Inorganic Phosphate Activates the AKT/mTORC1 Pathway and Shortens the Life Span of an α-Klotho–Deficient Model

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ABSTRACT

Inorganic phosphate (Pi) has been implicated in the pathogenesis of accelerated aging; however, the underlying mechanisms remain elusive. Herein, we demonstrated in cultured cells and in vivo that increased levels of extracellular Pi activated the AKT/mammalian target of rapamycin complex 1 (mTORC1) pathway by suppressing membrane-bound phosphatase and tensin homolog (PTEN) levels in a manner requiring the sodium-dependent Pi transporter PiT-1. High levels of extracellular Pi also led to phosphorylation of Ser/Thr clusters in the C-terminal tail of PTEN, which has been shown to dissociate PTEN from the membrane. Notably, blockade of mTORC1 activity by rapamycin treatment prolonged the life span of hyperphosphatemic α-Klotho–deficient (Kl²/²) mice. Dietary correction of hyperphosphatemia or treatment with rapamycin also rescued the brown adipose tissue dysfunction and oxidative damage observed in Kl²/² mice. Furthermore, rapamycin treatment partially rescued these effects and extended the life span of Kl²/² mice when Kl²/² mice were maintained on a high-phosphate diet. Finally, rapamycin reduced circulating Pi levels in Kl²/² mice, apparently by decreasing the localization of sodium-dependent Pi transport protein 2a at the renal brush border membrane. Therefore, the activation of mTORC1 may create a vicious loop that exacerbates the retention of Pi, which in turn may enhance oxidative damage and ultimately shorten the life span of Kl²/² mice. These results demonstrate that Pi has important roles in the aging process, and the blockade of mTORC1 may have therapeutic potential for premature aging-like symptoms associated with hyperphosphatemia.
gene have consistently exhibited severe oxidative damage and apoptotic changes, and recovery from apoptotic changes was achieved when circulating Pi levels were restored to normal ranges.5,10,11 These findings suggest that the increases in oxidative damage by hyperphosphatemia are responsible for the short life span of Kl−/− mice; however, the underlying molecular mechanisms remain unclear.

The truncated life span of kl hypomorphic mice (kl/kl) mice was previously reported to be partially improved by the deletion of one allele of the Irs1 gene,12 suggesting that the activation of signaling pathways downstream of insulin receptor substrate 1 is responsible for the short life span of kl/kl mice. Mechanistic target of rapamycin complex 1 (mTORC1) is a downstream molecule of insulin receptor substrate 1 and plays a role in life span determination.13 Furthermore, the activation of mTORC1 is known to enhance oxidative stress, in part, by suppressing the expression of antioxidant genes.14–16 These findings led us to speculate that hyperphosphatemia augments oxidative stress and shortens life spans by stimulating the Akt/mTORC1 pathway. Therefore, we herein analyzed the signaling pathway exerted by Pi and its roles in Pi-induced accelerated aging using Kl−/− mice.

RESULTS

Hyperphosphatemia Decreases Phosphatase and Tensin Homolog Expression Associated with Activation of the Akt/mTORC1 Pathway in Kl−/− Mice

In order to determine whether Akt/mTORC1 activation plays crucial roles in regulating the longevity of Kl−/− mice, we analyzed phosphorylated Akt (pAkt) and phosphorylated S6K (pS6K) levels, and found the tissue-specific activation of this pathway (Figure 1A, Supplemental Figure 1). Because the suppression of pAkt by a phosphoinositide 3-kinase inhibitor reduced pS6K levels, the activation of mTORC1 was considered to be downstream of that of Akt in Kl−/− mice (Figure 1B). Phosphatase and tensin homolog (Pten) protein levels were decreased in Kl−/− mice despite the presence of similar Pten transcript levels (Figure 1, C and D, Supplemental Figure 1A). The correction of hyperphosphatemia by a low-phosphate diet (LPD) reversed the expression of Pten/pAkt/ pS6K; however, growth retardation was still clearly observed in LPD-fed Kl−/− mice (Figure 1, E–G), suggesting that hyperphosphatemia is responsible for the altered Pten/Akt/mTORC1 pathway in Kl−/− mice.

