Elevated (Pro)renin Receptor Expression Contributes to Maintaining Aerobic Metabolism in Growth Hormone Deficiency

Yasufumi Seki,[1] Midori Yatabe,[1] Chikahito Suda,[1] Satoshi Morimoto,[1] and Atsuhiro Ichihara[1]

[1]Department of Medicine II, Endocrinology and Hypertension, Tokyo Women's Medical University, Tokyo 162-8666, Japan

Context: Growth hormone deficiency (GHD) leads to obesity and may induce tissue hypoxia. As (pro)renin receptor [(P)RR] is reported to contribute to the aerobic metabolism by stabilizing pyruvate dehydrogenase (PDH), it may play a substantial role in GHD.

Objective: We aimed to investigate serum soluble (P)RR [s(P)RR] concentration, the origin of s(P)RR, and significance of (P)RR in GHD.

Design, Setting, and Participants: Serum s(P)RR concentration was examined in 72 patients with pituitary diseases, including 32 patients with severe GHD (SGHD) and after GH replacement in 16 SGHD patients. Leptin-deficient *ob/ob* obese mice were treated with pegvisomant, a GH receptor antagonist, to explore the source of elevated serum s(P)RR in GHD. Adipocytes were cultured with 5% O_2 to examine the effects of hypoxia.

Results: Serum s(P)RR concentration was higher in patients with SGHD than in those without SGHD. Obesity was the important determinant of s(P)RR concentration. Serum s(P)RR concentration significantly decreased after GH replacement in SGHD patients. (P)RR mRNA expression was increased specifically in the adipose tissue (AT) of pegvisomant-treated obese mice compared with that of control obese mice. Hypoxia in cultured adipocytes increased (P)RR expression without affecting the PDH E1 β subunit (PDHB) expression; however, with (P)RR knockdown by small interfering RNA, hypoxia significantly decreased the expression of PDHB.

Conclusion: GHD patients showed increased serum s(P)RR concentration, possibly caused by obesity and hypoxia. (P)RR expression in AT of GHD patients may be elevated to help maintain aerobic metabolism under hypoxia. Thus, the elevated serum s(P)RR level may reflect hypoxia in ATs.

Growth hormone deficiency (GHD) is the most common hormone deficit in hypopituitarism. GHD not only causes obesity [1], glucose intolerance [2], and dyslipidemia [3] but also decreases aerobic exercise ability [4, 5], as a result of restricted oxygen delivery capacity [6]. A current method of diagnosis for GHD depends on multiple GH stimulation tests, such as by insulin and glucagon [7], and these tests place a burden on patients in terms of economy and time. Therefore, an easier and more effective biomarker of GHD has been desired.

Previous studies showed that (pro)renin receptor [(P)RR] contributes to tissue renin-angiotensin system-related pathogenesis, such as hypertension and diabetes [8–10]. Furthermore, there is a report that (P)RR is expressed in pituitary adenoma cells and regulates secretion of GH [11]. In addition, a recent study demonstrated an important role of (P)RR under hypoxic condition [12], in which (P)RR binds to pyruvate dehydrogenase (PDH) protein and maintains the PDH activity through inhibition of its degradation. In fact, it has been reported that serum concentration of the N-terminal domain of (P)RR, called the soluble form of (P)RR [s(P)RR], is elevated in various hypoxic conditions, such as hypertension [13], sleep apnea syndrome [14], and chronic heart failure [15]. Furthermore, hypoxia in adipose tissue (AT) of obese mice [16–19] and increased expression of (P)RR in AT of obese mice have also been reported [20].

This study was designed to evaluate if the serum s(P)RR level is elevated in GHD and if so, to assess the origin of s(P)RR and determine the significance of elevated s(P)RR concentration in GHD.

1. Subjects and Methods

A. Study Participants

In this prospective observational study, 72 adult patients with pituitary or hypothalamic diseases at Tokyo Women's Medical University Hospital were enrolled between December 2011 and December 2013. The study protocol was approved by the Ethics Committee of Tokyo Women's Medical University (2303-R5) and registered in the University Hospital Medical Information Network Clinical Trial Registry (UMIN000006222) on 1 October 2011, and all patients provided written, informed consent. Medical records were used to obtain patient characteristics (such as age, sex, body weight, and history of hypertension) and metabolic parameters (such as creatinine). All patients with hypopituitarism or diabetes insipidus received appropriate supplementation of hydrocortisone, levothyroxine, gonadal steroids, and/or desmopressin. Obesity was defined as body mass index (BMI) of higher than 25 kg/m^2, according to the criteria by the Japan Society for Study of Obesity [21]. All patients underwent the GH-releasing peptide 2 (GHRP-2) test to diagnose adult severe GHD (SGHD), defined as peak GH concentration <9 ng/mL [22]. A GH cutoff value of 9 ng/mL with GHRP-2 corresponded to a GH value of 1.8 ng/mL with the insulin tolerance test when the GH value was calibrated with recombinant World Health Organization 98/574 standard [22]. Although the GHRP-2 test is not included in the Endocrine Society Guidelines [23], the GHRP-2 test is considered to be safe [24] and widely used to diagnose SGHD in Japan because of its high sensitivity and specificity compared with the insulin tolerance test [25]. The peak GH concentration after GHRP-2 stimulation was ≥9 ng/mL in 40 patients (SGHD$^-$) and <9 ng/mL in 32 patients (SGHD$^+$). Etiologies of the SGHD$^-$ patients included 11 nonfunctioning pituitary adenoma, 10 Rathke cyst, eight prolactinoma, two Cushing disease, two acromegaly, and others. Etiologies of the SGHD$^+$ patients included 17 nonfunctioning pituitary adenoma, five craniopharyngioma, four Rathke cyst, and others. In all patients with hormonally functioning tumor, their remissions were confirmed. Sixteen of the SGHD$^+$ patients received GH replacement therapy for an average of 8.4 months (6 to 18 months). The GH doses were titrated to maintain their insulin-like growth factor 1 (IGF-1) levels to age-adjusted IGF-1 levels unless adverse effects manifested.

B. Animals

All procedures and animal care were approved by our Institutional Animal Research Committee and conformed to the animal care Guideline for the Care and Use of Laboratory Animals of Tokyo Women's Medical University. Male C57BL/6-Ham-Slc *ob/ob* and +/+ mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were fed a normal diet, and at 10 weeks of age, 40 mg/kg pegvisomant (Pfizer, Tokyo, Japan), a GH receptor antagonist, or equivalent volume of saline was injected subcutaneously for 5 consecutive days. One day after the final injection, the mice were killed by decapitation under isoflurane inhalation anesthesia (Intervet, Tokyo, Japan). The serum, liver, kidneys, perigonadal fat as white AT (WAT), and gastrocnemius muscles were obtained. WAT weight was measured, and the obtained organs were quickly frozen in liquid N_2 and stored at $-80°C$.

