Enhancing natriuretic peptide signaling in adipose tissue, but not in muscle, protects against diet-induced obesity and insulin resistance

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In addition to controlling blood pressure, cardiac natriuretic peptides (NPs) can stimulate lipolysis in adipocytes and promote the “browning” of white adipose tissue. NPs may also increase the oxidative capacity of skeletal muscle. To unravel the contribution of NP-stimulated metabolism in adipose tissue compared to that in muscle in vivo, we generated mice with tissue-specific deletion of the NP clearance receptor, NPRC, in adipose tissue (NprcMKO) or in skeletal muscle (NprcMKOr). We showed that, similar to Nprc null mice, NprcMKO mice, but not NprcMKOr mice, were resistant to obesity induced by a high-fat diet. NprcMKO mice exhibited increased energy expenditure, improved insulin sensitivity, and increased glucose uptake into brown fat. These mice were also protected from diet-induced hepatic steatosis and visceral fat inflammation. These findings support the conclusion that NPRC in adipose tissue is a critical regulator of energy metabolism and suggest that inhibiting this receptor may be an important avenue to explore for combating metabolic disease.

INTRODUCTION

Obesity, a result of calorie intake exceeding energy expenditure, is frequently associated with tissue inflammation and insulin resistance. It is a major risk factor for many metabolic disorders, including type 2 diabetes (T2D), cardiovascular diseases, and several types of cancer (1–3). The link between obesity and T2D depends largely on the activity and function of adipose tissue (4). In mammals, there are two major types of adipose tissue with distinct physiological roles. White adipose tissue (WAT) stores excess nutrients in the form of triglycerides, and brown adipose tissue (BAT) dissipates heat through an uncoupling protein 1 (UCP1)-dependent mechanism. The “rediscovery” of functional BAT in healthy adult humans (5) and the identification of UCP1-positive brown-like (beige) adipocytes in white adipose suggests that UCP1 is a potential target for increasing energy expenditure to control body weight (6–9).

The cardiac-derived natriuretic peptides (NPs), atrial NP (ANP), and the related B-type NP (BNP) are key factors that control blood pressure by acting on the kidney (10). The physiological actions of ANP and BNP are mediated by binding to NP receptor A (NPRA), which activates its guanylyl cyclase domain to produce cyclic guanosine 3’,5’-monophosphate (cGMP), leading to activation of cGMP-dependent protein kinases [protein kinase G (PKG)] (11, 12). The other receptor for these cardiac NPs, NP receptor C (NPRC), functions to clear the NPs from circulation through receptor-mediated internalization (13). Thus, the ability of the NPs to elicit a biological response depends on the relative ratio of the functional receptor NPRA to the “clearance receptor” NPRC.

NP receptors are present in adipose tissue (14). ANP stimulates lipolysis in cultured human adipocytes, with potency similar to the β-adrenergic agonist isoproterenol (15). We have shown that NPs can also induce the adipose “browning” program through the same NPRA-cGMP-PKG signaling cascade (16). We have previously shown that ANP treatment of cultured human adipocytes increases mitochondrial biogenesis, UCP1 abundance, and uncoupled respiration (16). Clinical studies in humans report that NPs can increase energy expenditure and fat oxidation independent of the β-adrenergic axis (17). Compared with lean individuals, obese subjects have increased NPRA abundance in adipose tissues, resulting in a decreased NPRA/NPRC ratio and blunted cellular responses (18–20). This increase in NPRC in adipose tissue also has been posited to contribute to the lower circulating BNP concentration observed in obese subjects, possibly due to increased peptide uptake (21).

The ability of NPs to stimulate lipolysis is reported to be primate-specific and not to occur in rodent adipose tissues (22). This species difference is likely due to the fact that NPRC abundance in the rodent is 100-fold higher (22, 23). In line with this hypothesis, primary adipocytes from Nprc null mice (Nprc−/−) show a lipolytic response to ANP (16). In addition, Nprc−/− mice have markedly reduced body weight and fat mass as well as higher amounts of UCP1 in their white adipose depots. We therefore proposed that NPRC plays a critical role in modulating the metabolic effect of NPs, particularly in the adipose tissue (16). However, because studies in rodents and humans suggest that NPs may be involved in skeletal muscle metabolism (24–27) and whole-body Nprc−/− mice also exhibit a skeletal overgrowth phenotype (23, 28), it is unclear whether adipose tissue is the major site by which NPs increase energy expenditure. Here, we showed that deletion of Nprc in adipose tissue (NprcMKO), but not in skeletal muscle (NprcMKOr), protected against diet-induced obesity and insulin resistance. NprcMKO mice exhibited increased energy expenditure, reduced inflammation, and a redistribution of lipid storage from liver to visceral fat. These findings suggest that the adipose tissue is the major site of NP-mediated metabolic effects in mice lacking NPRC under high-fat diet (HFD)–feeding conditions.

