Nuclear Receptor Coactivator PNRC2 Regulates Energy Expenditure and Adiposity*

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PNRC2 was identified in our laboratory as a general cofactor for nuclear receptors. To better characterize the physiological function of PNRC2, we used gene-targeting technology to generate PNRC2-null mice (PNRC2−/− mice). These PNRC2−/− mice are viable and fertile. PNRC2-null mice, especially male mice, are lean and are resistant to high fat diet-induced obesity but without the induction of insulin resistance. Male mice devoid of PNRC2 protein have a higher metabolic rate than wild-type mice. They consume more oxygen and produce more heat. Consistent with reduced adipose mass, the levels of leptin are lower in PNRC2−/− mice. This study provides evidence that PNRC2 plays one or more important roles in controlling the energy balance between energy storage and energy expenditure. PNRC2 may be a new target in the treatment of obesity and related metabolic diseases.

Energy homeostasis is accomplished by mechanisms that control food intake, energy expenditure, and energy storage. Energy imbalance over time results in obesity. Identification of the regulatory pathways that govern the balance between energy intake, energy storage, and energy expenditure is the key to understanding diseases such as obesity and its related disorders, including insulin resistance, type II diabetes, hyperlipidemia, and atherosclerosis. The tight matching of energy intake and energy expenditure is governed by the central nervous system, primarily in the hypothalamus (1, 2). Leptin and insulin are primary effectors that activate receptors located on secondary effector neurons in the hypothalamus. The hypothalamic centers also monitor energy storage through the action of leptin and insulin (3). The subsequent manipulation of energy storage is governed by a wide variety of enzymes, transcription factors, cytokines, and receptors. Among them, members of the nuclear receptor superfamily and their coactivators and corepressors have been shown to play critical roles in regulating metabolic processes and energy homeostasis by responding to an array of biological active substances, including hormones, vitamins, and drugs (4–6). For example, the peroxisome proliferator-activated receptors can promote lipid synthesis and storage in adipocytes (PPARγ) (7–9) or activate oxidation pathways in liver (PPARα) (10) and muscle and brown adipocytes (PPARβ) (11, 12). The liver X receptors α and β can regulate genes that decrease total body cholesterol (13, 14). ERRα activates genes involved in multiple key energy production pathways, including cellular fatty acid uptake, fatty acid oxidation, and mitochondrial electron transport/oxidation phosphorylation (15–17), whereas another orphan receptor, small heterodimer partner, functions as a negative regulator of energy production in brown adipose tissue (BAT) by functioning as a negative regulator of PGC-1α expression (18). Likewise, the coactivators or corepressors of these nuclear receptors also play essential roles in regulating energy metabolism (19–21). For example, the PPARγ coactivators, PGC-1α and PGC-1β, play a critical role in the maintenance of glucose, lipid, and energy homeostasis and in the regulation of mitochondrial oxidative metabolism (22). Several members in the p160 coregulator family also have specific functions in energy metabolism. Mice deficient in SRC2/TIF2/GRIP1 have higher lipolysis in white fat, have higher energy expenditure in brown fat, and are resistant to obesity; whereas SRC-1−/− mice are prone to obesity due to reduced energy expenditure (20). Fibroblasts isolated from the TIF2−/− mice do not differentiate into adipocytes as effectively as WT fibroblasts, suggesting that TIF2 is required for adipogenesis (20). The nuclear receptor corepressor RIP140 has been reported to regulate the balance between energy storage and energy expenditure. Mice devoid of RIP140 protein are lean and are resistant to high fat diet-induced obesity (19).

Our studies to elucidate the regulatory mechanisms of human aromatase expression in breast cancer have identified and characterized a new family of coactivator proteins, proline-rich nuclear receptor coregulatory protein (PNRC) (23) and PNRC2 (24). PNRC and PNRC2 were identified as steroidogenic factor 1-interacting proteins in a yeast two-hybrid screening of a human mammary gland cDNA expression library.

The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; PGC, PPARγ coactivator; T, tesla; BAT, brown adipose tissue; WAT, white adipose tissue; WT, wild type; PNRC, proline-rich nuclear receptor coregulatory protein; ES, embryonic stem cell; UCP1, uncoupling protein 1; FFA, free fatty acid.

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These proteins were found to interact with the ligand-binding domains of all the nuclear receptors tested, including ERα, PR, GR, TR, RXR, and RXR, in a ligand-dependent manner. They were also found to interact in a ligand-independent manner with orphan receptors such as steroidogenic factor 1 and ERR. These two coactivators are unique in that they are significantly smaller than most of the coregulatory proteins previously identified and are proline-rich. Unlike most of the coactivators that interact with nuclear receptors through their LXXLL motif, these new coactivators interact with nuclear receptors through a proline-rich Src homology domain-3-binding motif, SD(E)PPSPS (23, 24).

PNRC2 was also isolated from a brain cDNA phage display library by its interaction with the AF1 domain of ERRγ (25). Recently, PNRC2 and PNRC2 were also repeatedly isolated as multiple nuclear receptor interacting proteins in a large interaction-screening project using an automated version of the yeast two-hybrid system (26). In their screening, PNRC2 was isolated as a prey with the ligand-binding domains of 10 different nuclear receptor baits, including ERα, ERβ, ERRγ, HNF4α, HNF4γ, LRH1, PXR, RARγ, RORα, and RORβ. In the same study, PNRC was isolated as the interacting partner of RARβ, RARγ, RORα, RORβ, HNF4α, HNF4γ, ERβ, ERRγ, and LRH1. Thus, PNRC2 and PNRC appear to be more general cofactors for nuclear receptors than previously thought.

