The sodium phosphate cotransporter family and nicotinamide phosphoribosyltransferase contribute to the daily oscillation of plasma inorganic phosphate concentration

see commentary on page 1033

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Circulating inorganic phosphate exhibits a remarkable daily oscillation based on food intake. In humans and rodents, the daily oscillation in response to food intake may be coordinated to control the intestinal absorption, renal excretion, cellular shifts, and extracellular concentration of inorganic phosphate. However, mechanisms regulating the resulting oscillation are unknown. Here we investigated the roles of the sodium phosphate cotransporter SLC34 (Npt2) family and nicotinamide phosphoribosyltransferase (Nampt) in the daily oscillation of plasma inorganic phosphate levels. First, it is roughly linked to urinary inorganic phosphate excretion. Second, expression of renal Npt2a and Npt2c, and intestinal Npt2b proteins also exhibit a dynamic daily oscillation. Analyses of Npt2a, Npt2b, and Npt2c knockout mice revealed the importance of renal inorganic phosphate reabsorption and cellular inorganic phosphate shifts in the daily oscillation. Third, experiments in which nicotinamide and a specific Nampt inhibitor (FK866) were administered in the active and rest phases revealed that the Nampt/NAD^+ system is involved in renal inorganic phosphate excretion. Additionally, for cellular shifts, liver-specific Nampt deletion disturbed the daily oscillation of plasma phosphate during the rest but not the active phase. In systemic Nampt$^{+/-}$ mice, NAD levels were significantly reduced in the liver, kidney, and intestine, and the daily oscillation (active and rest phases) of the plasma phosphate concentration was attenuated. Thus, the Nampt/NAD^+ system for Npt2 regulation and cellular shifts to tissues such as the liver play an important role in generating daily oscillation of plasma inorganic phosphate levels.

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Hyperphosphatemia is linked to vascular calcification with chronic kidney disease (CKD), and is an independent risk factor for cardiovascular mortality in hemodialysis patients.[1–3] Serum inorganic phosphate (Pi), even within the normal range, is associated with cardiovascular events, cardiovascular mortality, and all-cause mortality, and exhibits a daily oscillation in both healthy individuals and patients with CKD.[4–9] Observational studies assessing the relationship between dietary intervention and serum Pi levels are confounded by the lack of standardization regarding the time of day that serum Pi was assessed.[10] Serum Pi levels exhibit a well-described daily oscillation in normal and CKD patients.[11] Pi peaks between 02:00 and 04:00 (rest phase), and the lowest levels are detected between 08:00 and 10:00 (active phase).[11–14] Most epidemiologic studies have demonstrated that the fasting morning serum Pi concentration is linked to cardiovascular events and mortality. The factors regulating this link, however, are not known.[15,16]

Plasma Pi concentrations and renal Pi excretion display significant daily oscillations in animals[17–19] as well as in humans.[12,13,20–23] The daily oscillation of plasma Pi levels in nocturnal rodents (rats) is roughly inverse to that in humans.[14,17–20,24] In humans and rodents, plasma Pi levels are decreased during the active phase and increased in the resting phase.[14,17–19] On the other hand, in humans and rodents, changes in urinary Pi excretion levels are roughly the reverse of the changes in the plasma Pi concentrations.[14,17–20,24] Prolonged fasting abolishes the nocturnal peak in serum Pi,[25,26] indicating that dietary intake contributes to the daily changes in serum Pi. Changes in parathyroid hormone (PTH), growth hormone, $1,25(OH)_2D_3$, and fibroblast growth factor 23 (FGF23) cannot fully explain the daily oscillation of plasma Pi concentrations.[12,14,27–29]

Pi homeostasis is predominantly regulated by sodium-dependent Pi transporters of the solute carrier family SLC34, including Npt2a, Npt2b, and Npt2c. Npt2a and Npt2c are responsible for reabsorption of approximately 70% to 80% of the Pi filtered by the kidney.[3,30] Small intestinal Npt2b also has an important functional role.[3,31–33] Serum Pi is a function of Pi homeostasis as well as the balanced movement of Pi between intracellular and extracellular spaces.[34] Detailed mechanisms of the cellular Pi shift are unknown, but cellular energy metabolism (ATP and nicotinamide adenine dinucleotide $[NAD]^+$) may be involved in Pi utilization.[35] The role of the SLC34 family in the daily oscillation remains unknown.

In a previous study, we investigated a partial hepatectomy-induced hypophosphatemia model and found that the nicotinamide phosphoribosyltransferase (Nampt)/NAD^+ system is important for systematic regulation of Npt2a, Npt2b, and Npt2c transporters.[36] Nampt acts via enzymatic activity to synthesize nicotinamide mononucleotide and to maintain homeostasis of NAD, which plays a dual role in energy metabolism and biologic signaling.[37,38] We hypothesized that the Nampt/NAD^+ system controls the daily oscillation. Here we investigated the roles of Npt2 and Nampt in the daily oscillation of plasma Pi concentrations.

RESULTS

Daily oscillation of plasma Pi levels and urinary Pi excretion in wild-type mice

First, we investigated the daily oscillation of plasma Pi and urinary Pi excretion in wild-type (WT) mice. Plasma Pi levels were lower at 08:00 AM (Zeitgeber time [ZT], light/dark cycle ZT0, lights on; ZT12, lights off) and gradually increased, peaking at around ZT10 (Figure 1a). Thereafter, the plasma Pi concentrations gradually decreased from ZT10 to ZT18. Renal Pi excretion values were highest from ZT10 to ZT14 (Figure 1b). Fractional excretion of phosphate (FEPi, %) values was highest at ZT14 (Figure 1c). We used brush border membrane vesicle (BBMV) total protein as a loading control because actin appears to undergo some time-of-day variation (data not shown). Renal and intestinal BBMV (20 μg/lane) were analyzed by immunoblotting. SDS–PAGE analysis revealed almost the same protein levels in each lane. Npt2a protein levels in the BBMVs gradually decreased from ZT2 to ZT14 and then increased to ZT22 (Figure 1d). The pattern of Npt2c protein levels was not as prominent as that of Npt2a. Daily oscillations of Npt2b protein were similar to those of renal Npt2a (Figure 1e).

Renal and intestinal Na/Pi transport activities in the BBMVs were significantly reduced at ZT14 compared with ZT2 (Figure 1f). Plasma PTH and FGF23 levels did not change between ZT2 (rodent rest phase) and ZT14 (rodent active phase; Figure 1g and h). These findings revealed that renal Npt2a protein and intestinal Npt2b protein levels exhibit daily oscillations, like plasma and urinary Pi levels, independent of the plasma FGF23 and PTH levels.

Effects of fasting on renal Pi excretion

Next, we investigated the effect of food deprivation on Pi excretion and plasma Pi levels (Supplementary Figure S1). Animals were analyzed during food deprivation and compared with those fed *ad libitum* (Supplementary Figure S1A). We analyzed 2 groups (feeding group and food-deprived group) beginning at ZT14 (Supplementary Figure S1B). In the food-deprived group, urinary Pi excretion levels gradually increased. Plasma Pi levels were significantly higher compared with the feeding group in all periods (Supplementary Figure S1B). The Npt2a protein levels were markedly decreased in the food-deprived mice (Supplementary Figure S1C). These findings suggest that the daily oscillation of plasma Pi concentrations depends on food intake, which is consistent with previous findings.[39]

Roles of Npt2 in the daily oscillations of plasma and urinary Pi levels

To investigate whether renal Npt2 proteins are involved in the daily oscillation of the plasma Pi concentration, we analyzed the daily oscillations of Npt2a$^{-/-}$, Npt2a$^{-/-}$/Npt2c$^{-/-}$, and intestine-specific Npt2b deletion mice (Npt2b$^{flox/flox}$-vCre) (Figure 2). Food intake behavior did not differ between groups (Figure 2c). Npt2a$^{-/-}$ mice have hypophosphatemia and hyperphosphaturia. During the diurnal phase (ZT2−ZT10), the increase in the plasma Pi concentration observed in Npt2a$^{+/+}$mice was not observed in Npt2a$^{-/-}$ mice (Figure 2a). During the active phase (ZT14−ZT22), however, the reduced plasma Pi concentration in Npt2a$^{+/+}$ mice was also observed in Npt2a$^{-/-}$ mice (Figure 2a). In contrast, during the active phase, renal Pi excretion levels were significantly increased in Npt2a$^{-/-}$ mice and Npt2a$^{+/+}$ mice (Figure 2b). Npt2a protein levels in Npt2a$^{+/+}$ mice were markedly decreased at ZT14 compared with ZT2 (Figure 2d). Npt2c protein levels were highest at ZT2 and ZT14 in Npt2a$^{-/-}$ mice. Unlike Npt2a$^{+/+}$ mice, Npt2a$^{-/-}$ mice showed no increase in the plasma Pi concentration during the rest phase (ZT2−ZT10), whereas the plasma Pi concentration was reduced during the active phase.