RESULTS

HFD-fed Nprc−/− mice were leaner and exhibited improved glucose tolerance

NPRC was greatly increased at the mRNA and protein levels in the adipose tissue of Nprc−/− mice after 12 weeks on an HFD (Fig. 1, A to C), suggesting that the resulting increase in NPRC may attenuate the ability
Fig. 1. *Nprc* is increased at the mRNA and protein levels in adipose tissue by HFD feeding. (A and B) *Nprc<sup>fl/fl</sup>* and *Nprc<sup>AKO</sup>* mice were fed with a chow diet (CD) or an HFD for 12 weeks. qRT-PCR for the expression of *Nprc* mRNA relative to 36B4 in gonadal WAT (gWAT) (A) and BAT (B) of CD-fed (*n* = 3) and HFD-fed (*n* = 5) *Nprc<sup>fl/fl</sup>* mice. (C) Western blotting analysis for NPRC protein performed on the lysates from gWAT and BAT of *Nprc<sup>AKO</sup>* and *Nprc<sup>fl/fl</sup>* mice. Blots are representative of three separate cohorts. ***P < 0.001, unpaired two-tailed Student’s *t* test.

Fig. 2. HFD-fed *Nprc<sup>−/−</sup>* mice are leaner and exhibit improved glucose tolerance. (A) Image of *Nprc<sup>−/−</sup>* and wild-type (WT) mice after 12 weeks on HFD. (B to E) Body weights (BW) (B), fat mass (C), lean mass (D), and tissue weights (E) of *Nprc<sup>−/−</sup>* (*n* = 5) and WT (*n* = 6) mice after 12 weeks on HFD. (F) Daily food intake of *Nprc<sup>−/−</sup>* (*n* = 3) and WT (*n* = 4) mice on HFD. (G) Representative images and hematoxylin and eosin (H&E) staining of BAT, inguinal WAT (iWAT), gWAT, and liver from WT (*n* = 3) and *Nprc<sup>−/−</sup>* (*n* = 3) mice after 12 weeks on HFD. Scale bars, 100 μm. (H) Immunostaining and quantification of UCP1 in the BAT of WT (*n* = 2) and *Nprc<sup>−/−</sup>* (*n* = 2) mice after 12 weeks on HFD, as described in Materials and Methods. Scale bar, 200 μm. A.U., arbitrary units. (I and J) Plasma glucose concentration and area under the curve (AUC) during intraperitoneal glucose tolerance test (GTT) (I) and insulin tolerance test (ITT) (J) of *Nprc<sup>−/−</sup>* (*n* = 12) and WT (*n* = 8) mice after 12 weeks on HFD. *P < 0.05; **P < 0.01; ***P < 0.001, unpaired two-tailed Student’s *t* test.
of NPs to stimulate lipolysis and thermogenesis. Nprc−/− mice on the HFD gained less weight than wild-type mice, which was primarily due to decreased fat mass (Fig. 2, A to E), without changes in food intake (Fig. 2F). In addition to smaller depots of WAT, Nprc−/− mice also accumulated less lipid in both the interscapular brown fat (BAT) and the liver (Fig. 2G) and had more UCP1 in the BAT (Fig. 2H). Nprc−/− mice also showed better glucose and insulin tolerance (Fig. 2, I and J). Consistent with a previous report (23), concentrations of circulating NPs were not altered in Nprc−/− mice (fig. S1A). Together, these results suggest that Nprc−/− mice are metabolically healthier than the wild-type mice.

Several studies suggest a role of NPs in regulating skeletal muscle metabolism (24–27). In wild-type mice, the expression of Nprc and Npra in skeletal muscle was lower than in adipose tissues, but the ratio of Npra to Nprc was comparable (fig. S2, A and B). To investigate the contribution of adipose tissue and muscle to the leaner phenotype of HFD-fed Nprc−/− mice, we next generated tissue-specific Nprc knockout mice. Skeletal muscle–specific knockout mice (NprcMKO) were generated by crossing floxed Nprc mice (Nprcfl/fl) with myogenin-Cre mice (29). NprcMKO mice were born in the expected Mendelian and sex ratios. Circulating NP concentrations and blood pressure were comparable between the two genotypes (fig. S1, B and D). Nprc expression was efficiently reduced in the skeletal muscle tissues examined, with no changes in expression of Npra, as indicated by quantitative real-time polymerase chain reaction (qRT-PCR) (fig. S2, C and D). However, we found that compared to Nprcfl/fl mice, HFD-fed NprcAKO mice had comparable body weight and composition (Fig. 3, A to D), energy expenditure (Fig. 3E and fig. S3, A and B), food consumption (Fig. 3F), physical activity (Fig. 3G), and glucose and insulin tolerance (Fig. 3, H and I). Therefore, contrary to expectation, these results suggest that skeletal muscle did not contribute to the metabolic improvements seen in HFD-fed Nprc−/− mice.

