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Low dietary inorganic phosphate affects the brain by controlling apoptosis, cell cycle and protein translation

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Abstract

Inorganic phosphate (P_i) plays a key role in diverse physiologic functions. In a previous study, we showed that high dietary P_i perturbs brain growth through Akt/ERK signaling in developing mice. However, no study has investigated the response of the brain to low dietary P_i. In this study, we addressed this question by studying the effects of low dietary P_i on the cerebrum of developing mice. Two-week-old weaned mice were fed with a low phosphate diet for 4 weeks. At the end of the study, their cerebrum was dissected and signals important for protein translation, apoptosis and cell cycle were examined. The low phosphate diet did not cause physiologically significant changes; it increased the protein expression of phosphatase and tensin homolog deleted on chromosome 10 but decreased Akt activity. In addition, expression of eukaryotic translation initiation factor binding protein coupled with increased complex formation of eukaryotic translation initiation factor 4E/eukaryotic translation initiation factor binding protein 1 was induced in the cerebrum by low phosphate, leading to reduced cap-dependent protein translation. Finally, low phosphate facilitated apoptosis and suppressed signals important for the cell cycle in the cerebrum of dual-luciferase reporter mice. In summary, our results showed that a low phosphate diet affects the brain by controlling protein translation, apoptosis and cell cycle in developing mice. Our results support the hypothesis that P_i works as a stimulus capable of increasing or decreasing several pivotal genes for normal development and suggest that regulation of P_i consumption is important in maintaining a healthy life.

Keywords: Inorganic phosphate; Cerebrum; Protein translation; Apoptosis; Cell cycle

1. Introduction

Inorganic phosphate (P_i) is present in bacterial, fungal, plant and animal cells. P_i plays a critical role in diverse cellular functions involving intermediary metabolism and energy transfer mechanisms. It is a vital component of phospholipids in membranes and of nucleotides, both of which provide energy and serve as components of DNA, RNA and phosphorylated intermediates in cellular signaling [1]. Regulation of P_i balance is accomplished by family members of the sodium-dependent P_i transporter (NPT),

Abbreviations: P_i, Inorganic phosphate; NPT, Sodium-dependent P_i transporter; eIF4E-BP1, Eukaryotic translation initiation factor 4E binding protein 1; Akt1, *Homo sapiens* v-akt murine thymoma viral oncogene homolog 1; PTEN, Phosphatase and tensin homolog deleted on chromosome 10; LucR, Renilla luciferase; LucF, Firefly luciferase; mTOR, Mammalian target of rapamycin; IRES, Internal ribosome entry site; HRP, Horseradish peroxidase; TUNEL, Terminal deoxynucleotidyl transferase-mediated dUTP-biotin end labeling.

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which regulates entrance into the cellular membrane [2]. Recently, the existence of a constitutively active NPT has been known to play a key role in ATP (adenosine triphosphate) biosynthesis; therefore, it is essential for neuronal cell survival [3]. Moreover, brain-specific NPT1 has been found to exist principally in the cerebrum and cerebellum [4], whereas NPT2 has been identified in the third ventricle and amygdala [5]. Although the biologic significance of brain-specific NPT is still unknown, its role appears to be of paramount importance. Previous research had identified a wide range of nutritional and mineral risk factors that relate to cognitive function in children [6–8]. Additionally, studies have suggested that disturbances in ionic homeostasis may contribute to cerebral excitability [9] and secondary complications after brain injury [10]. An enhanced understanding of the responses of the developing brain of young animals to stimulation may provide critical clues for coping with diverse changes, subtle or dramatic, in brain function.

The serine/threonine protein kinase Akt, also termed protein kinase B, controls key cellular processes such as glucose metabolism [11], cell cycle progression [12] and apoptosis [13]. Akt has been identified as a key effector of the PI3K (phosphatidylinositol-3-phosphate) signaling pathway and functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets. Binding of PI3K-generated phospholipids to Akt results in translocation of Akt from the cytoplasm to the inner surface of the membrane, in which Akt is phosphorylated [14]. In fact, recent lines of evidence have suggested that Akt is involved in cerebral ischemia [15], Alzheimer's disease [16], seizures [17] and many other neurodegenerative diseases [18].

Surveys conducted in various countries have indicated that dietary intake of P_i has increased steadily as P_i-containing food increased by approximately 17% in the decade leading to 1993. These surveys also suggested that the use of P_i as a food additive may continue to increase [19]. To date, many studies involving P_i have focused mainly on its effect on bones and kidneys. In a previous study, we showed that high dietary P_i can perturb normal brain growth possibly through Akt/ERK signaling in developing mice [20]. However, no study has investigated homeostatic maintenance of the brain in response to low dietary P_i. In this study, we addressed this question by studying the potential effects of low P_i on the brain of developing weaned mice, with a special focus on the cerebrum. Our results demonstrated that low P_i affects the brain in young mice by decreasing cap-dependent protein translation, enhancing apoptosis and perturbing the cell cycle. Our results support the hypothesis that P_i works as a stimulus capable of increasing or decreasing several pivotal genes for normal growth and suggest that optimal regulation of P_i consumption may be one of the most cost-effective approaches to maintain a healthy life.

