Article

Cell Metabolism

PTH/PTHrP Receptor Mediates Cachexia in Models of Kidney Failure and Cancer

Graphical Abstract

Highlights
- 5/6 nephrectomy and LLC tumors trigger cachexia and adipose tissue browning
- Elevated circulating PTH and PTHrP stimulate browning through their receptor PTHR
- PTHR function in fat is required for adipose tissue browning and wasting
- Loss of PTHR in fat tissue also attenuates skeletal muscle atrophy

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In Brief
Kir et al. reveal a role for the PTH/PTHrP pathway in cachexia driven by kidney failure or cancer and show how PTH and PTHrP stimulate adipose tissue browning through their receptor PTHR. Loss of PTHR in adipocytes blocks atrophy of not only fat tissue, but also skeletal muscle in mice.

Authors
Serkan Kir, Hirotaka Komaba, Ana P. Garcia, ..., Beate Lanske, Richard A. Hodin, Bruce M. Spiegelman

Correspondence
bruce_spiegelman@dfci.harvard.edu

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PTh/PThrP Receptor Mediates Cachexia in Models of Kidney Failure and Cancer

Seren, H. Komaba, Ana P. Garcia, Konstantinos P. Economopoulos, Wei Liu, Beate Lanske, Richard A. Hodin, and Bruce M. Spiegelman

1Department of Cancer Biology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02215, USA
2Department of Oral Medicine, Infection, and Immunity, Harvard School of Dental Medicine, Boston, MA 02115, USA
3Department of Surgery, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA
4Correspondence: bruce_spiegelman@dfci.harvard.edu

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SUMMARY

Cachexia is a wasting syndrome associated with elevated basal energy expenditure and loss of adipose and muscle tissues. It accompanies many chronic diseases including renal failure and cancer and is an important risk factor for mortality. Our recent work demonstrated that tumor-derived PThrP drives adipose tissue browning and cachexia. Here, we show that PTh is involved in stimulating a thermogenic gene program in 5/6 nephrectomized mice that suffer from cachexia. Fat-specific knockout of PThr blocked adipose browning and wasting. Surprisingly, loss of PTh in fat tissue also preserved muscle mass and improved muscle strength. Similarly, PThr knockout mice were resistant to cachexia driven by tumors. Our results demonstrate that PThrP and PTh mediate wasting through a common mechanism involving PThr, and there exists an unexpected crosstalk mechanism between wasting of fat tissue and skeletal muscle. Targeting the PTh/PThrP pathway may have therapeutic uses in humans with cachexia.

INTRODUCTION

Cachexia is a wasting syndrome associated with several illnesses including cancer, chronic kidney disease (CKD), and heart failure. Elevated basal energy expenditure in these disorders leads to wasting of adipose tissue and skeletal muscle through enhanced fat and protein catabolism. Approximately half of all patients with cancer suffer from cachexia (Argilés et al., 2014). Up to 75% of patients with CKD who are undergoing dialysis show signs of wasting (Mak et al., 2011). Cachexia leads to poor outcomes and is an important risk factor for mortality. Cachexia is very different from malnutrition because it cannot be overcome by nutritional supplementation. There are currently few, if any, effective therapies against cachexia (Fearon et al., 2013; Penna et al., 2011).

There are now known to be at least two types of uncoupling protein 1 (UCP1)-expressing adipocytes (Peirce et al., 2014). Classical brown fat is located mainly in the interscapular region in rodents, whereas pockets of such cells can also be found in white adipose tissues upon cold exposure or with certain hormones. These latter cells come from a distinct cell lineage from classical brown fat and are termed beige adipocytes (Wu et al., 2012). The thermogenic activity of brown/beige fat contributes significantly to energy expenditure in rodents. Both brown and beige fat cells are also found in humans and play a role in energy homeostasis (Cypess et al., 2013; Virtanen et al., 2009). Several studies have described activation of brown fat in rodent models of cancer cachexia; anecdotal reports also show activated brown fat in at least some cachectic patients (Bianchi et al., 1989; Bing et al., 2000; Brooks et al., 1981; Roe et al., 1996; Shelloch et al., 1986; Tsoli et al., 2013). “Browning” of the white fat depots drives wasting in rodent models of cancer cachexia (Kir et al., 2014; Petruzzelli et al., 2014; Tsoli et al., 2012).

