BPB Reports

Report

TIC10/ONC201 Enhances Phosphate Uptake in the Human Neuroblastoma Cell Line SH-SY5Y

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The type-III sodium-dependent phosphate transporters, SLC20A1 and SLC20A2, are distributed throughout the body, including the central nervous system. Various neurodegenerative diseases, including primary basal ganglia calcification (PBGC), involve the disruption of phosphate homeostasis. Patients with PBGC harbor a mutated *SLC20A2*. Previously, we demonstrated that the phosphate transport activity of SLC20A2 was involved in PBGC pathology. Thus, we hypothesized the activation of phosphate transport as one of the therapeutic targets for PBGC. It was previously reported that *SLC20A1* and *SLC20A2* were increased at vascular smooth muscle cell of ATF4-overexpression mice. This study investigated the effect of TIC10/ONC201, a potential activator of ATF4, on phosphate transport in SH-SY5Y, a neuronal cell model. Treatment with 3 μM TIC10, which did not cause cell death, increased phosphate uptake along with the *ATF4* and *SLC20A1* but not *SLC20A2*. Treatment with 3 μM TIC10 also enhanced phosphate uptake in SLC20A2-knockdowned cells but not SLC20A1-knockdowned cells. In conclusion, TIC10 enhanced phosphate uptake in SH-SY5Y cells via SLC20A1 but not SLC20A2.

Key words phosphate, sodium dependent phosphate transporter, primary basal ganglia calcification

INTRODUCTION

Inorganic phosphate (Pi) is involved in the maintenance of biological functions, including intracellular signal transduction, membrane dynamics, mineralization, nucleic acid synthesis, and regulation of phosphate homeostasis in mammalians.[1)] Disruption of phosphate homeostasis in the neurovascular unit (NVU) is involved in several neurodegenerative diseases, such as Alzheimer's disease (AD)[2)] and Parkinson's disease (PD).[3)] Abnormal protein aggregation is thought to be a pathogenic factor in these diseases, which are also associated with integrated stress responses (ISRs).[4)] ISR is mainly regulated by the transcription factor ATF4.[4)] ATF4 is highly involved in the pathology of neurodegenerative disease.[5,6)] ATF4 is a stress-responsive transcription factor belonging to the ATF/CREB family.[7)] ATF4 mitigates endoplasmic reticulum stress by inducing defective proteolysis-associated molecules, autophagy-related factors, and amino acid transporters.[8)] Based on the stress responses, ATF4-regulated ISR is activated by stopping translation, as stress, may lead to defects such as the accumulation of denatured proteins. In response to this environmental stress, mitochondria initiate an ISR, which is beneficial for healthy aging and neuroprotection.[9)]

Phosphate homeostasis may be controlled by ATF4-regulated ISR. The overexpression of *ATF4* induced *SLC20A1* and *SLC20A2* in mouse vascular smooth muscle cells.[10)] Phosphate uptake in the brain is primarily dependent on the two phosphate transporters, SLC20A1 and SLC20A2.[11)] Furthermore, *SLC20A2* is thought to be the main causal gene for primary basal ganglia calcification (PBGC), also so many called as Fahr's disease, idiopathic basal ganglia calcification, primary familial brain calcification, primary brain calcification. Patients with PBGC present a disruption of phosphate homeostasis in the NVU.[12)] Thus, phosphate regulation in the brain may play an essential role in PBGC, AD and PD. However, the regulatory mechanism of phosphate homeostasis in the NVU, particularly the neuron, had not been reported previously.

TIC10, also known as ONC201, is a small molecule with promise in cancer therapeutic research. TIC10 has a variety of target proteins, including ATF4 and tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL).[13,14)] The compound can be administrated orally and intravenously. Furthermore, it penetrates the blood-brain barrier (BBB) and is a therapeutic tool for glioblastoma which is an intractable condition.[14)] The present study investigated the potential of TIC10 in regulating phosphate homeostasis in a human neuroblastoma cell line, SH-SY5Y.

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MATERIALS AND METHODS

Cell Culture and Knockdown Experiments SH-SY5Y (CRL-2266) was cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C and 5% CO_2 in a humid environment. For the *SLC20A1* and *SLC20A2* knockdown experiments, SH-SY5Y cells were transfected with MISSION® siRNA Universal Negative Control (non-targeting control; NC), esiRNA human *SLC20A1* (siSLC20A1), or esiRNA human *SLC20A2* (siSLC20A2) (Sigma-Aldrich).

