Prokineticin Receptor 1 as a Novel Suppressor of Preadipocyte Proliferation and Differentiation to Control Obesity

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Abstract

Background: Adipocyte renewal from preadipocytes occurs throughout the lifetime and contributes to obesity. To date, little is known about the mechanisms that control preadipocyte proliferation and differentiation. Prokineticin-2 is an angiogenic and anorexigenic hormone that activate two G protein-coupled receptors (GPCRs): PKR1 and PKR2. Prokineticin-2 regulates food intake and energy metabolism via central mechanisms (PKR2). The peripheral effect of prokineticin-2 on adipocytes/preadipocytes has not been studied yet.

Methodology/Principal Findings: Since adipocytes and preadipocytes express mainly prokineticin receptor-1 (PKR1), here we explored the role of PKR1 in adipose tissue expansion, generating PKR1-null (PKR1−/−) and adipocyte-specific (PKR1ad−/−) mutant mice, and using murine and human preadipocyte cell lines. Both PKR1−/− and PKR1ad−/− had excessive abdominal adipose tissue, but only PKR1−/− mice showed severe obesity and diabetes-like syndrome. PKR1ad−/− mice had increased proliferating preadipocytes and newly formed adipocyte levels, leading to expansion of adipose tissue. Using PKR1−/− knockdown in 3T3-L1 preadipocytes, we show that PKR1 directly inhibits preadipocyte proliferation and differentiation. These PKR1 cell autonomous actions appear targeted at preadipocyte cell cycle regulatory pathways, through reducing cyclin D, E, cdk2, c-Myc levels.

Conclusions/Significance: These results suggest PKR1 to be a crucial player in the preadipocyte proliferation and differentiation. Our data should facilitate studies of both the pathogenesis and therapy of obesity in humans.

Introduction

Obesity causes many serious diseases such as type-2 diabetes mellitus, cardiovascular diseases and certain types of cancer, and has contributed to increases in mortality and morbidity rates [1]. Identification of factors regulating white fat tissue growth provides an important strategy to combat these diseases. Obesity is characterized by an expansion of adipose tissue mass due to hypertrophy, an increase in adipocyte size [2], and hyperplasia, an increase in cell number [3]. The expended adipose tissue plays a key role for the induction of insulin resistance commonly seen in obesity [4]. The adipocytes increase in size is due to lipid accumulation [5]. Mature adipocytes are postmitotic [6]. Thus, adipocyte hyperplasia in adults requires the generation of new adipocytes from precursor cells (preadipocytes) and stem cells resident in the stromal-vascular compartment of white adipose tissues (WATs). Preadipocytes are capable of proliferating and differentiating into an adipose deposit [7]. Stimulation of the proliferation of these cells may therefore result principally in an increase in adipocyte number. In mice, preadipocytes can proliferate and subsequently differentiate into mature adipocytes [8]. Thus, understanding the mechanisms controlling preadipocyte proliferation and conversion to adipocyte provides insights into the etiology and prevention of obesity and its associated pathologies.

Prokineticin-2 is a potent angiogenic [9] and anorexigenic hormone [10]. It binds two similar G protein-coupled receptors (GPCRs): PKR1 and PKR2 [11]. This hormone, which is widely distributed in mammalian tissues [12], has HIF-1 binding sites and is induced by low oxygen levels [13]. It is involved in diverse effects in peripheral systems, including angiogenesis in the ovary, testis [14,15] and heart [16]. It also stimulates hematopoiesis [17] and neurogenesis [18]. Prokineticins induce the differentiation of...
murine and human bone marrow cells into the monocyte/macrophage lineage [19] and activate monocyte proliferation and differentiation [20] and macrophage migration [21]. Prokineticin-2/PKR1 restores the pluripotency of epicardial progenitor cells and triggers the differentiation of endothelial and vascular smooth muscle cells [16]. By binding to PKR1, prokineticin-2 directly promotes angiogenesis, by activating MAPK and Akt [16].

Prokineticin-2 [22] is involved in appetite suppression [10] and energy homeostasis, thermoregulation [15], via a direct hypothalamic mechanism [23]. The intracranial or peripheral injection of prokineticin-2 has been shown to reduce food intake and body weight in lean and obese mice at the levels of hypothalamus (central effect via PKR2) [10] and dorsal vagal complex (peripheral effects via PKR1) [24]. However, the role of prokineticin-2 and its receptors in adipocyte and preadipocyte function is unknown.

PKR1 is the principal receptor expressed by preadipocytes and adipocytes [12]. In this study, we explored the role of PKR1 in adipocyte function, in vivo using PKR1-null (PKR1<sup>−/−</sup>) and adipocyte-specific (PKR1<sup>ad−/−</sup>) mutant mice, and in vitro, using murine (3T3-L1) and human preadipocyte cell lines (SGBS). Here we described multiple PKR1 functions regulates preadipocyte proliferation and differentiation, controlling adipose tissue expansion.

Materials and Methods

Ethics statement

The animal study was approved by the Animal Care and Use, and ethics committees of the Perfection du Bas-Rhin (Permit Number: B67–274) with the recommendations in the Guide for the Care and Use of Laboratory Animals of the French Animal Care Committee, with European regulation-approved protocols. The animal experimentation and housing were conducted at the accredited Animal Experimentation and housing Facility of the Institut de recherche de l’Ecole de biotechnologie de Strasbourg (Register number: C67-218-19).

The human study was approved by the ethics committee of Toulouse-Rangueil and Nancy-J. d’Arc Hospitals. Human adipose tissues were collected according to the guidelines of the Ethical Committee of Toulouse-Rangueil and Nancy-J. d’Arc Hospitals. All subjects gave their informed consent to participate to the study and investigations were performed in accordance with the declaration of Helsinki as revised in 2000 (http://www.wma.net/e/policy/b3.htm). Human subcutaneous adipose tissue samples were obtained from patients undergoing abdominal liposuction for plastic surgery or before a bariatric surgery and immediately frozen in OCT compound (Sakura) and sectioned at −80 °C. Written informed consent was obtained from all subjects and samples were coded and treated anonymously.