We further investigated the role of intestinal Npt2b in the daily oscillation of plasma Pi concentrations using Npt2b$^{flox/flox}$-vCre mice (intestine-specific Npt2b deletion mice). Npt2b$^{flox/flox}$-vCre mice had normal plasma Pi levels, but decreased renal Pi excretion, as reported previously.[33] Our established Npt2b$^{flox/flox}$-vCre mice, however, had lower plasma Pi levels than vCre$^+$ (control) mice at only 8 weeks. Increased plasma Pi concentrations were observed in intestinal vCre$^+$ mice and intestinal Npt2b$^{flox/flox}$-vCre mice during ZT2 to ZT10 (Figure 2e). Furthermore, plasma Pi concentrations were reduced during ZT14 to ZT22 in vCre$^+$ mice and Npt2b$^{flox/flox}$-vCre mice (Figure 2e). Urinary Pi excretion was suppressed in Npt2b$^{flox/flox}$-vCre mice compared with vCre$^+$ mice (Figure 2f). The daily oscillation pattern of plasma and urine Pi in Npt2b$^{flox/flox}$-vCre mice was similar to that in intestinal Npt2b vCre$^+$ mice.

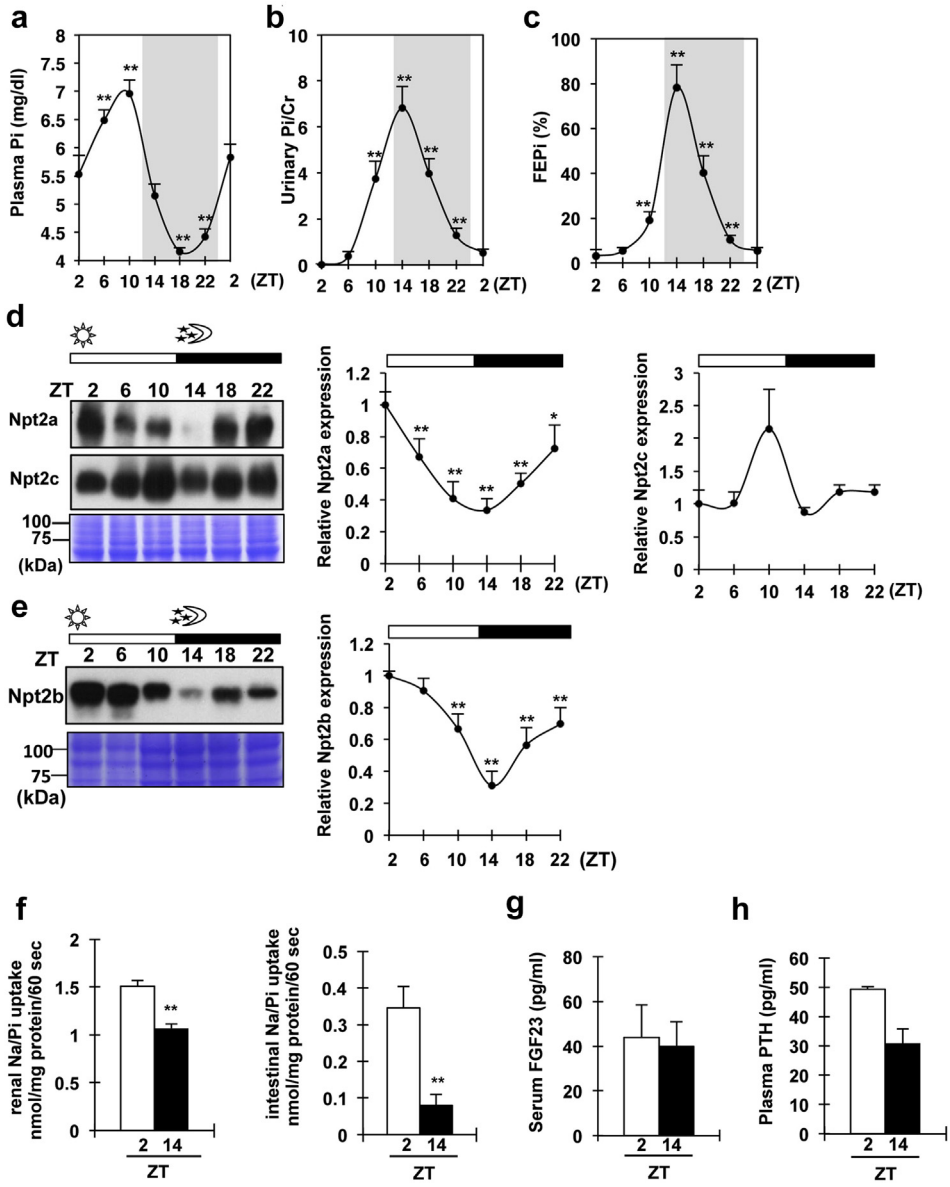

Figure 1 | Daily oscillations in inorganic phosphate (Pi) metabolism. Wild-type (WT) mice were maintained under a light-dark regimen (12 h:12 h cycle) and fed *ad libitum*. Samples were collected every 4 hours from Zeitgeber time (ZT) 2 (10:00). (**a**) Plasma Pi. (**b**) Urinary Pi/urinary creatinine (Cr). (**c**) Fractional excretion of phosphate (FEPi; %). (**a–c**) Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ (n = 10 each group). (**d**) Immunoblotting analysis of Npt2a and Npt2c in renal brush border membrane vesicles (BBMVs) from mice. (**e**) Immunoblotting analysis of Npt2b in distal intestinal BBMVs from mice. BBMVs were isolated at ZT2, 6, 10, 14, 18, and 22. White and black bars represent the light and dark phases, respectively. Values are expressed as the mean ± SEM (error bars). ZT: 08:00, ZT12: 20:00, *n* = 10 each group. (**d,e**) Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ (n = 5 each group). (**f**) Renal Na/Pi transport activity in mice. Na/Pi transport activity was determined by ^{32}P uptake in kidney BBMVs. Data are presented as mean ± SEM. $^{**}P < 0.01$ (*n* = 5–7/group). Intestinal Na/Pi transport activity in mice. Na/Pi transport activity was determined by ^{32}P uptake in distal intestinal BBMVs. Samples were collected from WT mice at ZT2 or ZT14. (**g**) Serum fibroblast growth factor 23 (FGF23). (**h**) Plasma parathyroid hormone (PTH). Open column shows ZT2, and closed column shows ZT14. (**g,h**) Data are presented as mean ± SEM (n = 8–10/group). $^{*}P < 0.05$, $^{**}P < 0.01$ (*n* = 8–10/group). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Next, we analyzed Npt2a$^{-/-}$/Npt2c$^{-/-}$ mice. The plasma Pi concentration was markedly decreased and the renal Pi excretion was conversely increased (Figure 2g and h), as reported previously.[40] The daily oscillation of the plasma Pi concentration observed in WT mice (Figure 1a) was not observed in Npt2a$^{-/-}$/Npt2c$^{-/-}$ mice. These findings suggest that the 2 transporters are indispensable to the daily oscillation of plasma Pi concentration.