C. Mouse Adipocyte Experiments

The 3T3-L1 mouse preadipocyte cells were obtained from the Japanese Collection of Research Bioresources [Kyoto, Japan; JCRB9014, Research Resource Identifier (RRID): CVCL_0123]. Cells were seeded on collagen-coated plates (Corning, Corning, NY) at 4000 cells/cm^2, incubated for 6 days with Dulbecco's modified Eagle medium (DMEM; Life Technologies, Tokyo, Japan) with 10% bovine serum albumin (Life Technologies, Tokyo, Japan), and then incubated for 3 days in the differentiation medium: DMEM with 10% fetal bovine serum (Life Technologies, Tokyo, Japan), 0.5 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO), 1 μM dexamethasone (Sigma-Aldrich, St. Louis, MO), and 10 μg/mL insulin (Wako, Osaka, Japan). Thereafter, maintenance medium of DMEM, including 10% fetal bovine serum and 10 μg/mL insulin, was replaced every 3 days after differentiation. The cells were used for experiments, 5 to 8 days after seeding. All medium contained 50 U/mL penicillin and 50 μg/mL streptomycin (Nacalai Tesque, Kyoto, Japan). Cells were maintained in humidified air with 5% CO_2 at 37°C.

For small interfering RNA (siRNA) experiments, differentiated cells in maintenance medium were transfected as previously described [26]. In brief, cells were replated at 116,000 cells/cm^2, and (P)RR siRNA (MSS230245:ATP6AP2 siRNA) or scrambled siRNA (Thermo Fisher Scientific, Waltham, MA) was transfected using Lipofectamine RNAiMAX Transfection Reagent (Thermo Fisher Scientific, Waltham, MA) and OptiMEM (Life Technologies, Tokyo, Japan), according to the manufacturer's protocol.

At 48 hours after transfection, the medium was replaced to serum-free maintenance medium, and cells were placed into the Hypoxic Incubator Chamber (Stemcell Technologies, Vancouver, Canada) to be incubated under an hypoxic or a normoxic condition at 37°C for 8 hours. To maintain an hypoxic condition, the chamber was filled with 5% oxygen, 5% CO_2, and 90% nitrogen.

For Western blot, whole cell lysates were prepared using lysis buffer [Tris-buffered saline (TBS; 25 mM Tris, 137 mM NaCl, 2.68 mM KCl), 1% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitor cocktail (Nacalai Tesque, Kyoto, Japan)]. Protein samples (10 μg) were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Membranes were blocked in 5% (w/v) nonfat dry milk in TBS containing 0.1% Tween 20 at room temperature for 1 hour, incubated overnight at 4°C with primary antibodies, washed in TBS containing 0.1% Tween 20, and incubated for 1 hour with appropriate secondary immunoglobulin G (IgG) horseradish peroxidase-linked antibodies. Blots were visualized using chemiluminescence reagent (Nacalai Tesque, Kyoto, Japan) and LuminoGraph I (Atto, Tokyo, Japan). Antibodies used were anti-ATP6AP2 (HPA003156, RRID: AB_1078245; Atlas Antibodies, Bromma, Sweden), anti-PDH E1 β subunit (PDHB; WH0005162M3, RRID: AB_2236961; Sigma-Aldrich, St. Louis, MO), anti-β-actin (#4980, RRID: AB_2223172; Cell Signaling Technology, Danvers, MA), anti-rabbit IgG (A0545, RRID: AB_257896; Sigma-Aldrich, St. Louis, MO), and anti-mouse IgG (NA9310, RRID: AB_772193; GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Densitometry was performed using ImageJ software (NIH, Bethesda, MD).

D. Assays

Creatinine, plasma glucose, [hemoglobin A1c (HbA1c) National Glycohemoglobin Standardization Program (NGSP)], low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, and triglyceride were measured by standard laboratory methods at our clinical laboratory center. Estimated glomerular filtrating rate (GFR) was calculated using the formula developed by the Japanese Society of Nephrology [27]. Serum GH concentrations of patients were measured using enzyme immunoassay (Tosoh Bioscience, Tokyo, Japan), calibrated with recombinant WHO 98/574 standard. Serum IGF-1 concentrations of patients were measured using the immunoradiometric assay "Daiichi" (Fujirebio, Tokyo, Japan). IGF-1 standard deviation (SD) score was calculated based on the age- and sex-specific normative data of IGF-1 in the Japanese population [28]. Serum IGF-1 concentrations of mice were measured using the insulin-like growth factor I enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN). Serum s(P)RR concentrations of patients and mice were measured using the Soluble (Pro)renin Receptor ELISA Assay Kit (Takara Bio, Shiga, Japan) [29]. Each ELISA assay was performed according to the manufacturer's protocol, and absorbance was measured by Chameleon V (Hidex, Turku, Finland) for s(P)RR and Spectra Max i3 (Molecular Devices, San Jose, CA) for IGF-1.

E. Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from mouse tissue and cell lysate using TRIzol (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was performed with total RNA using the High Capacity cDNA Reverse Transcription Kit (Life Technologies, Tokyo, Japan). Expressions of (P)RR—furin, and a disintegrin and metalloprotease (ADAM)19—mRNA were determined by quantitative real-time reverse transcriptase-polymerase chain reaction using TaqMan Gene Expression Assay (Life Technologies, Tokyo, Japan) on StepOnePlus (Life Technologies, Tokyo, Japan). Target mRNA expression was corrected by 18S ribosomal mRNA expression, shown as relative expression ratio. All samples were analyzed in duplicate.

F. Statistical Analyses

All data were shown as means $\pm$ SD except for peak GH response to GHRP-2 which was expressed as median (range). Baseline characteristics of the patients were compared between SGHD$^-$ and SGHD$^+$ patients by unpaired t test, whereas levels of GH peak response to GHRP-2 were by Mann-Whitney U test. Categorical variables were compared using the Pearson's χ^2 test. Serum s(P)RR and IGF-1 concentration and IGF-1 SD score of the patients were compared between before and after GH replacement therapy by paired t test. Multiple comparisons were analyzed using Tukey-Kramer test. Because of skewed distribution, levels of GH peak response to GHRP-2 were log transformed for regression analyses. In multivariate regression analysis, explanatory factors were selected from the results of univariate regression analysis. The threshold for significance was $P < 0.05$. All statistical analyses were performed using JMP Pro 12 (SAS Institute, Tokyo, Japan).

3. Results

A. Characteristics of Patients

Characteristics of the patients with (SGHD$^+$) or without (SGHD$^-$) GHD are shown in Table 1. BMI and serum levels of creatinine and triglyceride were significantly higher, and estimated GFR and serum HDL-cholesterol level were significantly lower in the SGHD$^+$ patients than in the SGHD$^-$ patients. Number of deficit anterior pituitary hormones and frequency of diabetes insipidus were higher in the SGHD$^+$ patients than in the SGHD$^-$ patients, and IGF-1, IGF-1 SD scores, and GH peak response to GHRP-2 were significantly lower in the SGHD$^+$ patients than in the SGHD$^-$ patients.