In this study, we have generated and characterized PNRC2-deficient mice (PNRC2−/− mice) to better understand the physiological function of PNRC2. PNRC2-null mice, especially male mice, are lean with reduced fat mass and are resistant to high fat diet-induced obesity. The results of the present study indicate that the lean phenotype of PNRC2−/− mice is largely a consequence of the increased energy expenditure. This study provides evidence that PNRC2 plays important roles in controlling the energy balance between energy storage and energy expenditure. The PNRC2−/− mouse is an important model for the study of metabolic regulatory roles of this coactivator protein.

EXPERIMENTAL PROCEDURES

Animals—Breeding stock strains 129S1 and C57Bl/6J were obtained from Jackson Labs (Bar Harbor, ME). PNRC2-KO mice, as B6 and 129SvJ hybrids, and all other mice were bred at the City of Hope Animal Resources Center under Association for Assessment and Accreditation of Laboratory Animal Care-approved conditions. All mice were housed at the City of Hope Animal Resources Center in ventilated cage racks (Allentown Caging Equipment, Allentown, NJ), had free access to water, were exposed to a 12- h light/dark cycle, and received standard rodent chow diet (chow 5001, Purina Mills, Richmond, IN), except for high fat diet experiments where mice were fed a 45% (wt/wt) fat diet (TestDiet). All institutional guidelines for animal care and use were followed.

Generation of PNRC2-null Mice—The targeting vector for PNRC2 gene knockout, pTg3-PNRC2, was constructed by inserting fragments of the murine PNRC2 gene, amplified by PCR from 129si genomic DNA by PCR, into the targeting vector pKSLoxpNT (27) (hereafter, we have named it pTg3 for simplification). This targeting vector contained the phosphoglycerate kinase promoter/neomycin gene (PGK-Neo), as well as the herpes simplex thymidine kinase (hsv-tk) gene to allow the use of a positive-negative selection scheme. The neo gene in pTg3-PNRC2 was flanked by a 2.24-kb fragment (that served as the 5′-targeting arm), a 5′-flanking region ending at 67 bp upstream of exon I, and a 4.86-kb fragment (served as the 3′-targeting arm), starting from 200 bp downstream of stop codon in exon III of the mouse PNRC2 gene. The 2.24-kb 5′-arm was inserted into a BamH1 site between the tk and neo expression cassettes of pTg3 vector by blunt end ligation, and the 4.86-kb 3′-arm was subcloned into ClaI and SalI sites localized 3′ to PGK-Neo. The final targeting construct is illustrated in Fig. 1A. Homologous recombination in mouse embryonic stem cells by this targeting vector should result in the deletion of exon1, intron1, exon II, intron II, and part of exon III that contains the entire coding sequence, as shown in Fig. 1A. The SalI-linearized targeting vector pTg3-PNRC2 (100 μg) was electroporated into embryonic stem (ES) cells derived from the 129S1 mouse strain. The transfected ES cells were selected in the medium containing 300 μg/ml G418 for 2 weeks and then further selected in the medium containing both G418 and 2 μM ganciclovir. A total of 168 surviving colonies was screened for homologous recombination event by Southern blotting on the HindIII-digested genomic DNAs isolated from the selected positive clones. Two out of four correctly targeted clones, ES14 and ES95, were injected into C57BL/6 blastocysts, and the resulting chimeric males were then bred to C57BL/6 females to obtain germ line transmission of the mutation and to produce F1 heterozygous (PNRC2+/−) mice, which were then interbred to generate mice homozygous for the mutation, i.e. PNRC2-null (PNRC2−/−) mice.

Genotyping of ES Cells, Mice, and Embryos by Southern Blot and PCR Analysis—The genomic DNA was isolated from ES cells, tail or embryo samples, after proteinase K digestion, by means of isopropanol precipitation (28). Briefly, tissues or cells were lysed in 0.5 ml of lysis buffer (100 mM Tris-HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 μg proteinase K/ml) and incubated at 37 °C (cells) or 55 °C (tissues) with agitation for several hours. One volume of isopropanol was added to the lysate, and the samples were mixed or swirled until precipitation was completed. Precipitated DNA was recovered and resuspended in 100 μl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5.

Extracted DNA was used as the template for PCR-based genotyping. Primers for amplification of the WT and targeted alleles were designed based on sequences external to the deleted region (within 5′-arm) (which exist in both WT allele and mutant allele) and within the deleted exon I or within the selection cassette (neo gene), respectively (see Fig. 1O). The primer pair, A (5′-GCAGGATTTCACCAATCAGGAC3′), from the end of 5′arm in the 5′-flanking region, and B (5′-GCTGAAGACCCCTCGACTTGTAAAA3′), from exon I, was used to detect the wild-type allele (Fig. 1C), and primer pair, A (same as above) and C (5′-GGTGGATGTTGAATAGTGTGGC-GAGG-3′), from the neomycin cassette, was used to detect the targeted allele (Fig. 1C). DNA in PCR-genotyping reactions was amplified in 40 cycles with melting, annealing, and extension temperatures of 94 °C, 59 °C, and 72 °C, respectively. The size of
the PCR product for wild-type allele should be 512 bp, whereas the PCR product for targeted allele should be 392 bp in length.