Effects of nicotinamide on the daily oscillation of urinary Pi levels

We then investigated the factors controlling the daily oscillation of the plasma Pi concentration. In the small intestine and kidney, NAD levels were significantly increased at ZT14 compared with ZT2 (Figure 3a and b). NAD levels in the liver, on the other hand, were significantly increased at ZT2 compared with ZT14. These data

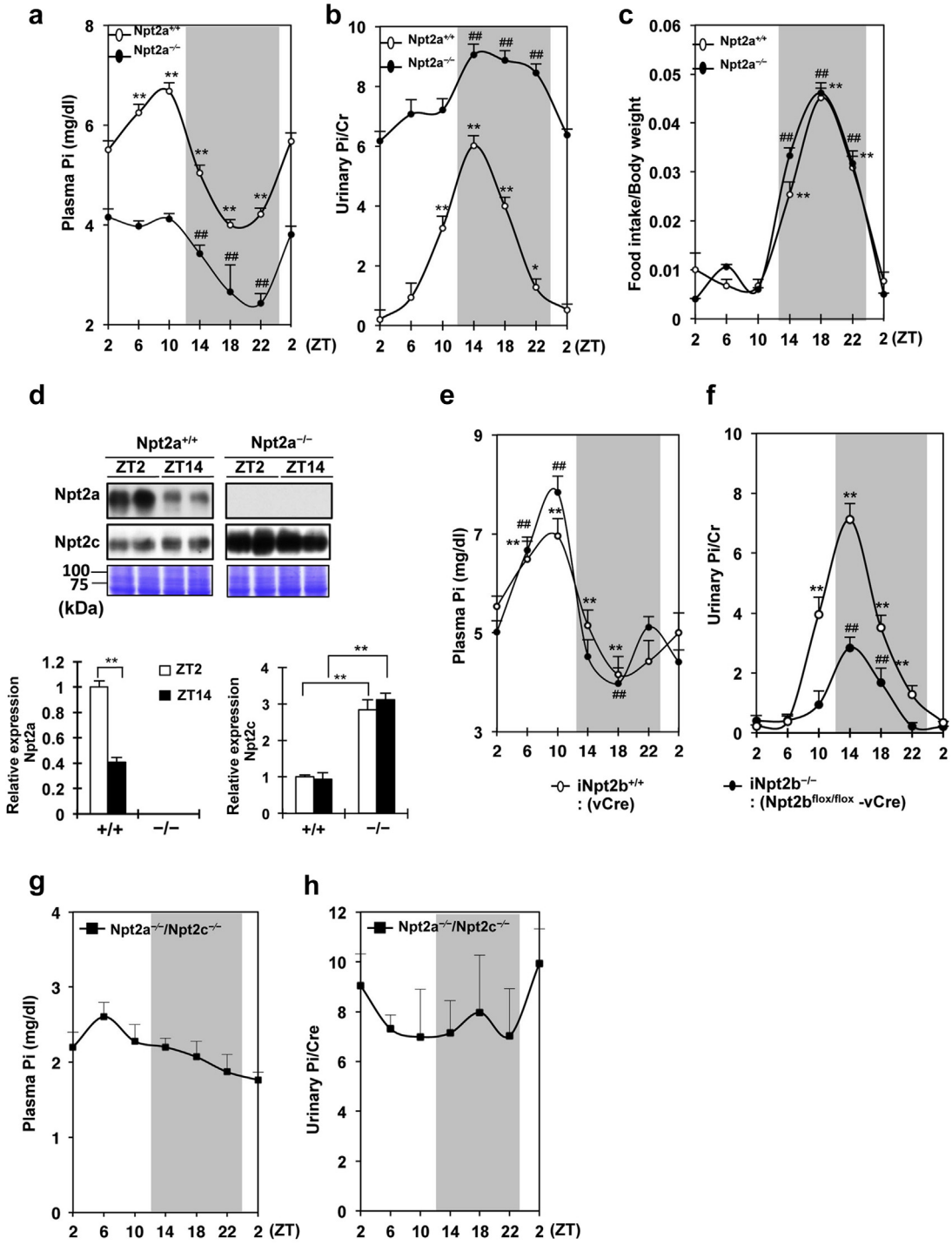

Figure 2 | Daily oscillations of plasma inorganic phosphate (Pi) and Pi excretion depended on renal expression of Npt2a and Npt2c and intestinal expression of Npt2b. $Npt2a^{+/+}$, $Npt2a^{-/-}$, $Npt2a^{-/-}Npt2c^{-/-}$, villin Cre transgenic mice (vCre; control), and intestine-specific Npt2b conditional knockout mice ($Npt2b^{flox/flox}$-vCre) were maintained under a light/dark cycle (ZT0, light on; ZT12, light off) and fed *ad libitum*. Samples were collected every 4 hours from ZT2 (10:00). (**a**) Plasma Pi. (**b**) Urinary Pi/urinary creatinine (Cr). (**c**) Ratio of food intake to body weight in $Npt2a^{+/+}$ and $Npt2a^{-/-}$mice. (**a–c**) Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#\#}P < 0.01$ ($n = 5$ each group). * = versus ZT2 in $Npt2a^{+/+}$; # = versus ZT2 in $Npt2a^{-/-}$ mice. (**d**) Immunoblotting analysis of Npt2a and Npt2c proteins in renal and intestinal brush border membrane vesicles (BBMVs) from $Npt2a^{+/+}$ and $Npt2a^{-/-}$ mice. BBMVs were isolated at ZT2 (open column), and ZT14 (closed column). Data are presented as mean ± SEM. $^{**}P < 0.01$ ($n = 10$ each group). (**e**) Plasma Pi. (**f**) Urinary Pi/urinary creatinine (Cr) in vCre and $Npt2b^{flox/flox}$-vCre mice. (**e,f**) Data are presented as mean ± SEM. $^{**}P < 0.01$, $^{\#\#}P < 0.01$ ($n = 5$ each group). * = versus ZT2 in vCre; # = versus ZT2 in $Npt2b^{flox/flox}$-vCre mice. (**g**) Plasma Pi. (**h**) Urinary Pi/urinary creatinine (Cr) in $Npt2a^{-/-}$/$Npt2c^{-/-}$mice ($n = 10$). Data are presented as mean ± SEM. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