Table 1. Baseline Characteristics of the Study

	SGHD⁻ (n = 40)	SGHD⁺ (n = 32)	*P* Trend
Male, n (%)	10 (25)	14 (44)	0.094
Age, y	47.0 ± 17.0	51.2 ± 18.2	0.321
BMI, kg/m^2	21.6 ± 3.4	25.7 ± 5.3	<0.001
Hypertension, n (%)	12 (30)	9 (28)	0.862
Systolic BP, mmHg	122 ± 19	120 ± 15	0.638
Diastolic BP, mmHg	72 ± 13	70 ± 10	0.389
Creatinine, mg/dL	0.68 ± 0.18	0.82 ± 0.15	0.001
Estimated GFR, mL/min/1.73 m^2	83.6 ± 17.1	69.4 ± 15.2	<0.001
Plasma glucose, mg/dL	93.2 ± 10.9	98.5 ± 38.0	0.406
HbA1c (NGSP), %	5.6 ± 0.5	5.9 ± 1.0	0.108
LDL-cholesterol, mg/dL	111.5 ± 34.2	125.7 ± 35.3	0.099
HDL-cholesterol, mg/dL	66.0 ± 15.9	52.7 ± 13.6	<0.001
Triglyceride, mg/dL	88.6 ± 36.5	159.3 ± 69.2	<0.001
Number of deficit anterior pituitary hormones	0.2 ± 0.7	2.9 ± 1.1	<0.001
Diabetes insipidus, n (%)	2 (5)	11 (34)	0.001
IGF-1, ng/mL	137.1 ± 43.9	76.6 ± 32.7	<0.001
IGF-1 SD score	-0.5 ± 1.1	-2.4 ± 1.5	<0.001
GH peak response to GHRP-2, ng/mL	30.3 (9.9–114.6)	1.4 (0.03–8.56)	<0.001

Data are shown as means ± SD except for GH peak response to GHRP-2 which is shown as median (range).
Abbreviation: BP, blood pressure.

B. Serum s(P)RR Concentration in Obese and Lean Patients With or Without SGHD

Serum s(P)RR concentration was significantly higher in the SGHD⁺ patients (23.2 ± 2.3 ng/mL) than in the SGHD⁻ patients [21.7 ± 3.5 ng/mL; Fig. 1(a)]. When the patients were divided into four groups by obesity and SGHD, the serum s(P)RR concentration in the SGHD⁺ patients with obesity (Obese SGHD⁺; 25.2 ± 3.2 ng/mL) was significantly higher than that in the SGHD⁺ patients without obesity [Lean SGHD⁺; 21.9 ± 2.9 ng/mL; Fig. 1(b)]. No significant effect of obesity on the serum s(P)RR concentration was observed in the SGHD⁻ patients (Obese SGHD⁻ 23.0 ± 6.2 ng/mL vs Lean SGHD⁻ 21.5 ± 3.0 ng/mL). Serum s(P)RR concentration in Lean SGHD⁺ was not significantly different from that of Lean SGHD⁻. Obese SGHD⁺ patients showed an insignificant tendency for an increased serum s(P)RR level than Obese SGHD⁻ patients. In the SGHD⁺ patients who received GH replacement therapy (average 2.3 ± 0.5 mg/week), the GH replacement therapy significantly increased their serum IGF-1 concentrations (87.6 ± 42.1 to 166.6 ± 51.8 ng/mL) and IGF-1 SD scores (-2.3 ± 1.6 to 0 ± 1.4) and decreased their serum s(P)RR concentrations [30.3 ± 2.7 to 26.9 ± 4.6 ng/mL; Fig. 1(c)–1(e)]. There was no significant change in body weight after GH replacement (before GH: 77.6 ± 13.1 vs after GH: 78.6 ± 13.3 kg). Changes in serum s(P)RR concentration did not significantly correlate with GH dose or changes in BMI [Fig. 1(f)].

C. Regression Analyses of Serum s(Pro)Renin Receptor Level

Univariate regression analyses of serum s(P)RR concentration showed significant positive correlations with BMI and creatinine and negative correlations with HDL-cholesterol and natural logarithm (ln) of GH peak response to GHRP-2 (Table 2). BMI was the only significant explanatory variable in a multivariate regression analysis testing BMI, HDL-cholesterol, creatinine, and ln (GH peak response to GHRP-2; Table 3).

D. (P)RR in Obese and Lean Mice With or Without Growth Hormone Receptor Blockade

Five-day treatment of pegvisomant significantly decreased serum IGF-1 levels compared with treatment with saline in both lean and obese mice [Fig. 2(a)]. The IGF-1 levels were similar between the pegvisomant-treated lean and obese mice. Blood glucose level [Fig. 2(b)],
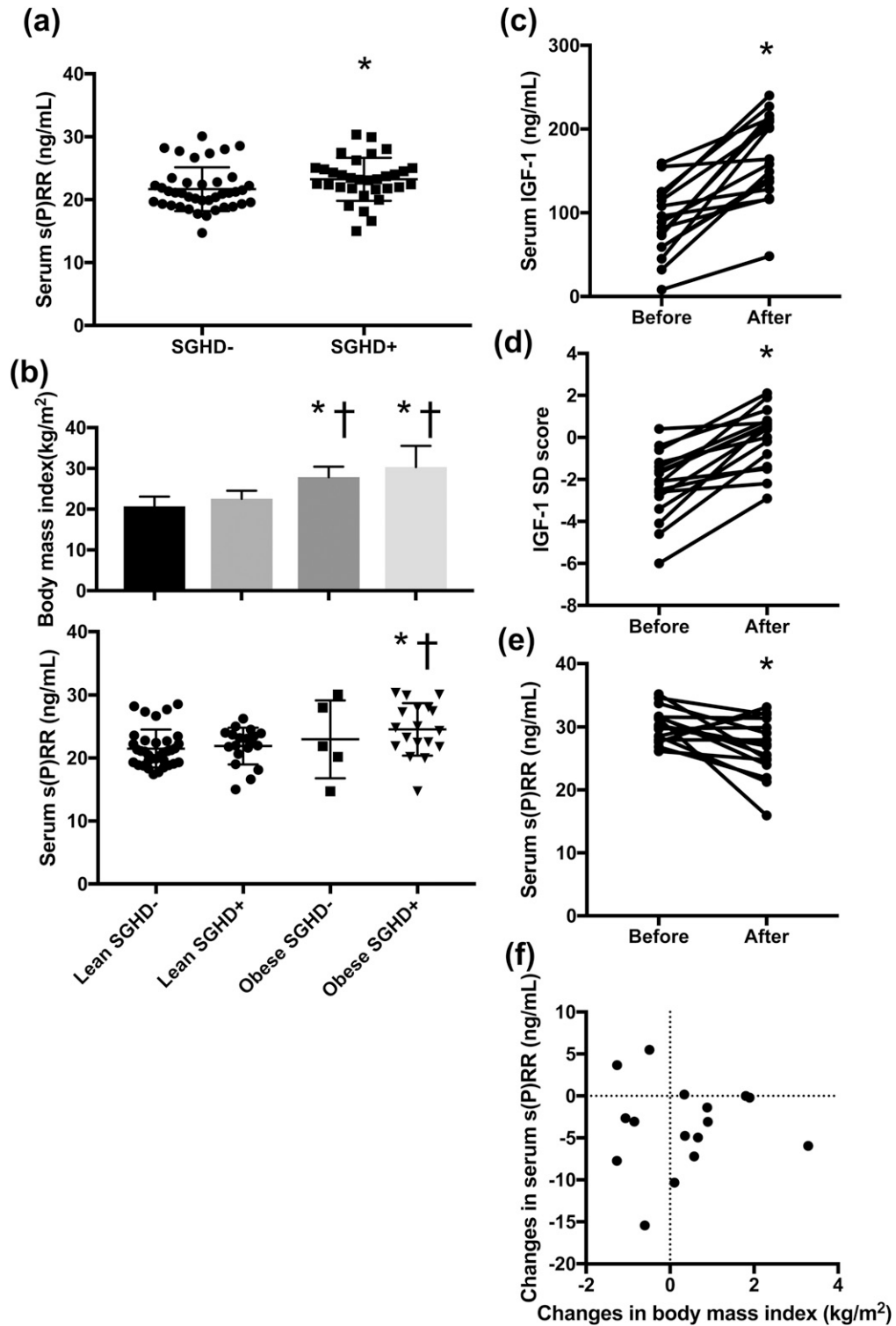
Figure 1. Effect of SGHD and obesity on serum s(P)RR concentration in humans. (a) Serum s(P)RR concentration of the patients with SGHD (SGHD$^+$, n = 32) or without SGHD (SGHD$^-$, n = 40). *$P < 0.05$ vs SGHD$^-$. (b, upper) BMI and (lower) serum s(P)RR concentration of the patients classified based on obesity and SGHD. *$P < 0.05$ vs Lean SGHD$^-$; †$P < 0.05$ vs Lean SGHD$^+$. (c–e) Change of serum (c) IGF-1 levels, (d) IGF-1 SD score, and (e) serum s(P)RR concentration, before and after GH replacement therapy (n = 16). *$P < 0.05$ vs before the therapy. (f) Correlation between the changes in serum s(P)RR concentration and the changes in BMI.