**HFD-fed NprcAKO mice gained less body weight and exhibited higher energy expenditure and improved glucose tolerance**

Adipocyte-specific Nprc knockout mice (NprcAKO) were generated by crossing the floxed Nprc mice with adiponectin-Cre mice (30). NprcAKO mice were born in the expected Mendelian and sex ratios. Their circulating NP concentrations and blood pressure were comparable to littermate Nprcββ mice (fig. S1, C and D). NPRC mRNA and protein were absent from the WAT and BAT of the NprcAKO mice, but not in the liver, kidney, or heart, whereas there was no change in Npra and NPro in any of the tissues examined (fig. S2, E to G). When fed a HFD, NprcAKO mice gained less weight than Nprcββ mice (Fig. 4, A and B). Total fat mass was reduced in NprcAKO mice (Fig. 4C), whereas lean mass was not different between genotypes (Fig. 4D).

Indirect calorimetry analysis showed that oxygen consumption, carbon dioxide production, and energy expenditure increased in HFD-fed NprcAKO mice (Fig. 4E and fig. S3, C and D) without alterations in food intake or physical activity (Fig. 4, F and G). After 12 weeks on an HFD, NprcAKO mice had a significantly lower fasting plasma insulin concentration compared with Nprcββ mice (Fig. 4H). GTT revealed that NprcAKO mice had lower blood glucose concentrations with a significantly reduced AUC (Fig. 4I). Similarly, ITT showed that the NprcAKO mice had improved insulin sensitivity, although the decrease in AUC was not statistically significant (Fig. 4J).
In both genotypes, glucose appearance rate (Fig. 5C) was significantly higher rate of glucose uptake (Fig. 5B) in Nprc AKO mice compared to Nprc fl/fl control mice, UCPI was more abundant in the BAT of Nprc AKO mice (Fig. 5G). In addition, the transcription of several genes encoding BAT-derived adipokines, including Fgf21, Nrg4, and Bmp8b, also increased in the BAT of Nprc AKO mice (Fig. 5G). Although the absence of NPRC in iWAT and gWAT might be expected to favor increased expression of thermogenesis-related genes in those tissues (16), the two genotypes did not show differences for the expression of any of these genes except for Cidec (fig. S4, A and B). We also saw no histological evidence of “beige” or UCPI-positive cells (fig. S4C). Thus, contrary to expectation, deletion of Nprc promoted thermogenesis in the BAT but not in iWAT or gWAT for Nprc AKO mice, at least in the setting of diet-induced obesity.

Nprc deficiency in adipocytes results in a healthy redistribution of lipid storage and prevents inflammation in adipose tissue

Upon dissection, gross examination of tissues showed that despite an overall reduced body fat (Fig. 4C), the mass of the iWAT fat pad was not different between genotypes (Fig. 6A). However, Nprc AKO mice had increased gWAT mass, whereas liver weight was significantly reduced (Fig. 6A). The size of the adipocytes in the iWAT depot from Nprc AKO mice was smaller than those from Nprc fl/fl mice (fig. S5, A and B). The BAT of Nprc AKO mice did not change in overall mass but had a slightly darker complexion and smaller lipid droplets compared with that of Nprc fl/fl mice (Fig. 6A and B). In addition, the livers of Nprc AKO mice were essentially devoid of steatosis (Fig. 6A, A and B), which was associated with reduced expression of lipogenic and fatty acid uptake genes (such as Lpl, Acox1, and Srebf1) (Fig. 6C). In the gWAT depot of Nprc fl/fl mice, there was a high density of crown-like structures (Fig. 6B), indicative of inflammatory macrophage infiltration into the adipose tissue that is associated with insulin resistance (31). In contrast, few crown-like structures were detected in Nprc AKO gWAT. Immunohistochemical staining for the macrophage-specific marker F4/80 confirmed the robust presence of macrophages in the gWAT from Nprc fl/fl mice. 