Our recent study identified parathyroid hormone-related protein (PThrP), a tumor-derived small polypeptide, as an inducer of thermogenic gene expression and wasting in adipose tissue (Kir et al., 2014). Interestingly, neutralization of PThrP by a specific antibody attenuated wasting of both fat tissue and skeletal muscle in tumor-bearing mice (Kir et al., 2014). PThrP is overexpressed by many tumors and its presence in the circulation correlates with a greater degree of wasting in patients with metastatic cancer (Kir et al., 2014). PThrP and parathyroid hormone (PTh) share the same cell surface receptor, PTh/PThrP receptor or PThr (Villardaga et al., 2011). Although PTh-secreting tumors are very rare, secondary hyperparathyroidism is frequently observed among patients with CKD (Bayne and Ilidige, 2001; Levin et al., 2007; Tentori et al., 2015). Here, using fat-specific PThrP-deficient mice, we investigated the role of the adipose PTh/PThrP pathway in cachexia associated with both CKD and cancer. Our results demonstrate that mice lacking PThr in their fat tissue are resistant to cachexia driven by renal failure and tumors.

RESULTS

5/6 Nephrectomy Causes Adipose Tissue Browning and Cachexia

5/6 Nephrectomy is a common experimental model for kidney failure; this involves removal of one kidney and two-thirds of the other (DeBoer, 2009). Nephrectomized mice suffer cachexia and have elevated circulating PTh. We used this model to investigate the roles of PTh in CKD-associated cachexia.
Nephrectomized mice developed uremia as assessed by blood urea nitrogen (BUN) levels and displayed elevated circulating PTH (Figures 1A, 1B, and S1A). These mice showed reduced body weight compared to sham-operated controls (Figure 1C). The weight loss phenotype was accompanied by increased energy expenditure, as shown by elevated O₂ consumption and elevated heat production (Figures 1D and S1B). CO₂ production was also elevated with no changes in respiratory quotient (Figures S1C and S1D). Importantly, the weight loss was not due to increased physical activity or reduced food intake (Figures 1E and S1E). We further documented the cachexia phenotype by measuring weight of fat and skeletal muscle tissues. Epididymal white adipose tissue (eWAT; a visceral fat depot), interscapular brown fat (iBAT), and gastrocnemius muscle all exhibited significant decreases in total mass (Figures 1F, S2A, and S2B). We performed gene expression analysis to understand the mechanisms involved. Expression of the thermogenic genes Ucp1, Dio2, Cidea, and Pgc1a were induced in iWAT, iBAT, and to a lesser extent in eWAT (Figures 1G, 1H, and S2C). Skeletal muscle wasting of the nephrectomized mice was also accompanied by a decrease in expression of the pro-growth hormone Igf1 and induction of the muscle atrophy-related genes Murf-1, Atrogin-1, and Myostatin (Figure 1).
and deep cervical fat samples were collected from patients undergoing parathyroidec-tomy for PHPT. The control group samples were collected from patients undergoing thyroidec-tomy for benign pathologies (i.e., Graves disease, benign goiter, and Hurtle-cell neoplasm). Importantly, none of the patients had hyperparathyroidism, which is known to be involved in thermogenic regulation (Table S1). We first compared subcutaneous and deep cervical fat samples from all patients and tested mRNA levels of some marker genes previously described by a study examining gene expression profiles of these anatomical locations (Cypess et al., 2013). We found that expression of UCP1 and LHx8 is elevated in deep cervical fat (Figure 3A) while expression of LEPTIN and SHOX2 is enriched in subcutaneous samples (Figure 3B). We next compared mRNA levels of thermogenic genes in the PHPT and control groups. Although expression of these genes in subcutaneous fat does not differ between the two groups (Figure 3C), we observed significant up-regulation of CIDEA and PGClA and an upward trend for UCP1 and DIO2 expression in PHPT deep cervical samples (Figure 3D). The deep cervical fat has characteristics of both brown and beige fat with significant thermogenic capacity (Cypess et al., 2013). Therefore, it is likely that upregulation of thermogenesis in such fat depots by PTH may contribute significantly to hypermetabolism.