Extracellular Pi Activates the Akt/mTORC1 Pathway by Suppressing Pten

We investigated the mechanisms responsible for Pi-induced activation of the Akt/mTORC pathway. Although the Pi treatment did not reduce Pten transcript levels, it significantly decreased Pten protein levels in 3T3-L1 preadipocytes in a dose- and time-dependent manner, and this was associated with increased pAkt/pS6K levels (Figure 2, A–D). The Pi treatment also reduced the expression of Pten in 3T3-L1 adipocytes, hibernoma-derived T37i cells, and primary brown adipocytes (Supplemental Figure 2A). Membrane-bound Pten expression, which is critical for its phosphatase activity, was reduced by the Pi treatment (Figure 2E). Because Pi-induced Akt/S6K phosphorylation disappeared when Pten expression was knocked down, the activation of Akt/S6K by the Pi treatment was likely a downstream event of the downregulated expression of Pten (Figure 2, F and G, Supplemental Figure 2, B and C). The Pi treatment also did not increase the polyubiquitination of Pten (Figure 2H); therefore, decreases in Pten expression by Pi are unlikely to have been due to enhanced proteasomal degradation. These results suggest that Pi reduces membrane-bound Pten expression by altering its intracellular localization and activating the Akt/mTORC1 pathway.

Extracellular Pi Reduces Pten Expression in a PiT 1-Dependent Manner

We determined whether PiT-1 is involved in this regulation because it is a predominant sodium (Na+)/Pi cotransporter in 3T3-L1 cells (Supplemental Figure 3A). PiT-1 and PiT-2 expression in 3T3-L1 cells was not altered by the Pi treatment, and no significant differences were observed in their expression between wild-type (WT) and Kl−/− mice (Supplemental Figure 3, B and C). Knockdown experiments for PiT-1 were performed to determine the involvement of PiT-1. Although the knockdown of PiT-1 slightly enhanced PiT-2 expression, it was still lower than that of PiT-1, suggesting that PiT-2 did not compensate for the decreased expression of PiT-1 (Figure 3A). Membrane-bound Pten levels were not significantly reduced by the Pi treatment in PiT-1 knocked-down cells, whereas they were in control cells (Figure 3B). Furthermore, the overexpression of WT-PiT-1 or S132A-PiT-1, the latter of which localizes to the plasma membrane but lacks transporter activity,17 significantly reduced Pten expression without affecting Pten transcript levels (Figure 3, C–E, Supplemental Figure 3D). These results indicate that PiT-1 is involved in the suppressive effects of Pi on Pten expression in a transporter activity-independent manner.

Extracellular Pi Maintains the Phosphorylation Status of Ser/Thr Clusters in the C-tail of Pten

We examined the involvement of ERK activation, a known downstream molecule of the Pi signaling pathway,1 in this regulation, and found that an MEK inhibitor did not inhibit Pi-induced reductions in Pten (Supplemental Figure 3, E–G), thereby indicating that the downregulation of Pten by Pi was independent of ERK activation. We then investigated the phosphorylation of Ser/Thr clusters in the C-tail of Pten (pPten) because the phosphorylation of these residues was previously reported to dissociate Pten from the membrane.18 The results obtained showed that the Pi treatment enhanced the phosphorylation status of these residues (Figure 3F). pPten levels were consistently higher in Kl−/− mice (Figure 3G, Supplemental Figure 3H). Furthermore, the overexpression of WT-PiT-1 or S132A-PiT-1 enhanced pPten levels.
in 3T3-L1 cells (Figure 3H). A PTEN mutant (PTEN-4A), in which Ser/Thr clusters were mutated to alanine, was protected from Pi-induced downregulation (Figure 3I). Pi-induced Akt/S6K activation also disappeared in cells stably expressing PTEN-4A (Figure 3J). These results indicate the importance of PTEN phosphorylation in the Pi-induced activation of the Akt/mTORC1 pathway.

**Rapamycin Extends the Life Span of Kl/2/2 Mice**

We treated mice with rapamycin (Rapa treatment) to investigate the role of mTORC1 activation in the short life span of Kl/2/2 mice, and found that it significantly prolonged their life span by approximately 40%; however, growth retardation was still noted in Rapa-treated Kl/2/2 mice (Figure 4, A and B). In line with similar Pten/pAkt/pS6K levels in the lung and ileum of Kl/2/2 and WT mice (Supplemental Figure 1B), the Rapa treatment did not improve the expansion of the alveolar space (emphysema) or atrophic changes in ileal villi in Kl/2/2 mice (Figure 4, C and D), indicating that the life span extension by Rapa treatment was not associated with improved pulmonary or intestinal functions.
Brown Adipose Tissue Function Is Impaired in \( K\ell^{2/2} \) Mice