Table 2. Univariate Regression Analysis of Serum s(P)RR Level and Baseline Characteristics and Metabolic Parameters (n = 72)

Factors	r	*P* Trend
Age	−0.008	0.946
BMI	0.409	<0.001
Creatinine	0.328	0.005
Estimated GFR	−0.176	0.145
HbA1c (NGSP)	0.063	0.625
LDL-cholesterol, mg/dL	0.201	0.100
HDL-cholesterol, mg/dL	−0.291	0.015
Triglyceride, mg/dL	0.211	0.080
IGF-1 SD score	−0.089	0.465
ln (GH peak response to GHRP-2, ng/mL)	−0.284	0.017

body weight [Fig. 2(c)], and WAT weight/body weight [Fig. 2(d)] were significantly higher in obese mice than in lean mice but were not significantly different between the mice treated with saline and pegvisomant. The serum s(P)RR level was significantly higher in the obese mice than that in the lean mice regardless of the treatments [Fig. 2(e)]. Pegvisomant treatment significantly increased WAT (P)RR mRNA expression only in obese mice [Fig. 3(a)]. Expression of (P)RR mRNA in the gastrocnemius muscle, liver, or kidneys was not significantly different among the four groups. The mRNA expression of (P)RR processing enzymes, furin and ADAM19, in WAT was similar among the four groups [Fig. 3(b)].

E. Effects of Hypoxia on (P)RR Expression and PDH Activity in 3T3-L1 Cells

In the differentiated 3T3-L1 cells, 5% hypoxia treatment of 8 hours significantly increased (P)RR protein expression by 52% without altering (P)RR mRNA expression [Fig. 4(a) and 4(b)]. (P)RR siRNA treatment knocked down the (P)RR mRNA expression to 10.3 ± 4.9% compared with scrambled siRNA [Fig. 4(c)]. Hypoxia treatment did not alter PDHB expression in the cells treated with scrambled siRNA. However, hypoxia treatment significantly decreased PDHB expression in the cells treated with (P)RR siRNA [Fig. 4(d)].

3. Discussion

In this study, we found an elevated serum s(P)RR concentration in SGHD patients, especially with obesity and elevated (P)RR expression in the WAT of obese mice with GH receptor blockade. We also showed the possibility of tissue hypoxia as an additional factor contributing to elevated (P)RR expression in the AT of GHD patients.

SGHD patients showed higher serum s(P)RR concentration than those without SGHD, and GH replacement therapy ameliorated the elevated s(P)RR concentration. Higher serum s(P)RR concentration was observed in obese SGHD patients than in lean SGHD patients, and regression analyses revealed that BMI was the only substantial explanatory variable for serum s(P)RR concentration. As AT weight is increased in GHD patients [30], we considered AT as a possible source of high-serum s(P)RR in obese SGHD patients.

Table 3. Multivariate Regression Analysis of Serum s(P)RR Level (n = 72)

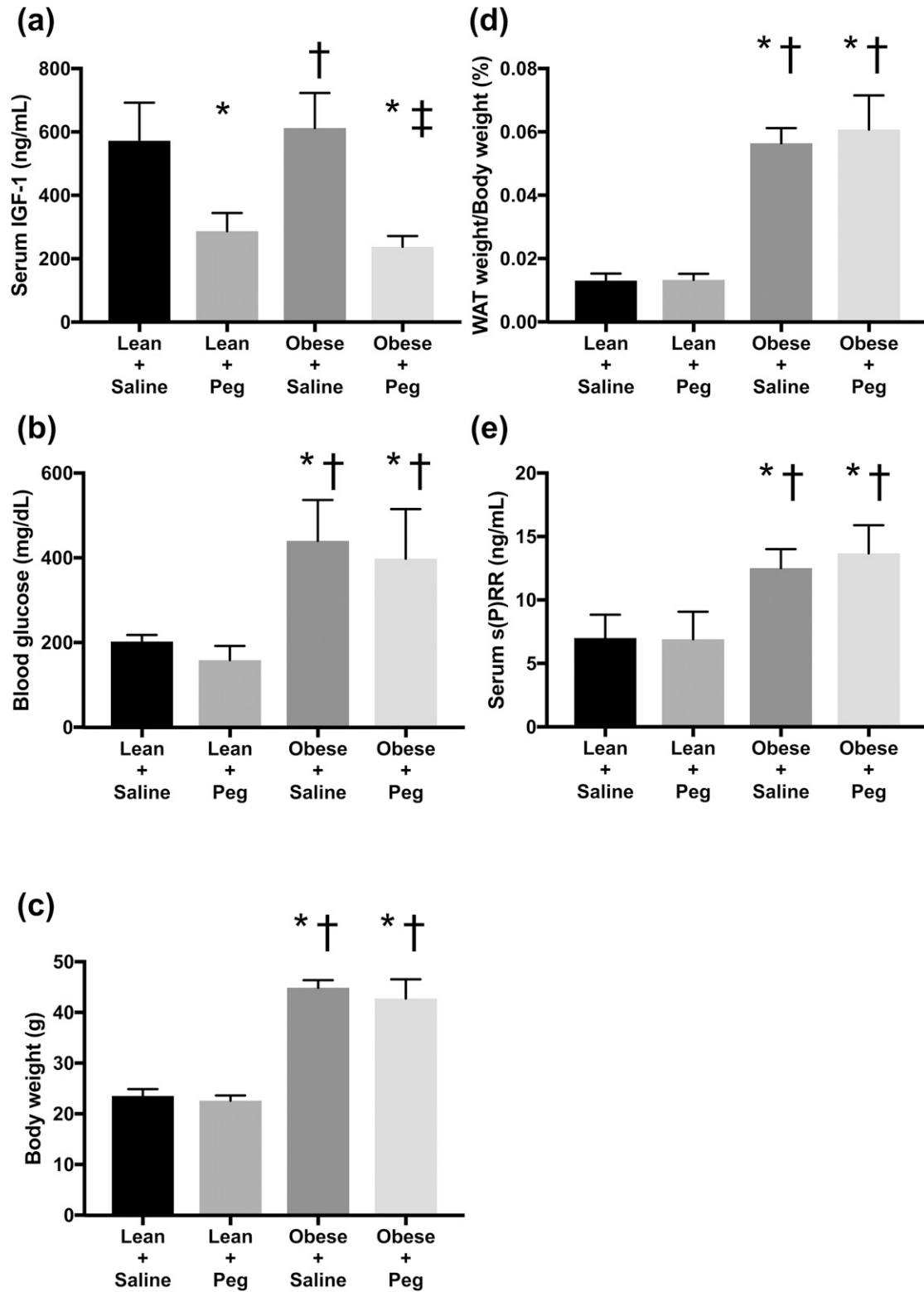| Factors	*β* Value	*P* Trend
BMI	0.210	0.037
HDL-cholesterol	−0.016	0.560
Creatinine	4.023	0.105
ln (GH peak response to GHRP-2)	−0.136	0.538