2. Materials and methods

2.1. Animals and diets

Recruitment of ribosomes to messenger RNAs (mRNAs) is the rate-limiting step in translation initiation and a frequent target for translation control. Two mechanisms of ribosome binding exist in mammalian cells. Cap-dependent translation is mediated by the mRNA 5′ cap structure and represents the standard mode of translation used by most cellular mRNAs. Cap-independent protein translation is used by some RNA viruses, including picornavirus and hepatitis C virus, as well as by some cellular mRNAs and involves the binding of ribosomes to an mRNA structural element termed an internal ribosome entry site (IRES) [21]. The mode of translation plays an important role in cell survival and growth [20,22]. Transgenic mice expressing the CMV–renilla luciferase (LucR)–c-Myc–IRES–firefly luciferase (LucF) reporter gene (2-week-old weaned male mice) were used to analyze cap-dependent and cap-independent protein translations since LucR and LucF provide the level of cap-dependent protein translation and that of cap-independent (IRES) protein translation, respectively [20,22]. Previous studies suggested that an abnormal level of P_i may affect posttranscriptional protein translation [20,23]. The weaned mice were separated into two dietary groups of five mice; one group received an AIN (American Institute of Nutrition)-93-based diet containing 0.5% P_i (normal P_i), whereas the other group received the same diet with 0.144% P_i (low P_i). All diets were prepared according to the guidelines of the AIN and thus fulfill the requirement for normal growth as described by Reeves et al. [24]. The mice were on the specified diet for 4 weeks until their complete physical maturation (6 weeks after birth). At the end of the experiment, all mice were sacrificed and their blood was taken for analysis. For effective focusing on the effects of low P_i on the brain, we selected the cerebrum as a representative of the brain; we dissected it under a stereomicroscope and stored it in liquid nitrogen for further use. All animal experiments were performed according to the guidelines for the care and use of laboratory animals of the Seoul National University.

2.2. Serum biochemical and hematologic analyses

The levels of calcium, phosphorous and other important biochemical values in the serum were determined using a biochemical autoanalyzer (VITALAB, Merck, The Netherlands). Hematologic parameters consisting of erythrocyte, leukocyte, neutrophil, lymphocyte, monocyte, eosinophil, basophil, platelet, hemoglobin and hematocrit levels were determined with a hematologic autoanalyzer (Coulter T540 Hematology System, Fullerton, CA, USA).

2.3. Luciferase assay

Luciferase activities in the tissue extracts were measured by an EG&G Berthold luminometer (Bundoora, Victoria, Australia). Briefly, each dissected cerebrum was homoge-

nized in a passive lysis buffer (Promega, Madison, WI, USA). The homogenates were centrifuged for 20 min at 4500 rpm at 4°C, and the supernatant was centrifuged for an additional 15 min at 13,000 rpm at 4°C. LucF and LucR activities were measured using a dual-luciferase assay kit (Promega).

2.4. Western blot analysis

After measuring the protein concentrations of the homogenized lysates using a Bradford kit (Bio-Rad, Hercules, CA, USA), we separated equal amounts (50 μg) of protein on SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred them to nitrocellulose membranes. The membranes were blocked for 1 h in TBST (Tris-buffered saline with Tween 20) containing 5% skim milk, and immunoblotting was performed by incubating the membranes overnight with their corresponding primary antibodies at 4°C. Antibodies raised against NPT1, p-Akt (Thr308), eukaryotic translation initiation factor 4E (eIF4E), eIF4E binding protein 1 (BP1), phosphatase and tensin homolog deleted on chromosome 10 (PTEN), Bad, Bcl-xL, cytochrome *c*, apoptotic protease activating factor 1 (APAF1), caspase 3, cyclin D1, cyclin-dependent kinase 4 (CDK4), cyclin E, CDK2, p53, p27, p21 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against the mammalian target of rapamycin (mTOR) was obtained from Cell Signaling (Beverly, MA, USA). Monoclonal antibodies against *Homo sapiens* v-akt murine thymoma viral oncogene homolog 1 (Akt1) and p-Akt (Ser473) were raised using general methods described elsewhere. After washing them in TBST, we incubated the membranes with a horseradish peroxidase (HRP)-labeled secondary antibody; the bands of interest were detected using a luminescent image analyzer (LAS-3000, Fujifilm, Japan). Results were quantified using the Multi Gauge Version 2.02 Program of the LAS-3000.

2.5. Immunoprecipitation

To determine whether low P_i may affect protein translation by affecting the complex formation between eIF4E and eIF4E-BP1, we performed immunoprecipitation assay. Briefly, cell lysates were dissolved in a homogenization buffer (20 mM of Tris, 150 mM of NaCl, 1 mM of EGTA, 1 mM of EDTA, 0.5% Triton X-100, 0.5% NP-40 and inhibitors of proteases and phosphatases) and precleaned by incubation with Protein A-Sepharose (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 4°C. The supernatant was then incubated with anti-eIF4E-BP1 antibody overnight. Immune complexes were separated by 15% SDS-PAGE gels. Visualization of eIF4E and that of eIF4E-BP1 were carried out by immunoblotting using specific anti-eIF4E and eIF4E-BP1 antibodies, respectively.

2.6. TUNEL assay

Formalin-fixed and paraffin-embedded brain tissue slides were deparaffinized in xylene and rehydrated through alcohol gradients. The slides were washed with PBS (phosphate-buffered saline), and nicked DNA ends were labeled by the TUNEL method using an in situ cell death detection kit (ROCHE, Basel, Switzerland) following the manufacturer's protocol. As a final step, tissue sections were counterstained with methyl green (Trevigen, Gaithersburg, MD, USA).