We examined the phenotypes of \( K\ell^{2/2} \) mice in more detail and identified decreases in rectal temperatures, even when maintained with WT or heterozygous (Het) mice (Figure 5A). A markedly larger decrease was observed in rectal temperatures when \( K\ell^{2/2} \) mice were single-caged after weaning, and these mice died earlier than those group-housed with WT/Het mice (Figure 5, B and C), suggesting that heat transfer from WT/Het mice to \( K\ell^{2/2} \) mice was critical for their survival, particularly in the early stage of life. Because brown adipose tissue (BAT) is central to maintaining body temperature in rodents, we analyzed its function in \( K\ell^{2/2} \) mice. Although BAT weight was lower in \( K\ell^{2/2} \) mice, this difference disappeared when normalized to body weight (Figure 5D). No significant changes were noted in hematoxylin and eosin (H&E) staining between the two groups (Supplemental Figure 4A); however, as shown previously, \(^\text{19} \) Ucp1 expression was reduced in \( K\ell^{2/2} \) mice (Figure 5, E and F), indicating decreased heat generation. The induction of \( Ppargc1a \) by a \( \beta3 \)-adrenergic agonist was...
impaired in Kl2/2 mice (Figure 5G). When mice were placed at 6°C, rectal temperatures decreased in Kl2/2 mice, whereas WT mice tolerated the cold (Figure 5H). In order to examine the role of BAT dysfunction further, single-caged Kl2/2 mice were maintained at a warm temperature (30°C). The percentage of surviving mice was greater in Kl2/2 mice at 30°C compared with those at 22°C, although the difference did not reach statistical significance by chi-squared test (Figure 5I). BAT dysfunction in Kl2/2 mice was rescued by the correction of Pi levels (Figure 6, A–C). Although kl transcripts were weakly expressed in BAT, BAT dysfunction was unlikely caused by the lack of kl in BAT because brown adipogenesis and the induction of Ucp1 and Ppargca by forskolin were not affected by the lack of kl in primary brown adipocytes (Supplemental Figure 4, B–D).

**ROS Generation Is Enhanced in Kl2/2 Mouse BAT**

In order to elucidate the mechanisms responsible for BAT dysfunction in Kl2/2 mice, we performed 8-hydroxy-2'-deoxyguanosine staining as a marker of oxidative DNA damage, and found increases in oxidative stress in Kl2/2 mouse BAT that were accompanied by enhanced apoptosis...
Cleaved caspase 3 expression was consistently elevated in Kl2/2 mice (Figure 5K). Recovery from enhanced oxidative damage in Kl2/2 mice was achieved by the correction of Pi levels (Figure 6D), suggesting that hyperphosphatemia-induced oxidative stress resulted in tissue damage and caused BAT dysfunction. BAT dysfunction is rescued by the compensatory beige conversion of white adipocytes through sympathetic activation20; therefore, we determined why compensatory mechanisms are not operative in Kl2/2 mice. Sympathetic activity was increased in Kl2/2 mice, and the amount of lipid droplets in iWAT was markedly decreased (Supplemental Figure 5, A–C). Although Ppargc1a expression was increased, that of beige adipocyte markers including Ucp1, Tmem26, and CD137 was not induced in Kl2/2 mice (Supplemental Figure 5D), suggesting that the beige conversion of iWAT was not operative. Furthermore, reductions in lipid accumulation were not caused by a lipodystrophic phenotype based on the lack of fibrotic changes and reduced F4/80 expression (Supplemental Figure 5, C and E). The correction of Pi levels partially reversed these abnormal phenotypes (Supplemental Figure 5F–H). Immunohistochemical analyses revealed the massive accumulation of oxidative stress with enhanced apoptosis in iWAT of Kl2/2 mice (Supplemental Figure 5I), suggesting that cellular damage by oxidative stress blocked the beige conversion of white adipocytes.

**Rapamycin Improves BAT Function in Kl2/2 Mice**

We investigated whether mTORC1 activation was involved in BAT dysfunction. The Rapa treatment suppressed pS6K levels, improved rectal temperatures, and reversed Ucp1 expression in Kl2/2 mice (Figure 7, A–C). The induction of Ppargc1a by a β3-adrenergic agonist was significantly rescued in Rapa-treated Kl2/2 mice (Figure 7D). H&E staining of BAT was not affected by the Rapa treatment (Supplemental Figure 6). The Rapa treatment significantly increased the expression of antioxidant genes including Superoxide dismutase 1 (Sod1), Sod2, and Glutathione reductase (Gsr) (Figure 7E) in Kl2/2 mice, and this was associated with the amelioration of oxidative damage in BAT (Figure 7F). Furthermore, an in vitro analysis revealed that the mitochondrial membrane potential was reduced by the Pi treatment, and this was antagonized by a coincubation with rapamycin (Figure 7G). These results indicate that the hyperphosphatemia-induced activation of the mTORC1 pathway causes mitochondrial dysfunction and oxidative damage in Kl2/2 mice.