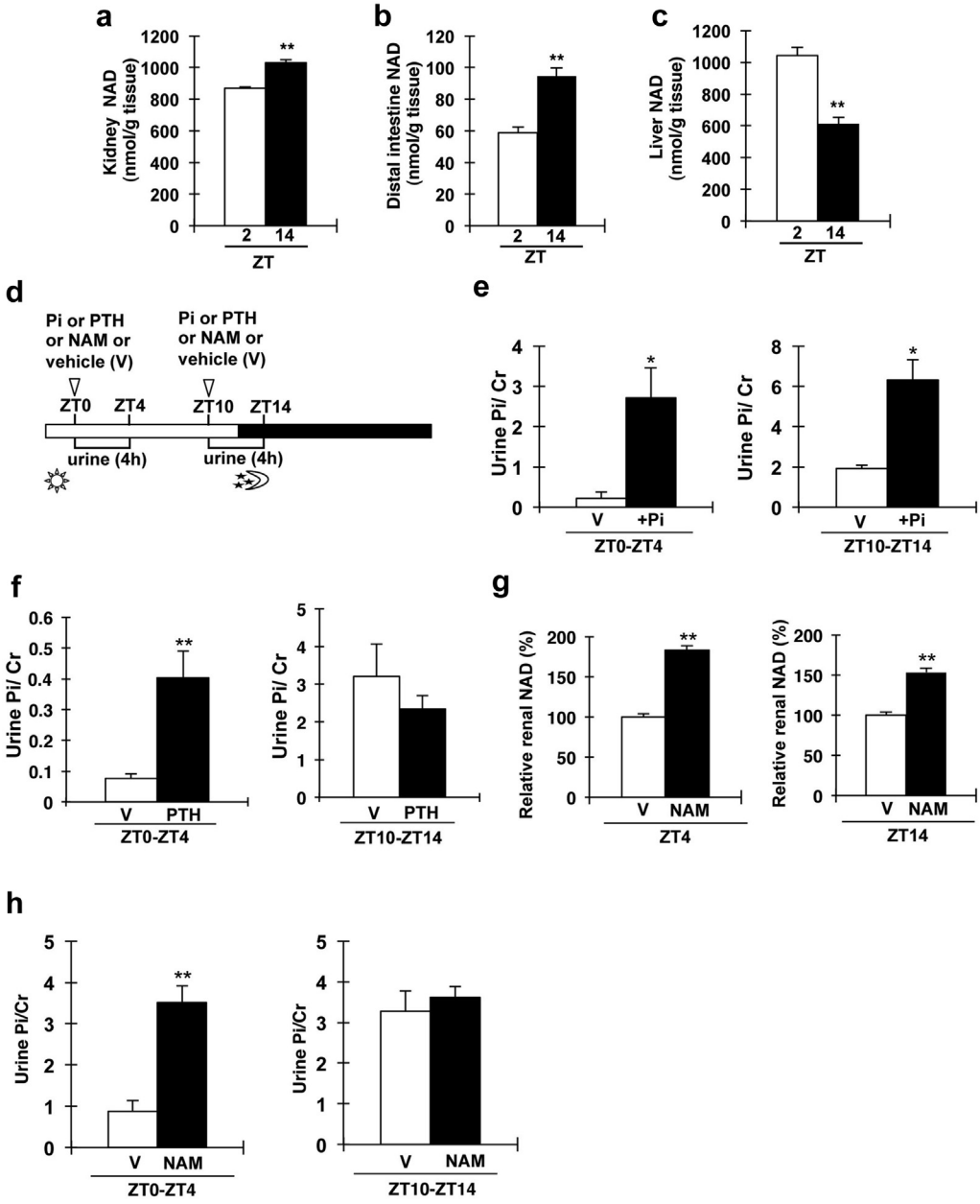

Figure 3 | Effect of nicotinamide and high phosphate loading on the daily oscillations of plasma inorganic phosphate (Pi) and Pi excretion. Samples were collected from wild-type (WT) mice (C57BL6 mice) at Zeitgeber time (ZT) 2 or ZT14. (**a**) NAD of the kidney cortex. (**b**) NAD of the intestine. (**c**) NAD of the liver. Open column shows ZT2, and closed column shows ZT14. (**a–c**) Data are presented as mean ± SEM. $^{**}P < 0.01$ ($n = 8$–10/group). (**d**) Experimental design. NAM (0.5g/kg body weight, i.p.), parathyroid hormone (PTH; 75 μg/kg body weight, i.v.), or Pi (100 μmol Pi oral administration) were administered to WT mice at ZT0 or ZT10, and samples were collected 4 hours after injection. (**e,f,h**) Urinary Pi/creatinine (Cr). (**g**) Relative NAD of the kidney cortex (vehicle: 100%). (**e–h**) Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ ($n = 8$–10/group). NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide.

suggest that cellular NAD levels affect Npt2a and Npt2b levels (Figure 3c).

We further investigated the effect of phosphaturic factors (Pi load, PTH, and nicotinamide [NAM]) on Pi excretion between the rest and active phases (Figure 3d). Pi load significantly stimulated renal Pi excretion to the same extent in the rest and active phases (Figure 3e). PTH injection significantly increased the Pi load in the rest phase, but not the active phase (Figure 3f). Changes in the cellular NAD concentration affect PTH responsiveness.[41] Next, we analyzed the effect of NAM on Pi excretion in the rest and active phases (Figure 3g and h). Injection of NAM increased the cellular NAD concentration (Figure 3g). In the active phase, NAM injection did not affect phosphaturic activity (Figure 3h). Npt2a protein levels were decreased in the rest phase, but not in the active phase (data not shown). These findings indicate

that the effect of NAM differs between the rest and active phases.

Effect of a Nampt inhibitor on renal Pi excretion during rest and active phases

The Nampt enzyme is a rate-limiting step of cellular NAD synthesis and has a circadian rhythm.[37,38] We analyzed the effect of a Nampt inhibitor (FK866) on C57BL6 mice (WT) (Figure 4). We injected FK866 into WT mice at ZT0 (rest phase) and ZT16 (active phase), and measured cellular NAD concentrations 4 hours later (Figure 4a). FK866 treatment significantly decreased renal NAD concentrations at ZT20 (active phase), but not at ZT4 (rest phase) (Figure 4b).

Intestinal NAD contents tended to decrease similarly (Figure 4c). At ZT4, the liver NAD contents were significantly decreased (Figure 4d). Thus, fluctuations in the NAD levels in the intestine and kidney tended to be opposite those in the liver.

FK866 treatment during the rest phase did not affect renal Pi excretion or plasma Pi levels. In contrast, FK866 significantly blocked Pi excretion at ZT16 to ZT20 (active phase) (Figure 4e). In addition, FK866 significantly increased plasma