For Southern blot analysis, genomic DNA (5–10 μg) isolated from ES cells, mice tissues, or embryos, was digested overnight at 37 °C with restriction enzymes HindIII for 5′ probe and Xba1 for 3′ probe, separated on a 0.8% agarose gel (1 × TBE (89 mM Trisborate, 2 mM EDTA, pH 8.3), 25 V overnight), and transferred to a Hybond-N+ nylon membrane (Amer sham Biosciences) by capillary blotting with 0.4 M NaOH overnight. The membrane was baked for 1 h at 80 °C in a vacuum oven and then hybridized with 5′ probe (900 bp) or 3′ probe (420 bp), which is within 5′-homologous arm, or is outside of 3′-homologous arm region, respectively. The probes were labeled with [32P]dCTP through random priming using the Megaprime DNA Labeling System (Amer sham Biosciences). The labeled probes were purified using probe-Quant G-50 Micro Column (Amer sham Biosciences). Prehybridization, hybridization, and washing were carried out according to the protocols described in the “Gene Images random primer labeling module” from Amer sham Biosciences. Briefly, the dried membrane was placed in a glass hybridization vial, pre-wet with 5× SSC and prehybridized at 60 °C for 1 h in the prehybridization buffer (5× SSC, 0.1% (w/v) SDS, 5% (w/v) dextran sulfate, 1:20 diluted liquid block (Amer sham Biosciences), and 100 μg/ml salmon sperm DNA (denatured)). At the end of prehybridization, the denatured probe was added to the same prehybridization buffer at 2.5 × 107 cpm/ml and continued to incubate at 60 °C overnight. The membrane was washed once first with 1× SSC/0.1% SDS and then with 0.5× SSC/0.1% SDS at 60 °C, each for 15 min. If necessary, the membrane was washed in 0.1× SSC/0.1% SDS at 60 °C for additional 5–10 min.

**Northern Blot Analysis**—To confirm the absence of PNRC2 expression in homozygous mice, Northern blot analysis was performed as previously described (32) on the total RNA isolated from WT, PNRC2+/- and PNRC2−/- mice tissues, using the ExpressHyb solution (Clontech Laboratories, Mountain View, CA). A 900-bp 3′ end-fragment of mouse PNRC2 cDNA was generated by PCR, using the following forward and reverse primers, respectively: 5′-GTACAGGTTAAAGTTATAGTG-3′ and 5′-TGAGAACTAATTCTGTT-3′. The PCR product was radioactively labeled with [32P]dCTP through random priming using the Megaprime DNA Labeling System (Amer sham Biosciences). Prehybridization, hybridization, and washing were carried out according to the protocols described in the “Gene Images random primer labeling module” from Amer sham Biosciences. Briefly, the dried membrane was placed in a glass hybridization vial, pre-wet with 5× SSC and prehybridized at 60 °C for 1 h in the prehybridization buffer (5× SSC, 0.1% (w/v) SDS, 5% (w/v) dextran sulfate, 1:20 diluted liquid block (Amer sham Biosciences), and 100 μg/ml salmon sperm DNA (denatured)). At the end of prehybridization, the denatured probe was added to the same prehybridization buffer at 2.5 × 107 cpm/ml and continued to incubate at 60 °C overnight. The membrane was washed once first with 1× SSC/0.1% SDS and then with 0.5× SSC/0.1% SDS at 60 °C, each for 15 min. If necessary, the membrane was washed in 0.1× SSC/0.1% SDS at 60 °C for additional 5–10 min.

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**Quantitative NMR**—To determine the body composition of mice, quantitative NMR analysis was performed on PNRC2-null mice and their WT littermates. This was done in the Small Animal Imaging Center at the Children’s Hospital of University of Southern California, according to the procedures described previously (29). Briefly, mice were anesthetized with ketamine/xylazine (100 mg/kg/10 mg/kg) and placed in a tubular imaging restraint device designed to hold the mouse’s torso in a comfortable position. Either hot air or a warm water jacket was used to maintain the animal’s body temperature. Ophthalmic ointment was placed on the surface of the eyes to prevent corneal drying. The animal’s heart and respiratory rate were monitored throughout the imaging period through use of noninvasive monitoring equipment. All images were obtained using a 500-MHz AMX MR (11.7 T) system (Bruker Instruments, Inc., Billerica, MA) equipped with microimaging gradients (1 T/m maximum gradient strength, 2 Kt/m/s maximum slew rate). Metamorph software, Version 6.0 (Universal Imaging Corp., West Chester, PA), was used for image processing and analysis.

**Determination of Body Weight, Body Length, Food Intake, Body Mass Index, and Fecal Lipid Content**—When the mice were 8 weeks of age, the age-matched mice were divided into two groups under each diet condition (chow or high fat diet), PNRC2-null and WT littermates, at 12 mice/per group and 4 mice/per cage. Body weight was recorded once a week at the same time of day. For the food intake experiment, each mouse was housed in a separated metabolic cage. Food intake was determined by weighing the food given and the food remaining in the grid of the cages during a period of 4 days. The amount of food consumed per mouse per day was calculated by dividing the food consumed by the number of days in the feeding interval. To measure lipid absorption, feces of mice at 12 months of age were collected from PNRC2-null mice and WT mice, and the fecal lipid content in these feces was measured and compared. The fecal lipid content was measured gravimetrically following a Folch extraction, as described (30, 31). At 12 months of age mice were sacrificed under CO2 euthanasia. Immediately, total body length (from the tip of the nose to the anus) was measured.
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Measurement of Energy Expenditure by Indirect Calorimetry—Metabolic rates were measured by indirect calorimetry in 12-month-old PNRC2-null and WT mice by using an 8-chamber open-circuit Oxymax system (CLAMS, Columbus Instruments, Columbus, OH) at Mouse Metabolic Phenotyping Center in the University of Cincinnati. Briefly, mice were individually housed in acrylic calorimeter chambers through which air of known O₂ concentration was passed at a constant flow rate. The system automatically withdrew gas samples from each chamber hourly for 24 h. The system then calculated the volume of O₂ consumed (vO₂) and CO₂ generated (vCO₂) by each mouse in 1 h. The RQ, ratio of the vCO₂ to vO₂, was calculated. Energy expenditure measurements were carried out in both light and dark cycles at both fed and fasting conditions. Mice were maintained at 25 °C and had free access to water in all conditions.

Rectal Body Temperature Measurement before and after Fasting—To measure the adaptive thermogenesis in PNRC2-null and WT mice, 6-week-old WT and PNRC2<sup>−/−</sup> mice were placed in individual cages with normal chow diet. Initial body temperature was recorded by inserting a small thermoprobe into the rectum of the mouse. Mice were then fasted (without food but with free access to water) overnight, and rectal body temperature was recorded again after fasting for 24 h. The body weights of the mice were also recorded before and after overnight fasting.