**Rapamycin Reduces Circulating Pi Levels in Kl2/2 Mice**

Because serum Pi levels were significantly decreased by the Rapa treatment in Kl2/2 mice (Figure 8A), Rapa-treated Kl2/2 mice were maintained on a high-phosphate diet (HPD) to reverse the Pi-lowering effects of Rapa (Figure 8B). Under this condition, Rapa partially improved rectal temperatures,
reduced oxidative stress, and elongated the life span (Figure 8, C–E) despite higher Pi concentrations (P=0.06; control diet [CD]-Kt–/–+Vehicle versus HPD-Kt–/–+Rapa by the t test). The expression of Na+/Pi cotransporters was analyzed to elucidate the molecular mechanisms by which Rapa decreases Pi levels. Akt/mTORC1 activation in the kidney was associated with the suppression of Pten and the Rapa treatment improved tissue calcification in Kt–/– mice (Figure 9, A–D). Npt2a
protein expression was decreased in the renal brush border membrane (BBM) of Rapa-treated $Kl^{-/-}$ mice despite the comparable Npt2a transcripts levels, and this was associated with reduced BBM Pi transport (Figure 9, E–H, Supplemental Figure 7A). Additionally, the Pi treatment decreased the polyubiquitination of the Npt2a protein, and this was antagonized by the Rapa cotreatment (Figure 9I), suggesting that Pi-induced mTORC1 activation partly increases Npt2a protein levels by suppressing proteasomal degradation. Npt2b expression in the ileum was not affected by Rapa (Supplemental Figure 7, B and C). These results indicate that mTORC1 activation creates a vicious circle that exacerbates the retention of Pi, which may further augment the accumulation of oxidative damage, resulting in the short life span of $Kl^{-/-}$ mice (Figure 9J).

**DISCUSSION**

We demonstrated that extracellular Pi downregulated PTEN expression, which may be linked to reduced longevity in $Kl^{-/-}$ mice through activation of the Akt/mTORC1 pathway. These results are consistent with the widely accepted tenet that suppression of the insulin/IGF-1 signaling pathway elongates longevity.\textsuperscript{21}–\textsuperscript{23} The involvement of PTEN in life span determinations is also evident in a mouse model with an increased life span in which Pten is exogenously overexpressed.\textsuperscript{24} Although the pathogenesis of the short life span of $Kl^{-/-}$ mice is complex and includes multiple levels of biologic abnormalities, accumulating evidence indicates that oxidative damage by hyperphosphatemia plays important roles in the development of the short life span of these mice\textsuperscript{5,10,11}; however, the underlying mechanisms remain unclear.
Extracellular Pi is incorporated into the cytosol through Pi transporters and transported into mitochondria, where it is used as a substrate for ATP synthesis.25 Pi also serves as a regulator of mitochondrial functions such that it stimulates oxidative phosphorylation.26 Hence, increases in extracellular Pi result in the enhanced generation of ROS. A previous study reported that Pi increased ROS generation in human endothelial cells8 and murine osteoblastic cells.9 Based on the long-standing extracellular Pi is incorporated into the cytosol through Pi transporters and transported into mitochondria, where it is used as a substrate for ATP synthesis.25 Pi also serves as a regulator of mitochondrial functions such that it stimulates oxidative phosphorylation.26 Hence, increases in extracellular Pi result in the enhanced generation of ROS. A previous study reported that Pi increased ROS generation in human endothelial cells8 and murine osteoblastic cells.9 Based on the long-standing...
free radical theory of aging,⁴² these findings suggest a causative role for Pi-induced ROS generation in premature aging-like symptoms in Klotho−/− mice; however, emerging evidence has revealed the context-specific function of ROS as a prosurvival signal such that adequate increases in ROS may extend longevity.⁴⁸,⁴⁹ Klización that the Rapa treatment enhanced antioxidant gene expression in activity, which is known to regulate ROS production, and found the localization of Npt2a to the renal BBM in WT mice. We identified a previously unrecognized signaling pathway exerted by Pi, which includes the downregulation of PTEN associated with Akt activation. This result suggests the existence of a novel mechanism for Akt activation in Klotho−/− mice besides the antagonistic effects of soluble α-Klotho on the insulin/IGF-1 signaling pathway.¹² In order to determine whether the activation of Akt in Klotho−/− mice is involved in ROS generation, we assessed mTORC1 activity, which is known to regulate ROS production, and found that the Rapa treatment enhanced antioxidant gene expression in Klotho−/− mice. This result suggests that the increased ROS levels generated by hyperphosphatemia were not appropriately detoxified, resulting in their massive accumulation in Klotho−/− mice. We proposed that impaired BAT function plays a causative role in the short life span of Klotho−/− mice. BAT was initially considered to be nonfunctional in adult humans; however, BAT and/or beige adipose tissue is present and involved in energy homeostasis in adult humans.³¹–³³ The amount of BAT/beige adipose tissue decreases with age and is positively associated with bone mass in humans.³⁴,³⁵ In experimental models, Ortega-Molina et al. reported a positive relationship between BAT function and life span in mouse models.²⁴ Although these lines of evidence underline the important role of BAT function in the regulation of aging, further analyses are needed in order to unravel the relationship between BAT function and the aging process. We also demonstrated that the Rapa treatment decreased circulating Pi levels in Klotho−/− mice. In clinical settings, Rapa treatments after kidney transplantation have been shown to induce hypophosphatemia, suggesting the involvement of mTORC1 in Pi homeostasis.³⁶ The introduction of mTOR in Xenopus oocytes has consistently been shown to enhance Npt2a-mediated Pi incorporation.³⁷ We mechanistically demonstrated that Rapa reduced Npt2a expression in the renal BBM of Klotho−/− mice. However, consistent with previous findings, Rapa did not affect Npt2a expression levels in WT mice, indicating the critical role of mTORC1 activation in enhancing the localization of Npt2a to the renal BBM in Klotho−/− mice. In conclusion, we herein propose a previously unrecognized function for extracellular Pi in the regulation of the aging process. Because the blockade of calpain 1 or a plasminogen activator inhibitor-1 deficiency in Klotho−/− mice is known to extend longevity,³⁹,⁴⁰ further analyses are warranted to determine the importance of the proposed pathway in this context. Our results are clinically important because they provide a