Figure 2. Effect of GH receptor blockade and obesity on serum s(P)RR concentration in mice. Serum (a) IGF-1 concentration, (b) blood glucose concentration, (c) body weight, (d) WAT weight ratio to body weight, and (e) s(P)RR concentration in lean and obese mice treated with saline or pegvisomant (Peg). *$P < 0.05$ vs Lean + Saline mice; †$P < 0.05$ vs Lean + Peg mice; ‡$P < 0.05$ vs Obese + Saline mice.
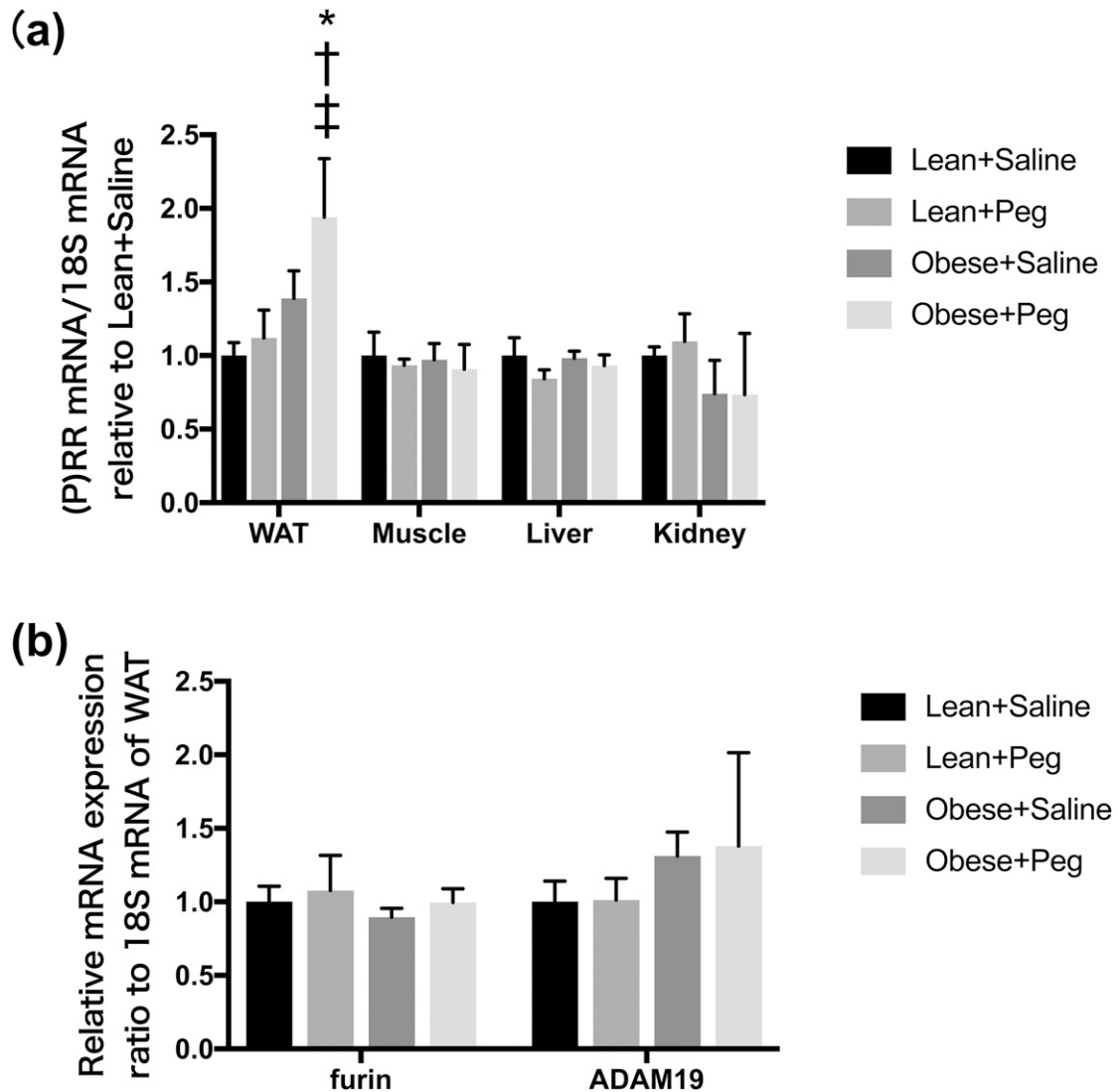
Figure 3. Expression of (P)RR mRNA and its processing enzyme mRNA in lean and obese mice treated with saline or pegvisomant (Peg). (a) Relative mRNA expression of (P)RR of WAT, gastrocnemius muscle, liver, and kidneys of the lean and obese mice treated with or without the pegvisomant treatment. (b) Relative mRNA expression of furin and ADAM19 of the WAT. *$P < 0.05$ vs Lean + Saline mice; †$P < 0.05$ vs Lean + Peg mice; ‡$P < 0.05$ vs Obese + Saline mice.

To mimic GHD, we performed GH receptor blockade in lean and obese mice. Serum s(P)RR concentration was increased in obese mice, with and without GH receptor blockade, compared with lean mice. Whereas the mRNA expression of (P)RR was comparable in the muscle, liver, and kidneys among control and obese mice regardless of GH receptor blockade, (P)RR mRNA expression in AT after treatment with the GH receptor antagonist was significantly higher than that with saline in obese mice. The mRNA expression of (P)RR processing enzymes—furin [31] or ADAM19 [32]—was unchanged by GH receptor blockade in AT. These results suggested that increased expression, but not cleavage of (P)RR in AT, may be the cause of elevated serum s(P)RR concentration in GHD patients.