2.7. Immunohistochemistry

Formalin-fixed and paraffin-embedded tissue sections (5 μm) were transferred to plus slides (Fisher Scientific, Pittsburgh, PA, USA). The tissue sections were deparaffinized in xylene and rehydrated through alcohol gradients and then washed and incubated in 3% hydrogen peroxide (AppliChem, Darmstadt, Germany) for 30 min to quench endogenous peroxidase activity. After washing them in PBS, we incubated the tissue sections with 5% bovine serum albumin in PBS for 1 h at room temperature to block unspecific binding sites. Primary antibody (Bad) was applied on tissue sections overnight at 4°C. On the following day, tissue sections were washed and incubated with secondary HRP-conjugated antibody for 1 h at room temperature. After careful washing, we counterstained tissue sections with Mayer's hematoxylin (DAKO, Carpinteria, CA, USA) and washed them with xylene. Cover slips were mounted using Permount (Fisher Scientific), and the slides were reviewed using a light microscope (Carl Zeiss, Thornwood, NY, USA).

2.8. Data analysis

Quantification of Western blot analysis was performed using the Multi Gauge Version 2.02 Program. All results are given as mean±S.E. Results were analyzed by Student's *t* test (Graphpad Software, San Diego, CA, USA) or two-way analysis of variance (SAS User Guide Statistics, SAS Institute, Cary, NC, USA). Two-way analysis of variance was used to analyze body weight changes. A P value $<.05$ was considered to be significant, whereas that $<.01$ was considered to be highly significant as compared with the corresponding control.

3. Results

3.1. Low dietary P_i does not cause physiologically significant growth change in young dual-luciferase reporter mice

The different nutritional requirements during development and diverse functional roles of P_i suggest that appropriate in vivo nutritional studies at specific stages of growth are needed. Therefore, the potential effects of low P_i on the growth of young weaned mice were investigated. As mentioned earlier, we used dual-luciferase reporter mice, which allowed for analysis of cap-dependent and cap-independent protein translations efficiently [20,22]. As shown in Fig. 1A, the low P_i diet caused a slight reduction

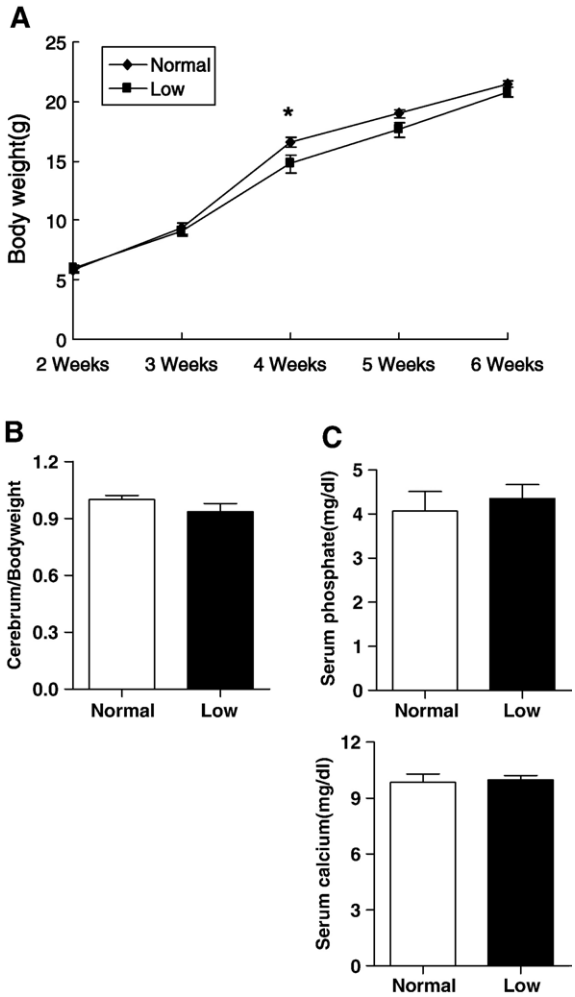

Fig. 1. Changes in body weight, serum phosphate and serum calcium in response to low P_i. Two-week-old weaned transgenic mice were fed with a normal diet (0.5%) or a low P_i diet (0.144%) for 4 weeks. (A) Changes in body weight. (B) Changes in the relative weight of the cerebrum. (C) Changes in serum calcium and phosphate levels. *$P<.05$ compared with the normal diet group (mean±S.E., $n=5$).

in body weight gain temporarily at 2 weeks, with rapid recovery as a function of time despite the fact that food intake was equivalent to normal food consumption (food consumption data not shown). In addition, the relative weight of the cerebrum remained unchanged (Fig. 1B). The low P_i diet did not cause any change in the serum phosphate and calcium concentrations as compared with those of the normal diet group (Fig. 1C). Other serum biochemical and hematologic values remained within the reference range (data not shown).

3.2. Low dietary P_i decreases brain-specific NPT1

To confirm the hypothesis that phosphate transport in the brain may play a key role in the homeostasis of brain function as mentioned earlier, we evaluated the potential effect of low dietary P_i on brain-specific NPT protein expression by Western blotting. Results revealed that low dietary P_i significantly decreased NPT1 protein expression in the cerebrum of young dual-luciferase reporter mice (Fig. 2A). Densitometric analysis clearly reconfirmed the reduction in NPT1 protein expression in the test group (Fig. 2B).