**PTHR and PTH Signal through PTHR to Induce Ucp1**

To investigate the role of PTHR in cachexia in the models of kidney failure and cancer, we crossed PTHR-floxed mice (Pthr1<sup>lox/lox</sup>) (Kobayashi et al., 2002) with Adiponectin-Cre mice to generate fat cell-specific knockout animals (Adipo-PTHR-KO) (Figure S3). We isolated primary fat cells from these mice and treated them with PTHrP, PTH, or norepinephrine. Whereas norepinephrine robustly induced Ucp1 mRNA expression in the knockout cells, PTHrP and PTH completely failed to upregulate Ucp1 (Figure 4A). Similarly, PTHrP treatment of the knockout mice did not induce expression of the thermogenic genes in white and brown fat depots (Figures 4B–4D). These findings indicate that PTH and PTHrP depend entirely on the well-defined receptor PTHR to drive the thermogenic gene program. Of note, we also tried to generate skeletal muscle-specific PTHR knockout mice using human skeletal alpha (HSA)-Cre driver. To our surprise, we were unable to deplete PTHR in skeletal muscle tissue despite obtaining very robust Cre expression. This is in agreement with a previous report demonstrating PTHR localization in Pax7+ satellite cells and CD34+ hematopoietic stem cells but not in the mature myotubes (Kimura and Yoshikawa, 2014).

**Adipo-PTHR-KO Mice Are Resistant to 5/6 Nephrectomy-Driven Cachexia**

Next, we examined the contribution of elevated circulating PTH to cachexia associated with 5/6 nephrectomy. We examined metabolic phenotypes of 5/6 nephrectomized Adipo-PTHR-KO and their wild-type (WT) littermates. Both groups developed similar uremia and secondary hyperparathyroidism upon 5/6 nephrectomy (Figures 5A, 5B, and S4A). As shown in Figures 1D and S1B, energy expenditure and heat production of the WT mice significantly increased upon nephrectomy while their physical activity was significantly decreased (Figures 5C, 5D, and S4B). Interestingly, nephrectomy-driven hypermetabolism was greatly suppressed in Adipo-PTHR-KO mice while their physical activity was improved (Figures 5C, 5D, and S4B). CO2 production, respiratory quotient, and food intake were unchanged among Adipo-PTHR-KO mice (Figures S4C–S4E). In agreement with the metabolic phenotypes, nephrectomized Adipo-PTHR-KO mice lost weight to a lesser extent compared to the WT nephrectomy group, which exhibited severe cachexia (Figure 5E). Wasting of fat tissue and, surprisingly, wasting of skeletal muscle were improved in the Adipo-PTHR-KO group (Figure 5F).

Histological examination of adipose tissues and skeletal muscle showed that deletion of PTHR in fat tissue prevented fat droplet shrinkage and muscle fiber atrophy observed in the WT nephrectomy group (Figure 6A). PTHR deletion also blocked upregulation of thermogenic genes in fat tissues (Figures 6B–6D). In addition to preserving muscle mass, nephrectomized Adipo-PTHR-KO mice also maintained their muscle strength, as evidenced by improved grip strength compared to the WT nephrectomy group (Figure 6E).
nephrectomized WT mice (Figure 6E). In accordance, induction of atrophy-related genes Murf-1, Atrogin-1, and Myostatin was inhibited in the Adipo-PTHR-KO mice (Figure 6F). These findings suggest existence of a PTH-PTHR signaling axis mediating cachexia in kidney failure and an important crosstalk between fat and skeletal muscle wasting. One possible explanation for this crosstalk is that PTH-induced, fat tissue-derived circulating factors may trigger muscle atrophy. Using microarrays, we investigated global gene expression profiles of fat cells treated with PTH or PTHrP. Expression of 13 genes that encode secreted proteins were upregulated by both PTH and PTHrP (Figure S5). These include the circulating bioactive cytokines Il6, Il33, Cxcl1, Cxcl5, and Cxcl14.