Genetically manipulated animal models

Male PKR1<sup>−/−</sup> mice in C57BL/6 gene background were originally made by homologous recombination [23]. For diet-induced obesity, 6-week-old PKR1-deficient mice and their wild-type littermates (C57/BL/6) were kept in micro isolation cages and fed a high-fat diet containing 60% fat or normal cow diet (5% fat) (Research Diet, New Brunswick, NY) for 34 weeks. PKR1 floxed mice [25] and p2C-CreERT2 [26] on a C57BL6/J background were intercrossed to generate the following genotypes: PKR1L2/L2 × p2C-CreERT2 (L2<sup>Tg</sup>/0) and PKR1L2/L2 × p2C-CreERT2 (L2<sup>Tg</sup>/0). Mice in which PKR1 was inactivated after tamoxifen injection will be referred to as PKR1<sup>ad−/−</sup> and their controls will be referred to as L2/L2. Tamoxifen was injected 1 mg/day (i.p.) for 5 days to the L2/L2 and PKR1<sup>ad−/−</sup> mice at the 3 weeks of age. The second tamoxifen injection was realized at the age of 13 weeks. All the analyses were performed when the mice were 24 and 40 weeks old. Mice were placed in metabolic cages (1 mouse/cage) and acclimated for 2 days before the experiment. The body weight, water and food intake, and urine volume were performed daily for an additional 3 days. The primers utilized in these studies for the genotyping analyses were shown in the Table 1.

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

Prior to studies, mice were fasted for 5 h. For GTT, mice received an intraperitoneal injection of glucose (1 mg/g body weight). In ITT studies, mice received an intraperitoneal injection of 0.75U of insulin per kg of body weight according to methods described elsewhere [27]. Blood samples were collected from the tail vein (tail-snip technique) at various times after the glucose or insulin load, as indicated. Blood glucose was immediately determined on a Contour blood glucose monitoring system (Bayer).

Insulin stimulation of Akt activity detected by Western Blot assay

Animals were anaesthetized with 1% pentobarbital and were followed by an IP injection of insulin (150 mU/g, Umaline®, Rapide). 0 and 20 min after injection mice were scarified and the adipose tissues were harvested for protein extraction. Extracted proteins were transferred to nitrocellulose membranes and immunoblotted with the Phospho Akt (Ser-473 and Thr 308) Antibody kit (Cell Signaling Technology), following incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG. Phosphorylated protein was visualized by enzyme-linked chemiluminescence (Amersham Biosciences) and quantified by scanning laser densitometry, normalizing to total amounts of the Akt proteins.

Histological and electron microscopy analyses

Organs were removed from 24 or 40 week-old mice, dissected and frozen for the cutting of frozen sections (5 μm), which were stained with Mallory tetrachrome. For electron microscopy, all organs were fixed by and embedded in epoxy resin according to methods described elsewhere [27]. Adipocyte diameters were analyzed on cryosectioned adipose tissue (3 independent sections) and 6–7 pictures were taken for each section. We counted more than 50 adipocytes per piece of WAT, resulting in a total of 3–400 adipocytes counted per mouse (total 3 mice). Quantification of adipocyte size was done with ImageJ software (http://rsbweb.nih.gov/ij/). All images were converted into binary files using a unique threshold value that separated positively labeled cells from background.

For succinic dehydrogenase (SDH) stain, excised adipose tissues were snap frozen in OCT compound (Sakura) and sectioned at 50 μm. Subsequent staining procedure was performed by standard protocols [28]. The staining intensity was quantified using ImageJ program. Microscope images were converted to gray scale and inverted. Pixels between the threshold 95–255 were selected to avoid the background signal, and the integrated densities were measured.

Immunostaining analyses

Frozen tissue sections for the immunofluorescence staining of structural proteins were fixed, blocked and stained with primary antibodies against PECAM-1, (Santa Cruz), PKR1 (IGBMC,
was subtracted. Analyzed using the pixel data from which background intensity compared pixel intensity and pixel distribution. These data were software. The laser was chosen according to the sample and confocal microscope and images were controlled by LEICA number. Some of the fluorescent signals were obtained with fluorescent microscope. Signal intensity was quantified on per high-power field or per dapi positive total nucleus digitalized images and calculated as the product of averaged pixel fluorescent microscope. Binding was detected by incubation with Fluorescein, Alexa 555- , Alexa 488-conjugated secondary antibodies [27].

Finally, the nuclei were stained with DAPI. Fluorescence was obtained and then, apoptosis was detected by the TdT-mediated flurescein in situ apoptosis detection kit (Milipore) according to the manufacturer’s protocol [16]. Proliferation was evaluated with prokineticin-2 at 5 nM concentration. Hemocytometer [29]. Note that maximum effect was observed number per well was daily counted from d 0–4 with a or absence of prokineticin-2 (peprotech) (1, 5, 10 nM). Total cell were plated in DMEM containing 0.5 or 10% FBS in the presence and 10 m medium containing 0.01 mg/ml transferrin, 100 nM cortisol, (day 0) were induced to differentiate in DMEM/Ham's F12 (1:1) American Type Culture Collection, and cultured in DMEM, for 48 h then cell number was counted. In the other settings growth capacity of 3T3-L1 cells was determined in which 6 x 104 cells

### Evaluation of proliferation and apoptosis

Cryosectioned mice adipose tissue samples or cultured cells were obtained and then, apoptosis was detected by the TdT-mediated dUTP nick end-labeling (TUNEL) assay utilizing Apoptag fluorescein in situ apoptosis detection kit (Milipore) according to the manufacturer’s protocol [16]. Proliferation was evaluated with Ki67 Labeling (Santa Cruz), as previously described protocol [27]. Cryosectioned mice adipose tissue samples or cells were fixed, blocked and stained with primary antibodies against Ki67. Antibody binding was detected by incubation with Fluorescein, Alexa 488-conjugated secondary antibody. Cells were scored for Tunel or Ki67-positive nuclei corresponding to DAPI stained nucleus in the each high power field (20 minimum) containing at least 30 nuclei at ×40 magnification.

