PAQR3 Has Modulatory Roles in Obesity, Energy Metabolism, and Leptin Signaling

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Diet-induced obesity is commonly associated with leptin resistance, and attenuated leptin signaling contributes to the progression of obesity. PAQR3 is a member of the progesterone and AdipoQ receptor (PAQR) family with close homology to adiponectin receptors. We hypothesized that PAQR3 is implicated in the regulation of obesity and energy homeostasis. To address this hypothesis, we fed Paqr3-deleted mice with high-fat diet (HFD), followed by analyses to evaluate obesity, hepatic steatosis, insulin resistance, metabolic rate, and leptin signaling. We found that mice with deletion of Paqr3 are resistant to HFD-induced obesity and hepatic steatosis, accompanied by improvement of insulin resistance and insulin signaling. Paqr3-deleted mice have an increased energy expenditure and physical activity. HFD-induced leptin resistance is reversed by Paqr3 ablation. Overexpression of PAQR3 reduces leptin signaling whereas deletion of Paqr3 enhances leptin signaling in the hypothalamus. In conclusion, this study reveals that PAQR3 has an important physiological function in modulating obesity, energy metabolism, and leptin signaling.

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Obesity, mainly caused by an imbalance between energy intake and expenditure, has become a worldwide epidemic and imposes a major threat to human health in modern society (1). Obesity is categorized as one of the major components of metabolic syndrome that is associated with insulin resistance, inflammation, and increased risk of developing type 2 diabetes and cardiovascular diseases (2–4). Energy balance relies on a tightly regulated homeostatic system that orchestrates energy intake with energy expenditure in order to maintain a stable body weight (5). The central nervous system (CNS) plays a key role in energy homeostasis regulation (5, 6). Molecules secreted by peripheral tissues, such as adipose tissue, impart on the CNS to regulate energy intake and expenditure, and one of the central players in the regulatory circuit is leptin (6). Leptin signaling in the hypothalamus is critical for sensing the availability of fuel in the body and preventing excessive energy stores, thereby reducing obesity. Leptin deficiency results in obesity in rodents and humans, owing to hyperphagia and inappropriate metabolism elicited by CNS. In diet-induced obesity, leptin levels are elevated, reflecting increased fat stores. Importantly, injection of additional leptin in obese individuals fails to reverse obesity, a phenomenon coined as leptin resistance (7). Therefore, it has been proposed that an improvement of leptin sensitivity or an increase in the responsiveness to the action of leptin could be a promising strategy to alleviate obesity.

Leptin regulates a broad spectrum of homeostatic functions following its binding to its receptor (6, 8, 9); LepRb is the major functional isoform of leptin receptor in the CNS (9, 10). Leptin binding alters the conformation of the preformed LepRb homodimer, enabling transphosphorylation and activation of LepRb-associated JAK2 (9). The activated JAK2 then phosphorylates other tyrosine residues within the LepRb/Janus family of tyrosine kinases.
(JAK)2 complex to mediate downstream signaling. Activated LepRb recruits signal transducer and activator of transcription 3 (STAT3), a latent transcription factor, which subsequently becomes tyrosine phosphorylated by JAK2, enabling its nuclear translocation and promoting its transcriptional effects (9, 11, 12).

PAQR3 is a member of the progesterone and AdipoQ receptor (PAQR) family in which AdipoR1 and AdipoR2 (ie, PAQR1 and PAQR2) function as cell surface receptors for adiponectin, an adipocyte-secreted cytokine that regulates glucose and lipid metabolism (13). PAQR3, or Raf kinase trapping to Golgi, is a Golgi-localized membrane protein that modulates intracellular signaling by sequestering proteins onto the Golgi apparatus. Through spatial regulation of Raf kinase and Gβ subunit of G protein-coupled receptors (14–16), PAQR3 is actively implicated in the regulation of cell proliferation and migration and plays an important role in tumor development (17–20). Lately, our group demonstrated that PAQR3 also regulates insulin signaling by tethering the p110α subunit of phosphatidylinositol 3-kinase (PI3K) to the Golgi apparatus to modulate insulin sensitivity (21), because p110α is the primary molecule within the PI3K family that mediates insulin signaling (22). However, whether or not PAQR3 impinges on energy metabolism and obesity is currently unknown. Here we used a high-fat diet (HFD)-induced mouse model to investigate the functions of PAQR3 in regulating energy homeostasis and metabolism.