Adipo-PTHR-KO Mice Are Resistant to Lewis Lung Carcinoma-Driven Cachexia

Results from the 5/6 nephrectomy study prompted us to examine tumor-driven cachexia in the Adipo-PTHR-KO mice using the Lewis Lung Carcinoma (LLC) model. Remarkably, 16 days after tumor inoculation, mice with PTHR knockout in adipose tissues did not suffer significant weight loss whereas WT mice displayed evident cachexia (Figure 7A). PTHR depletion in fat tissues attenuated wasting of both adipose tissue and skeletal muscle without changing average tumor mass (Figures 7B and 7C). Histological examination of these tissues showed larger fat droplets and muscle fibers in the tumor-bearing Adipo-PTHR-KO mice (Figure 7D). Similar results were also obtained from Adipo-PTHR-KO mice killed 14 days after tumor inoculation when WT controls were experiencing moderate cachexia (Figure S6). Gene expression analysis on these mice indicated that Adipo-PTHR-KO group is resistant to browning induced by LLC tumors (Figures 7E–7G). Muscle function of the knockout mice was also significantly improved as atrophy-related gene expression in their muscle tissue was significantly suppressed (Figures 7H and 7I). These observations are similar to our published results on PTHrP neutralization by a specific antibody given systemically (Kir et al., 2014), but these new data indicate that cachectic effects of tumor-derived PTHrP are essentially entirely mediated by PTHR expressed in fat tissues.

DISCUSSION

Cachexia is a debilitating disease leading to poor outcomes in cancer and other chronic disorders. There is an urgent need for therapeutics against the wasting syndrome. PTHrP is produced by many tumors and it is often involved in the hypercalcemia of malignancy (Iguchi et al., 2001; Mundy and Edwards, 2008). Our recent work demonstrated that tumor-derived PTHrP drives adipose tissue browning and cachexia in tumor-bearing mice even when it is present at levels not sufficient to increase plasma calcium. PTH and PTHrP share the same receptor specificity and therefore may both be involved in cachexia. Circulating PTH has been reported to be elevated in many chronic diseases, particularly, in CKD (Childs et al., 2012; Dolecek et al., 2003; Jackson et al., 2013; Levin et al., 2007; Visser et al., 2003). Taken together, our new findings describe an important role for PTH in CKD-related cachexia. Like PTHrP, PTH potently stimulates thermogenic gene expression in fat tissue. Importantly and surprisingly, loss of PTHR selectively in fat tissues blocks fat and muscle wasting in both CKD and cancer cachexia models. Because loss of PTHR in fat tissue also improves muscle mass and strength, these data argue that there must be indirect
mechanisms (involving PTHR function in fat tissue) through which pathological signals go from fat to skeletal muscle.

Chronic kidney failure is a very complex disease. Although malnutrition or various comorbid events may contribute to weight loss in patients with CKD, increased energy expenditure in certain patients correlates with increased mortality and cardiovascular disease (Neyra et al., 2003; Wang et al., 2004). Interestingly, patients undergoing dialysis with hyperparathyroidism were shown to have increased resting energy expenditure that may be reduced after parathyroidectomy (Cuppari et al., 2004). Increased Ucp1 expression in brown fat was previously implicated in the weight loss of 5/6 nephrectomized mice (Cheung et al., 2007, 2014). Our findings support the involvement of adipose tissue thermogenesis in this cachexia model and identify PTH as an important driver of thermogenesis and hypermetabolism. As shown above, PTH treatment potently induces thermogenic genes in fat tissue of mice. Furthermore, primary hyperparathyroidism in humans, although not associated with cachexia, led to increased expression of thermogenic genes in deep cervical fat, which possesses some characteristics of brown/beige fat tissue, including significant thermogenic capacity. Therefore, it is possible that secondary hyperparathyroidism associated with CKD and other chronic diseases in humans may lead to inappropriate thermogenesis and trigger wasting in the presence of other contributing factors. Our findings also showed that the PTH/PTHrP pathway stimulates expression of a number of secreted factors in fat cells. Therefore, this pathway has multiple effects on the fat tissue, which may trigger hypermetabolism and skeletal muscle atrophy. Further studies are needed to establish a role for these other secreted factors in PTH/PTHrP-driven muscle atrophy.