For FACS analyses, epididymal WATs of 40-week-old male mice were digested with 1 mg/ml collagenase Type IV (Sigma-Aldrich), filtered through sterilized lens paper, and centrifuged. Enriched adipocytes floating on the top were removed. Cell pellets were permeabilized (20 h at −20°C in 70% ethanol). At least 1 x 10^5 cells in PBS/0.5% BSA/2 mmol/liter EDTA were incubated with fluorescein isothiocyanate (FITC)-conjugated Ki-67 antibody. The labeled cells were analyzed by multiparameter flow cytometry using a FACSCalibur flow cytometer and the CellQuest Pro software (BD Bioscience).

### Cell counting

Murine preadipocytes, 3T3-L1 cells (10^5) were obtained from American Type Culture Collection, and cultured in DMEM, for 48 h then cell number was counted. In the other settings growth capacity of 3T3-L1 cells was determined in which 6 x 10^4 cells were plated in DMEM containing 0.3 or 10% FBS in the presence or absence of prokineticin-2 [peprotech] (1, 5, 10 nM). Total cell number per well was daily counted from d 0–4 with a hemocytometer [29]. Note that maximum effect was observed with prokineticin-2 at 5 nM concentration.

### Adipocyte differentiation by oil red O staining

Confluent 3T3-L1 were differentiated into adipocytes by the DMEM supplemented with 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich), 1 mM dexamethasone (Sigma-Aldrich), and 10 μg/ml insulin (Sigma-Aldrich), as previously described [30]. After 2 days the medium was replaced with the medium containing only 10 μg/ml insulin. The human cell strain, derived from an adipose depot of an infant with Simpson-GolabieBehmel syndrome (SGBS), was cultured as follows: briefly, confluent cells (day 0) were induced to differentiate in DMEM/Ham's F12 (1:1) medium containing 0.01 mg/ml transferrin, 100 nM cortisol, 0.2 nM triiodothyronine, and 20 nM insulin. To trigger the

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**Table 1. Oligonucleotides used for PCR analyses.**

| Genes      | Sequence Forward (5'-3') | Sequence Reverse (5'-3') |
|------------|--------------------------|--------------------------|
| Beta actin | CATCTTGCCGTACCTGTCCA     | GGCGCCGACTCATGACT       |
| PPAR alpha | ACCGATGCTGTCTCCTGATG     | GTGTGATAAAGCCATTGCCT     |
| PPAR gamma | CGAGAAGGAAGAACTGGTGG     | GAAACTGCGACCTTTGAAA     |
| C/EBP alpha| CAAGAACGCACAGCTTCC       | GCTACTGCTCACTCCGACAC    |
| Adiponectin| AAATCTGCGCTACGCTCGGAGG   | TCTCCAGAGTGGCCATCTGCCC   |
| Resistin   | AGAAGGCCAGACAGCTTCC      | TGGCAGCTCATATCTCCTC     |
| Cyclin D1  | GAGATTGTGCACTCATATG      | CTCTCTCTCGCACTCTGCT     |
| Cyclin E1  | TTTCGACGCACTCTCCTCT      | TGGGACCTTATAGCTCAGCCACA |
| Cdk-2      | CACAGCCGTGGCATATCTGG     | CATGGTGTGGTGATACACCT     |
| c-Myc      | CCAATGTCGTCATGAGGAGA     | TCTCTCTCTCCGTTCTCTC     |
| HIF1 alpha | TACAAGGCCAGACAACTCACC    | TGGCAATTTGGGCTACC       |
| mPKR1      | GCCATCGCCAATGGAGGATG     | TGGTGAAGGAGGACAGTGA      |
| UCP        | CTTGTCACAACTTTGGAAAGG    | CTTGTCAGACTAGGAACAT     |
| GLUT 4     | GTGTGGCTCAACAGCTTGGATTG  | CGCAGTCTGAGTCAAGG       |
| TNFalpha   | AGCCGATGATCTCCTAAACAG    | AGATAGCAATTCGGCTAGC     |
| hPKR2      | CTTGCTCTCCTCCTCCCAA     | TGGCAAGAGGAGGAAGAAGAA   |
| hPKR1      | CGGCAATGGAATCTTCCCTT    | GATGACAGGTGTTGGAGTG     |
| Cre        | ATC TTC CAG CAG GCC CAC CAT TGC CCC TGT | TGA CGG TGG GAG AAT GTT AAT CCA TAT TGG |
| L2/L2      | GAG TGG ACA TCT AGT GGT AGT CAG G | GGG TGT GAG GTG GSA TTA AGT CAC |

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PKR1 Controls Preadipocyte Function

Intestinal lipid absorption
WT and PKR1−/− mice were fasted overnight. Mice received 500 μl of olive oil by gavage. Mice were sacrificed 1 hour after gavage feeding and tissues were harvested for histological or oil-red oil analyses [34].

Fecal fat quantification
Mice were caged individually in metabolic cages with inserts that permit collection and prevent ingestion of fecal material. Mice were maintained for 3 days and feces were collected at days 2 and 3. Fat was extracted from 1 g of feces with 20 ml of chloroform/methanol (2:1) for 20 min at room temperature as previously described[34].