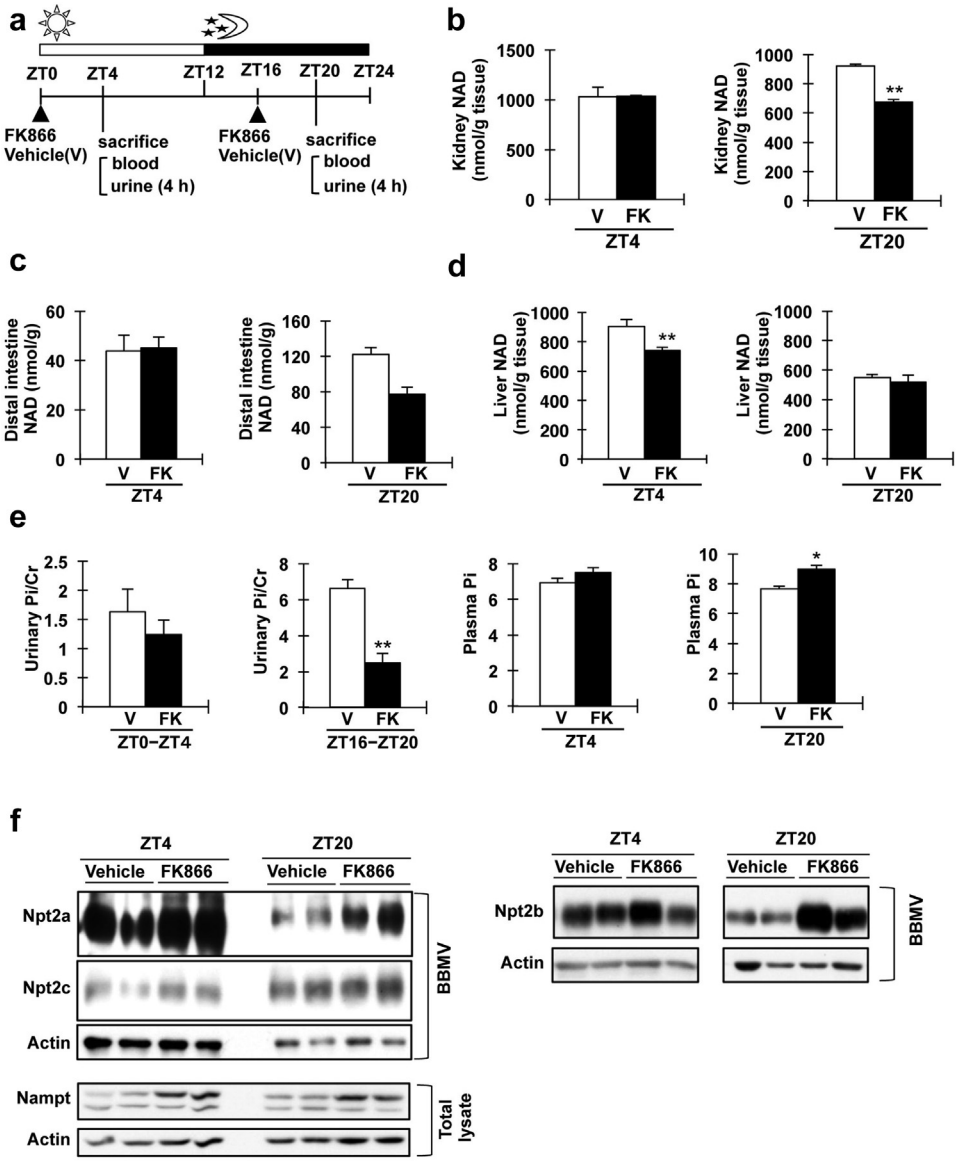

Figure 4 | FK866 affected inorganic phosphate (Pi) reabsorption during the active and rest phases. (**a**) Experimental design. Wild-type (WT) mice (10 weeks old) were treated with FK866 (70 mg/kg body weight) by i.p. injection at Zeitgeber time (ZT) 0 or ZT16, and samples were collected 4 hours after injection. The concentration of FK866 was selected according to a previous report.[31] (**b**) NAD concentration in kidney cortex. (**c**) NAD concentration in the distal intestine. (**d,e**) Urinary Pi/creatinine (Cr), plasma Pi concentration. (**f**) Immunoblotting of Npt2a and Npt2c proteins in kidney brush border membrane vesicles (BBMVs) and Npt2b in intestine BBMVs from FK866-injected mice. All membranes were reprobed for actin. Actin was used as an internal control. ($n = 8$/group.) Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ ($n = 8$/group). NAD, nicotinamide adenine dinucleotide. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

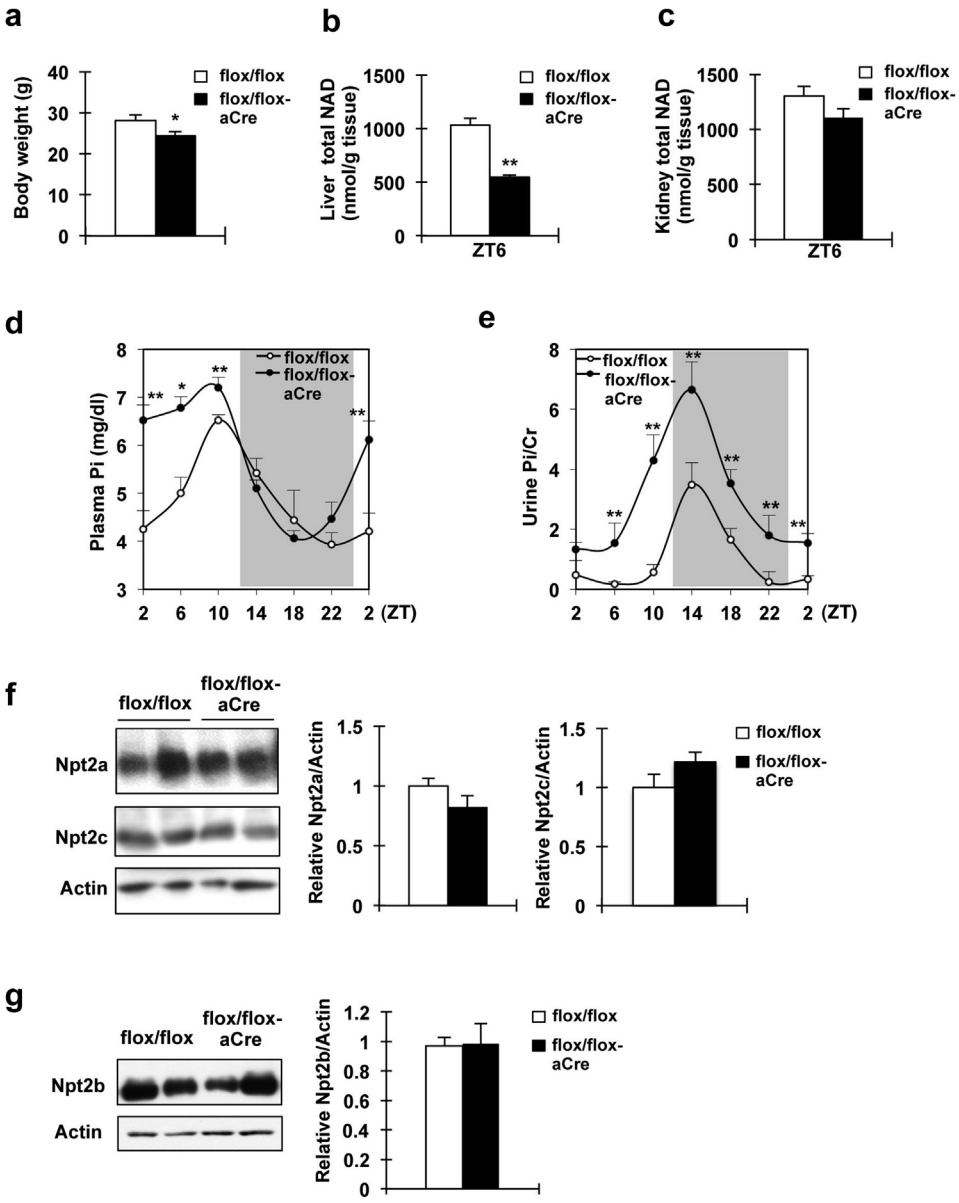

Figure 5 | Effect of liver nicotinamide phosphoribosyltransferase (Nampt) on daily oscillations of plasma inorganic phosphate (Pi) and Pi excretion. (**a**) Body weight of male liver-specific Nampt conditional knockout mice (Nampt$^{flox/flox}$-albuminCre: flox/flox-aCre) and control mice (Nampt$^{flox/flox}$; flox/flox) at 11 weeks ($n = 8$/group). (**b,c**) NAD concentrations in the liver and kidney cortex were measured in flox/flox-aCre and flox/flox mice at Zeitgeber time (ZT) 6 ($n = 8$/group). Samples were collected every 4 hours from ZT2 (10:00). (**d**) Plasma Pi. (**e**) Urinary Pi/urinary creatinine (Cr). (**f,g**) Immunoblotting analysis of Npt2a and Npt2c in renal brush border membrane vesicles (BBMVs) and Npt2b in intestine BBMVs from flox/flox-aCre and flox/flox mice. BBMVs were collected at ZT6. All membranes were reprobed for actin. Actin was used as an internal control ($n = 8$/group). Data are presented as mean ± SEM. *$P < 0.05$ (flox/flox-aCre vs. flox/flox), **$P < 0.01$ ($n = 8$/group). NAD, nicotinamide adenine dinucleotide. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Pi concentrations at ZT20. These observations suggest that Nampt inhibition is involved in the daily oscillation of plasma Pi and Pi excretion. Inhibition of the nocturnal (active phase) increase in Nampt activity prevented the reduction of renal Npt2a and intestinal Npt2b protein levels during the active phase (Figure 4f). The results shown in Figures 3 and 4 suggest that renal and intestinal Pi transport is controlled by the Nampt activity in the resting and active phases, respectively.

