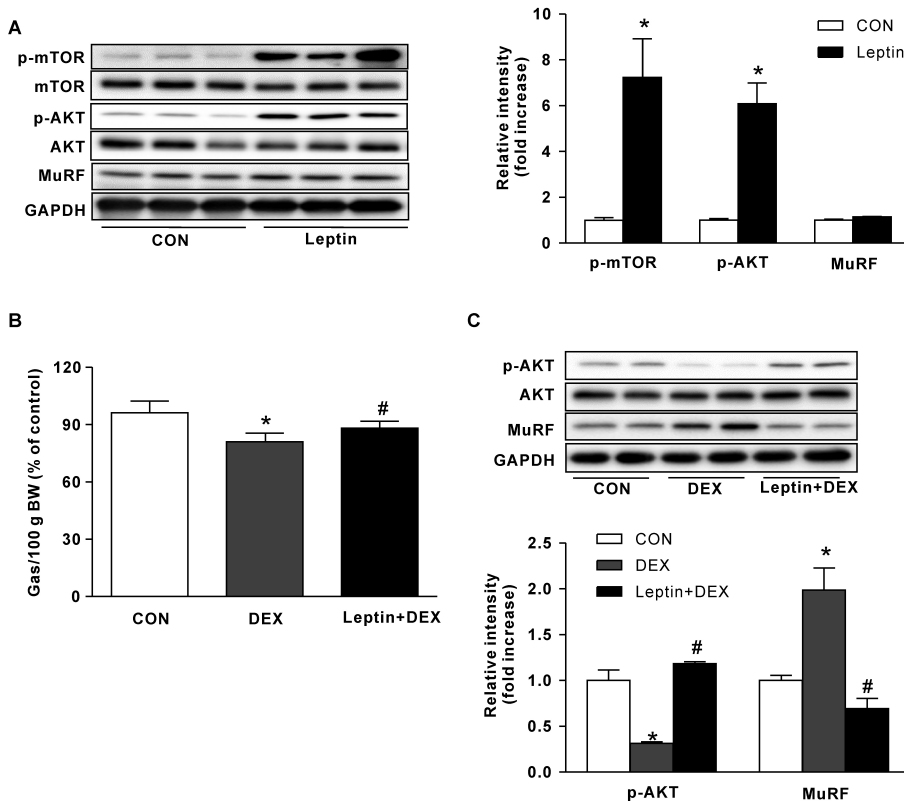


Fig. 4. Exogenous leptin induces muscle resistance to dexamethasone (Dex). (A) The expression of proteins in the gastrocnemius muscle of WT mice with or without leptin treatment. Leptin (5 μ g/g) was administered i.p. 45 min before sample collection ($n = 3$). Changes in muscle weight (B, $n = 8$) and protein expression (C, $n = 3$) in the muscle after 7 days of Dex (25 mg/kg/day) and/or leptin (5 μ g/g/day) treatment. * $P < 0.05$ compared to WT mice without any treatment. # $P < 0.05$ compared to dexamethasone-treated WT mice. Data represent the mean \pm SEM.

neurons of ARH in MC4R-dependent manner. Leptin-mediated SNA for the increase in EE may occur via ARH (24). However, the dorsomedial hypothalamus is essential for transmitting the thermogenic signal bypassing ARH and exploiting MC4R-independent pathway. Based on this, the present results suggest that endurance to cold stress in Epac2a-KO mice is primarily due to leptin signaling in the hypothalamic SNA, consistent with previous findings (4).

In skeletal muscle, leptin directly exerts its action, which is like the action of insulin, by facilitating glycogen synthesis and glucose uptake, and promoting cell survival. Leptin deficiency (e.g., *ob/ob* mice) caused decreased muscle mass (25-27) and increased expression of the muscle-wasting protein myostatin (28, 29), which was recovered by administration of exogenous leptin. Because the leptin receptor is expressed in human skeletal muscle, leptin may be an essential autocrine and paracrine muscle trophic factor (30). In addition, the sympathetic nervous system, mainly via β_2 receptors, increases muscle mass by activating the Akt/mTOR pathway (31-33), which has therapeutic potential against age-related muscle wasting, muscular dystrophy, and various muscle catabolic stresses (34). In Epac2a-KO mice, the skeletal muscle is more anabolic in nature, and it can endure when treatment is prolonged with the glucocorticoid, dexamethasone. Therefore, it may be plausible that augmented leptin signaling

in Epac2a-KO mice fortifies the skeletal muscle through its own leptin receptors, as well as its own adrenergic receptors via hypothalamic leptin signaling.

In conclusion, like arterial blood pressure, which is elevated in diet-induced obesity, hypothalamic leptin resistance in the absence of Epac2a cannot block leptin signaling to enhance sympathetic outflow. This suggests that Epac2a in the hypothalamus only affects leptin-mediated signaling for appetite control (22). The present results also suggest that leptin receptor-induced signaling in skeletal muscle and BAT might be normal in Epac2a-KO mice, unlike the leptin receptor signaling in the hypothalamus.

MATERIALS AND METHODS

Detailed information is provided in the Supplementary Material.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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