3.3. Low dietary P_i decreases Akt expression but increases PTEN expression

Akt is involved in a wide range of important functions in diverse organs, including the brain, and has been demonstrated to be an important signaling protein in response to nutrient metabolism [25]. This fact and the results of our previous study indicating that high P_i changed the pattern of Akt protein expression in mouse brain [20] prompted us to investigate the effects of low P_i on Akt signals in vivo. Low dietary P_i significantly increased PTEN expression but decreased total Akt expression with no significant change in phosphorylation at Ser473 and a slight decrease in phosphorylation at Thr308 in Western blotting (Fig. 3A). Such changed patterns of PTEN and Akt could be clearly observed in densitometric analysis (Fig. 3B). In this study, however, phosphospecific antibodies were used to measure Akt activation, and Akt is activated by phosphorylation [20,25]. Therefore, the level of Akt activation is represented as a ratio of the phosphorylated form to total protein. As shown in Fig. 3C, low P_i increased Akt phosphorylation at Ser473 but did not affect Akt phosphorylation at Thr308 in the cerebrum, although a significant suppression of total Akt protein was observed in the cerebrum (Fig. 3A and B). Such data suggest that low P_i suppresses Akt activity by disturbing Akt phosphorylation in murine cerebra.

3.4. Low dietary P_i reduces cap-dependent protein translation in the cerebrum

We have previously shown that high dietary P_i increased cap-dependent protein translation in the cerebrum of young dual-luciferase reporter mice [20]. Modification of cap-dependent protein translation is known to

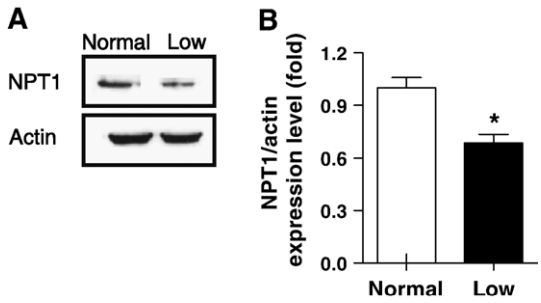

Fig. 2. Western blot analysis of NPT1 protein in the brain of mice fed with a low P_i diet and those fed with a normal phosphate diet. Two-week-old weaned transgenic mice were fed with a normal diet or a low P_i diet for 4 weeks. Their cerebrum was dissected, and tissue homogenates were subjected to Western blot analysis. (A) Expression of NPT1 protein in the cerebrum. (B) The bands of interest were further analyzed by densitometry. *$P<.05$ compared with the normal diet group (mean±S.E., $n=3$).

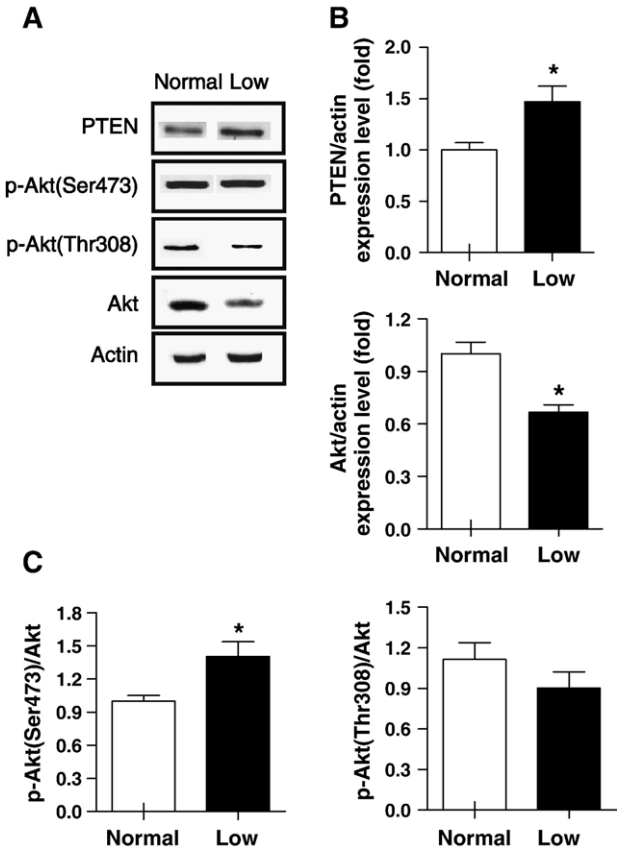

Fig. 3. Western blot analysis of PTEN, Akt and phospho-Akt protein in the cerebrum. Two-week-old weaned transgenic mice were fed with a normal diet or a low P_i diet for 4 weeks. Their cerebrum was dissected, and tissue homogenates were subjected to Western blot analysis. (A) Expression of PTEN, Akt and phospho-Akt proteins in the cerebrum. (B and C) The bands of interest were further analyzed by densitometry. *$P<.05$ compared with the normal diet group (mean±S.E., $n=3$).

affect various processes, such as cell growth and development, oncogenic transformation and even the function of the central nervous system [26]. We were therefore interested in the potential effects of low dietary P_i on protein translation in the brain of young dual-luciferase reporter mice. Low P_i did not affect mTOR protein expression but triggered significant augmentation of eIF4E-BP1 expression (Fig. 4A and B). To determine the differential levels of cap-independent and cap-dependent protein translations, we performed LucF and LucR assays. In contrast to the effect of high P_i on protein translation, low P_i decreased cap-dependent protein translation, thus, increasing cap-independent (IRES-dependent) protein translation in the cerebrum significantly (Fig. 4C). Since a complex of eIF4E and eIF4E-BP1 plays a key role in protein translation, immunoprecipitation analysis was performed. Our results clearly demonstrated that low dietary P_i increased immune complex formation between eIF4E and eIF4E-BP1, thus reducing cap-dependent protein translation in the cerebrum of dual-luciferase reporter mice (Fig. 4D).