Daily oscillation of plasma Pi concentrations in liver-specific Nampt-knockout mice

In a previous study, we found that Pi release from the liver may contribute to maintaining the plasma Pi concentration.[36] Liver NAD levels exhibited a prominent daily rhythm that depended on food intake, as reported previously.[42] Next, we investigated liver-specific Nampt-deletion mice (Nampt$^{flox/flox}$-aCre). Liver Nampt levels exhibit a remarkable diurnal rhythm.[43] The body weight of

liver-specific Nampt$^{flox/flox}$-aCre mice was decreased compared with Nampt$^{flox/flox}$ mice (Figure 5a). In liver-specific Nampt$^{flox/flox}$-aCre mice, the liver NAD levels were significantly decreased during the rest phase (ZT6), but renal NAD levels were similar to those of the Nampt$^{flox/flox}$ mice (Figure 5b and c). Compared with Nampt$^{flox/flox}$ mice, the daily oscillation was not as prominent in the rest phase in liver-Nampt$^{flox/flox}$-aCre mice. In the rest phase, plasma Pi levels were higher in the liver-Nampt$^{flox/flox}$-aCre mice than in the Nampt$^{flox/flox}$ mice (Figure 5d). Urinary Pi excretion was markedly increased in the liver of Nampt$^{flox/flox}$-aCre mice at ZT12 to ZT16, but not at ZT0 to ZT4 (Figure 5e). Animals with deletion of Nampt in the liver exhibited no changes in the Npt2a and Npt2c protein levels (Figure 5f) and intestinal Npt2b levels (Figure 5g). The plasma Pi concentration was decreased during the active phase as in the Nampt$^{flox/flox}$ mice.

Daily oscillation of plasma Pi concentrations in Nampt$^{+/-}$ knockout mice

The findings of the present study suggest that liver Nampt contributes to increased plasma Pi levels from ZT2 to ZT10 (rest phase), but not to reduced plasma Pi levels during the active phase (ZT14–ZT20). Nampt expressed in other tissues such as fat, muscle, and bone might be involved in the daily oscillation of plasma Pi concentrations.

Because Nampt knockout is lethal in mice, we investigated the daily oscillation of plasma Pi concentrations in Nampt$^{+/-}$ mice. Male and female Nampt$^{+/-}$ mice have a normal body weight and almost normal fed and fasted glucose levels.[44] We also conducted insulin tolerance tests in male mice, and detected no difference between Nampt$^{+/-}$ and control mice as reported previously[44] (data not shown). Therefore, we used male Nampt$^{+/-}$ mice for analysis of the daily oscillation of the plasma Pi levels (Figure 6).

Body weight of Nampt$^{+/-}$ and Nampt$^{+/+}$ mice did not differ (Figure 6a). Compared with Nampt$^{+/+}$ mice, no prominent daily oscillation was observed in Nampt$^{+/-}$ mice. At ZT4 and ZT16, Nampt protein levels in the liver, kidney, and intestine of Nampt$^{+/-}$ mice were reduced by approximately 30% to 50% compared with those in Nampt$^{+/+}$ mice (Figure 6d and e). In the rest phase, plasma Pi levels were lower in Nampt$^{+/-}$ mice than in Nampt$^{+/+}$ mice (Figure 6b). Urinary Pi excretion was markedly decreased in Nampt$^{+/-}$ mice at ZT12 to ZT16, but not ZT0 to ZT4 (Figure 6c). Plasma NAD concentrations were significantly decreased in both groups at ZT4 and ZT16 (Figure 6f). Cellular NAD concentrations in the liver, distal intestine, and kidney were significantly decreased in Nampt$^{+/-}$ mice at ZT16, but not ZT4 (Figure 6f). Pi transport activities in the BBMVs of Nampt$^{+/-}$ mice were significantly increased in Nampt$^{+/+}$ mice at ZT16, but not ZT4 (Figure 7a). Npt2a and Npt2b protein levels were also increased at ZT16 in Nampt$^{+/-}$ mice (Figure 7b and c).

DISCUSSION

Daily oscillations of plasma Pi levels have been described for decades.[11–13,17–20,22,34] While food intake is an important factor for controlling these oscillations,[14,24] the mechanisms remain unknown. Renal excretory rhythms are driven by circadian changes in both glomerular filtration and tubular reabsorption/secretion in renal tubular function.[45–47] A large number of genes essential for water and solute homeostasis follow a well-marked circadian expression pattern.[46] In addition, Npt2a and Npt2c transcript levels in the kidney show no circadian variations, like NHE3.[48,49] In an animal study, Shinoda and Seto showed that daily variations in plasma Pi depend on food intake.[19] During the active phase (ZT12–ZT24), food intake stimulates the mobilization of extracellular Pi into the intracellular space and accelerates renal Pi excretion.[19] Thereafter, the plasma Pi concentration gradually decreases.[19] In humans and rodents, the daily oscillation of plasma Pi depending on food intake may be coordinated to control the plasma Pi balance; intestinal Pi absorption, renal Pi excretion, and cellular Pi shifts (e.g., between bone, liver, and muscle). Serum Pi levels fall in response to insulin administration.[50] Changes in serum Pi levels may thus be secondary to changes in insulin secretion. Insulin, however, does not induce renal Pi excretion.[50]

In the diurnal phase (rodent rest phase, no access to food) in WT mice, plasma Pi concentrations gradually increase from ZT0 to ZT10 (Figure 1a). In a previous study, Bielesz *et al.* reported a relationship between Pi excretion and serum Pi levels in rats.[17] Renal Pi excretion gradually increased from the rest phase (ZT0–ZT10), and Na/Pi transport activities significantly decreased at ZT9 to ZT10 compared with that at ZT0 to ZT1.[17] The findings indicate that the diurnal increase in renal Pi excretion in rats is not mediated by apparent changes in the Npt2a BBM protein abundance and localization.[17] In contrast, changes in the tubular Pi load and tubular threshold for reabsorption appear sufficient to explain the massive phosphaturia observed during the day.[17] Therefore, it is possible that the increase in plasma Pi levels during the rest phase (from ZT0 to ZT10) are due to Pi release or a reduction of the Pi influx in the soft tissues (for example, liver). Our findings led us to the same conclusion.

We found in a previous study that NAD metabolism in the liver-kidney axis is an important regulator of the plasma Pi concentration.[36] Dousa and coworkers reported the mechanism of Pi transport inhibition by NAD.[35,51] They demonstrated that NAD inhibits renal Na/Pi transport mainly in response to metabolic stimuli.[35,51] NAD acts indirectly by first being converted to cyclic ADP-ribose (cADPR), a potent stimulator of intracellular Ca^{2+} mobilization. According to this hypothesis, the increase in cellular NAD levels is an important factor for renal Na/Pi transport activity and Npt2a.[35]

Furthermore, hepatic NAD^+ levels dynamically change in a circadian manner, and are tightly related to nutritional states.[43] In contrast, fluctuations of NAD levels in the intestine and kidney tend to be opposite those in the liver. Liver NAD levels are significantly increased during the rest phase compared with the active phase. During the metabolically inactive phase in rodents (diurnal phase), the consumption of