**Figure 8.** Rapa reduces serum Pi levels in Klotho−/− mice. (A) Serum Pi levels in WT and Klotho−/− mice treated with Vehicle (Veh) or Rapa (n=10). (B and C) Serum Pi levels (n=5–9) (B) and rectal temperatures (n=5–7) (C) in Veh-treated WT mice fed CD or HPD, Veh-treated Klotho−/− mice fed CD or HPD, and Rapa-treated Klotho−/− mice fed HPD. (D) Representative images from three independent experiments of 8-OHdG staining in BAT. (E) The survival rates of Veh-treated Klotho−/− mice fed CD (n=13), and Rapa-treated Klotho−/− mice fed HPD (n=15) were plotted. Animal studies were performed when mice were 6 weeks old. Data are expressed as the mean±SEM. *P<0.001; **P<0.01; ***P<0.05. s-Pi, serum phosphate; Tm, temperature.
Figure 9. Suppression of mTORC1 by Rapa reduces BBM Pi transport in Kl--/mice. (A–C) The expression levels of pS-Akt (Ser473) (A), Pten (B), and pS6K (C) in the kidneys of WT and Kl--/mice were determined by a western blot analysis (n=5). (D) Representative images of Von Kossa staining from three independent experiments in the kidneys of Rapa (R)- or Vehicle (V)-treated WT or Kl--/mice. (E) Representative images of immunohistochemistry for Npt2a from three independent experiments in the kidney of R- or V-treated WT or Kl--/mice. (F) The expression of Npt2a in the renal BBM of R- or V-treated WT or Kl--/mice was determined by a western blot analysis.
novel insight into the therapeutic potential of the blockade of mTORC1 activity for the treatment of hyperphosphatemia-associated premature aging-like symptoms.

**CONCISE METHODS**

**Animal Studies**

α-Klotho knockout mice (Kt<sup>−/−</sup> mice) (B6.129-Kt<sup>minYin</sup>/Jcl) were kindly provided by Dr. Yoichi Nabeshima (Institute of Biomedical Research and Innovation, Kobe, Japan) and the generation of Kt<sup>−/−</sup> mice was as described previously. WT littermate mice were used as controls for Kt<sup>−/−</sup> mice. Mice were maintained with free access to water and standard chow (CE-2, CLEA Japan, Inc.) on a 12-hour light/dark cycle in a pathogen-free animal facility. Kt<sup>−/−</sup> mice were maintained with WT or Het unless otherwise stated. A CD containing 0.6% phosphate and 1.0% calcium, a LPD containing 0.2% phosphate and 1.0% calcium, and a HPD containing 1.65% phosphate and 1.0% calcium were purchased from CLEA Japan, Inc., and mice were fed these diets after weaning. None of the diets included phytate as the source of phosphate. Although food intake was not significantly affected by the type of diet, food intake in HPD-fed mice was slightly lower than that in CD-fed mice (Supplemental Figure 8A). Experimental procedures for the glucose tolerance test, cold exposure treatment, and centriﬁcation were performed as described elsewhere.