Statistic analysis
The results are expressed as means ± SEM. Unless otherwise noted, statistical comparisons for all experiments were performed using Mann–Whitney (for 2 groups) and Kruskal–Wallis (for >2 groups) tests. Statistical comparisons for staining with Oil Red O for adipocytes, CD68 macrophages, PECAM for endothelial cells, Pref-1 for preadipocytes, Tunel and Ki67 for apoptotic nd proliferating cells were performed using the unpaired Student t test and ANOVA. P<0.05 was considered statistically significant for all tests.

Results
PKR1 null mutant (PKR1−/−) mice develop obesity
PKR1 null mutant (PKR1−/−) mice become severely obese on a normal chow diet with body weights ~40% higher than those of wild-type mice at the age of 40 weeks (Fig. 1A and Fig. S1). However, the mutant and wild-type mice consumed identical amounts of food on the chow diet, ruling out the possibility that PKR1 deficiency results in a greater body weight gain due to hyperphagia (Fig. 1A, right). The epididymal fat pads (subcutaneous and visceral WATs) were also ~4 times heavier in the PKR1−/− mice than in their wild-type littermates on a normal chow diet (Fig. 1A, middle and Fig. 1B). However, there is no difference in brown adipose tissue mass between two groups of mice. Note that no PKR1 expression was detected in brown tissues. We investigated whether PKR1 deficiency increased WAT mass by the hypertrophy or by hyperplasia of white adipocytes, by measuring the numbers and diameters of WAT in the visceral region of PKR1−/− and wild-type mice. The staining of semi-thin sections revealed that PKR1−/− adipocytes were smaller than wild-type adipocytes, but that their density was higher (Fig. 1C and 1D). However, proliferating Ki67+ cells were clearly abundant in PKR1−/− adipose tissue (Fig. 1E and FACS analysis). Most of the Ki67+ cells were also positive for the Pref-1 marker of preadipocytes (Fig. 1F). Higher transcript levels for the transcription factors involved in adipogenesis, (PPARα, PPARγ, C/EBPα, cyclin D, E, cdk2, c-Myc) were used (Table 1). The real-time PCR was carried out in an iCycler myiQ apparatus (Bio-Rad, Life Science Thermogenesis
Rectal temperature was measured in 40-weeks-old WT and PKR1−/− mice [33]. Mice were caged individually and fasted for 3 h before they were placed in a room maintained in 4 °C for cold-exposure experiments. The initial temperature measurements were performed 3 hours before cold exposure (before fasting), at time point 0 (just before cold exposure) and after 2 hours in cold environment.

RNA interference
The RNA interference technique was used for down-regulating PKR1 gene expression as previously described [32]. Transfection was performed by using siPORT Amine transfection reagent (Ambion, Austin, TX, USA) with 10 nM siRNA for mouse PKR1 (Ambion siRNA #181827), according to the manufacturer’s instructions (Ambion). Negative control siRNA transfection was also performed by using non-specific siRNA (Ambion). After 48 hours medium was replaced with induction medium to induce differentiation into mature adipocyte or to isolate RNA for detection of PKR1 levels by PCR. In this condition reduced expression of PKR1 lasts at least 48 h. Note that PKR1 expression was reduced after adipogenic cocktail treatment. Thus, the down regulation of PKR1 remained constant before and after adipogenic induction.

Analysis of gene expression by quantitative PCR
Total RNA was prepared from 3T3-L1 cells or collected tissues using a Tri-Reagent (MRC, Cincinnati, OH) according to the manufacturer’s instructions [29]. Total RNA (2-5 μg) was reverse-transcribed with Super Script II Reverse Transcription Reagents (Invitrogen). The resultant cDNA was subjected to real-time quantitative PCR, in which a specific primers for mouse adiponectin, resistin, TNFα, PPARα, PPARγ, C/EBPα, cyclin D, E, cdk2, c-Myc were used (Table 1). The real-time PCR was carried out in an iCycler myQ apparatus (Bio-Rad, Life Science Research, Hercules, CA) and SYBR green (Bio-Rad). Relative values of mRNAs were analyzed by the ΔΔC(T) method, normalized to GAPDH mRNA and shown as fold change in expression over control.

Thermogenesis
Rectal temperature was measured in 40-weeks-old WT and PKR1−/− mice [33]. Mice were caged individually and fasted for 3 h before they were placed in a room maintained in 4 °C for cold-exposure experiments. The initial temperature measurements were performed 3 hours before cold exposure (before fasting), at time point 0 (just before cold exposure) and after 2 hours in cold environment.

Expression over control.
Normalized to GAPDH mRNA and shown as fold change in expression over control.

Carried out in an iCycler myQ apparatus (Bio-Rad, Life Science Research, Hercules, CA) and SYBR green (Bio-Rad). Relative
Figure 1. PKR1 null mutant mice (PKR1<sup>−/−</sup>) exhibit obesity at the later age. A) Body weight (g), and WAT and pancreas (pancr) weight (g), and food intake (g/24 h) at the age of 40 weeks (*p<0.05, n = 6). B) Representative illustration of abdominal obesity in PKR1<sup>−/−</sup> mice. C) Representative illustration of semi-thin sections of adipocyte tissue derived from PKR1<sup>−/−</sup> and WT mice. D) Histograms show cell number and diameter differences between these two groups of mice. E) Representative illustration of Ki67 positive cells (green) and dapi positive total cells (blue) in adipose tissue of PKR1<sup>−/−</sup> and WT mice. Quantification of Ki67<sup>+</sup> cell numbers in the high power field (HPF, ×40 objective) (*p<0.05, n = 20 HPF). F) Flow cytometry for Ki67 positive nuclei in the cells that were prepared from PKR1<sup>−/−</sup> and WT type abdominal (epididymal and visceral) WATs tissue after removal of adipocytes. F) Representative illustration and quantification of pref-1<sup>−/−</sup>/Ki67<sup>+</sup> positive cells (orange) and dapi positive total cells (blue) in adipose tissue of PKR1<sup>−/−</sup> and wild type mice (*p<0.05, n = 20 HPF, ×40 objective). G) qPCR analyses of transcript levels of adipogenic genes of PKR1<sup>−/−</sup> mice over WT mice (fold increase). H) Representative illustration of electron microscopic analyses of adipose tissues derived from PKR1<sup>−/−</sup> and WT mice demonstrating cytoplasmic lipid droplets (lp) and swollen mitochondria (mtc). Nc: nucleus, bv: blood vessel. I) Illustration and histogram shows the PECAM-1 staining and capillary formation are similar between the adipose tissues of PKR1<sup>−/−</sup> and WT mice (*p<0.05, n = 20 HPF, ×40 objective). J) Illustration and histogram shows the succinyl dehydrogenase (SDH) staining changes between the adipose tissues of PKR1<sup>−/−</sup> and WT mice (*p<0.05, n = 20 HPF, ×40 objective). K) Western blots analyses reveal a high level of HIF-1α levels in PKR1<sup>−/−</sup> mice (*p<0.05, n = 3).