Morphological Analysis of WAT, BAT, and Liver from WT and PNRC2-null Mice Fed Chow Diet or a High Fat Diet—To determine how the PNRC2-null mice respond to the effects of being fed a high fat diet, morphology of inguinal WAT, BAT, and liver from WT and PNRC2-null mice fed chow diet or a high fat diet was examined. At the end of the experiment for body weight evaluation, food intake, and fecal lipid content measurement, several of the mice (WT and PNRC2-null mice) were sacrificed. WAT,
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BAT, and liver tissues were collected from these mice, fixed in neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm onto poly-L-lysine-coat slides. Sections were stained with hematoxylin/eosin. Paraffin section preparation and staining were carried out by the Anatomic Pathology Laboratory at City of Hope. Frozen sections (10 μm) of formalin-fixed liver were mounted on poly-L-lysine-coated slides and stained with Oil red O (0.15% in 60% isopropanol) for 5 min. Sections were counterstained with hematoxylin and mounted in glycerol gelatin (Sigma) to examine lipid accumulation.

Plasma Analyses, Glucose, and Insulin Tolerance Tests—To further investigate the biological basis of lean phenotype of PNRC2-null mice, a series of physiological tests were performed on the PNRC2-null mice and WT control mice. Different quantitative assays were performed, including total cholesterol (Roche Diagnostics, Alameda, CA), plasma triglycerides (Thermo Trace), free fatty acids (Roche Applied Science), insulin (Crystal Chem Inc., Downers Grove, IL), leptin (Crystal-Chem), and total thyroid-stimulating hormone (DPC Co., Los Angeles, CA). For glucose tolerance test, mice were fasted overnight before receiving an intraperitoneal injection of 20% d-glucose (1 g/kg body weight, Sigma-Aldrich). At 0, 15, 30, 45, 60, and 120 min after injection, 5–10 μl of blood was drawn from the tail, and blood glucose levels were assayed using an Elite XL Glucosemeter (Bayer HealthCare LLC, Tarrytown, NY). For insulin tolerance test, WT and PNRC2-null mice were fasted for 5 h and given 1.7 units/kg insulin (Humulin R, Lilly, Toronto, Ontario, Canada) by intraperitoneal injection. Blood glucose was monitored for 4 h at 15-min intervals after the insulin challenge.

Measurement of Free Glycerol in WAT— Epididymal fat tissue was dissected from male PNRC2+/− and WT littermates and incubated in the KRH buffer (118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, and 10 mM HEPES, pH 7.4) plus 2.5% bovine serum albumin at 37°C, 5% CO2 incubator for 2 h with gentle shaking. Released glycerol was assayed using an enzymatic glycerol assay kit (Sigma) according to the manufacturer’s instruction.

RESULTS

Generation of PNRC2-null Mice—One allele of the PNRC2 gene was disrupted in 129s1 ES cells, using the targeting vector pTG3-PNRC2 shown in Fig. 1A. The mouse PNRC2 gene is organized in three exons, with the entire coding region in exon 3. The neomycin resistance gene (neo) in this vector was designed to replace the almost complete PNRC2 gene, beginning from 67-bp upstream exon 1 and extending into exon 3, right after the stop codon. Details of the construction of the pTG3-PNRC2 vector are described under “Experimental Procedures.” The pTG3-PNRC2 targeting vector was introduced into 129 S1 ES cells by electroporation and, following selection in G418 and ganciclovir, cell clones were screened by Southern blotting analysis of their DNA.

DNA was isolated from 168 G418/ganciclovir-resistant ES clones, and homologous recombinants were identified by Southern analysis of HindIII-digested DNA, using a probe derived from 5′-flanking region of the PNRC2 gene (and present in the 5′-arm of the targeting vector). The probe hybridized to a HindIII fragment of 3380 bp from the WT PNRC2 allele, whereas the mutant allele, created by homologous recombination between pTG3-PNRC2 targeting vector and the endogenous PNRC2 gene, yielded a 2487-bp fragment (Fig. 1B). Of 168 G418/ganciclovir-resistant ES clones screened, four were found to be heterozygous for the PNRC2 mutation. The structure of the mutant allele in these putative heterozygous clones was further confirmed using a 3′ probe that hybridized to an XbaI fragment of 7133 bp from the wild-type PNRC2 allele, whereas the mutant allele yielded a 6600-bp fragment (Fig. 1B). The calculated herpes simplex virus thymidine kinase counter-selection was 6-fold. Thus, the frequency of homologous recombination at the PNRC2 genomic locus, after correction for the thymidine kinase-mediated enrichment, was 1 in 200 of G418-resistant clones.

Two of four cell lines carrying a targeted PNRC2 gene were injected into WT C57BL/6 blastocytes and transferred into the uteri of pseudo pregnant recipients. Chimeric male mice generated from injections with these two ES clones transmitted the ES cell genome through the germ line, as indicated by the agouti coat color of the offspring after breeding with C57BL/6 females. About 50% of these agouti pups were heterozygous for the mutant PNRC2 allele when examined by Southern blot (data not shown). These mice were indistinguishable from their WT littermates and displayed no discernable abnormalities. Male and female heterozygous mice were then intercrossed to obtain homozygous PNRC2-null mice and to determine the viability of homozygous mutant animals. We separately interbred heterozygous mutant mice derived from two independent cell lines (E14 and E95) and observed no differences between them, and therefore the results were pooled. PNRC2−/− mice are viable and fertile. The predicted 1:2:1 Mendelian distribution of WT (PNRC2+/+), heterozygous (PNRC2+/−), and homozygous mutant (PNRC2−/−) mice was found in the offspring of heterozygous crosses. A representative PCR analysis of tail DNA from three letters of mice is shown in Fig. 1C.