3.5. Low dietary P_i facilitates apoptosis in the cerebrum

Since Akt signaling pathways are known to be associated with apoptosis and growth inhibition [13,27], we were interested in the potential effects of low P_i on apoptosis in the cerebrum of weaned mice fed with low dietary P_i. Our results indicated that levels of proapoptotic proteins such as Bad, cytochrome *c*, Apaf-1 and caspase 3 increased significantly while antiapoptotic Bcl-xL decreased significantly (Fig. 5A and B), suggesting that low dietary P_i facilitated apoptosis in the cerebrum of developing mice. Such enhanced apoptosis was reconfirmed clearly by the densitometric analysis (Fig. 5C and D). Moreover, TUNEL assay (Fig. 5E) and immunohistochemical analysis of Bad protein as a representative signal of proapoptosis (Fig. 5F) further demonstrated that an apoptotic process was active in

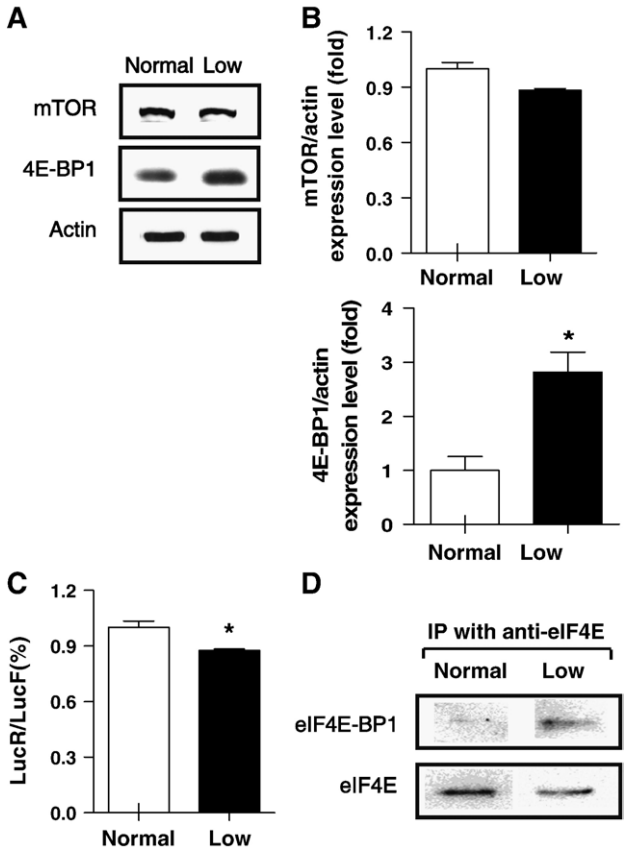

Fig. 4. Western blot analysis of mTOR and eIF4E-BP1, measurement of dual-luciferase activity and immunoprecipitation assay in the cerebrum of mice fed with a normal diet and those fed with a low P_i diet. Two-week-old weaned transgenic mice were fed with a normal diet or a low P_i diet for 4 weeks. Their cerebrum was dissected, and tissue homogenates were subjected to Western blot analysis. (A) Expressions of mTOR and eIF4E-BP1 protein in the cerebrum. (B) The bands of interest were further analyzed by densitometry. (C) Luciferase activities were measured for the determination of the ratios between cap-dependent (LucR) and cap-independent (LucF) protein translations in the cerebrum. (D) Immunoprecipitation analysis of eIF4E and eIF4E-BP1 levels. *$P<.05$ compared with the normal diet group (mean±S.E., $n=3$).