**HFD-fed Nprc AKO mice showed increased glucose uptake and thermogenesis-related gene expression in the BAT**

We next examined insulin action in conscious, unrestrained mice by hyperinsulinemic-euglycemic clamp. Nprc AKO mice had a higher glucose infusion rate than Nprc fl/fl mice (Fig. 5A), indicating enhanced insulin sensitivity. In line with this finding, there was a statistically significant increase in the rate of endogenous glucose disappearance (Rd) in Nprc AKO mice (Fig. 5B), suggesting increased glucose disposal. In both genotypes, glucose appearance rate (Ri) was comparably suppressed by the insulin clamp, illustrating that hepatic insulin action was not different between genotypes (Fig. 5C). Furthermore, there was a significantly higher rate of glucose uptake (Ri) into BAT than Nprc fl/fl mice but not into other tissues examined (Fig. 5D).
inflammatory M2 macrophage markers (such as Cd68, F4/80) expression was significantly reduced in gWAT of NprcAKO mice, as indicated by increased AKT phosphorylation and glucose transporter type 4 (GLUT4) protein (Fig. 7A). In addition, the abundance of acyl–coenzyme A carboxylase (ACC) and fatty acid synthase (FASN), markers of de novo lipogenesis, was also higher in the gWAT of NprcAKO mice. 

NprcAKO mice on an HFD gained less body weight and showed improved insulin sensitivity, despite a larger gWAT depot than in the Nprcfl/fl control mice. Basal insulin signaling was increased in gWAT of the NprcAKO mice, as evidenced by increased AKT phosphorylation and glucose transporter type 4 (GLUT4) protein (Fig. 7A). In addition, the abundance of acyl–coenzyme A carboxylase (ACC) and fatty acid synthase (FASN), markers of de novo lipogenesis, was also higher in the gWAT of NprcAKO mice. 

The abundance of key transcriptional regulators of adipogenesis, peroxisome proliferator–activated receptor α (PPARα), and CCAAT/ enhancer binding protein (C/EBPα), were also higher in gWAT tissue samples from NprcAKO mice (Fig. 7A). Furthermore, the abundance of adiponectin also increased at the protein level in gWAT of NprcAKO mice (Fig. 7A). Active adipogenesis and lipogenesis in the gWAT depot of NprcAKO mice are consistent with the increased mass of this depot.

The amount of NPRA did not change, but PKG activity, as indicated by the phosphorylation of vasodilator-stimulated phosphoprotein (VASP), was greater in the gWAT of NprcAKO mice (Fig. 7A). Consistent with increased PKG activity, there was an increase in phosphorylated hormone-sensitive lipase (HSL) and total adipose triglyceride lipase (ATGL), suggesting that in the NprcAKO gWAT tissue there was also a higher degree of lipolysis in addition to increased lipogenesis and triglyceride deposition. In the iWAT of the NprcAKO mice, basal insulin signaling activity increased (Fig. 7B). However, there was still an increase in phosphorylated VASP and phosphorylated HSL, suggesting higher PKG signaling and lipolysis. In the iWAT of the NprcAKO mice, there were no apparent changes in markers of insulin signaling, de novo lipogenesis, or adipogenesis but a moderate increase in phosphorylated VASP and phosphorylated HSL (Fig. 7C).

The increased PKG activity in NprcAKO adipose tissues was further confirmed in vitro using primary adipocytes differentiated from the stromal vascular fraction of the iWAT depots from the two genotypes. Cells from both genotypes differentiated into adipocytes to a similar extent (Fig. S6A). When stimulated by a cocktail containing ANP and BNP, Nprc-deficient adipocytes showed greater PKG activity, as indicated by phosphorylation of VASP, and higher lipolytic activity (Fig. S6, B to D), indicating that Nprc-deficient adipocytes were more
The link between obesity and the cardiac NPs originates with reports by Sarzani et al. showing that receptors for ANP and BNP are present in adipose tissue (14) and that obese human subjects often have substantially higher amounts of the NP clearance receptor NPRC in adipose tissue and lower circulating NPs (18, 37). An increase in NPRC relative to the signaling receptor NPRA renders the tissue less responsive to NPs (fig. S6, E and F). The high amount of NPRC in the adipose tissue of obese subjects gave rise to the notion that adipose tissue might be a “sink” for circulating NPs, which could contribute to the hypertension that is often associated with obesity (18, 38). It is noteworthy also that lower plasma NP concentrations in obese human subjects could also be the result of reduced secretion of NPs because obesity is also associated with lower concentrations of N-terminal pro-BNP, a precursor fragment that is not cleared by NPRC (39).