**Cell Culture**

3T3-L1 cells were obtained from the Human Science Research Resources Bank (Osaka, Japan) and maintained in high-glucose DMEM supplemented with 10% FCS. Experimental procedures for the adipogenic induction of 3T3-L1 cells, culture of T37i cells, and preparation of primary brown adipocytes are described in the Supplemental Material.

**In Vitro Pi Treatment**

A 500 mM stock Pi solution (pH 7.2) was prepared by combining 500 mM Na<sub>2</sub>HPO<sub>4</sub> and 500 mM Na<sub>2</sub>HPO<sub>4</sub>. In <i>in vitro</i> Pi treatment experiments, Pi was added to Pi-free media supplemented with 10% of calf serum or FCS containing 0.28 mM and 0.37 mM of Pi, respectively.

**Western Blot and Real-Time RT-PCR Analyses**

Western blot and real-time RT-PCR analyses were performed as described in the Supplemental Material. Whole-cell lysates were used for the western blot analysis unless otherwise stated.

**Detection of Polyubiquitinated Proteins**

In order to detect polyubiquitinated proteins, cells were treated with MG132 (10 μM) for 3 hours and solubilized in IP buffer (5 mM EDTA, 150 mM NaCl, 0.5% Nonidet P-40, 10% glycerol, and 10 mM Tris-HCl, pH 8.0) containing a protease inhibitor cocktail (Complete TM, EDTA-free; Roche Diagnostics, Indianapolis, IN). After centrifugation, supernatants were incubated with the antibody of interest, followed by immunoprecipitation with protein A/G-Sepharose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight. Samples were washed five times with IP buffer and then subjected to a western blot analysis for ubiquitin.

**Constructs and Mutagenesis**

The expression vector containing human PTEN (pCMV_Flag-hPTEN) was purchased from RIKEN (Tsukuba, Japan) and generation of the Flag-hPTEN4A (S380A, T382A, T383A, and S385A) mutant was performed by site-directed mutagenesis using QuickChange II XL (Agilent Technologies, Santa Clara, CA) according to the manufacturer's protocol. The oligonucleotides used for site-directed mutagenesis were prepared as described previously. The pFLC1-mouse PIT-1 vector was obtained from RIKEN, and PIT-1 cDNA was subcloned into a pENTR vector using the pENTR Directional TOPO cloning kit (Invitrogen, Carsbad, CA) and then transferred to the pDNA3.2/V5 vectors using the LR recombination reaction system (Invitrogen). Site-directed mutagenesis was performed to create the pDNA3.2-PIT-1-S132A mutant, which harbors a point mutation leading to the lack of its transporter activity, using QuikChange II XL. Npt2a cDNA was purchased from RIKEN and subcloned into the pCMV2-Flag vector.

**Generation of the Lentivirus**

The cDNA of interest was inserted into a TOPO pENTR vector using the pENTR Directional TOPO cloning kit and recombined with the CSII-EF-RfA–IRES-puro vector (a kind gift from Dr. Hiroyuki Miyoshi, RIKEN BRC) with the LR recombination reaction system (Invitrogen). The lentivirus was generated according to the protocol provided by RIKEN BRC. 3T3-L1 cells were infected with the lentivirus in the presence of 5 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO) and cells expressing the gene of interest were selected by adding puromycin (1 μg/ml) to the culture medium.

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**Supplemental Figure 8B**

The rapamycin treatment did not affect food intake (Figure 8A). Experimental procedures for the glucose tolerance test, cold exposure treatment, and centriﬁcation were performed as described elsewhere. Analyzed data are expressed as the mean ± SEM. *P < 0.001; **P < 0.01; ***P < 0.05. DAPI, 4',6-diamidino-2-phenylindole; IP, immunoprecipitation.
Generation of the Adenovirus
The cDNA of interest was inserted into the TOPO pENT vector using the pENT Directional TOPO cloning kit and recombined with the pAd-CMV-V5 vector (Invitrogen) using the LR recombination reaction system (Invitrogen). The adenovirus was generated using the ViraPower Adenovirus Expression System (Invitrogen) according to the manufacturer’s protocol. The infection of 3T3-L1 cells with the adenovirus was performed in the presence of 4 μg/ml of poly-l-lysine.