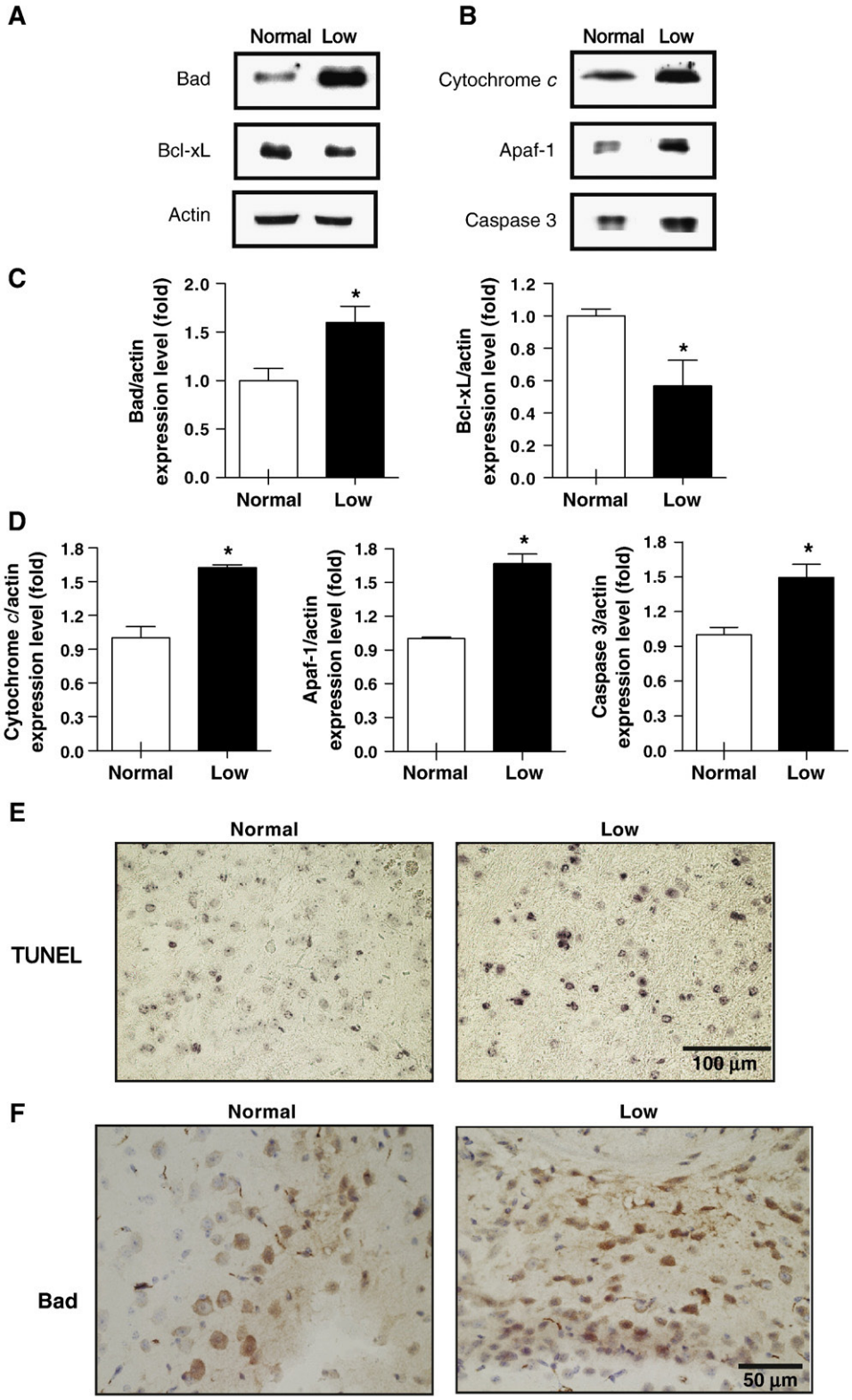

Fig. 5. Western blot analysis of apoptotic signal molecules, TUNEL and immunohistochemical analysis in the cerebrum. Two-week-old weaned transgenic mice were fed with a normal diet or a low P_i diet for 4 weeks. Their cerebrum was dissected, and tissue homogenates were subjected to Western blot analysis. (A and B) Expression levels of Bad, Bcl-xL, cytochrome *c*, Apaf-1 and caspase 3 in the cerebrum. (C and D) The bands of interest were further analyzed by densitometry. $*P<.05$ compared with the normal diet group (mean±S.E., $n=3$). (E) TUNEL assay. Scale bar = 100 μm. (F) Immunohistochemical analysis of Bad in the cerebrum. Apoptotic signals (dark blue) were clearly detected in the brain of mice from the low phosphate group. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

the cerebrum of dual-luciferase reporter mice treated with low dietary P_i.

3.6. Low dietary P_i suppresses signals important for the cell cycle

Akt is known to regulate cell cycle progression [12], and P_i is a signaling molecule capable of regulating global gene expression and cellular functioning in multiple cell types [28]. We thus evaluated the effects of low levels of P_i on the cell cycle in the cerebrum by analyzing the expression of various regulators of the cell cycle by Western blot analysis. Low P_i increased the protein expression of p53 but did not change the levels of p27 and p21 (Fig. 6A and B). Moreover, low dietary P_i suppressed signals important for cell cycle progression because significant reductions in cyclin A1, CDK4 and cyclin E were clearly observed (Fig. 7A and B).