ANP stimulates lipolysis in cultured human adipocytes through increases in cGMP and PKG activity, in a manner parallel to β-adrenergic receptors (βARs) and cyclic adenosine 5’-monophosphate/protein kinase A [reviewed in (40)]. NPRA activation also leads to uncoupled respiration in brown adipocytes and increases browning of WAT, in a parallel pathway to the βARs (41). Because a growing body of evidence places the NP system at the center of “cardiometabolic” disease (42), including the adipocyte response to early pathological stress on the heart (43), we studied their tissue-specific effects by manipulating NPRC abundance.

Mice with naturally occurring mutations that disrupt the expression of the Nprc gene are longer due to delayed bone ossification and have very little body fat, giving rise to their original names of “longjohn” and “strigous” (28). We have shown that the WAT and BAT of Nprc−/− mice have smaller lipid droplets and an increased ability to respond to NPs for lipolysis (16). Here, we showed that Nprc−/− mice were also protected from HFD-induced obesity and glucose intolerance. This metabolic benefit was retained in NprcMKO mice but not in NprcAKO mice. These findings indicated that the role of NPRC in regulating NP-mediated metabolism during HFD-feeding conditions responsive to NP stimulation. We used human embryonic kidney (HEK) 293 cells that stably express NPRA [also known as HEK293-GCA cells (36)] to further test, in a controlled way, the role of NPRC in the ANP dose-response curve. The presence of NPRC resulted in a shift of the cGMP dose-response curve to the right, compared to HEK293-GCA cells without NPRC (fig. S6, E and F). Together, these data indicated that NPRC deletion from adipose tissue increased the potency of ANP, thereby increasing PKG signaling and leading to an improvement in the metabolic phenotypes.

**DISCUSSION**

Fig. 6. HFD-fed NprcAKO mice are protected from hepatic steatosis and adipose tissue inflammation. (A) Tissue weight of NprcAKO (n = 14) and NprcMKO (n = 7) mice after 12 weeks on HFD. (B) Representative images and H&E staining of BAT, iWAT, gWAT, and liver from NprcMKO (n = 3) and NprcAKO (n = 3) mice. Scale bars, 100 μm. (C) qRT-PCR for the expression of markers of de novo lipogenesis and fatty acid uptake in the liver of HFD-fed NprcAKO (n = 8) and NprcMKO (n = 7) mice. Data were relative to 36B4 (n = 2) and NprcAKO (n = 2) mice. Crown-like structures are indicated by arrows. Scale bars, 100 μm (top) and 50 μm (bottom). (E and F) qRT-PCR for the expression of genes coding macrophage markers and inflammatory cytokines in gWAT (E) and iWAT (F) of HFD-fed NprcAKO (n = 14) and NprcMKO (n = 7) mice. Data were relative to 36B4. (G and H) Picro-sirius staining of gWAT (G) and liver (H) sections from NprcMKO (n = 2) and NprcAKO (n = 2) mice. Collagen fibrils were stained red (H; arrows). Scale bars, 100 μm (top) and 50 μm (bottom). (I) Plasma concentrations of adiponectin of HFD-fed NprcMKO (n = 8) and NprcMKO (n = 7) mice. *P < 0.05; **P < 0.01, unpaired two-tailed Student’s t test.

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largely depended on the adipose tissue but not on the skeletal muscle. This finding is consistent with previous findings that the improvement of insulin sensitivity in obese and diabetic subjects is associated with an increased NPRA/NPRC ratio in adipose tissue but not in skeletal muscle (19). Because NPs promote oxidative metabolism in human skeletal muscle (17, 27), the role of NPRC in skeletal muscle metabolism in the Nprc<sup>−/−</sup> mice may need to be investigated under other physiological settings, such as exercise performance or in response to direct NP infusions, as has been done in humans (25).

We showed here that targeted Nprc deficiency in adipose tissue increased oxygen consumption and carbon dioxide production without altering food intake and physical activity. Notably, UCP1 and other mitochondrial proteins increased in the BAT of mice consuming either a chow diet or an HFD (26). An important observation from our findings that needs to be further studied is that the expression of Fgf21, Nrg4, and Bmp8b, which encode BAT-enriched adipokines, increased in the BAT of Nprc<sup>−/−</sup> mice. Fgf21 (fibroblast growth factor 21) regulates energy expenditure, glucose homeostasis, and insulin sensitivity, potentially through the action of adiponectin (44, 45). Neuregulin 4 (Nrg4) is a brown fat–enriched secreted factor that preserves metabolic homeostasis through attenuation of hepatic lipogenesis (46). Bone morphogenetic protein 8B (BMP8B) increases BAT thermogenesis through both central and peripheral actions (47). It is possible that one of the effects of NP signaling in the BAT is the synthesis and release of these “batokines.” Consequently, the increased production of these batokines in Nprc<sup>−/−</sup> mice may also contribute to the overall improved metabolic profile of these mice in an endocrine and/or paracrine manner. The reduced liver fibrosis that we observed in Nprc<sup>−/−</sup> mice is reminiscent of previous findings that continuous ANP intravenous infusion or transgenic BNP overexpression prevents liver fibrosis (48, 49). Because our Nprc<sup>−/−</sup> mice did not show differences in NPRC or NPRA abundance in the livers compared with Nprc<sup>fl/fl</sup> mice (fig. S2, E to G), we favor the interpretation that the absence of steatosis was the primary reason for reduced fibrosis. Moreover, whether NRG4 is responsible for the reduced hepatic steatosis and fibrosis of Nprc<sup>−/−</sup> mice is still to be explored.