Finally, our work indicates that the PTH/PTHrP pathway is a generally important player in cachexia and targeting this pathway may be useful in fighting cachexia. In fact, our earlier study showed that neutralization of PTHrP by a specific antibody blocks tumor-driven cachexia (Kir et al., 2014). It will be interesting to test if similar approaches may be beneficial against CKD-driven cachexia. Unlike PTHrP, PTH is also involved in regulation of mineral metabolism. Therefore, therapeutic targeting of PTH or PTHR may be limited by their crucial roles in calcium and phosphate regulation.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Synthetic mouse PTHrP(1–34), rat PTH(1–34), and rat PTH(1–84) were purchased from Bachem. Mouse intact PTH (1–84) ELISA assay kit was from ALPCO. Norepinephrine was purchased from Sigma. Total-ERK1/2 antibody (#9102) and anti-UCP1 (ab10983) were purchased from Cell Signaling and Abcam, respectively.

**Animal Studies**

All animal experiments were approved by the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center. In all studies, lean, 6- to 8-week-old, male, C57BL/6 mice were used. Pth1r-floxed mice were a gift from Dr. Henry Kronenberg (MGH) (Kobayashi et al., 2002). Pth1r-floxed mice (±Adiponectin-Cre) were maintained on a pure C57BL/6 background. All other mice were obtained from Charles River Laboratories. Mice were maintained in 12 hr light/dark cycles (6 am to 6 pm) at 24 °C and fed standard irradiated rodent chow diet; 5 million LLC cells per mouse were injected subcutaneously over the flank. Non-tumor-bearing control mice received the vehicle (PBS) only. Mice received subcutaneous injections of PTHrP and PTH peptides and all mice were killed between 4 pm and 7 pm. Plasma was collected into EDTA tubes for the PTH ELISA assay. Heparin tubes were used to collect plasma for BUN measurements, which were performed with a Vitros analyzer. Whole-body energy metabolism was evaluated using a Comprehensive Lab Animal Monitoring System (CLAMS, Columbia Instruments). Mice were put into metabolic cages between post-surgery weeks.
CaCl₂, 2.4 U/ml dispase II (Roche), and 1.5 U/ml collagenase D (Roche). Digested with PBS, minced, and digested for 45 min at 37°C. Inguinal fat tissue was dissected, washed in 10% FBS, filtered through a 40-μm cell strainer, centrifuged, and resuspended as a cell suspension. The cells were then cultured in adipocyte culture medium (DMEM/F12 plus glucagon [1:1; Invitrogen], pen/strep, and 10% FBS), filtered through a 40-μm cell strainer, centrifuged as above, resuspended in adipocyte culture medium and plated. 

**Grip Strength**

Forelimb grip strength was assessed on the same day that the mice were killed. Each mouse was allowed to grab a bar attached to a force transducer (Model DFX II; Chatillon) as it was pulled by the tail horizontally away from the bar (Cabe et al., 1978).