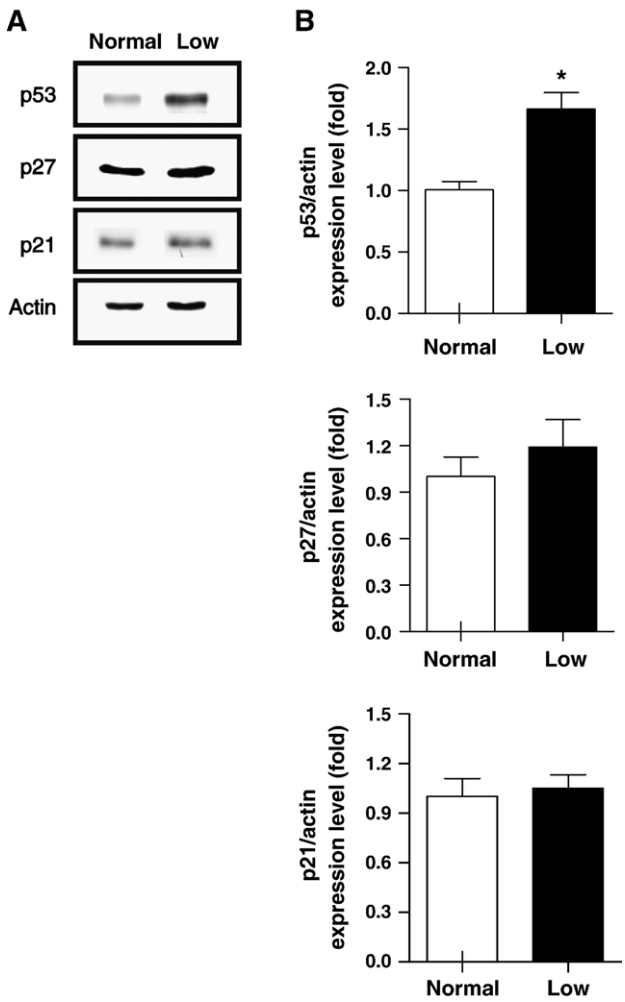

Fig. 6. Western blot analysis of p53, p27 and p21 in the brain of mice fed with a low P_i diet and those fed with a normal phosphate diet. Two-week-old weaned transgenic mice were fed with a normal diet or a low P_i diet for 4 weeks. Their cerebrum was dissected, and tissue homogenates were subjected to Western blot analysis. (A) Expression of p53, p27 and p21 proteins in the cerebrum. (B) The bands of interest were further analyzed by densitometry. *P<.05 compared with the normal diet group (mean±S.E., n=3).

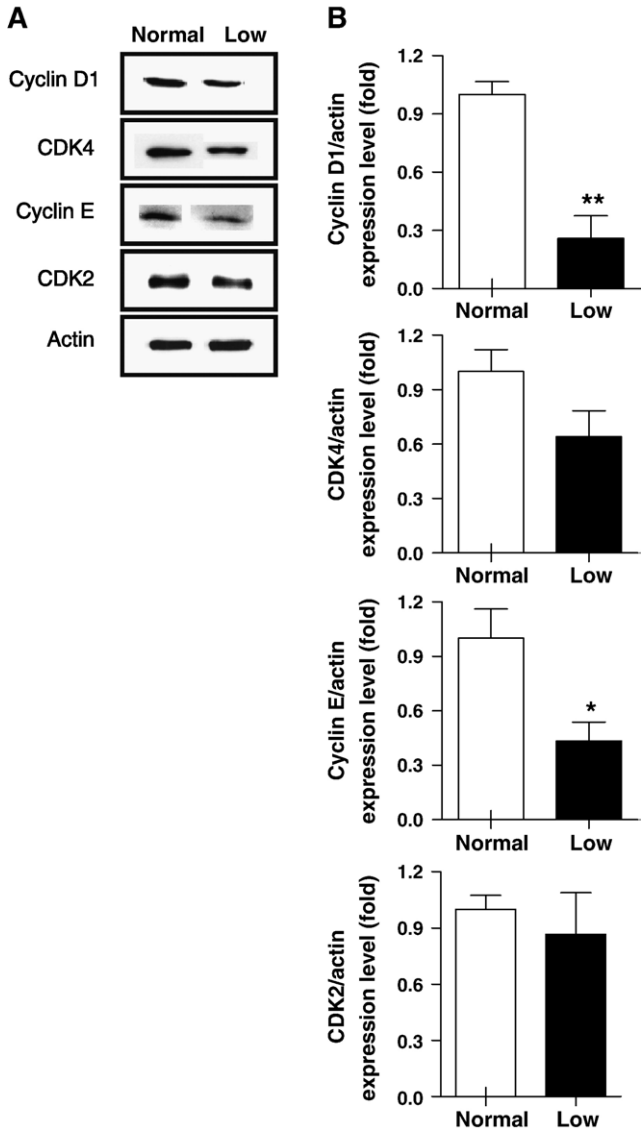

Fig. 7. Western blot analysis of cyclin D1, CDK4, cyclin E and CDK2. Two-week-old weaned transgenic mice were fed with a normal diet or a low P_i diet for 4 weeks. Their cerebrum was dissected, and tissue homogenates were subjected to Western blot analysis. (A) The expression levels of cyclin D1, CDK4, cyclin E and CDK2. (B) The bands of interest were further analyzed by densitometry. *P<.05 compared with the normal diet group (mean±S.E., n=3).

4. Discussion

Serum P_i level is maintained within a narrow range through intestinal absorption, exchange with intracellular as well as bone storage pools and renal tubular reabsorption. Recently, brain-specific NPT has been identified and considerable progress has been made in our understanding of its function and regulation [29,30]. P_i transport into the brain is regulated mainly by dietary and serum levels [1]. P_i

works as a stimulus capable of increasing or decreasing the expression of a number of pivotal genes such as those involved in the regulation of transcription, signal transduction and cell cycle [23,31]. Our previous work also showed that an excess amount of dietary P_i perturbed normal brain growth through Akt/ERK signaling in developing mice [20]. Thus, appropriate P_i consumption seems to be important in maintaining a healthy life. As mentioned earlier, no study has investigated the homeostatic maintenance of the brain in response to low dietary P_i. Therefore, in this study, we tried to evaluate the potential effects of low P_i on the brain of developing weaned mice, with a special focus on the cerebrum. The low P_i diet induced a slight decrease in body weight gain in the middle of the study (Fig. 1A); however, this small change may not be physiologically significant because other factors (e.g., serum phosphate, calcium, cerebral weight and many other factors) remained normal. Altogether, the data suggest that low dietary P_i may not induce any significant physiologic change in young mouse brain development.