CCK-8 Assay SH-SY5Y cells were seeded in 24-well plates. After 24 h, the medium was replaced with DMEM without FBS but containing 0, 1, 3, 5, and 10 μM TIC10. TIC10 was diluted in DMSO. After another 24 or 48 h, the cell counting reagent of the Cell Counting Kit 8 (CCK-8) was added to each well following the manufacturer's instructions and as previously described.[15]

RNA Preparation and Quantitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR) SH-SY5Y cells were treated with 20 nM siRNAs for 48 h, after which reverse-transcription was conducted using ReverTra Ace™ qPCR RT Master Mix (TOYOBO, Osaka, Japan), following the manufacturer's instructions and as previously described.[16] The primers used were human *GAPDH* (forward: 5′-TGGTGAAGACGCCAGTGGA-3′; reverse: 5′-GCACCGTCAAGGCTGAGAAC-3′), human *SLC20A1* (forward: 5′-CTGTGGGGTACCATCCTCATC-3′; reverse: 5′-AGGGGCTTTCAGAAGGACTACAC-3′), human *SLC20A2* (forward: 5′-TTTTGTGTGGCTCTTCGTGTG-3′; reverse: 5′-ATACTGGGGACTCTGCTTCCTG-3′), human *ATF4* (forward: 5′-GGCCAAGCACTTCAAACCTC-3′; reverse: 5′- GAGAAGGCATCCTCCTTGCT-3′). Target gene mRNA levels were normalized to those of *GAPDH*.

Phosphate Uptake Assay A phosphate uptake assay was conducted on treated cells grown to confluency in 24-well plastic plates as previously described.[17] The transport rate was expressed as nmol Pi per min per mg protein.

Statistical Analysis Data are presented as the means ± standard errors. Significance was determined using analysis of variance. Further statistical analysis for post hoc comparisons was performed using the Bonferroni-Dunn test. $P < 0.05$ was considered statistically significant. All statistical tests were conducted by employing the SigmaPlot 13 software (Systat Software Inc, San Jose, CA, USA)

RESULTS

TIC10 Increased the Transcription of SLC20A1, but not SLC20A2 TIC10 induces ATF4 as well as TRAIL and causes cancer cell apoptosis.[18] Therefore, to determine the condition that impedes SH-SY5Y neuronal cell death, the effect on cell viability was examined using the CCK-8 assay. SH-SY5Y cells were treated with 0, 1, 3, 5, and 10 μM TIC10 for 24 h and then the cell viability was ascertained, which was unchanged at all concentrations (Fig. 1A). Next, to confirm that TIC10 enhanced the transcription of *ATF4*, its mRNA level was evaluated using qRT-PCR in SH-SY5Y cells treated with 0, 1, 3, and 5 μM TIC10 for 24 h. The results showed that *ATF4* expression was significantly upregulated at 3 μM (Fig. 1B).

The influence of TIC10 on the expression of the phosphate transporter-related genes *SLC20A1* and *SLC20A2* was quantified using qRT-PCR. The expression of *SLC20A1* showed the same trend as that of *ATF4* and was markedly increased at 3 μM TIC10 (Fig. 1C), while that of *SLC20A2* was unchanged (Fig. 1D).

TIC10 Increased Phosphate Uptake in siSLC20A2-Treated SH-SY5Y Cells SH-SY5Y cells were incubated with 0, 1, 3, and 5 μM TIC10 for 24 h, and the intracellular phosphate transport activity was measured to investigate the influences of TIC10 on phosphate uptake. Figure 2A shows the scheme of the phosphate uptake assay. The results showed a significant concentration-dependent (up to 3 μM) increase in phosphate uptake of TIC10 (Fig. 2B). SH-SY5Y cells were treated with siSLC20A1 or siSLC20A2 for 24 h to ascertain whether this effect of TIC10 was due to an increase in *SLC20A1* transcription. Following this treatment, 3 μM TIC10 was administrated, and the cells were incubated for a further 24 h. After this, intracellular phosphate transport activity was measured using ^{32}P. *SLC20A1* and *SLC20A2* transcription was reduced by ~ 70% in the siSLC20A1 and siSLC20A2-treated cells, respectively (Figs. 2C and D). Thus, it was confirmed that *SLC20A1* and *SLC20A2* were knocked down by siRNA treatment. Additionally, *SLC20A1* transcription did not alter in siSLC20A2-treated cells and *vice versa*, suggesting the absence of a compensatory expression mechanism between *SLC20A1* and *SLC20A2* in SH-SY5Y cells. Finally, phosphate uptake in siSLC20A1-treated cells was unaltered by TIC10 treatment but was remarkably elevated in the siSLC20A2-treated cells (Fig. 2E). These results suggest that TIC10 enhanced phosphate uptake in SH-SY5Y cells via increaseing *SLC20A1* transcription.