Hypoxia in ATs of obese model animals has been reported [16–19]. GHD also can cause hypoxia in AT. Patients with GHD have impaired aerobic exercise capacity, as decreased cardiac function [33], lung volume [34, 35], and red cell mass [6] reduce oxygen delivery to muscles. These suggest that AT of obese patients with GHD may also be hypoxic. Previous
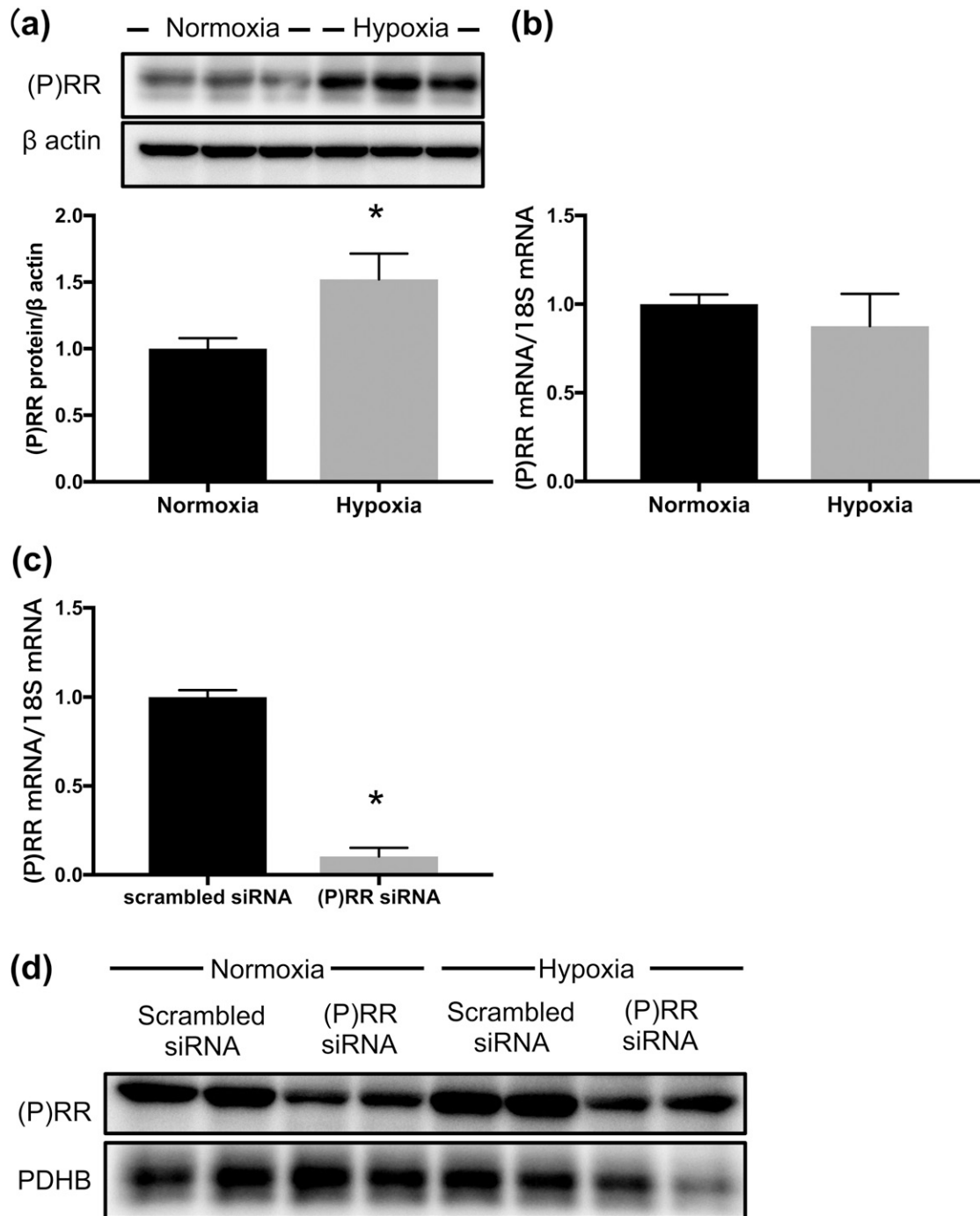
Figure 4. Effects of hypoxia on (P)RR and PDHB expression in 3T3-L1 cells. (a) Representative Western blot image and protein levels for (P)RR and β-actin expression after normoxia or 5% hypoxia treatment of 8 hours. *$P < 0.05$ vs normoxia. (b) Relative mRNA expression of (P)RR after normoxia or 5% hypoxia treatment of 8 hours. (c) Relative mRNA expression of (P)RR after the 48-hour treatment with scrambled or (P)RR siRNAs. *$P < 0.05$ vs scrambled siRNA. (d) Representative Western blot image for (P)RR, PDHB, and β-actin after the 48-hour treatment with scrambled or (P)RR siRNAs, followed by 8-hour treatment of normoxia or 5% hypoxia.

studies showed that hypoxia stimulates PDH kinase (PDK) expression [36, 37]. Generally, hypoxia increases PDK activity and decreases PDH activity. As illustrated in Fig. 5, a recent study showed that (P)RR is capable of maintaining PDH activity through stabilizing a PDHB protein [12]. In the current study, knockdown of (P)RR decreased expression of PDHB only in
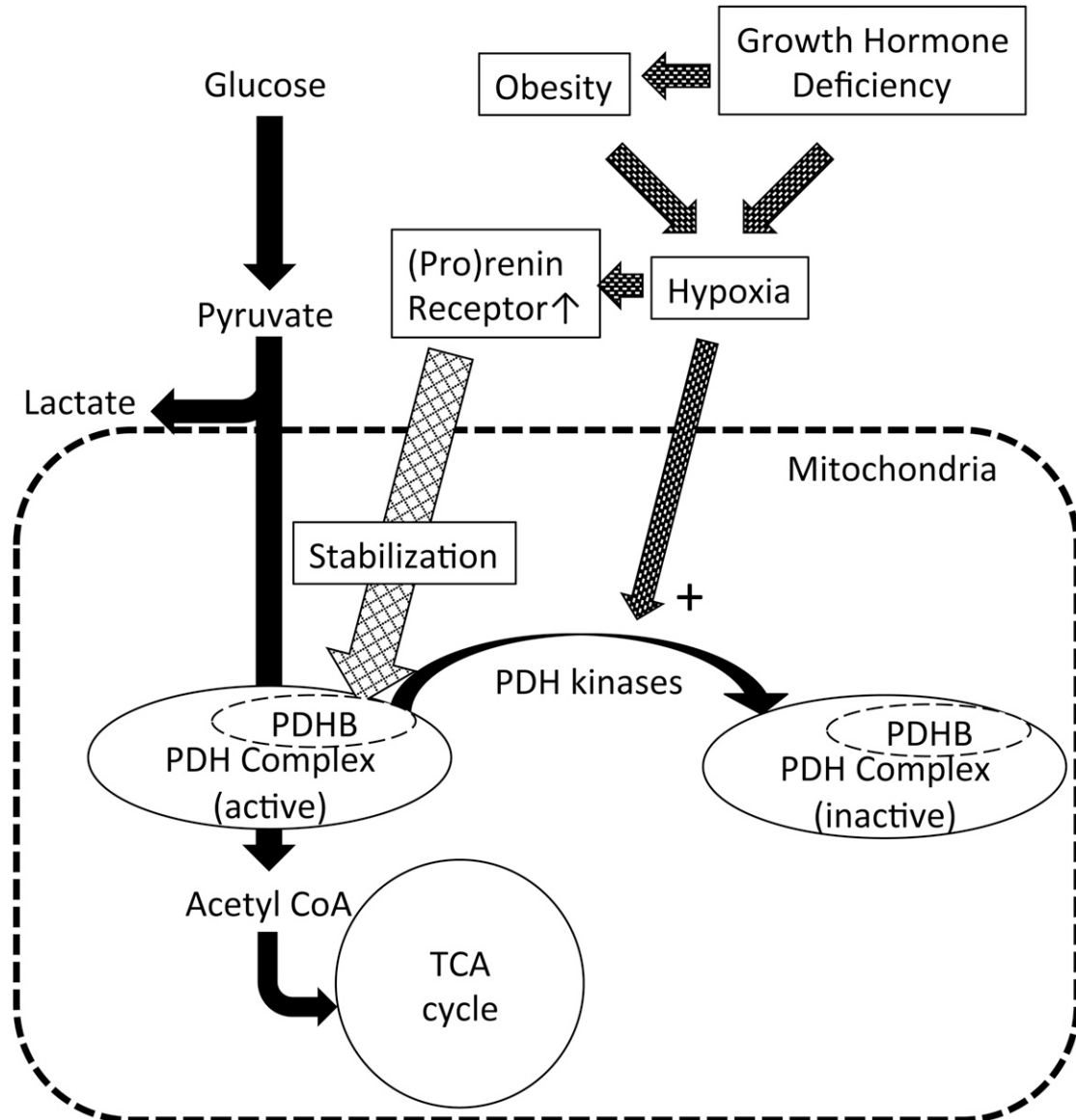
Figure 5. Hypothetic regulation of PDH activity by (P)RR in ATs. Under hypoxic condition and GHD, PDH activity tends to decrease through the hypoxia-induced stimulation of PDK. However, hypoxia also enhances the (P)RR expression. As (P)RR binds to PDHB and thus maintains the PDH activity, PDH activity is actually unchanged even under hypoxic conditions if the expression of (P)RR is intact. CoA, coenzyme A; TCA, tricarboxylic acid.