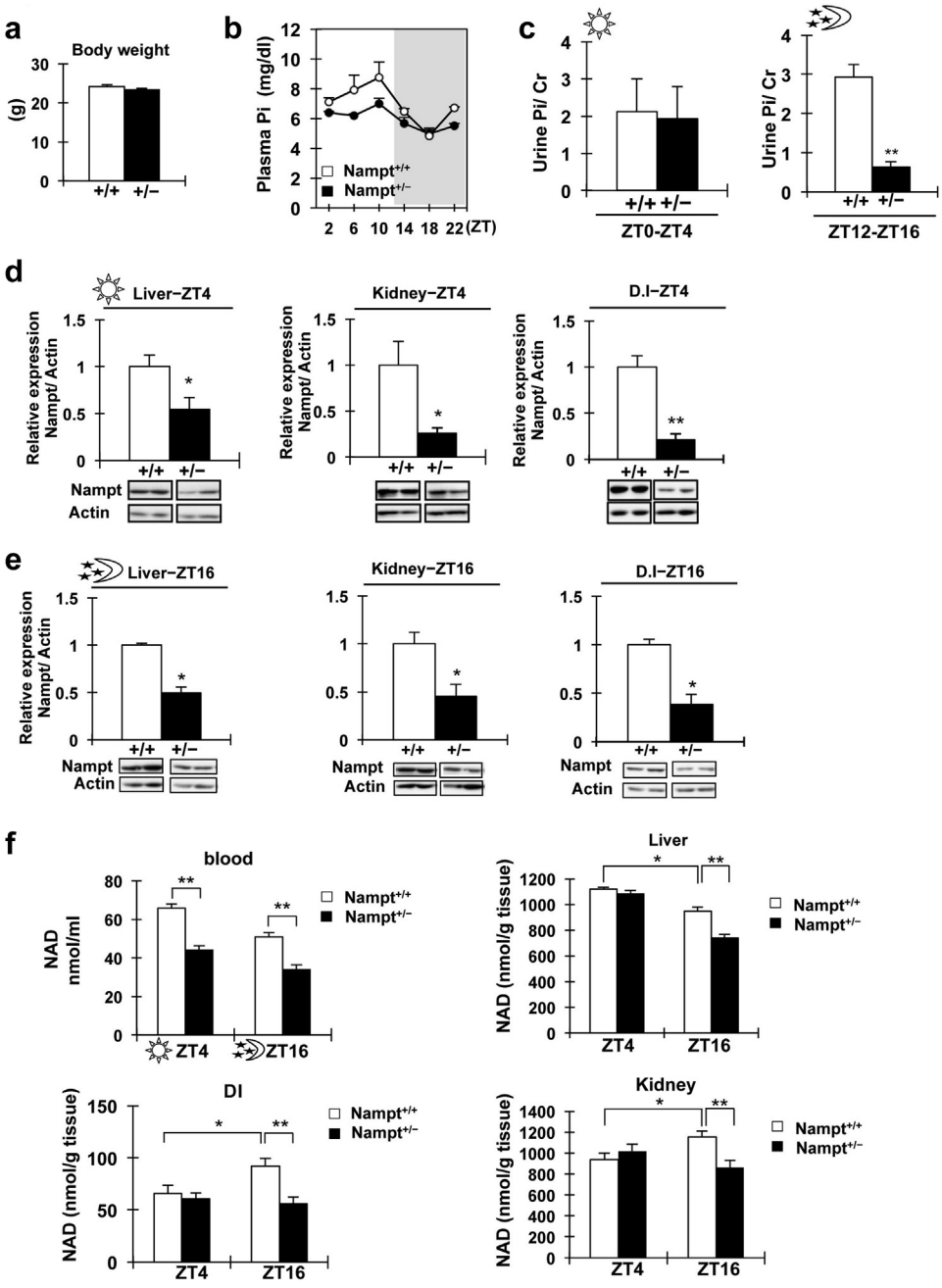

Figure 6| Characterization of nicotinamide phosphoribosyltransferase (Nampt) gene heterozygous (Nampt$^{+/-}$) mice. (**a**) Body weight of male Nampt$^{+/+}$ and Nampt$^{+/-}$ mice at 10 weeks ($n = 8$/group). (**b**) Plasma inorganic phosphate (Pi). Samples were collected from Nampt$^{+/+}$ and Nampt$^{+/-}$ mice at Zeitgeber time (ZT) 2, 6, 10, 14, 18, and 22. (**c**) Urinary Pi/creatinine (Cr). Samples were collected from Nampt$^{+/+}$ and Nampt$^{+/-}$ mice at ZT4 or ZT16. (**d**) Immunoblotting of Nampt protein in liver, kidney, and distal intestine total lysate of Nampt$^{+/+}$ and Nampt$^{+/-}$ mice at ZT4 ($n = 5$/group). (**e**) Immunoblotting of Nampt protein in liver, kidney, and distal intestine total lysate of Nampt$^{+/+}$ and Nampt$^{+/-}$ mice at ZT16 ($n = 5$/group). (**f**) NAD concentration in the blood, liver, distal intestine (DI), and kidney cortex were measured in Nampt$^{+/+}$ and Nampt$^{+/-}$ mice ($n = 5$/group). Data are presented as the mean ± SEM. *$P < 0.05$; **$P < 0.01$. NAD, nicotinamide adenine dinucleotide. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

cellular NAD levels is decreased compared with that during the active phase (nocturnal phase). Cellular NAD levels show tissue specificity, and NAD levels are controlled by many enzymes and metabolic factors (e.g., ATP, ADP, and Pi).[52–54] In the present study, liver-specific Nampt knockout mice had markedly increased renal Pi excretion and disrupted daily oscillation of plasma Pi during the rest phase, but not during the active phase. Npt2a and Npt2c levels in the liver-specific Nampt knockout mice were not increased in the rest phase compared with the control mice. Based on these data, we suggest that the Nampt/NAD$^+$ system in the liver is important for generating the daily oscillation of the plasma Pi