Akt is a potent survival-promoting molecule that has been shown to be necessary for the survival of a variety of neurons and nonneuronal cells. A recent study demonstrated that adhesion molecules on glia play an important role in the regulation of brain growth through Akt phosphorylation [32]. Thus, a disruption in Akt activity may affect normal brain development. Akt is activated by phosphorylation mainly at two important sites, Thr308 and Ser473 [33]. Our results clearly indicated that low P_i reduced Akt activity since we observed decreased total Akt expression and imbalanced Akt phosphorylation (Fig. 3A–C). Our results showing reduced Akt activity and increased PTEN expression by low P_i can be further supported by the recent lines of evidence that Akt requires phosphorylation of both Thr308 and Ser473 for full activity and that Akt activity is negatively regulated by PTEN [34]. Recent evidence indicates that the Akt family contains three highly conserved members, namely, Akt1, Akt2 and Akt3, the first two of which are required for normal growth and metabolism, respectively. Akt1 is required for normal brain development. We monitored Akt activity and demonstrated that low P_i intake reduced Akt activity, which was associated with abnormal brain development. Such a correlation was noticed by Easton et al. [35], who showed that Akt1- and Akt3-deficient brains were reduced in size. Furthermore, absence of Akt1 led to a reduction in cell numbers, whereas the lack of Akt3 resulted in smaller and fewer cells. Additionally, attenuated mTOR signaling was observed in the brains of Akt3-deficient mice but not in those of Akt1-deficient mice. In our study, low dietary P_i did not have a significant effect on the levels of mTOR but increased eIF4E-BP1 protein expression significantly in the cerebrum (Fig. 4A and B). Such selective increase of eIF4E-BP1 strongly suggests that low P_i may affect the status of protein translation because eIF4E-BP1 is known to be a key factor determining cap-dependent/cap-independent protein translation [36]. In fact, our data clearly demonstrate that low dietary P_i inhibits cap-dependent protein translation by affecting the interaction between eIF4E and eIF4E-BP1 (Fig. 4C and D). Altogether, low levels of P_i might facilitate cap-independent protein translation in the cerebrum, possibly through Akt signaling. The mechanisms by which low P_i induces change in Akt activity in the cerebrum are under investigation.

Akt signaling is closely associated with apoptosis [13,27]. We showed that low levels of P_i induce significant up-regulation of signals important for apoptosis in the cerebrum (Fig. 5). An increasing body of evidence indicates that mitochondria play a pivotal role in apoptotic processes in mammalian cells. Disruption of mitochondrial membranes indicates mitochondrial damage and is generally defined as the early stage of apoptosis followed by the efflux of small molecules such as Apaf-1 [37]. Our results clearly showed that low dietary P_i increased the apoptosis by facilitating the signals important for proapoptosis and suppressing the signals important for antiapoptosis (Fig. 5), thus suggesting that a mitochondria-mediated pathway might be involved in low dietary P_i-triggered apoptosis in the cerebrum of dual-luciferase reporter mice. This observation is consistent with the finding of a recent study on the enhanced susceptibility of erythrocytes to apoptosis following phosphate depletion [38]. Phosphate depletion is a common disorder caused by dietary restriction, decreased intestinal absorption, renal wasting or cellular redistribution of phosphate. Its primary causes include starvation, severe diarrhea, PO4-binding antacids, hyperparathyroidism, vitamin D deficiency, leukemia, hepatic coma, alcohol withdrawal and glucose and/or insulin administration [39]. Sequels of phosphate depletion include impaired production of ATP, which impedes the function of Ca^{2+}-ATPase (adenosine triphosphatase) and thus leads to increases in cytosolic Ca^{2+} activity [40]. However, in our study, a low P_i diet (0.144% and not 0.02% as described in Ref. [38]) with AIN-93 was not supposed to cause any side effect associated with phosphate depletion and, indeed, values of serum calcium, phosphate and many other important factors remained unchanged (Fig. 1). Consequently, the low dietary P_i-mediated apoptosis observed in our study is probably not associated with phosphate depletion. Moreover, our results indicated that low P_i increased p53 protein expression significantly (Fig. 6A and B). Recent lines of evidence suggest that p53, as an integrated stress signaling protein, induces the production of such proapoptotic proteins as Bad and PTEN in fetal mouse cerebral wall [41]. As cell cycle control is closely associated with apoptosis, we investigated whether we could observe changes in the expression of several cell cycle regulators in response to low P_i in the cerebrum of dual-luciferase reporter mice. Initiation of cell cycle control via extracellular signals induces the transcription of several proteins, including cyclin D1, which, when complexed with CDK4, allows progression toward the cell cycle [42]. We observed that low dietary P_i led to repressed expressions of CDK4, cyclin D1 and cyclin E (Fig. 7A and B). Reduced cyclin D1

expression might also be explained by low Akt activity, whose accurate level of expression is required to regulate cyclin D1 and c-Myc IRES-dependent translation [43]. The reduced levels of cyclin D1 and cyclin E as well as the other important regulators of cell cycle progression that we observed in low P_i diet-treated cerebra might in turn favor apoptosis [44]. Altogether, the findings of highly expressed p53 with increased PTEN, facilitated apoptosis and suppressed cell cycle progression strongly suggest that low P_i may affect the normal brain by mediating apoptosis and regulating the cell cycle.

In summary, this study suggests that low dietary P_i affects young mouse brain development through disturbances in required interactions of protein translation, apoptosis and cell cycle regulation. The control of dietary P_i on such pivotal signaling pathways may be involved in numerous biologic processes during development, and its deregulation may cause various neurodegenerative diseases of the brain. Extensive studies to elucidate the precise effects and mechanisms — including the shift in cap-dependent translation versus cap-independent translation — of such activated signals on development and pathogenesis in the brain are currently underway.

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