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activity (Fig. 1J), and higher protein levels of hypoxia-inducible factor-1, a marker of hypoxia (Fig. 1K), clearly demonstrating the occurrence of hypoxia in the mature PKR1$^{-/-}$ adipocytes with defective capillary formation.

**PKR1 null mutant mice displayed diabetes-like disorders**

We then investigated possible impairment of the insulin response in hypoxic PKR1$^{-/-}$ WAT tissues. We found that the insulin-stimulated phosphorylation of Akt (at serine and threonine) were significantly lower in PKR1$^{-/-}$ adipose tissue as compared to wild (WT) adipose tissue ($^*$ $p<0.05$, $n=3$). (B) Glucose tolerance (GTT) and insulin tolerance tests (ITT) in 40 weeks old mice. PKR1$^{-/-}$ mice did not tolerate glucose load and displayed impaired ITT ($^*$ $p<0.05$, $n=8$). (C) Body weight increases in HFD-fed and NFD-fed mice. $^*$ shows a significant difference between NFD-fed PKR1$^{-/-}$ mice and WT mice, $n=8$. t shows difference between HFD-fed and NFD-fed WT mice (blue). (D) GTT and ITT in HFD-fed mice at the age of 40 weeks. No significant alteration was observed between the groups, due to severe increase in GTT and ITT of WT mice treated with HFD ($^*$ $p<0.05$, $n=8$). (E) Representative illustration of semi-thin sections of adipocyte tissue derived from PKR1$^{-/-}$ and WT mice after HFD feeding showing the hypertrophic adipocytes in both groups ($\times40$). (F) Histogram shows cell numbers and diameters are similar between these two groups of mice (ns = no significance, $p>0.05$, $n=20$).

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hypertrophic visceral adipocytes (Fig. 2E) without alterations in cell number and diameter (Fig. 2F).

Adipocyte specific loss of PKR1 in mice exhibit enhanced abdominal adiposity

To eliminate an endothelial-adipocyte interation and possible central regulation of the body weight in mutant mice, we generate tamoxifen-inducible adipocyte/preadipocyte-specific PKR1 knockout mice (Fig. S3A, B and C). PKR1ad−/− mice had a 13±2% higher body weight (Fig. 3B left) and 1.5 times higher epididymal mass (Fig. 3B middle) and an increased number of adipocytes at the age of 24 weeks (Fig. 3A and 3B right). The PKR1ad−/− and L2/L2 mice ingested similar amounts of food (4.56±0.35 and 4.9±0.39 g/day, respectively). Semi-thin sections and electron microscopy showed interstitial macrophage deposition in PKR1ad−/− adipose tissue, which was confirmed by macrophage-specific, CD68 staining (Fig. 3E). Capillary formation, as detected by PECAM-1 staining, was also elevated in PKR1ad−/− adipose tissues (Fig. 3E). The PKR1ad−/− adipose tissues contained a 3 times higher number of Ki67+/Pref-1+ positive cells (orange) and dapi positive total cells (blue) in adipose tissue of PKR1ad−/− and L2/L2 mice (p<0.05, n=20 HPF, ×40 objective). F) Representative illustration and quantification of pref-1+/Ki67+ positive cells (orange) and dapi positive total cells (violet) in adipose tissue of PKR1ad−/− and L2/L2 mice (p<0.05, n=20 HPF, ×40 objective). G) Representative illustration and quantification of PPARγ/Ki67+ positive cells (orange) and PPARγ/dapi positive total cells (violet) in adipose tissue of PKR1ad−/− and L2/L2 mice (p<0.05, n=20 HPF, ×40 objective). H) GTT and ITT in 24 weeks old mice (p<0.05, n=6). doi:10.1371/journal.pone.0081175.g003

Figure 3. Abdominal obesity in PKR1ad−/− mice is due to preadipocyte proliferation and differentiation. A) Representative illustration of semi thin analyses of adipose tissues derived from control (L2/L2) and PKR1ad−/− mice. Blue shows macrophage accumulation. B) Body (g) and WAT weight (g), and adipocyte number of the PKR1ad−/− and L2/L2 mice (p<0.05, n=6). C) Representative illustration of electron microscopic analyses of adipose tissues derived from PKR1ad−/− and L2/L2 mice, showing macrophage deposition in PKR1ad−/− mice. D) Macrophage specific CD68 staining and quantification of macrophages on cryosectioned adipose tissues derived from PKR1ad−/− and L2/L2 mice (p<0.05, n=20 HPF, ×40 objective). E) Illustration and histogram shows the PECAM-1 staining and capillary formation changes between the adipose tissues of PKR1ad−/− and L2/L2 mice (p<0.05, n=20 HPF, ×40 objective). F) Representative illustration and quantification of pref-1+/Ki67+ positive cells (orange) and dapi positive total cells (blue) in adipose tissue of PKR1ad−/− and L2/L2 mice (p<0.05, n=20 HPF, ×40 objective). G) Representative illustration and quantification of PPARγ/Ki67+ positive cells (orange) and PPARγ/dapi positive total cells (violet) in adipose tissue of PKR1ad−/− and L2/L2 mice (p<0.05, n=20 HPF, ×40 objective). H) GTT and ITT in 24 weeks old mice (p<0.05, n=6). doi:10.1371/journal.pone.0081175.g003