**Materials and Methods**

**Animal studies**

All animals were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences. All of the experimental procedures were carried out in accordance with the CAS ethics commission with an approval number 2010-AN-8. Paqr3-null mice in C57BL/6J background were generated by crossing with Paqr3+/− mice and wild-type littermates were fed with HFD (Research Diets, Inc.) for 16 weeks. Male mice fed with HFD for 14 weeks were injected ip twice daily (at 8:30 AM and 5:30 PM) with PBS for 3 days for acclimation, followed by injection with recombinant mouse leptin (R&D Systems) at 1.5 mg/kg/d for 3 days and then PBS injection for 3 more days. Body weight was monitored at 8:00 AM daily and food intake was measured at 5:00 PM daily.

**Plasmids**

The full-length human leptin receptor LepRb expression plasmid was described as previously reported (24). The Myc-tagged PAQR3 was described previously (14).

**Cell culture and cell transfection**

Human embryonic kidney 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Transient transfection was performed with PolyJet Reagent (SignaGen Laboratories). For leptin treatment, the cells were maintained in DMEM for 5 hours, and then incubated with leptin (100 ng/mL) for different lengths of time.
Antibodies and immunoblotting analysis

The antibodies were purchased as follows: phospho-IRβ(Tyr-1150/1151), total IRβ, phospho-AKT(Ser-473), total AKT, phospho-glycogen synthase kinase 3 (GSK3)β(Ser-9), phospho-STAT3(Tyr-705), total STAT3, phospho-JAK2(Tyr-1007/1008), and total JAK2 from Cell Signaling Technology; phospho-insulin receptor substrate 1 (IRS-1)(Tyr-608) from Millipore Corp (Upstate Biotechnology); Myc and β-actin from

Figure 1. Ablation of Paqr3 abrogates HFD-induced obesity. A and B, Body weight and food intake of the mice with different genotype fed with normal chow (NC) or HFD (n = 12–18 for each group). HFD was started at the eighth week. All mice were in C57BL/6J background. The data are shown as means ± SEM. * and **, P < .05 and P < .01, respectively, between wild-type (wt) and Paqr3-deleted mice in HFD group. The inset in panel A is representative pictures of the mice with different genotype after HFD. C, Nuclear magnetic resonance analysis of the body fat and lean mass in 24-week-old wild-type and Paqr3-deleted mice fed with normal chow or HFD for 16 weeks (n = 7–10 for each group). The data are shown as means ± SEM; **, P < .01 between the 2 groups with the same feeding scheme. D, Representative hematoxylin-eosin staining of white adipose tissue in 24-week-old mice fed with either normal chow or HFD for 16 weeks. E, Serum levels of triglycerides, cholesterol, high-density lipoprotein and low-density lipoprotein were measured in 24-week-old mice fed with either normal chow or HFD for 16 weeks. ND, data not determined. Data are shown as means ± SEM. * and **, P < .05 and P < .01, respectively, between the 2 groups under the same feeding scheme.
Santa Cruz Biotechnology; α-tubulin from Sigma-Aldrich; goat antimouse IgG and goat antirabbit IgG from Jackson ImmunoResearch Laboratories. For immunoblotting, hypothalamic protein extracts were prepared by homogenizing with radioimmunoprecipitation assay lysis buffer supplemented with 1% protease inhibitor cocktail (Sigma-Aldrich). Protein extracts were subjected to SDS-PAGE for immunoblotting analyses.

**Statistical analysis**

The unpaired and two-tailed Student’s *t* test was used for all the statistical analyses.

**Results**

**Paqr3-deficient mice are resistant to HFD-induced obesity and hepatic steatosis**

To investigate the potential role of PAQR3 in obesity and energy homeostasis, we investigated the contribution of PAQR3 to HFD-induced obesity. We first analyzed the body weight of Paqr3-null (Paqr3−/−) mice and their wild-type littermate controls, under normal chow and HFD. When the mice were fed with normal chow, both wild-type and Paqr3−/− mice had similar gains in body weight (Figure 1A). Interestingly, Paqr3−/− mice were resistant to HFD-induced body weight gain starting from the fourth week upon HFD (Figure 1A). Paqr3-deleted mice had a similar food intake to their wild-type littermate controls, under both normal chow or HFD-fed conditions (Figure 1B). Furthermore, after 16 weeks of HFD, Paqr3−/− mice had a markedly decreased total body fat mass and increased lean body mass in comparison with wild-type littermate controls (Figure 1C). Histologic analysis of white adipose tissue revealed that Paqr3−/− mice had smaller adipocytes than wild-type animals (Figure 1D). Interestingly, we found that deletion of Paqr3 is associated with reduction of total cholesterol, high-density lipoprotein, and low-density lipoprotein in the serum under HFD condition (Figure 1E), indicating that HFD-induced hypercholesterolemia is also relieved by Paqr3 deletion.