To demonstrate that the homozygous recombination introduced in the PNRC2 gene resulted in abolishing the expression of the PNRC2 gene in WAT, we measured the amount of free glycerol in the epididymal fat tissue of the PNRC2−/− mice. The amount of free glycerol was found to be significantly lower in the PNRC2−/− mice compared to WT control mice (Fig. 1D). This result suggests that the PNRC2 gene plays a role in the regulation of energy expenditure and adiposity.
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A. WT PNRC2-/-

B. Body length (cm)

- Male
- Female

C. Male

WT, male, fat 49.2%

PNRC2-/-, male, fat 32.8%

Female

WT, female, fat 33.6%

PNRC2-/-, female, fat 23.9%

FIGURE 2. PNRC2 knockout mice have a lean phenotype. A, a photograph of representative WT and PNRC2-/- male mice on a normal chow diet at age of 12 months. B, body length (from nose to anus) at age of 12 month (n = 5 for each group of mice). C, MRI analysis of body fat content. All images were obtained using a 500-MHz AMX MR *11.7T System. Slice shown are immediately below the lower ends of the right kidney in PNRC2 KO and WT male mouse. Metamorph software was used for image processing and analysis. The percentages of fat area over the whole area were calculated. The representative images and the calculated fat content for WT and PNRC2-/- mice are shown. D, summary of combined MRI data of male and female mice (n = 8 for each group of male mice, n = 8 for each group of female mice, *p < 0.05). E, ratios of weights of WAT to whole body weights at age of 12 months (n = 6 for each group of male mice, and n = 8 for each group of female mice, *p < 0.05). F, decreased white adipocyte cell size in gonadal fat pad in PNRC2-/- mice, relative to wild-type mice on normal chow diet. Fat pads were collected from 12-month-old mice, fixed, and subjected to hematoxylin and eosin (HE) staining. A representative image is shown (10 × magnifications). Lower levels of blood leptin (G) and triglycerides (H), and higher levels of FFA (I) were found in PNRC2-/- male mice. Mice were 12 months old (n = 6 for each group of male mice and n = 8 for each group of female mice; *, p < 0.05 and **, p < 0.01). J, epididymal fat tissue was incubated in the KRH buffer plus 2.5% bovine serum albumin at 37°C 5% CO2 incubator for 2 h. Released glycerol was assayed using an enzymatic glycerol assay kit (Sigma) according to the manufacturer’s instruction and expressed as micromoles/g fat tissue. The data shown are the mean ± S.D. of five mice for each genotype.

PNRC2-null Mice Exhibit Lean Phenotype—On a regular chow diet, we observed a tendency for the PNRC2 KO mice to remain smaller than their WT littermates when we followed the body weights of the progressively older PNRC2-null mice (data not shown). At around 12 months of age, as shown in Fig. 2A, the phenotype of PNRC2-/- male mice was visually distinguishable from that of their WT littermates. By 12 months of age, PNRC2-/- male mice (n = 8 for each group) weighed 20% less than WT male mice (37.75 ± 5.68 versus 46.75 ± 7.09 g, respectively), and PNRC2-/- female mice showed the trend for lower weight than WT females (26.25 ± 3.2 versus 28.75 ± 3.3 g, respectively), but without reaching statistical significance for both male and female mice. No difference was detected in the body length between PNRC2-null and WT mice (Fig. 2B), suggesting that the lower body weight of PNRC2-null is not due to the general developmental delay or retardation.

To assess whether the reduced body weight was due to reduced adiposity in PNRC2-/- mice, body composition of PNRC2-/- mice and WT mice was determined at 12 months of age via MRI. Fig. 2C shows a representative abdominal cross-section of a 12-month-old male/female WT and a PNRC2-/- male/female mouse fed regular chow diet. Because lipids are rich in proton content and are present in high quantity in WAT, the majority of the signal observed in MRI is fat mass (indicated as white) and water. The fat content in PNRC2-/- mice
and WT mice was calculated as the ratio of fat area (white area) to the whole area of the MRI image. The areas were calculated using MetaMorph software, Version 6 (Universal Imaging Co., Downingtown, PA). As shown in Fig. 2D, the fat content (calculated based on MRI images) in PNRC2-null mice (male and female combined) was significantly less than that in WT mice (26.2 ± 8.89 versus 38.4 ± 8.24%, respectively; \( p < 0.05 \)). The difference in fat content between WT and knockout mice is in agreement with the difference in body weight, suggesting that the inability to gain weight is due to reduced adiposity in PNRC2-null mice.

To more accurately quantify the regional fat pad differences between PNRC2−/− and WT mice, we dissected and measured the weights of two major WAT fat pads (inguinal and gonadal) of 8 of the 12-month-old male (4 of PNRC2−/− and 4 of WT) mice and 8 female (4 of PNRC2−/− and 4 of WT) mice, and the ratios of fat weights to whole body weights were calculated (Fig. 2E). There was an average of 57% reduction of inguinal fat weight in male and 47% reduction in female PNRC2−/− mice, as compared with that in WT controls (male, 0.40 ± 0.14 versus 0.94 ± 0.3 g; female, 0.38 ± 0.13 versus 0.72 ± 0.33 g, respectively). Similar reduction in gonadal fat mass of PNRC2−/− mice was observed (male, 0.71 ± 0.09 versus 1.11 ± 0.58 g; female, 0.68 ± 0.28 versus 1.27 ± 0.94 g, respectively). The diameter of adipocytes in inguinal WAT of PNRC2−/− mice was much smaller than that of adipocytes in the WT control mice (Fig. 2F). Consistent with a lower amount of WAT, PNRC2−/− male mice had 60% less leptin than WT mice when fed normal chow diet (1.5 ± 0.59 versus 4.01 ± 0.24 ng/ml; \( p < 0.01 \)) (Fig. 2G). Taken together, the above data suggest that PNRC2 plays an important role in regulating adiposity.