In Vitro Overexpression and Knockdown Experiments
The forced expression of Flag-WT-hPTEN and Flag-hPTEN-4A was performed using lentiviral vectors. WT-PiT-1-V5 and PiT-1-S132A-V5 were overexpressed in cells using adenoviral vectors. Knockdown experiments for PiT-1 were performed based on the lentivirus-mediated expression of the microRNA system using the BLOCK-iT Pol II miR RNAi Expression Vector Kit with EmGFP (Invitrogen).

Histologic Analysis
Tissue samples were fixed in 10% buffered formalin and paraffin-embedded samples were prepared. H&E staining and Azan staining were performed according to conventional methods. Regarding Von Kossa staining, deparaffinized sections were incubated with 5% silver nitrate solution under ultraviolet light for 60 minutes. After sections were rinsed, they were incubated with 5% sodium thiosulfate for 10 minutes and counterstained with hematoxylin.

Immunohistochemistry
Tissue samples were fixed in 10% buffered formalin and paraffin-embedded samples were prepared. In 8-OHdG staining, following deparaffinization and rehydration, antigen retrieval was performed using citrate buffer at 95°C for 60 minutes and endogenous peroxidase activity was not quenched in order to avoid the unexpected production of 8-OHdG via reactions between hydroxyl radicals with endogenous dG. After blocking, sections were incubated with an anti-8OHdG antibody (1:20; MOG-020P, JaICA, Japan) at 4°C overnight. Sections were then incubated with a biotinylated secondary antibody, followed by an incubation with the streptavidin-biotinylated horseradish peroxidase complex, and visualized with 3,3′-diaminobenzidine using the ImmunoCruz Staining System (Santa Cruz Biotechnology). The expression of Npt2a and Npt2b in the kidney and ileum, respectively, was analyzed using paraffin-embedded sections. Sections were processed as described above; however, endogenous peroxidase activity was quenched after antigen retrieval based on the ImmunoCruz Staining System (Santa Cruz Biotechnology). Sections were incubated with an anti-Npt2a antibody (rabbit polyclonal antibody raised against an Npt2a peptide sequence [MMSYSERLGGPAVSP] in the N-terminal region⁴⁴) or anti-Npt2b antibody (1:100, NPT2B11-A, Alpha Diagnostic Intl., Inc.) at 4°C overnight. The expression of Npt2a and Npt2b was visualized using Alexa Fluor 488-conjugated secondary antibodies (Invitrogen). Phaloidin (Alexa Fluor 555 phalloidin, A34055, Invitrogen) and 4,6-diamidino-2-phenylindole (340–07971, DOJINDO) were used to stain F-actin and the nucleus, respectively. Images were captured by confocal microscopy (TCS SP8, Leica Microsystems, Buffalo Grove, IL). The TUNEL assay was performed using an in situ apoptosis detection kit (Takara, Kyoto, Japan) according to the manufacturer’s manual.

Immunocytochemistry
3T3-L1 cells expressing V5-tagged PiT-1 or PiT-1-S132A were cultured on coverslips in a six-well plate. Regarding immunofluorescence, cells were fixed with 10% buffered formalin and then permeabilized with 0.2% Triton X-100 for 10 minutes. Following blocking with 5% FBS in PBS for 1 hour, cells were incubated with an anti-V5 antibody (1:200, 46–0705, Invitrogen) in 0.5% BSA in PBS for 1 hour, and the expression of V5 was then visualized using Alexa Fluor 555-conjugated secondary antibodies (Invitrogen). Images were captured by confocal microscopy (TCS SP8, Leica Microsystems).

Mitochondrial Membrane Potential Analysis by Flow Cytometry
The mitochondrial membrane potential was determined using the MitoProbe JC-1 (5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazolycarbocyanide iodide) assay kit (Life Technologies, Carlsbad, CA). JC-1 aggregates in intact mitochondria and emits red fluorescence. When the mitochondrial membrane potential is disrupted, JC-1 remains in the cytoplasm as a monomer and emits green fluorescence. Primary preadipocytes that expanded from the stromal vascular fraction of BAT were treated with Pi (0 or 4 mM) for 16 hours in the presence of 0.5% FCS, and collected after trypsinization. A total of 1×10⁶ cells were incubated with 5 μM of JC-1 at 37°C for 20 minutes, and 10,000 cells were subjected to a flow cytometric analysis on a FACSCalibur flow cytometer. The percentage of JC-1 monomers (green fluorescence) was determined. In order to confirm the sensitivity of JC-1 to alterations in the mitochondrial membrane potential, 50 μM of carbonyl cyanide m-chlorophenyl hydrazine was used as a chemical uncoupler.