We next analyzed the effect of Paqr3 ablation on HFD-induced hepatic steatosis, a common complication of obesity. Under HFD condition, the liver triglyceride level during either constant feeding or fasting was significantly reduced by Paqr3 deletion (Figure 2A). Whereas HFD induced extensive lipid deposition manifested as macro- and microvesicular steatosis in wild-type mice, Paqr3 deletion significantly reduced fatty liver development upon HFD

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**Figure 2.** Paqr3 deletion reduces HFD-induced hepatic steatosis. A, Measurement of liver triglyceride concentration. The liver samples from 24-week-old mice fed with either normal chow (NC) or HFD for 16 weeks were used in measurement of triglyceride concentration. The number of animals used (n) is shown at the top of each bar. Data are means ± SEM. * and ** indicate \( P < .05 \) and \( P < .01 \), respectively, between the two groups of mice. B, Representative hematoxylin-eosin staining (upper panel) and Oil Red O staining (lower panel) with liver sections from 24-week-old mice fed with either normal chow or HFD for 16 weeks.
These data, therefore, suggest that HFD-induced hepatic steatosis is markedly alleviated by Paqr3 ablation. Deletion of Paqr3 ameliorates HFD-induced insulin resistance.

We previously reported that PAQR3 is able to modulate insulin signaling pathway by shunting p110\(\alpha\)/H9251 subunit of PI3K to the Golgi apparatus to inhibit its activation (21). We next addressed a question of whether PAQR3 had an effect on insulin resistance under HFD. As expected, HFD was able to induce glucose intolerance and insulin resistance in the mouse (Figure 3, A and B). Interestingly, glucose tolerance was apparently improved by Paqr3 deletion as revealed by GTT (Figure 3A). This result suggests that deletion of paqr3 relieved HFD-induced glucose intolerance. Consistently, HFD-induced insulin resistance was significantly ameliorated by Paqr3 deletion (Figure 3B); similarly, gluconeogenesis, as detected by PTT, was apparently reduced by Paqr3 (Figure 3C). Consistent with these observations, the fasting insulin level was significantly reduced in Paqr3\(^{-/-}\) mice under HFD (Figure 3D), indicating that the HFD-induced hyperinsulinemia, a physiologic hallmark of insulin resistance, is reduced by Paqr3 ablation. Furthermore, the insulin level of Paqr3\(^{-/-}\) mice under constant feeding condition was reduced under both normal chow and HFD (Figure 3E), further suggesting an improvement of insulin sensitivity by Paqr3 ablation. Collectively, these data point to the fact that HFD-induced insulin resistance is significantly ameliorated by Paqr3 deletion in vivo.

**HFD-induced inactivation of insulin signaling is reversed by Paqr3 ablation**

One of the major causes of insulin resistance is compromised insulin signaling in peripheral tissues such as liver and skeletal muscle. Because deletion of Paqr3 is able to improve HFD-induced insulin resistance, we hypothesized that Paqr3 should ameliorate the inactivated insulin signaling upon HFD. As expected, insulin signaling as measured by insulin-induced phosphorylations of insulin receptor-\(\beta\) (IR\(\beta\)), IRS-1, AKT, and GSK3\(\beta\) were all markedly compromised in both liver and skeletal muscle in HFD-fed mice (Figure 4). Interestingly, Paqr3 deletion appeared to have no effect at all on the phosphorylations of IR\(\beta\) and IRS-1 upon insulin administration in HFD-fed mice (Figure 4). However, the insulin-induced AKT and GSK3\(\beta\) phosphorylations were robustly enhanced by Paqr3 ablation in both liver and skeletal muscle in HFD-fed mice (Figure 4). Collectively, these data indicate that HFD-induced inactivation of insulin signaling can be significantly ameliorated by Paqr3 ablation. Furthermore, these results are consistent with our previous finding that PAQR3 negatively regulates insulin signaling by sequestrating PI3K p110\(\alpha\) subunits to the Golgi apparatus (21).