Here, we found no indications of “beiging” in the WAT of either Nprc<sup>−/−</sup> or Nprc<sup>−/−</sup> mice when fed an HFD. This finding may be due to additional unidentified factors induced by HFD feeding that could weaken the adipose browning program driven by NP. In addition to increased thermogenesis in the BAT, the gWAT mass of HFD-fed Nprc<sup>−/−</sup> mice unexpectedly increased, as did de novo lipogenesis and adipogenesis. PKG promotes brown adipocyte differentiation and mitochondrial biogenesis (50). Whether the cGMP pathway is involved in adipogenesis in WAT depots deserves further exploration (51). Moreover, the gWAT of HFD-fed Nprc<sup>−/−</sup> mice was metabolically healthier. This finding is reminiscent of a previous study showing that the capacity to expand adipose tissue can be associated with greater insulin sensitivity (52).

Fig. 7. Insulin signaling, de novo lipogenesis, adipogenesis, lipolysis, and PKG activity in adipose tissues of HFD-fed Nprc<sup>−/−</sup> mice. (A to C) Western blotting analysis for proteins involved in insulin signaling (p-Ser<sup>⁷³⁷</sup> AKT, p-Thr<sup>³⁰⁸</sup> AKT, and GLUT4), de novo lipogenesis (ACC and FASN), adipogenesis (PPARγ, C/EBPα, and ADIPOQ), PKG activity (p-Ser<sup>²³⁹</sup> VASP), and lipolysis (p-Ser<sup>⁵⁶³</sup> HSL and ATGL) performed on lysates from gWAT (A), iWAT (B), and BAT (C) of Nprc<sup>−/−</sup> and Nprc<sup>fl/fl</sup> mice after 12 weeks on HFD. p, phosphorylated. Blots are representative of three independent experiments.
Adipose tissue inflammation links obesity to insulin resistance (53). Along with improved insulin sensitivity, we found that in Nprc<sup>AKO</sup> mice, the gWAT did not contain macrophage infiltration, as seen in the gWAT of Nprc<sup>AKO</sup> mice. It is not clear whether the loss of NPRC from adipocytes is responsible for the reduced inflammation, which, in turn, improved insulin sensitivity or vice versa. PKG activity was increased, potentially through NP/NPRA signaling, in the visceral fat of Nprc<sup>AKO</sup> mice, and adiponectin in both plasma and gWAT increased accordingly. Although the effects on adipogenesis, lipogenesis, and inflammation together might be responsible for the improved insulin sensitivity in Nprc<sup>AKO</sup> mice, the mechanistic connection between the enhanced NP signaling and improved insulin sensitivity is yet to be investigated.

In conclusion, our results highlight a key role of cardiac NPs and their signaling in adipose tissue to regulate systemic energy expenditure and glucose homeostasis. Adipose-specific deletion of Nprc protects against diet-induced obesity and insulin resistance by activating thermogenesis and glucose uptake in the BAT, increasing lipolysis in subcutaneous iWAT, and alleviating inflammation while promoting tissue expansion in visceral gWAT. These various mechanisms of metabolic adaptation suggest the versatile functions of NP signaling in the control of energy metabolism in different adipose depots. These studies demonstrate that attenuation of NPRC activity in adipose tissue has the potential to be a therapeutic mechanism for controlling metabolic disease.