### Cell Culture

Inguinal stromal-vascular (SV) fractions were obtained from 30- to 35-day-old male mice by the following procedure. Inguinal fat tissue was dissected, washed with PBS, minced, and digested for 45 min at 37°C in PBS containing 10 mM CaCl₂, 2.4 U/ml dispase II (Roche), and 1.5 U/ml collagenase D (Roche). Digested tissue was filtered through a 100-μm cell strainer and centrifuged at 600 × g for 5 min to pellet the SV cells. These were then resuspended in adipocyte culture medium (DMEM/F12 plus glucagon [1:1; Invitrogen], pen/strep, and 10% FBS), filtered through a 40-μm cell strainer, centrifuged, and resuspended in adipocyte culture medium and plated. The SV cells were grown to confluence for differentiation, which was induced by the adipogenic cocktail containing 1 μM dexamethasone, 5 μg/ml insulin, 0.5 μM isobutylmethylxanthine (DMI), and 1 μM rosiglitazone in adipocyte culture medium. Two days after induction, cells were maintained in adipocyte culture medium containing 5 μg/ml insulin and 1 μM rosiglitazone. RT-qPCR

RNA was extracted from cultured cells or frozen tissue samples using TRIzol (Invitrogen), purified with QIAGEN RNeasy minicolumns, and reverse-transcribed using High Capacity cDNA Reverse Transcription kit (Applied Biosystems). Resulting cDNA was analyzed by qPCR using SYBR GreenER PCR Master Mix (Invitrogen). Reactions were performed in 384-well format using an ABI PRISM 7900HT instrument (Applied Biosystems). Relative mRNA levels were calculated using the comparative CT method and normalized to cyclophilin F (control) and muscle tissues were dissected and weighed (f).

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determined by Bio-Rad Protein assay and 30 μg of protein lysate was used in each SDS-PAGE run. PVDF membrane was blotted with antibodies in TBS containing 0.05% Tween and 5% BSA. For secondary antibody incubation, TBS-T containing 5% milk was used. ECL western blotting substrates from Pierce were used for demonstration of the results.

Human Study
This study was approved by the Human Studies Institutional Review Board of the Massachusetts General Hospital (MGH). Individuals who were scheduled to undergo neck surgery at MGH were identified by K.P.E. and W.L. and written informed consent was obtained by K.P.E. and W.L. before surgeries. Subcutaneous and deep cervical fat samples were collected from patients undergoing parathyroidectomy for PHPT. The control group samples were collected from patients undergoing thyroidectomy for benign pathologies (i.e., Graves disease, benign goiter and Hurtle-cell neoplasm). Samples collected included excess fat tissue that was resected as part of the surgical procedure.

Statistical Analysis
Values are expressed as mean ± SEM. Significant analysis was performed using two-tailed, unpaired t test for single variables and two-way ANOVA followed by Bonferroni post-tests for multiple variables. Two-way ANOVA with repeated measures was used when analyzing body weight and metabolic data such as oxygen consumption (VO₂), carbon dioxide consumption (VCO₂), physical activity, and heat output. Graphpad Prism software was used for ANOVA analysis.

ACCESSION NUMBERS
The accession number for the microarray dataset reported in this paper is GEO: GSE74082.
Figure 7. Adipo-PTHr-KO Mice Are Resistant to LLC Tumor-Driven Cachexia

(A–D) Mice inoculated with LLC cells were killed 16 days later (n = 6). Carcass weight (calculated by subtracting tumor weight from the total weight) (A) and tumor weight (B) are shown. Fat and muscle tissues were dissected and weighed (C). H&E staining of adipose tissues and gastrocnemius muscle is shown (D).

(E–G) Mice inoculated with LLC cells were killed 14 days later (n = 4–5). mRNA levels in iWAT (E) and iBAT (F) were measured with RT-qPCR. Total UCP1 and ERK1/2 protein levels in inguinal fat tissue were determined by western blotting (G).

(H and I) Mice inoculated with LLC cells were killed 16 days later (n = 6). Muscle function was analyzed by grip strength (G). mRNA levels in gastrocnemius muscle (H) were measured with RT-qPCR.

The values are mean ± SEM. *Differences between the LLC and non-tumor-bearing groups. #Differences between the WT-LLC and KO-LLC groups. *p < 0.05, **p < 0.005, ***p < 0.0005, #p < 0.05, ##p < 0.005, ###p < 0.0005. See also Figure S6.
SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2015.11.003.

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