**PNRC2 Is Highly Expressed in WAT of Mice**—Previously, PNRC2 transcripts had been detected by Northern analysis in a series of human cell lines (24), human tissues (25), and mouse tissues (32). However, its expression in fat tissue has not been reported. As shown above, homozygous PNRC2 knockout mice have a lean phenotype with much less fat mass. To investigate the biological basis for this phenotype, we further examined the expression of PNRC2 in adult mouse tissue, including the major metabolic tissues such as WAT, BAT, muscle and liver, by Northern analysis and quantitative real-time PCR. PNRC2 was found to express in WAT, BAT, muscle and liver, with strongly elevated expression in WAT compared with other metabolic tissues (Fig. 3), suggesting a role of this protein in adipose tissue biology.

**Male PNRC2-null Mice Are Resistant to High Fat Diet-induced Obesity**—As shown above, the PNRC2-deficient male mice are leaner than the WT mice on a normal chow diet, indi-
indicating a role of PNRC2 in lipid metabolism. To further assess this potential function of PNRC2, we challenged 6-week-old WT and PNRC2−/− male mice with a 45% (w/w) high fat diet for a total of 23 weeks. Weights of animals were monitored on a weekly basis. As shown in Fig. 4A, the weight gain in PNRC2−/− male mice was remarkably less than in WT littermates. The difference of weight gain between two groups of mice was observed as early as at the end of the first week of feeding. After 23 weeks of high fat diet feeding, WT mice showed 104.33 ± 11.56% weight gain, but the PNRC2−/− mice gained only by 34.64 ± 2.21%. No significant difference in weight gain between WT and PNRC2−/− female mice was observed (data not shown).

FIGURE 3. PNRC2 is highly expressed in WAT of mouse. Quantitative real-time reverse transcription-PCR analysis was performed to quantify PNRC2 expression in the metabolic tissues of adult WT mice. Data shown are mean ± S.D. of four mice in each group.

A.

B.

C.

D.

E.

FIGURE 4. Male PNRC2−/− mice are resistant to high fat diet induced obesity. A, body weight gain of male mice fed a high fat diet for 23 weeks. Eight-week-old male mice (n = 6 for each group) were fed a high fat diet (45% w/w) for 23 weeks. Body weights were recorded weekly. The increases in body weights were calculated based on the initial body weight at day 0 of high fat feeding and expressed as the percentage of increase. B, absolute fat mass of WAT was significantly reduced in 31-week-old male PNRC2−/− mice compared with WT mice at the end of high fat diet feeding. Data represent the mean ± S.D. (n = 6/ genotype). Histology of WAT (C), liver (D), and BAT (E) of mice fed high fat diet (*, p < 0.05 and **, p < 0.001).
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Similar to the normal chow diet feeding condition, the body weight difference between WT and PNRC2−/− male mice fed with a high fat diet was also largely due to a decrease in fat tissues of PNRC2−/− mice. The weights of gonadal, inguinal, and brown fat pads were 21%, 67, and 32% less in PNRC2−/− male mice than in WT mice, respectively (Fig. 4B). The size of adipocytes in the white fat tissue of WT mice fed the high fat diet was also characterized by the presence of numerous small lipid droplets, although the majority of the cells was still multi-polar (Fig. 4C). High fat diet also caused the accumulation of lipid droplets in the liver of WT mice, but not in the liver of PNRC2−/− mice (Fig. 4D). BAT cells from PNRC2−/− male mice were characterized by the presence of numerous small lipid droplets and were surrounded by many blood capillaries, which is typical of calorigenerically active BAT cells. Cells from WT mice fed a high fat diet contained much larger lipid droplets, although the majority of the cells was still multi-polar (Fig. 4E).

To examine whether the reduction in fat mass observed in PNRC2−/− mice was caused by decreased food intake or decreased fat absorption, both food intake and fat absorption were measured over a 4-day period. No significant difference was observed in the food consumption and fat absorption between PNRC2−/− and WT mice. Both male and female PNRC2−/− mice consumed similar amount of food (Fig. 5A) and absorbed fat as well as their wild-type littermates did (Fig. 5B).

**PNRC2−/− Mice Have Higher Energy Expenditure, Consume More Oxygen, and Produce More Heat**—The potential explanation for the lean phenotype of PNRC2−/− mice might be an increase in their metabolic rate. To test this hypothesis, we measured the VO₂ for 24 h by indirect calorimetry of 12-month-old mice on a standard diet or during fasting. Consistent with their reduction in body weight, male PNRC2−/− mice exhibited a significant increase in metabolic rate (VO₂) (Fig. 6A) and a significant increase in heat production (Fig. 6B) during the total 24-h period and during either the light or dark cycles, showing higher energy expenditure. No significant difference in metabolic rate and energy expenditure was observed between female PNRC2−/− mice and their WT littermates (Fig. 6B).

To further evaluate the adaptive thermoregulation of PNRC2−/− male mice in response to fasting, rectal body temperature of WT and PNRC2−/− mice was recorded before and after the 24-h fasting. Consistent with their high metabolic rate, male PNRC2−/− mice were found to be resistant to decrease of body temperature after fasting, compared with their WT littermates (Fig. 6C). Although the basal temperatures of the chow-fed WT and PNRC2−/− mice did not differ (37.58 ± 0.26 versus 37.25 ± 0.47 °C, respectively), the body temperatures of the fasted PNRC2−/− mice were significantly elevated relative to WT mice (37.00 ± 0.45 versus 35.40 ± 0.54 °C, respectively). This increased thermogenesis may explain the changes in energy balance observed in the PNRC2−/− male mice. The increased energy expenditure of PNRC2−/− mice was also reflected by the faster use of stored energy during fasting. In response to a 24-h fasting, PNRC2−/− male mice lost 11.36 ± 5.64% of their body weight, whereas WT mice lost only 4.38 ± 1.15% (Fig. 6D).