the hypoxic condition. These data suggest that the hypoxic condition in AT may be a contributing factor for elevated s(P)RR in obese GHD patients.

Potential clinical implications from this study may be illustrated as follows. First, the tissue renin-angiotensin system may be activated in AT in GHD patients. It has been shown that hypertensive patients have elevated serum s(P)RR, likely as a result of the activated intrarenal renin-angiotensin system [13]. In GHD patients, the prorenin-to-renin ratio was found to be increased [38], and we observed elevated (P)RR expression in the WAT of GHD model mice. As circulating prorenin can bind to (P)RR and acquire enzymatic activity without proteolytic cleavage [9], increased (P)RR in AT and prorenin in blood may synergistically activate the tissue renin-angiotensin system in AT in GHD patients. Second, (P)RR may contribute to the maintenance of PDH activity in adipocytes. The elevation of serum s(P)RR

concentration in GHD patients was likely a result of the increased (P)RR expression in AT. As (P)RR binding to PDH protein blocks phosphorylation and degradation of PDH, increased expression of (P)RR may preserve efficient energy metabolism but may also lead to increased oxidative stress in obesity and GHD, the effects of which remain to be determined.

This study has several limitations. First, it is unclear whether elevated serum s(P)RR concentration in GHD patients is a result of an increase in the volume of ATs or (P)RR expression in ATs. The changes in serum s(P)RR concentration did not correlate with changes in BMI after GH replacement, but the current study did not examine (P)RR expression in ATs in the patients. Second, the mechanism for the increased expression of (P)RR with hypoxia remains to be determined. Hypoxia induces glycolysis [39], adipogenesis [40], and adipokine production [41]. Whether these metabolic pathways affect the (P)RR expression in AT should be examined in future studies. Third, SGHD was diagnosed by a single test, the GHRP-2 test, in the current study. As GH cutoff values for mild to moderate GHD have not been determined for the GHRP-2 test, SGHD$^-$ patients in the current study include those with mild to moderate GHD. Whether serum s(P)RR concentration in the patients with mild to moderate GHD are increased compared with those without GHD cannot be determined from this study.

In conclusion, the current study showed that serum s(P)RR concentrations were elevated in obese patients with GHD. The origin of the elevated serum s(P)RR concentration was suggested to be the AT from the animal studies, and *in vitro* studies suggested that hypoxia may be one of the causes of elevated (P)RR expression in AT. The increased expression of (P)RR may contribute to the maintenance of energy metabolism in AT. Thus, the elevated serum s(P)RR levels may reflect hypoxic ATs caused by GHD and obesity. Further studies are needed to clarify the regulation of (P)RR expression under hypoxic conditions.

Acknowledgments

References and Notes

1. de Boer H, Blok GJ, Van der Veen EA. Clinical aspects of growth hormone deficiency in adults. *Endocr Rev*. 1995;**16**(1):63–86.

2. Johansson JO, Fowelin J, Landin K, Lager I, Bengtsson BA. Growth hormone-deficient adults are insulin-resistant. *Metabolism*. 1995;**44**(9):1126–1129.

3. Rosén T, Edén S, Larson G, Wilhelmsen L, Bengtsson BA. Cardiovascular risk factors in adult patients with growth hormone deficiency. *Acta Endocrinol (Copenh)*. 1993;**129**(3):195–200.

4. Woodhouse LJ, Mukherjee A, Shalet SM, Ezzat S. The influence of growth hormone status on physical impairments, functional limitations, and health-related quality of life in adults. *Endocr Rev*. 2006;**27**(3): 287–317.

5. Mossberg KA, Masel BE, Gilkison CR, Urban RJ. Aerobic capacity and growth hormone deficiency after traumatic brain injury. *J Clin Endocrinol Metab*. 2008;**93**(7):2581–2587.

6. Christ ER, Cummings MH, Westwood NB, Sawyer BM, Pearson TC, Sönksen PH, Russell-Jones DL. The importance of growth hormone in the regulation of erythropoiesis, red cell mass, and plasma volume in adults with growth hormone deficiency. *J Clin Endocrinol Metab*. 1997;**82**(9):2985–2990.

7. Fleseriu M, Hashim IA, Karavitaki N, Melmed S, Murad MH, Salvatori R, Samuels MH. Hormonal replacement in hypopituitarism in adults: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2016;**101**(11):3888–3921.

8. Ichihara A, Kaneshiro Y, Takemitsu T, Sakoda M, Suzuki F, Nakagawa T, Nishiyama A, Inagami T, Hayashi M. Nonproteolytic activation of prorenin contributes to development of cardiac fibrosis in genetic hypertension. *Hypertension*. 2006;**47**(5):894–900.

9. Ichihara A, Hayashi M, Kaneshiro Y, Suzuki F, Nakagawa T, Tada Y, Koura Y, Nishiyama A, Okada H, Uddin MN, Nabi AHMN, Ishida Y, Inagami T, Saruta T. Inhibition of diabetic nephropathy by a decoy peptide corresponding to the "handle" region for nonproteolytic activation of prorenin. *J Clin Invest*. 2004;**114**(8):1128–1135.

10. Seki Y, Ichihara A, Mizuguchi Y, Sakoda M, Kurauchi-Mito A, Narita T, Kinouchi K, Bokuda K, Itoh H. Add-on blockade of (pro)renin receptor in imidapril-treated diabetic SHRsp. *Front Biosci (Elite Ed)*. 2010;**2**:972–979.

11. Tani Y, Yamada S, Inoshita N, Hirata Y, Shichiri M. Regulation of growth hormone secretion by (pro)renin receptor. *Sci Rep*. 2015;**5**:10878.

12. Kanda A, Noda K, Ishida S. ATP6AP2/(pro)renin receptor contributes to glucose metabolism via stabilizing the pyruvate dehydrogenase E1 β subunit. *J Biol Chem*. 2015;**290**(15):9690–9700.

13. Morimoto S, Ando T, Niiyama M, Seki Y, Yoshida N, Watanabe D, Kawakami-Mori F, Kobori H, Nishiyama A, Ichihara A. Serum soluble (pro)renin receptor levels in patients with essential hypertension. *Hypertens Res*. 2014;**37**(7):642–648.