**Improved energy homeostasis in Paqr3-deficient mice**

To investigate the possible mechanism(s) underlying the protection from HFD-induced weight gain in Paqr3-
deficient mice, we analyzed energy expenditure and locomotor activity of the mice (Figure 5). In comparison with the wild-type mice, the energy expenditure, as assessed by oxygen consumption and carbon dioxide production corrected per body weight during indirect calorimetry, was significantly increased in Paqr3 deletion mice after 14 weeks of HFD (Figure 5, A and B). The RER (VCO2:VO2), an indicator for the source of energy expenditure, was also calculated with the mice (Figure 5C). The reduction of RER in Paqr3-null mice indicates that more lipids were consumed in these animals, consistent with our findings that Paqr3 ablation was able to reduce HFD-induced obesity. Moreover, there was consistent and significant increase in spontaneous locomotor activity in Paqr3 ablation mice (Figure 5D). Thus, the protection from HFD-induced obesity in Paqr3-null mice is likely caused by increased energy expenditure due to Paqr3 ablation.

**Paqr3-deleted mice exhibit an increase in leptin sensitivity**

Leptin resistance in the hypothalamus represents a hallmark during the development of high-caloric diet-induced weight gain and peripheral insulin resistance (25–28). Leptin functions as a very important hormone to reduce food intake and increase energy homeostasis. Given that Paqr3 deletion could protect the mice from HFD-induced obesity, we hypothesized that leptin action was likely altered in Paqr3−/− mice. Therefore, we analyzed the effect...
of Paqr3 deletion on leptin sensitivity. Paqr3−/− mice and their littermate controls were fed with HFD for 14 weeks, followed by injection of PBS for 3 days, leptin injection for 3 days, and then PBS injection for 3 more days (Figure 6A). As expected, leptin injection was able to slightly reduce body weight of the mice. However, the effect of leptin to reduce body weight in Paqr3−/− mice was much greater than that of their littermate controls (Figure 6A). Consistently, leptin failed to significantly reduce food intake in the control mice, but it significantly reduced food intake in Paqr3−/− mice (Figure 6B). Furthermore, the leptin level in the serum of Paqr3−/− mice was dramatically reduced when fed with HFD (Figure 6C). In addition, we analyzed the mRNA expression level of PAQR3 in hypothalamus and other tissues (Figure 6D). We found that PAQR3 was indeed expressed in the hypothalamus at a level higher than the cerebellum and lower than the cerebrum. Meanwhile, the PAQR3 mRNA level in the hypothalamus was comparable to that in the white adipose tissue and brown adipose tissue. These data indicate that HFD-evoked leptin resistance is ameliorated with Paqr3 ablation, consistent with our observations that Paqr3 deletion is associated with a reduction in obesity and an increase in energy expenditure of the mice.

**PAQR3 regulates leptin signaling**

Because the in vivo studies had revealed that PAQR3 was able to modulate leptin sensitivity, we next investigated whether PAQR3 could directly regulate leptin signaling. Both PAQR3 and a leptin receptor LepRb were expressed in human embryonic kidney 293T cells. As expected, leptin treatment was able to induce rapid phosphorylation of STAT3 and JAK2 (Figure 6A). Interestingly, overexpression of PAQR3 profoundly diminished leptin-induced phosphorylations of STAT3 and JAK2 (Figure 7A). We also investigated the potential mechanism that might...
underlie the modulatory effect of PAQR3 on leptin signaling. We found that suppressor of cytokine signaling 3 (SOCS3), a major negative regulator of leptin signaling (29–31), was significantly reduced by PAQR3 deletion (Figure 7C). Taken together, these data suggest that PAQR3 has a direct effect on modulation of leptin signaling in vitro and in vivo at least partially through its regulation on SOCS3.

**Discussion**

In this study, we report that PAQR3, a member of the progesterone and AdipoQ receptor (PAQR) family, has a significant function in the regulation of diet-induced obesity. HFD is a model of overnutrition to induce obesity in rodents. When fed with HFD for 16 weeks, wild-type mice are obese with a profound increase in body fat composition. However, Paqr3 ablation mice had significantly reduced obesity and body fat mass (Figure 1). Moreover, nonalcoholic fatty liver disease, a major complication of obesity, was markedly reduced by Paqr3 deletion (Figure 2). The HFD-induced glucose resistance, insulin resistance, hyperinsulinemia, and reduction of insulin signaling were all alleviated by Paqr3 deletion (Figures 3 and 4).

In addition to the improvement of insulin sensitivity in HFD-fed Paqr3-null mice, energy expenditure was significantly enhanced by Paqr3 ablation (Figure 5). Lastly, deletion of Paqr3 was associated with increases in leptin sensitivity and leptin signaling (Figures 6 and 7). Collectively, these data have provided convincing evidence that PAQR3 plays an important role in regulating obesity and energy homeostasis accompanied by modulation of leptin signaling.