PKR1 Controls Preadipocyte Function
Prokineticin-2 inhibits proliferation of 3T3-L1 cells

To gain further insights into the functional interactions governing adipose tissue expandability, we investigated whether PKR1 has a cell-autonomous role in preadipocytes 3T3-L1. For control 3T3-L1 cells, the number of cells began to increase about 24 h after induction with 10% FCS. However, there were fewer cells 24–72 h after induction following prokineticin-2 (5 nM or 10 nM) treatment than after treatment with 10% FCS alone (Fig. 4A). We then investigated the cell cycle by Ki67 staining (Fig 4B and 4C). Entry into S phase increased within 10 h, in cells grown in 10% FBS but not in cells grown in 0.5% FCS. However, the increase in the number of Ki67+ cells induced by 10% FCS was inhibited by prokineticin-2 treatment. No differences were observed between prokineticin-2-treated and control cells in culture medium supplemented with 0.5% FCS, indicating that prokineticin-2 had no cytotoxic effect (Fig. 4B). A direct apoptotic effect of prokineticin-2 was excluded by TUNEL staining, which revealed no detectable apoptosis 48 hours after treatment of the cells with prokineticin-2 (Fig. 4D). These data suggested that 3T3-L1 cells were at a similar degree of confluence and that there was no cytotoxicity or apoptosis induction in response to prokineticin-2 treatment. Strikingly, prokineticin-2 induced a marked decrease in the expression of molecular markers of the cell cycle, such as cyclin E, cyclin D (within 10 hours), Cdk2 and c-Myc (within 48 hours) as compare to 10% FCS alone (Fig. 4E). Pretreatment of the 3T3-L1 cells with prokineticin-2 also downregulated the cell cycle gene expression 48 hours after the administration of an adipogenic cocktail (insulin, dexamethasone and isobutylmethylxanthine) (Fig. 4F). Prokineticin-2 mediated this effect was completely abolished after acute PKR1 knockdown, achieved through the use of an siRNA targeting PKR1. From day 48, PKR1 mRNA levels were 70% lower after transfection with PKR1 siRNA than after transfection with a nonspecific siRNA (Fig. S5A and B).

Prokineticin-2 inhibits adipogenic differentiation of preadipocytes

To next explore the in vitro role of prokineticin 2 in conversion of preadipocyte to adipocyte, the 3T3-L1 cells were grown to confluence and their differentiation into adipocytes was induced with an adipogenic cocktail, following treatment of the cells with prokineticin-2 or vehicle. The prior treatment of the 3T3-L1 cells with prokineticin-2 (5 nM) induced a significant decrease in adipogenic cocktail-mediated lipid accumulation (Fig. 5A and histogram) and TG accumulation (Fig. 5C). We evaluated the consequences of PKR1 depletion for preadipocyte differentiation further, by studying the effects of prokineticin-2 on 3T3-L1 adipogenesis after acute PKR1 knockdown, achieved through the use of an siRNA targeting PKR1. From day 48, PKR1 mRNA levels were 70% lower after transfection with PKR1 siRNA than after transfection with a nonspecific siRNA (Fig. S5A and B).

PKR1 Controls Preadipocyte Function

To next explore the in vitro role of prokineticin 2 in conversion of preadipocyte to adipocyte, the 3T3-L1 cells were grown to confluence and their differentiation into adipocytes was induced with an adipogenic cocktail, following treatment of the cells with prokineticin-2 or vehicle. The prior treatment of the 3T3-L1 cells with prokineticin-2 (5 nM) induced a significant decrease in adipogenic cocktail-mediated lipid accumulation (Fig. 5A and histogram) and TG accumulation (Fig. 5C). We evaluated the consequences of PKR1 depletion for preadipocyte differentiation further, by studying the effects of prokineticin-2 on 3T3-L1 adipogenesis after acute PKR1 knockdown, achieved through the use of an siRNA targeting PKR1. From day 48, PKR1 mRNA levels were 70% lower after transfection with PKR1 siRNA than after transfection with a nonspecific siRNA (Fig. S5A and B).
were significantly reduced in obese visceral and subcutaneous human tissues. Together, these in vitro data provide a convincing demonstration that PKR1 plays a key role in suppressing preadipocyte conversion to adipocytes.

**Discussion**

The identification of factors involved in white fat tissue growth is important to elucidate the etiology of obesity. Albeit recent studies have provided insights into the anorexigenic and angiogenic properties of prokineticin-2, its physiological functions in preadipocytes and adipocytes are unknown. In this study, we provide in vivo and in vitro evidence for distinct roles of prokineticin receptor-1 (PKR1) in maintenance of proliferation and conversion of preadipocytes to adipocytes, controlling abdominal adipocyte numbers and adipose tissue mass.