14. Nishijima T, Tajima K, Yamashiro Y, Hosokawa K, Suwabe A, Takahashi K, Sakurai S. Elevated plasma levels of soluble (pro)renin receptor in patients with obstructive sleep apnea syndrome in parallel with the disease severity. *Tohoku J Exp Med*. 2016;**238**(4):325–338.

15. Fukushima A, Kinugawa S, Homma T, Masaki Y, Furihata T, Abe T, Suga T, Takada S, Kadoguchi T, Okita K, Matsushima S, Tsutsui H. Increased plasma soluble (pro)renin receptor levels are correlated with renal dysfunction in patients with heart failure. *Int J Cardiol*. 2013;**168**(4):4313–4314.

16. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am J Physiol Endocrinol Metab*. 2007; **293**(4):E1118–E1128.

17. Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K, Furukawa S, Tochino Y, Komuro R, Matsuda M, Shimomura I. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 2007;**56**(4):901–911.

18. Rausch ME, Weisberg S, Vardhana P, Tortoriello DV. Obesity in C57BL/6J mice is characterized by adipose tissue hypoxia and cytotoxic T-cell infiltration. *Int J Obes*. 2008;**32**(3):451–463.

19. He Q, Gao Z, Yin J, Zhang J, Yun Z, Ye J. Regulation of HIF-1α activity in adipose tissue by obesity-associated factors: adipogenesis, insulin, and hypoxia. *Am J Physiol Endocrinol Metab*. 2011;**300**(5): E877–E885.

20. Tan P, Shamansurova Z, Bisotto S, Michel C, Gauthier M-S, Rabasa-Lhoret R, Nguyen TM-D, Schiller PW, Gutkowska J, Lavoie JL. Impact of the prorenin/renin receptor on the development of obesity and associated cardiometabolic risk factors. *Obesity (Silver Spring)*. 2014;**22**(10):2201–2209.

21. Examination Committee of Criteria for 'Obesity Disease' in Japan; Japan Society for the Study of Obesity. New criteria for 'obesity disease' in Japan. *Circ J*. 2002;**66**(11):987–992.

22. Chihara K, Shimatsu A, Hizuka N, Tanaka T, Seino Y, Katofor Y; KP-102 Study Group. A simple diagnostic test using GH-releasing peptide-2 in adult GH deficiency. *Eur J Endocrinol*. 2007;**157**(1): 19–27.

23. Molitch ME, Clemmons DR, Malozowski S, Merriam GR, Vance ML; Endocrine Society. Evaluation and treatment of adult growth hormone deficiency: an Endocrine Society clinical practice guideline. *J Clin Endocrinol Metab*. 2011;**96**(6):1587–1609.

24. Fukuda I, Hizuka N, Muraoka T, Ichihara A. Adult growth hormone deficiency: current concepts. *Neurol Med Chir (Tokyo)*. 2014;**54**(8):599–605.

25. Kinoshita Y, Tominaga A, Usui S, Arita K, Sakoguchi T, Sugiyama K, Kurisu K. The arginine and GHRP-2 tests as alternatives to the insulin tolerance test for the diagnosis of adult GH deficiency in Japanese patients: a comparison. *Endocr J*. 2013;**60**(1):97–105.

26. Kilroy G, Burk DH, Floyd ZE. High efficiency lipid-based siRNA transfection of adipocytes in suspension. *PLoS One*. 2009;**4**(9):e6940.

27. Matsuo S, Imai E, Horio M, Yasuda Y, Tomita K, Nitta K, Yamagata K, Tomino Y, Yokoyama H, Hishida A; Collaborators developing the Japanese equation for estimated GFR. Revised equations for estimated GFR from serum creatinine in Japan. *Am J Kidney Dis*. 2009;**53**(6):982–992.

28. Isojima T, Shimatsu A, Yokoya S, Chihara K, Tanaka T, Hizuka N, Teramoto A, Tatsumi KI, Tachibana K, Katsumata N, Horikawa R. Standardized centile curves and reference intervals of serum insulin-like growth factor-I (IGF-I) levels in a normal Japanese population using the LMS method. *Endocr J*. 2012; **59**(9):771–780.

29. Maruyama N, Segawa T, Kinoshita N, Ichihara A. Novel sandwich ELISA for detecting the human soluble (pro)renin receptor. *Front Biosci (Elite Ed)*. 2013;**5**:583–590.

30. Beshyah SA, Freemantle C, Thomas E, Rutherford O, Page B, Murphy M, Johnston DG. Abnormal body composition and reduced bone mass in growth hormone deficient hypopituitary adults. *Clin Endocrinol (Oxf)*. 1995;**42**(2):179–189.

31. Cousin C, Bracquart D, Contrepas A, Corvol P, Muller L, Nguyen G. Soluble form of the (pro)renin receptor generated by intracellular cleavage by furin is secreted in plasma. *Hypertension*. 2009;**53**(6): 1077–1082.

32. Yoshikawa A, Aizaki Y, Kusano K, Kishi F, Susumu T, Iida S, Ishiura S, Nishimura S, Shichiri M, Senbonmatsu T. The (pro)renin receptor is cleaved by ADAM19 in the Golgi leading to its secretion into extracellular space. *Hypertens Res*. 2011;**34**(5):599–605.

33. Colao A, Marzullo P, Di Somma C, Lombardi G. Growth hormone and the heart. *Clin Endocrinol (Oxf)*. 2001;**54**(2):137–154.

34. Merola B, Sofia M, Longobardi S, Fazio S, Micco A, Esposito V, Colao A, Biondi B, Lombardi G. Impairment of lung volumes and respiratory muscle strength in adult patients with growth hormone deficiency. *Eur J Endocrinol*. 1995;**133**(6):680–685.

35. Merola B, Longobardi S, Sofia M, Pivonello R, Micco A, Di Rella F, Esposito V, Colao A, Lombardi G. Lung volumes and respiratory muscle strength in adult patients with childhood- or adult-onset growth hormone deficiency: effect of 12 months' growth hormone replacement therapy. *Eur J Endocrinol*. 1996; **135**(5):553–558.

36. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. *Cell Metab*. 2006; **3**(3):177–185.

37. Lee JH, Kim E-J, Kim D-K, Lee J-M, Park SB, Lee I-K, Harris RA, Lee M-O, Choi H-S. Hypoxia induces PDK4 gene expression through induction of the orphan nuclear receptor ERRγ. *PLoS One*. 2012;**7**(9): e46324.

38. Gordon MS, Caston-Balderrama AL, Gordon MB. Abnormal prorenin processing in growth hormone deficiency. *Growth Horm IGF Res*. 2005;**15**(4):251–255.

39. Yin J, Gao Z, He Q, Zhou D, Guo Z, Ye J. Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue. *Am J Physiol Endocrinol Metab*. 2009;**296**(2):E333–E342.

40. Yun Z, Maecker HL, Johnson RS, Giaccia AJ. Inhibition of PPAR γ 2 gene expression by the HIF-1-regulated gene DEC1/Stra13: a mechanism for regulation of adipogenesis by hypoxia. *Dev Cell*. 2002; **2**(3):331–341.

41. Lolmède K, Durand de Saint Front V, Galitzky J, Lafontan M, Bouloumié A. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *Int J Obes Relat Metab Disord*. 2003;**27**(10):1187–1195.