In both humans and rodents, obesity is commonly associated with leptin resistance (7). In our diet-induced obesity mouse model, HFD was clearly associated with development of leptin resistance (Figure 6). However, HFD-induced leptin resistance could be significantly alleviated by Paqr3 deletion (Figure 6). Furthermore, such reduction of leptin resistance is associated with an increase in leptin signaling in the hypothalamus (Figure 7B) upon Paqr3 deletion. We speculate that the increase in leptin action by Paqr3 deletion could partially explain the elevation of energy expenditure and reduction of obesity in Paqr3-null mice. It was proposed that many factors could underlie the compromised leptin action in the brain, such as impaired transport of leptin across the blood brain barrier (32), endoplasmic reticulum stress (33–35), local in-
flammation (28, 36), JAK inhibition by SOCS3 (29–31), and attenuation of leptin signaling by protein tyrosine phosphatases (37, 38). Interestingly, we found that deletion of PAQR3 is associated with down-regulation of SOCS3 (Figure 7C), indicating that PAQR3 may modulate leptin signaling through its regulation of SOCS3 expression. However, it will be imperative in the future to address whether PAQR3 exploits other mechanisms to reduce obesity-associated leptin resistance.

Is it possible that PAQR3 serves as an adiponectin receptor to mediate the regulatory effects of adiponectin on energy metabolism? Our current understanding about PAQR3 is not supportive of this possibility. PAQR3 is a Golgi-localized protein without appreciable localization on the plasma membrane; therefore, it is unlikely to function as an adiponectin receptor in the cell surface. As a matter of fact, we found that overexpression of PAQR3 in C2C12 cells could not alter adiponectin-mediated phosphorylations of AMP-activated protein kinase and acetyl-CoA carboxylase (ACC) (data not shown). In addition, adiponectin is commonly considered as a “beneficial” factor in energy metabolism and deletion of both adiponectin receptors AdipoR1 and AdipoR2 could lead to insulin resistance and glucose tolerance associated with elevated tissue triglyceride content and inflammation/oxidative stress, thus leading to insulin resistance and marked glucose intolerance (39). Deletion of AdipoR2 alone also promotes type 2 diabetes in the mouse (40). On the contrary, deletion of PAQR3 leads to an improvement of energy metabolism and insulin sensitivity, distinctive from the phenotype upon deletion of adiponectin receptors.

In addition to the improvement of leptin sensitivity by Paqr3 deletion, we observed that obesity-induced insulin resistance and reduction of insulin signaling are improved in Paqr3-null mice (Figures 3 and 4). The increase in insulin signaling could be explained by the regulation of PI3K activity by PAQR3. Our recent study indicates that PAQR3 actively modulates insulin signaling by spatial regulation of PI3K p110 and IRS-1. PAQR3 can sequester p110 subunit to the Golgi apparatus via interaction with a structural domain involved in its interaction with p85, the regulatory subunit of PI3K. Therefore, tethering of p110 subunit to the Golgi apparatus is also associated with dissociation of p110 from p85, leading to reduction of PI3K activity. As a matter of fact, the in vivo and in vitro experiments have pinpointed that about 30%–40% of endogenous PI3K activity is modulated by a PAQR3-mediated mechanism (21). Consistently, we found that Paqr3 deletion had no effect on the phosphorylations of IR and IRS-1 upon insulin administration in HFD-fed mice (Figure 4). However, the insulin-induced AKT and GSK3β phosphorylations were robustly enhanced by Paqr3 ablation in both liver and skeletal muscle in the mice (Figure 4).

Interestingly, the change of PI3K activity in the brain also plays a role in modulating energy balance. It was recently reported that reduction of PI3K activity by conditional deletion of p110α in the ventromedial hypotha-
lamic nucleus of the mouse is more sensitive to HFD-induced obesity due to reduced energy expenditure (41). Considering that the Paqr3 deletion is associated with an enhanced PI3K activity (21), we propose that the alteration of PI3K activity in the CNS in these mice may also contribute to the regulation of energy metabolism and obesity. It will also be interesting to investigate in the future whether modulation of PI3K activity in the brain is also directly associated with modulation of leptin signaling. Based on our current observation that the amelioration of HFD-induced obesity by Paqr3 deletion is associated with increases in metabolic rate, physical activity, and leptin signaling, we strongly suspect that PAQR3 impacts energy metabolism through the CNS. Such an issue can be addressed by establishment of mouse models with brain- or hypothalamus-specific deletion of Paqr3 gene in the future. Nevertheless, considering that PAQR3 plays an important role in controlling obesity, energy expenditure, and leptin signaling, we propose that modulating the expression and function of PAQR3 may comprise a new strategy to combat obesity.

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