DISCUSSION

This study aimed to investigate the effect of an ATF4 inducer, TIC10, on phosphate uptake in the human neuroblastoma cell line SH-SY5Y. In a previous report, the vascular smooth muscle cells-specific *ATF4* overexpression mice showed increased *SLC20A1* and *SLC20A2* aortic expression.[10] SH-SY5Y cells are valuable for investigating the genomic basis of the pathophysiology of neurodegenerative diseases,[19] and *SLC20A1* and *SLC20A2* were confirmed to be functional in them.[20] Therefore, it was evaluated whether TIC10 affected phosphate uptake in SH-SY5Y cells. The present study suggests that TIC10 increased phosphate uptake in SH-SY5Y cells *via* induction of *ATF4* and *SLC20A1* transcription.

Phosphate is an essential element involved in various functions in the body. In cells, it is a component of ATP, nucleic acids, and cell membranes. In addition, phosphate regulates various cellular functions by phosphate-signaling or regulating proteins through phosphorylation or dephosphorylation. Altered phosphate dynamics have also been observed in multiple neurodegenerative diseases, such as AD[2] and PD[3]. Altered expression of sphingosine 1-phosphate, a metabolite of sphingolipids, and other compounds is observed in these diseases. Thus, modified phosphate regulation is deeply involved in the neurodegenerative diseases.

PBGC is one such disease characterized by ectopic calcification in the brain. Patients with PBGC exhibits a variety of psychiatric and neurological symptoms. One of the genetic causes of PBGC is a mutated *SLC20A2*, which encodes a phos-