Results shown above clearly demonstrated higher energy expenditure in PNRC2−/− male mice than in WT mice. To investigate the possible mechanisms underlying the increased energy expenditure of PNRC2−/− mice, we compared the expression levels of uncoupling protein 1 (UCP1) in the metabolic tissues of PNRC2−/− and WT control mice. The expression level of UCP1 in BAT of PNRC2−/− male mice was found to be similar to that of WT male mice, whereas the levels of UCP1 in WAT, liver, and muscle of PNRC2−/− male mice were significantly higher than that of WT mice (Fig. 6E).

**DISCUSSION**

PNRC2 was previously identified as a nuclear receptor-interacting protein and was demonstrated to function as a coactivator for multiple nuclear receptors. The results presented in this study establish a new role for PNRC2 in energy homeostasis and adiposity. Previously, the PNRC2 gene was found to be widely expressed in human and mouse tissues. In the present study, PNRC2 was also found to be expressed in the metabolic tissues in mice, including WAT, BAT, liver, and muscle, with a high level in WAT, indicating a role of PNRC2 in adipose biology. To explore the physiological function of PNRC2 in vivo, we generated PNRC2-null mice through homologous recombination. The mice devoid of PNRC2 protein were viable and fertile, indicating that PNRC2 is not absolutely required in development, sexual maturation, and reproduction. Different from SRC-1-deficient mice that showed resistance to steroid hormone treatment (33), PNRC2−/− mice responded to hormones similar to WT mice (data not shown).

Rather surprisingly, PNRC2−/− mice, especially male mice,
FIGURE 6. Energy expenditure increases in male PNRC2\(^{-/-}\) mice. Oxygen consumption (vO\(_2\)) (A) and heat production (B) were analyzed by indirect calorimetry in WT and PNRC2\(^{-/-}\) mice under normal chow diet. Calorimetry was performed for 12 h during the light cycle (Light Cycle), 12 h during the dark cycle (Dark Cycle), and 24 h spanning both the light and dark cycles (Both Cycles), respectively. For A and B, *, p < 0.05, and the data points represent the mean ± S.D. of \(n = 4\)/genotype/gender mice. C, rectal body temperature changes of male mice in response to fasting (*, p < 0.05 of \(n = 6\)/genotype male mice). D, weight reduction of male PNRC2\(^{-/-}\) and WT mice after 24 h fasting (*, p < 0.05, \(n = 6\)/genotype). E, reverse transcription and quantitative real-time PCR analysis of UCP1 expression in metabolic tissues from wild-type and PNRC2\(^{-/-}\) mice (\(n = 3\)/genotype). BAT, brown fat tissue; WAT, white fat tissue. Data shown represent mean ± S.D. of three mice each group of genotype and triplicate reactions for each tissue sample.
were found to have lower body weight than their WT littermates under normal diet and were resistant to weight gain when challenged with a high fat diet for 23 weeks (Fig. 4A). The body weight differences were primarily due to reduced adiposity, as determined by quantification of the major fat pad weights (Fig. 2E) and by MRI (Fig. 2, C and D). Body mass and composition reflect the combined effects of three processes: energy intake, energy partitioning (storage), and energy expenditure. The energy required for maintaining body function, both at rest and during stress and exercise, is provided in the form of carbohydrates, fat, and protein nutrients that are consumed daily (34). Energy ingested in excess of immediate need is stored in adipose tissue (35). Energy expenditure consists of several components: obligatory basal activity (for performing cellular and organ functions), physical activity, and adaptive thermogenesis (heat production in response to environmental temperature or diet) (36). The lean phenotype of PNRC2~−/− mice indicates a divergence among energy intake, energy storage, and energy expenditure. PNRC2~−/− male mice were lean, but not due to diminished feeding behavior (Fig. 5A) or fat absorption (Fig. 5B). This indicates that less fat accumulation in PNRC2~−/− mice is, at least, partially due to the higher energy expenditure. This hypothesis was confirmed by measuring the metabolic rate of mice using indirect calorimetry. PNRC2~−/− male mice, but not female PNRC2~−/− mice, were found to consume more oxygen (Fig. 6A) and produce more heat (Fig. 6B) than their WT littermates.

Furthermore, these mice also have higher adaptive thermogenesis than the WT control. PNRC2~−/− male mice were resistant to decreased body temperature (Fig. 6C) and lost more body weight (Fig. 6D) in response to a 24-h fasting. Taken together, PNRC2~−/− mice have a higher energy expenditure that is probably an important cause for the lean phenotype of PNRC2~−/− mice. When energy expenditure is altered, there is either an “uncoupling” of one of the reactions of cellular metabolism or a change in the amount of biological work. For example, UCP1 has been shown to increase energy expenditure by uncoupling the relationship between protons entering the mitochondrial matrix and the synthesis of ATP (36). UCP1 is mainly expressed in BAT, a particular form of adipose tissue found in infants at birth, rodents, small mammals, and hibernators. In rodents, UCP1 and brown fat contribute importantly to whole body energy expenditure (37). Mechanisms underlying the increased energy expenditure of PNRC2~−/− male mice remain to be elucidated, because UCP1 expression in BAT has been found to be normal in PNRC2~−/− male mice, although the levels of UCP1 in WAT, liver, and muscle of PNRC2~−/− male mice have been shown to be significantly higher than that of WT mice (Fig. 6E). Because the overall expression level of UCP1 in WAT, liver, or muscle was very low compared with that in BAT, we were uncertain how significantly the increased UCP1 expression in WAT, liver, and muscle of PNRC2~−/− mice contributed to higher energy expenditure in PNRC2~−/− mice. PNRC2~−/− mice have a similar phenotype to nuclear receptor corepressor RIP140-null mice. RIP140-null mice were also lean with higher energy expenditure. Similarly, the UCP1 expression was not altered in the brown fat tissue of RIP140-null mice, although its expression in WAT was found to be markedly increased (19).