**Figure 5. Prokineticin-2 inhibits preadipocyte conversion to adipocytes.**

A) Oil-red O staining of 3T3-L1 cells treated with control (ctr), adipogenic cocktail (AC) for 8 days or pretreated with 5 nM of prokineticin-2 (+PK-2) 10 h before AC treatment (n = 20, *p < 0.05, HPF, ×40 objective). B) In the PKR1 knockdown 3T3-L1 cells (siRNA-PKR1), prokineticin-2 (5 nM) pretreatment did not inhibit adipogenesis induced by AC. Histogram shows pretreatment of adipocytes with prokineticin-2 reduced number of adipocytes detected by Oil red staining in the control siRNA transfected 3T3-L1 cells but not in the PKR1 siRNA transfected cells (n = 20, *p < 0.05, HPF, ×40 objective). C) Triglyceride (TG) accumulation in the cell lysates. * shows PK2 significantly diminished AC effect (n = 3, p < 0.05). D) qPCR analyses show increased levels of mature adipocyte markers, resistin and adiponectin by AC treated cells compared to that of AC (n = 3, p < 0.05). E) qPCR analyses reveal that PPARα and C/EBPα were significantly reduced by prokineticin-2 pretreatment as compare to that of AC (n = 3, p < 0.05). F) Representative illustration and histogram shows that prokineticin-2 reduced human preadipocyte SGBS cell differentiation into adipocytes and TG accumulation induced by AC (n = 3, p < 0.05). Quantitative PCR analyses show that transcript levels of human PKR1 (right panel) were reduced in obese visceral and subcutaneous human tissues (n is indicated in the figure, *p < 0.05).

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Both PKR1<sup>−/−</sup> and PKR1<sup>ad−/−</sup> mice displayed abdominal obesity, but only PKR1<sup>−/−</sup> mice had peripheral obesity accompanied by a diabetes-like syndrome, suggesting non-adipocyte PKR1-mediated events also contribute to development of peripheral obesity with diabetes-like syndrome. Angiogenesis and adipogenesis interact reciprocally via paracrine signaling systems in the development of adipose tissue [35]. In PKR1<sup>−/−</sup> mutants, the development of the new capillaries was obstructed due to loss of angiogenic PKR1 in endothelial cells as well. Therefore, the reciprocal regulation of adipogenesis via angiogenesis is impaired in the PKR1<sup>−/−</sup> mice, creating a hypoxic environment. High levels of HIF1α, an indicator of hypoxic stresses, in PKR1<sup>−/−</sup> adipocytes may also contribute to impaired adipocyte insulin signaling [36]. Consistently, PKR1<sup>−/−</sup>-WATs displayed impaired Akt phosphorylation in response to insulin. PKR1 deficient mice were obese and had poorer GTT and IITT responses than wild-type mice. In contrast to PKR1<sup>−/−</sup> mice, PKR1<sup>ad−/−</sup> mice had higher levels of capillary formation in fat tissues, indicating, the reciprocal regulation of adipogenesis via angiogenesis is not impaired in the PKR1<sup>ad−/−</sup> mice. Moreover, glucose and insulin tolerance remained unaffected in the PKR1<sup>ad−/−</sup> mice, indicating that a damaged endothelial-adipocyte interaction may involve in the impaired insulin response in PKR1-null mutant adipocytes. The obese phenotype of PKR1<sup>−/−</sup> mice did not seem to be modified by dietary fat content, because the high-fat diet resulted in smaller differences in body weight and GTT and IITT responses, due to the massive increase in body weight observed in wild-type mice. However, high-fat diet promoted hypertrophy in the both PKR1<sup>−/−</sup> and wild adipocytes, indicating that high calorie intake is the key factor in conversion of hyperplasia to hypertrophy in PKR1<sup>−/−</sup> adipocytes.

It seems unlikely that the hypothalamic control of energy metabolism is involved in the adipocyte hyperplasia observed in PKR1-null mice. PKR2, which is strongly expressed in the hypothalamus, is responsible for the central anorexic and thermoregulatory effect of prokineticin-2 [10]. Chronic continuous infusion of PK2 via the brainstem significantly reduced body weight and food intake in a mouse model of human obesity without altering energy expenditure [37]. Central regulation of the body weight was eliminated in our PKR1<sub>ad-/-</sub> mice that also had an increase in abdominal fat mass accumulation, due to accelerated preadipocyte proliferation promoting the formation of new adipocytes. Accordingly, preadipocyte hyperplasia in the adipose tissue [30] exacerbates the accumulation of white adipocytes, and is also a key event in the development of some types of obesity [39]. In this study, we also discovered that PKR1 also has essential functions in controlling preadipocyte proliferation largely through the downregulation of cell cycle genes (cyclin D, E, cdk2, c-Myc). Preadipocytes exhibit continual turnover in adult humans [5]. Increases in adipocyte number during aging have been implicated in the severity of obesity in the elderly [40]. The undifferentiated adipocyte precursors, preadipocytes residing in adipose tissue vascular stroma are capable of proliferating and differentiating into an adipose deposit in response to obesogenic triggers [41]. Recent loss of necdin in mice has been shown to promote a hyperplastic adipocyte due to increase in preadipocyte proliferation and differentiation [42]. Postconfluent DNA replication, cell division and cell proliferation during mitotic clonal expansion are required for initiation of the transcriptional cascade for preadipocyte differentiation [43]. Prokineticin-2 inhibited not only mouse embryonic preadipocyte but also adult human preadipocyte conversion to adipocyte. We also reported here that the expression of prokineticin-2 (Fig. S7) and its receptor PKR1 was altered in human WAT tissues. Possible mutations on these genes in human obesity remain to be determined.

A number of angiogenic factors, including epidermal growth factor, platelet-derived growth factor BB [44], basic fibroblast growth factor [45] and heparin-binding epidermal growth factor-like growth factor (HB-EGF) [46], inhibit the conversion of preadipocytes to adipocytes [47], much like we find with prokineticin-2.

Conclusively, this novel function of PKR1 as a major suppressor of preadipocyte proliferation and conversion to adipocytes will expand our knowledge on factors regulating adipocyte expansion in the alarming world-wide trend toward increasing obesity and its associated pathologies. Preadipocyte replication represents a new mechanism for expansion of fat mass in cases of human obesity and reducing preadipocyte replication may represent a new avenue of therapeutic intervention against obesity [48]. As 40% of drugs target GPCRs [49] PKR1 could be an effective target for treatment or prevention of obesity.