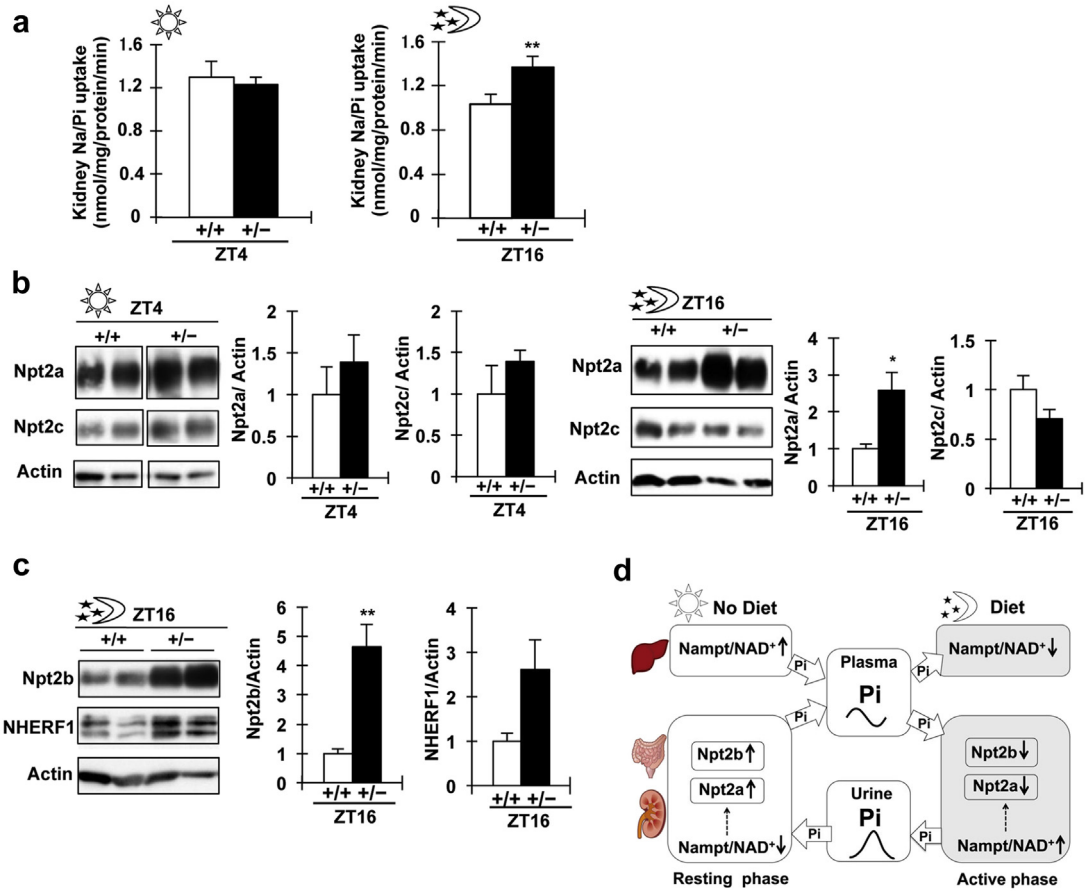

Figure 7 | Effect of nicotinamide phosphoribosyltransferase (Nampt) on daily oscillations of renal inorganic phosphate (Pi) uptake and Npt2 expression. (**a–c**) Samples were collected from Nampt$^{+/+}$ and Nampt$^{+/-}$mice at Zeitgeber time (ZT) 4 or 16. (**a**) Renal Na/Pi transport activity. Na/Pi transport activity was determined by ^{32}P uptake in kidney brush border membrane vesicles (BBMVs). (**b,c**) Immunoblotting analysis of Npt2a, Npt2c, Npt2b, and Na^+/H^+exchange regulatory cofactor (NHERF) proteins in renal and intestinal BBMVs from Nampt$^{+/+}$ and Nampt$^{+/-}$ mice. BBMVs were isolated at ZT4 and ZT16. All membranes were reprobed for actin. Actin was used as an internal control. Data are presented as mean ± SEM. $^{**}P < 0.01$, $^{*}P < 0.05$ ($n = 5$–7/group). (**d**) Roles of Npt2 and Nampt/NAD$^+$ pathway on the daily oscillation of plasma Pi levels. Based on this study, the predicted mechanism of the daily oscillation of plasma Pi in mice is as follows. Plasma Pi concentrations were increased during the rest phase (ZT0–ZT12, fasting) and decreased during the active phase (ZT12–ZT24, feeding). Plasma Pi levels during the rest phase (fasting phase) might be dependent on Pi release from the liver and the elevation of renal and intestinal Na^+-Pi transport activity through Npt2. Hepatic NAD$^+$ levels are known to dynamically change in a circadian manner, and are increased in the resting phase. The increase in cellular NAD$^+$ levels through Nampt activation may stimulate Pi release from the liver. On the other hand, the decrease in plasma Pi levels during the active phase (feeding phase) may be due to dietary intake. The changes in the tubular Pi load and tubular threshold for reabsorption appear sufficient to explain the massive phosphaturia observed during the active phase. Furthermore, activation of renal Nampt/NAD$^+$ activity suppresses renal and intestinal Npt2 protein levels. In addition to the liver, Nampt function in bone and muscle is expected to be involved in the daily oscillation. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

concentration, especially during the rest phase. We suggest that a hepatic Nampt-dependent NAD pathway may stimulate Pi efflux or block Pi influx.

In contrast, in the present study, we investigated the roles of Npt2a, Npt2b, and Npt2c in the daily oscillation of plasma Pi. Bielesz *et al.* evaluated only the relationship between the plasma Pi concentration and urinary Pi excretion from ZT0 to ZT9, and did not analyze the data at ZT10 to ZT24 (active phase).[17] For the active periods, we accurately measured the nocturnal (active phase) Pi rhythm and Pi excretion (Figure 1a and b). Plasma Pi concentration significantly decreased at ZT10 to ZT24 in the active phase. FEPi (%) values were also highest at ZT14 to ZT16. Npt2a protein levels were lowest at ZT14 and higher during the active phase (Figure 1e). Analysis of Pi metabolism in Npt2a$^{-/-}$and Npt2a$^{-/-}$/Npt2c$^{-/-}$ mice revealed that Npt2a and Npt2c proteins were essential for the daily oscillations of plasma Pi and Pi excretion. In this context, we suggest that the 3 transporters (intestine and kidney) are coordinated and regulate the daily oscillation of plasma Pi levels.

Interestingly, in Nampt$^{+/-}$ mice, we observed no prominent daily oscillation. It is possible that the Nampt/NAD$^+$ system controls renal Pi excretion and cellular shift. NAD$^+$ metabolism and Pi influx/efflux link the energy status with adaptive cellular responses.[35,55–57] Although classical experiments suggest that NAD$^+$ concentrations are held constant,

recent evidence indicates that cellular NAD^+ concentrations change under various conditions.[52,53] Importantly, cellular NAD levels fluctuate in a circadian manner through activation of the NAD^+ salvage pathway (Nampt pathway).[53,54] Further studies are necessary to elucidate the role of Nampt as a regulator of Pi metabolism in muscle and bone.

How does the daily oscillation of the plasma Pi concentration in such mice contribute to our understanding of the human serum Pi rhythm? Recent human studies suggest that the early morning nadir of serum Pi levels is due to shifts to the intracellular compartment or by Pi buffering by bone.[24] In humans and mice, the pattern of the circadian rhythm is different, but the cellular shift is considered to regulate serum Pi levels.[17,20,24] In hemodialysis patients, NAM effectively reduces serum phosphorus when co-administered with binders.[58,59] If the control mechanisms for serum Pi levels in dialysis patients during early morning fasting can be predicted based on the present findings in mice, the release of Pi from soft tissues (for example, liver) via Nampt/NAD may be important for serum Pi levels in dialysis patients. The results of this study are summarized in Figure 7d.

In conclusion, in the present study, we examined the mechanism of the daily oscillation of plasma Pi concentrations. There is a remarkable daily oscillation of plasma Pi levels due to dietary intake that is dependent on soft-tissue transfer and renal Pi excretion. The findings of the present study indicate that the formation of a daily oscillation of plasma Pi levels involves the Nampt/NAD^+ system of the soft tissues, including the liver, kidney, and intestine.

METHODS

Animal experiments

Details about the mice used in this study and the breeding methods are described in the Supplementary Methods.

Biochemical analyses

Plasma and urinary Pi was determined using the Phospha-C test (Wako Pure Chemical Industries Ltd., Osaka, Japan).[60] Urinary creatinine, serum-intact FGF23, and plasma intact PTH were determined using the creatinine-Wako test (Wako), FGF-23 ELISA kit (Kainos Laboratories Inc., Tokyo, Japan), and mouse PTH 1-84 ELISA kit (Immutopics, San Diego, CA), respectively.[60] Metabolic cages were used to collect urine samples and examine food intake every 4 hours.

Renal fractional phosphate excretion was calculated using the formula (U = urine, P = plasma):

$$FEPi(\%) = (\text{Pi U} \times \text{creatinineP})/(\text{creatinineU} \times \text{PiP}) \times 100^{17}$$

Preparation of BBMVs and transport assay

BBMVs were prepared from the kidney and jejunum by the Ca^{2+} precipitation method, and used for immunoblot analysis as described previously.[61,62] Levels of leucine aminopeptidase, Na^+-K^+-ATPase, and cytochrome c oxidase were measured to assess membrane purity. Uptake of ^{32}P into BBMVs was measured by the rapid filtration technique.[61,62]

Immunoblot analyses

Immunoblot analyses were performed using the following primary antibodies: affinity-purified anti-Npt2a[62] and anti-Npt2c,[62] which have been described previously. Anti-Npt2b (for mouse BBMVs; Alpha Diagnostics, San Antonio, TX)[31] and anti-Nampt (for mouse kidney, liver, and intestine total lysate; AdipoGen, Incheon, Korea) were analyzed using a commercial method. Mouse anti-actin monoclonal antibody (Chemicon, Temecula, CA) was used as an internal control. Membranes were exposed to standard X-ray films, and the densitometric quantification was performed using NIH Image imaging software. All experiments were repeated at least 5 times.

Total NAD (NAD^+ and NADH) analyses

The concentration of tissue and plasma NAD (NAD^+ and NADH) was measured using the colorimetric method.[63]

Statistical analyses

Statistical data are indicated as the means ± SEM. Statistical analysis was performed using an unpaired Student's t-test or analysis of variance followed by Dunnett's test. Values were considered statistically significant at $P < 0.05$.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Supplementary Methods.

Figure S1. Daily oscillation of Pi metabolism in fasted mice. (**A**) Experimental design. wild-type (WT; C57BL6 male) mice were maintained under a light/dark cycle (ZT0, light on; ZT12, light off) and either fasted or fed *ad libitum*. Samples were collected every 4 hours from ZT10 (18:00). (**B**) Plasma inorganic phosphate (Pi), urinary Pi/urinary creatinine (Cr), and urinary Pi. (**C**) Renal brush border membrane vesicles (BBMVs) were purified at ZT2. Immunoblotting analysis of Npt2a and Npt2c in renal BBMVs. Each lane was loaded with 20 μg of renal BBMVs. Actin was used as an internal control. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ versus feeding-fasting mice (n = 5–7/group).

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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