**MATERIALS AND METHODS**

**Reagents and antibodies**

The protease inhibitor cocktail (cOmplete Mini) and a phosphatase inhibitor cocktail (PhosSTOP) were obtained from Roche Diagnostics. The following antibodies were obtained from Cell Signaling Technology: Cox4 (4844), PPARγ (2345), C/EBPα (8178), p-Ser<sup>239</sup> VASP (3114), total VASP (3112), p-Ser<sup>147</sup> AKT (4060), p-Thr<sup>382</sup> AKT (9275), total AKT (9272), p-Ser<sup>63</sup> HSL (4139), total HSL (4107), ATGL (2439), ADIPOQ (2789), GLUT4 (2213), ACC (3676), FASN (3180), and β-actin (4967). Other antibodies used include NPRC (NBPI-31365, Novus Biologicals), NPRA (NBPI-31333, Novus Biologicals), NDUFS4 (PA5-21677, Pierce Biotechnology), UCP1 (ab23841, Abcam), and GAPDH (sc25788, Santa Cruz Biotechnology) and CYTOC (sc13156, Santa Cruz Biotechnology). Secondary antibodies against rabbit immunoglobulin G (IgG) (A3687) and mouse IgG (A3562), both conjugated with alkaline phosphatase, were from Sigma-Aldrich.

**Animal experiments**

Npr<sup>−/−</sup> mice (backcrossed to C57BL/6J for at least eight times) were a gift from N. Maeda and O. Smithies (Department of Pathology and Laboratory Medicine, University of North Carolina at Chapel Hill, Chapel Hill, NC, with the Mutant Mouse Regional Resource Center). Mice with a floxed Nprc allele were generated for this study by the Knockout Mouse Project at the University of California, Davis. Myogenin-Cre mice were a gift from E. Olson of University of Texas (UT) Southwestern Medical Center and bred to generate mice with skeletal muscle-specific disruption of the Nprc gene. Adiponectin-Cre mice (JAX-010803) were purchased from Jackson Laboratory and bred to generate mice with adipose-specific disruption of the Nprc gene. Tail DNA genotyping and qRT-PCR of lysate from different tissues were used to confirm the specific deletion of Nprc in skeletal muscles and adipose tissues. Mice were kept under a 12-hour light/12-hour dark cycle at constant temperature (23°C) with unlimited access to food and water. A 60% HFD (D12492, Research Diets) was provided starting at 7 weeks of age and maintained for 12 weeks, during which time body weight and body composition were monitored. After the 12-week feeding period, mice were euthanized, and tissues were carefully dissected, weighed, and either immediately frozen in liquid nitrogen or processed for histology. All animal studies were approved by the Institutional Animal Care and Use Committee of Sanford Burnham Prebys Medical Discovery Institute in accordance with the eighth edition of the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Primary adipocyte cell culture, oil red O staining, and glycerol release**

Primary adipocytes were prepared from iWAT and differentiated, as previously described (54). For oil red O staining, differentiated adipocytes were fixed with 3.7% formaldehyde for 10 min, stained with 0.5% oil red O for 1 hour at room temperature, and then washed with water. Glycerol release was evaluated with the free glycerol reagent from Sigma-Aldrich (F6428) according to the manufacturer’s instructions.

**Indirect calorimetry**

After 5 weeks (Nprc<sup>AKO</sup>) or 6 weeks (Nprc<sup>MKO</sup>) on HFD, mice were housed and monitored separately in open-circuit Oxymax chambers (Columbus Instruments) with free access to HFD and drinking water for 72 hours. Oxygen consumption, carbon dioxide production, and physical activity were monitored at 15- to 20-min intervals. Food and water intake was also recorded. Data from the first 24 hours were discarded because the mice adapted to the new housing.

**GTT and ITT and hyperinsulinemic-euglycemic clamps**

During the last week of HFD, mice were fasted for 5 hours, glucose (1 g/kg) was injected intraperitoneally, and blood glucose concentrations were measured through tail vein at 0, 15, 30, 45, 60, 90, and 120 min with Bayer CONTOUR blood glucose monitoring system. For the ITT, insulin (0.8 U/kg) was administered intraperitoneally, and blood glucose concentrations were measured at 0, 15, 30, 60, and 90 min. The AUC was calculated from the baseline and divided by the period of time. Hyperinsulinemic-euglycemic clamps were performed as previously described (55).

**Plasma insulin, adiponectin, and measurement of ANP and BNP**

Blood samples were collected by cardiac puncture after a 5-hour fast using heparin anticoagulation tube. Enzyme-linked immunosorbent assay kit was used to detect plasma concentrations of insulin (Merodia), adiponectin (AdipoGen), and ANP and BNP (Phoenix Pharmaceuticals) according to the respective manufacturer’s instructions.