Study Limitations: Recent studies suggest preadipocytes can differentiate to macrophages, dedifferentiate from adipocytes or macrophages, and differentiate back to adipocytes [50]. Since aP2-CreERT2 line has been found to induce recombination in the adipose tissue, capillary endothelium in the heart and in intermyofibrillar cells in the skeletal muscle, but not in macrophages in adipose tissue [51], thus we can exclude effect of macrophage on the observed phenotype of PKR1<sup>ad−/−</sup> mice. Our data show that the reciprocal regulation of adipogenesis via angiogenesis is not impaired in the PKR1<sup>ad−/−</sup> mice, eliminating possible contribution of endothelial cells in the PKR1<sup>ad−/−</sup> mice. The contribution of the endothelial-PKR1 signaling on regulation of metabolic homeostasis remains to be investigated.

Supporting Information

Figure S1 40 weeks old PKR1<sup>−/−</sup> null mutant mice exhibit hypoxic adipocytes. Representative illustration showing increased body weight of PKR1<sup>−/−</sup> mice (left) compare to wild type (right).

(TIF)

Figure S2 Metabolic changes on PKR1<sup>−/−</sup> null mutant mice at the 15 week-old age. GTT test shows PKR1-deficient mice have abnormal glucose clearance, beginning at 40 min postglucose treatment as compare to age matched wild type mice (n = 8).

(TIF)

Figure S3 Generation of PKR1<sup>ad−/−</sup> mice and Immunostainings on adipose tissues. A) Representative genotype analysis of PKR1<sup>ad−/−</sup> mice. Genomic DNA was amplified with oligonucleotide primers detecting aP2-Cre and PKR1<sup>floxed</sup> alleles. PKR1<sup>ad−/−</sup> mice harbor the aP2-Cre transgene and are homozygous for the PKR1 floxed allele (PKR1<sup>lox/lox</sup>). Control mice (L2/L2) are aP2-Cre negative and they are PKR1<sup>lox/lox</sup>. B) RT-PCR analyses on RNAs extracted from adipose tissue revealed that PKR1<sup>ad−/−</sup> mice had lower Pkd1 levels after tamoxifen treatment. C) Representative illustration of loss of PKR1 protein in the adipose tissue of PKR1<sup>ad−/−</sup> mice by immunostaining of the cytochrome oxidase adipose tissue with PKR1 antibody. D) Representative illustration of immunostaining of PKR1<sup>ad−/−</sup> adipocytes with dapi, Ki67 and pref-1 antibodies and corresponding secondary antibodies (upper). Pref-1 and PPARγ antibody stainings of the adipose tissues without secondary antibodies show no non-specific stainings of the adipose tissues with the first antibodies.
Figure S4 Glucose clearance after glucose and insulin treatment of 40 weeks old PKR1<sup>−/−</sup> mutant mice. GTT test showed that glucose clearance in PKR1<sup>−/−</sup> mice was similar to control group in response to glucose loading at the age of 40 weeks (left). The ITT remained similar in both groups at the 40 week-old ages (n = 6, p > 0.05) (right).

Figure S5 Prokineticin-2 mediated proliferation rate in 3T3-L1 cells, expressing low level of PKR1. A) Representative illustration of PCR analyses revealed that siRNA for PKR1 significantly reduced PKR1 expression 48 hours after siRNA-PKR1 transfection. Histogram shows quantification of the PKR1 expression levels in each group (n = 3, *p < 0.05). siRNA NS: nonspecific siRNA, siRNA PKR1: siRNA for PKR1. B) PK-2 was not able to inhibit proliferation induced by 10% FCS for 3 days in the 3T3-L1 cells transfected with siRNA for PKR1 (n = 3, *p > 0.05 different then initial 0 time). In the 3T3-L1 cells transfected with siRNA NS, PK-2 inhibits % FCS induced proliferation rate (*p < 0.05 different then initial 0 time ** p < 0.05 different than 10% serum).

Figure S6 Thermogenesis, expression profiles of energy metabolism-related genes in the muscle and lipid absorption in PKR1<sup>−/−</sup> null mutant mice. A) Body temperature of PKR1-deficient (PKR1<sup>−/−</sup>) and wild (WT) mice (n = 6), 3 hours before cold exposure (room temperature), the time of cold exposure (0) and 2 hours after cold (4°C) exposure. B) Total RNA was extracted from the muscle of 40-week-old mice. qRT-PCR was performed using primers listed in Table 1. The values in the muscle were normalized with those of β-actin. No statistically significant differences were noted between WT<sup>+/+</sup> and PKR1<sup>−/−</sup> mice (p > 0.05, n = 3). PPARs, peroxisome proliferators-activated receptor; GLUT4, glucose transporter type 4; UCP, uncoupling protein. All data are presented as mean ± SEM (n = 4).

C) Representative of semi-thin analyses of Jejunum derived from mutant and wild type mice 20 min after oil (vegetable oil) gavages indicating slightly higher levels of lipid absorption in the jejunum of PKR1<sup>−/−</sup> mice one hour after gavage with oil. C) Quantification of intestinal lipid levels after oil-red staining revealed a similar oil-red staining between the groups. D) The intestinal lipid/feces lipid ratio was not significantly altered between the groups (n = 6, p > 0.05).

Figure S7 Prokineticin-2 and its receptor PKR1 expression in Human adipose tissues. Quantitative PCR analyses show transcript levels of prokineticin-2, a ligand were increased in obese human WAT tissues.

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Author Contributions

Conceived and designed the experiments: CGN PV. Performed the experiments: CS JV MD MB PV CGN. Contributed reagents/materials/analysis tools: DM PC. Wrote the paper: CGN.

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