Isolation of the BBM Fraction from the Kidney and Intestine
The BBM of the kidney and ileum were isolated based on the divalent cation precipitation method. Briefly, the kidney cortex or ileum was homogenized in 30 vol (wt/vol) of homogenization buffer (50 mM mannitol, 2 mM Tris–HCl, pH 7.5). The homogenates were incubated in the presence of 100 mM CaCl₂ with rotation at 4°C for 10 minutes, and centrifuged at 3000×g at 4°C for 15 minutes. The supernatant was further centrifuged at 43,000×g at 4°C for 30 minutes. The pellet was resuspended in suspension buffer (300 mM mannitol, 10 mM HEPES/Tris, pH 7.5), homogenized, and centrifuged at 43,000×g at 4°C for 45 minutes. The pellet was solubilized in RIPA buffer and used as the BBM fraction.

BBM Pi Transport Assay
The BBM vesicle fraction solubilized in suspension buffer was further enriched by centrifugation at 43,000×g at 4°C for 45 minutes. The pellet was resuspended in suspension buffer and used for the BBM Pi transport assay. The transport of Pi into the renal BBM vesicles was measured by a rapid filtration technique. Twenty micrograms (10 μl) of BBM vesicles was mixed with 90 μl Na⁺-containing...
uptake solution (100 mM NaCl, 100 mM mannitol, 20 mM HEPES/Tris, and 0.1 mM KH$_2$PO$_4$ [20 μCi/ml], pH 7.5) or Na$^+$-free uptake solution (100 mM choline chloride, 100 mM mannitol, 20 mM HEPES/Tris, and 0.1 mM KH$_2$PO$_4$ [20 μCi/ml], pH 7.5) and incubated at room temperature for 60 seconds. The suspension containing BBM vesicles was quickly transferred to 0.45-μm premoistened filters (ADVANTEC, Tokyo, Japan) and washed with ice-cold stop solution (0.9% NaCl, 10 mM KH$_2$PO$_4$). The filters were then dissolved in scintillation cocktail (Filter-Count; PerkinElmer, Waltham, MA) and radioactivity was determined. Na$^+$-dependent Pi transport was determined by subtracting the incorporation of $^{32}$Pi in the absence of Na$^+$ from that in the presence of Na$^+$. All measurements were carried out in triplicate.

**Measurement of Serum Parameters**

Serum and urinary phosphate and urinary creatinine were measured using Phospha-C test Wako (270-49801, Wako Pure Chemicals, Tokyo, Japan) and LabAssay Creatinine (290-65901, Wako Pure Chemicals), respectively, following the manufacturer’s instructions. Urine epinephrine levels were measured using the Adrenalin/Epinephrine EIA Kit (RE 592 51) (82001, IBL) according to the manufacturer’s instructions.

**Statistical Analyses**

All data are expressed as the mean±SEM. Results were analyzed for significant differences using the t test or ANOVA followed by the Bonferroni multiple comparison post hoc test. A longitudinal analysis of body weight, the glucose tolerance test, and time-course analysis of body temperature under cold exposure were analyzed using a repeated measures ANOVA. Significant differences in survival rates were analyzed by the log-rank analysis of Kaplan–Meier plots. Significance was set at $P<0.05$.

**ACKNOWLEDGMENTS**

M.K. conceived the project and designed the research. M.K. performed most of the experiments. S.K. assisted in mouse genotyping, immunohistochemistry, and vector construction. All the authors analyzed the data. M.K. wrote the manuscript. The authors thank Drs. A. Imura and Y. Nabeshima (Institute of Biomedical Research and Innovation, Kobe, Japan) for $K_l^{−/−}$ mice and Dr. H. Miyoshi (RIKEN BRC, Ibaraki, Japan) for the lentiviral vectors. The authors are thankful to Dr. M. Lombe (Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine, France) for T37i cells.

This work was supported by grants from the Japan Osteoporosis Foundation, The NOVARTIS Foundation (Japan) for the Promotion of Science, Novo Nordisk Study Award for Growth and Development, the Osaka Medical Research Foundation for Intractable Diseases, and a grant from Nestle Nutrition Council, Japan to M.K.

**DISCLOSURES**

None.

**REFERENCES**