Analysis of plasma metabolites revealed that none of the glucose, insulin, or cholesterol levels were altered. No difference in glucose tolerance was detected between WT and PNRC2~−/− mice (data not shown). On the other hand, the level of triglycerides was ~37% lower (p < 0.001) (Fig. 2H), and the level of FFA was ~45% higher (p < 0.001) (Fig. 2I) in PNRC2~−/− male mice than in WT mice. Consistent with their lean phenotype, the plasma leptin levels of both male and female PNRC2~−/− mice were significantly reduced (Fig. 2G). The level of FFA in the blood reflects both the rate of release of fatty acids and the balance between the synthesis and breakdown of triglycerides in adipose tissue and liver. An increased level of FFA may be related to a decreased level of triglycerides, because FFA is a precursor of triglycerides. Another possibility for the increased FFA is that lipolysis is elevated in adipose tissue of PNRC2~−/− mice. However, no significant increase in glycerol release was detected in the epididymal fat pads isolated from PNRC2~−/− male mice compared with that from WT male mice (Fig. 2J). Increased plasma fatty acid concentrations are typically associated with many insulin-resistant states (38). However, the elevated circulating FFA did not promote increased insulin resistance in PNRC2~−/− mice. PNRC2~−/− mice have higher metabolic rate as demonstrated by indirect calorimetry and fasting. Therefore, a lower level of triglycerides and a higher level of FFA in the bloodstream of PNRC2~−/− mice suggest a higher fatty acid oxidation to produce energy in PNRC2~−/− mice to compromise the higher energy expenditure.

As noted above, the major phenotypes mainly occur in the male PNRC2~−/− mice. The causes for these gender differences are not totally clear at the present time. It might imply the involvement of sex hormones, such as estrogen, in the regulation of energy homeostasis by PNRC2. A growing body of evidence has demonstrated that sex hormones, including estrogen, progesterone, and androgen, play key roles in lipid metabolism and energy homeostasis through both genomic and non-genomic mechanisms (39). In the genomic mechanism, the sex hormone binds to its receptor and regulates the transcription of target genes. Leptin and lipoprotein lipase are two key proteins in adipose tissues that are regulated by transcriptional control with sex steroid hormones (40). In the non-genomic mechanism, the sex hormones bind to their receptors in the plasma membrane, and second messengers are formed. This involves both the cAMP cascade and phosphoinositide cascade. Activation of the cAMP cascade by sex hormones would activate hormone-sensitive lipase, leading to lipolysis in adipose tissues (41). Recently, it has been reported that sex hormones play important roles in adiposity and fuel partitioning (42, 43). By studying ovariectomized C57 BL/6 mice with estrogen or control pellet implants, D’Eon et al. (42) have demonstrated that estrogen regulates fat storage, FFA oxidation, and reduction of triglyceride storage, with effects that are very similar to what we have observed with PNRC2~−/− male mice. In addition, Moverare-Skrtic et al. (43) have found that the treatment of dihydrotestosterone (non-aromatizable androgen) resulted in obesity associated with reduced energy expenditure and fat oxidation in orchidectomized mice. These findings may explain why PNRC2~−/− female mice do not provide as clear a phenotype as PNRC2~−/− male mice, i.e. high estrogen levels.
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and low androgen concentrations in female mice prevent clear observation.

In addition to regulating energy expenditure, our preliminary data from this study also suggest a role of PNRC2 in adipogenesis. We found that the PNRC2 mRNA levels were markedly induced during the differentiation of 3T3-L1 preadipocytes into adipocytes (data not shown). The expression pattern of PNRC2 during the differentiation process was very similar to that of PPARγ (7), ap2 (44), and C/EBPα (45), key players in adipogenesis. We are now comparing the potency of mouse embryo fibroblasts isolated from WT and PNRC2−/− embryos to differentiate into adipocytes. Preliminary results showed that fewer numbers of mouse embryo fibroblasts from PNRC2−/− embryos differentiated into adipocytes, compared with mouse embryo fibroblasts from WT embryos under the exact same induction culture conditions (data not shown), suggesting a role of PNRC2 in adipogenesis. The differentiation of preadipocytes into mature fat cells is regulated by a cascade of transcriptional factors that interact in a complex fashion to control expression of many adipogenic genes. As noted earlier, PNRC2 interacts with a wide range of nuclear receptors. Many of PNRC2-interacting nuclear receptors, including ERRα1 (15–17), PR (39), AR (46), RARγ (45), RXR (47), TR (48), ERα (49), ERβ (49), HNF4α (50), LRH1 (51), PXR (52), RORα (53), RORβ (54), and PPARγ (55), play important roles in lipid metabolism and energy balance. Specifically, PNRC2 was found recently to interact with PPARγ and function as a coactivator of PPARγ in a ligand-dependent manner, indicating an involvement of PPARγ pathway in PNRC2 function in adipocyte differentiation.

In summary, PNRC2 deficiency protects mice from diet-induced obesity, possibly through interference with the energy expenditure and adipogenesis. As such, the results presented in this study demonstrate, for the first time, clear functional roles for PNRC2 in energy homeostasis and obesity. Importantly, although blood levels of FFA increased, analysis of plasma metabolites revealed that none of the glucose, insulin, or cholesterol levels were altered in male PNRC2-null mice. These results indicate that selective inhibition of PNRC2 may offer a therapeutic target for the treatment of obesity, especially in males. Further studies to identify the direct target genes that are regulated by PNRC2, using our knockout mouse model and embryonic fibroblasts isolated from PNRC2−/− embryos, will produce clearer pictures of mechanisms by which PNRC2 regulates adipogenesis and energy homeostasis.

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