**Tissue histology and microscopy**

Adipose tissues and liver were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS), dehydrated, embedded in paraffin, and cut into 5-μm sections. Sections were stained with H&E and examined under bright-field microscopy with a Nikon 80i. Adipocyte sizes were quantified using ImageJ software with the “Adipocyte” application tool. The UCP1 and F4/80 staining was performed on the Leica BOND RX automated system. Sections were deparaffinized, rehydrated, heated for antigen retrieval, and then incubated with UCP1 antibody (1:400 dilution; ab10983, Abcam) at room temperature for 2 hours and F4/80 antibody (1:50 dilution; HM1066, Hycult Biotech) for 1 hour. After washing in the Leica BOND wash buffer, slides were incubated with Rat Probe, followed by incubation with Rat-on-Mouse...
RNA was extracted from tissues with TRIzol reagent (Invitrogen) and purified by RNA Mini columns (Qiagen). Reverse transcription (Applied Biology) and SYBR green qRT-PCR (Life Technologies) were performed according to the manufacturer’s protocols. Target primer sequences are presented in table S1. qRT-PCR results were analyzed by ΔΔCt method, normalized to the internal control gene 36B4, and expressed as mean ± SEM.

Protein extraction and Western blotting

Protein was extracted from tissues, as previously described (56). For Western blotting analysis, 10 μg of protein, unless otherwise indicated, was resolved by 10% SDS–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Bio-Rad), and incubated overnight at 4°C with specific primary antibodies. Secondary antibodies conjugated with alkaline phosphatase were used for specific protein detection.

Mitochondrial DNA isolation and quantitative PCR

Mitochondrial DNA (mtDNA) was extracted from the skeletal muscle, as previously described (57). mtDNA and nuclear DNA were determined by evaluating the abundance of the DNA encoding NADH dehydrogenase subunit 1 and lipoprotein lipase by ΔΔCt methods, as previously described (57).

cGMP dose-response assay

HEK293 cells that were stably transfected with the green fluorescent protein–tagged NPRI plasmid (also known as HEK293-GCA cells) were a gift from J. Burnett (36). HEK293-GCA cells were transfected with NPrC–YPF (yellow fluorescent protein) or YFP plasmids by polyethylenimine when reaching 80 to 90% confluency and replated overnight with specific primary antibodies. Secondary antibodies conjugated with alkaline phosphatase were used for specific protein detection.

In vivo pressure hemodynamics

In vivo pressure hemodynamics was performed as described (58). Briefly, male mice (3 to 4 months old) were injected with etomidate (10 mg/kg intraperitoneally) and then placed in an induction chamber filled with 3.5% isoflurane mixed with 100% oxygen. Sedated mice were then intubated with a 20-gauge catheter sleeve, placed atop a heated surgical board, and connected to a small animal ventilator (model SAR-830/P; CWE Inc.). Respiration was set to 14- to 18-cm H2O at a rate between 140 and 160 breaths per minute, and isoflurane levels were adjusted using a vaporizer to attain a surgical plane of anesthesia (2 to 3%). A small area of skin in the neck region was removed, the sublingual salivary glands were gently separated, and a section (15 to 20 mm) of the right common carotid was isolated caudally from the bifurcation of the internal and external carotid. To aid in vessel cutoff and pressure catheter insertion, a 4-0 suture was tied at the bifurcation, a small vascular clamp was placed as far caudally as possible, and a second 4-0 suture was placed between the two. The vessel was then cut down, and a 1.4-F pressure catheter was inserted (model SPR-671, Millar Instruments) and advanced to near the aortic valve. Pressure traces were recorded using LabChart 8.0 (ADInstruments) software, and isoflurane was reduced to 1.5%. A rectal probe was then inserted to monitor core body temperature, which was held at 37 ± 1°C. After 10 min, which allowed the animal ample time to recuperate from surgery, consecutive pressure tracings from at least 30 to 60 s worth of stable recordings were analyzed using the built-in tools of the LabChart software.

Statistical analysis

All data are means ± SEM. Unpaired two-tailed Student’s t tests or two-way analysis of variance (ANOVA) followed by post hoc tests with Bonferroni’s correction for multiple comparisons tests was used to examine differences between groups, as appropriate. Glucose infusion rate was analyzed by one-tailed Student’s t test. Statistical significance was defined as P < 0.05.

SUPPLEMENTARY MATERIALS

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Fig. S1. Blood pressures and circulating NP concentrations of Nprc−/−, NprcΔNmo, and NprcΔmo mice.
Fig. S2. Expression of Nprc and Npra in skeletal muscle and adipose tissue.
Fig. S3. Alternative representation of CLAMS indirect calorimetry data of HFD-fed NprcΔNmo and NprcΔmo mice.
Fig. S4. HFD-fed NprcΔNmo and NprcΔmo mice show comparable expression of thermogenic-related genes in gWAT and iWAT.
Fig. S5. Adipocyte size distribution in iWAT and gWAT of HFD-fed NprcΔNmo and NprcΔmo mice.
Fig. S6. NPRC deficiency enhances NP signaling.

Table S1. PCR primer sequences.

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