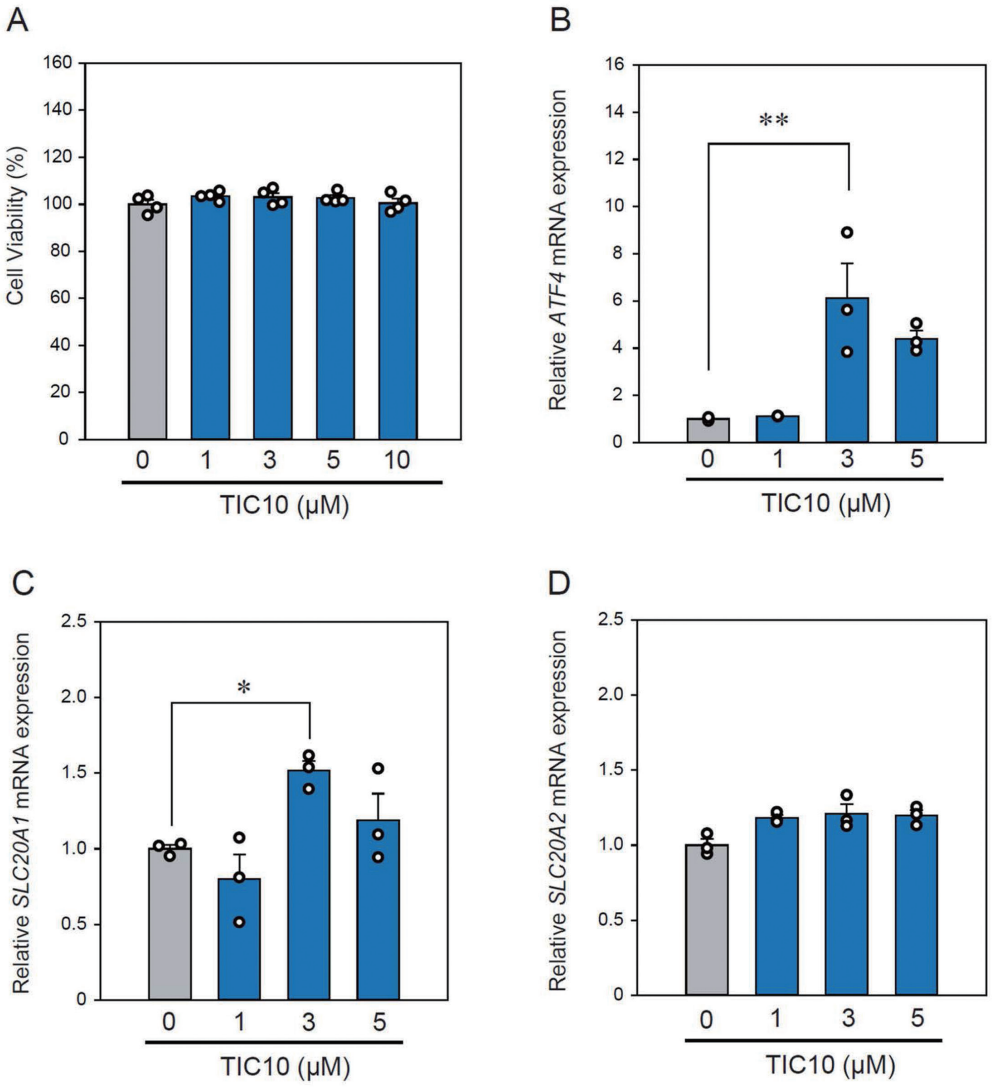

Fig. 1. TIC10 Increases the Expression of *ATF4* and *SLC20A1* in the SH-SY5Y Cell Line

(A) CCK-8 assay for cell viability was conducted 24 h after TIC10 treatment (1, 3, 5, and 10 μM). Data are expressed as the mean ± SEM (n = 4). (B–D) qRT-PCR analysis of *ATF4*, *SLC20A1*, and *SLC20A2* expression levels after TIC10 treatment for 24 h. Data are expressed as the mean ± SEM (n = 3). Significance was determined by a one-way ANOVA followed by Bonferroni's post hoc test (* $p < 0.05$ or ** $p < 0.01$ vs. 0 μM TIC10).

phate transporter. Phosphate levels in the cerebrospinal fluid (CSF) of PBGC patients with *SLC20A2* mutations were higher than those of healthy controls.[21] *Slc20a2*-KO mice showed elevated CSF phosphate levels,[22] suggesting a partial disruption of phosphate homeostasis in PBGC pathology. In addition, using cell-based assay, we have previously demonstrated that a mutation of *SLC20A2* did not affect PBGC pathology and maintained phosphate transport activity.[23] Thus, activation of phosphate transport may be a therapeutic concept in PBGC patients with *SLC20A2* mutations.

Here, we found that TIC10 activates phosphate transport. Sodium-dependent phosphate transporters can be classified into type-I (SLC17 family), type-II (SLC34 family), and type-III (SLC20 family). In SH-SY5Y cells, they are expressed mainly as SLC20A1 and SLC20A2, compared to other phosphate transporters.[20] Our results suggest that the activation of phosphate transport by TIC10 may be through an increased expression of *SLC20A1* but not *SLC20A2*, although the relationship between *SLC20A1* and *ATF4* at molecular level is unknown. Thus, it is necessary to identify whether *ATF4* binds to the promoter regions of *SLC20A1* and if *SLC20A1* expression is changed in neuronal cells where *ATF4* is deleted.

TIC10 is well-studied in the field of cancer research. TIC10 has a variety of target proteins, including ATF4, a regulator of ISR. Enhancing SLC20A1 expression *via* ATF4 would be one of the therapeutic targets for PBGC because phosphate transport disruption is a cause of PBGC pathophysiology. Activating phosphate transport could lead to therapies that include inhibition of calcification and subsequent neurodegeneration. However, TIC10 also activates TRAIL and leads to the death of not only cancer cells but also normal cells in their vicinity. However, TIC10 may not cause any significant toxicity to the normal tissue.[14] Taken together, TIC10 might be future seed compounds in PBGC if the appropriate concentration and timing of therapeutic intervention can be cleared using an appropriate mice model of PBGC.

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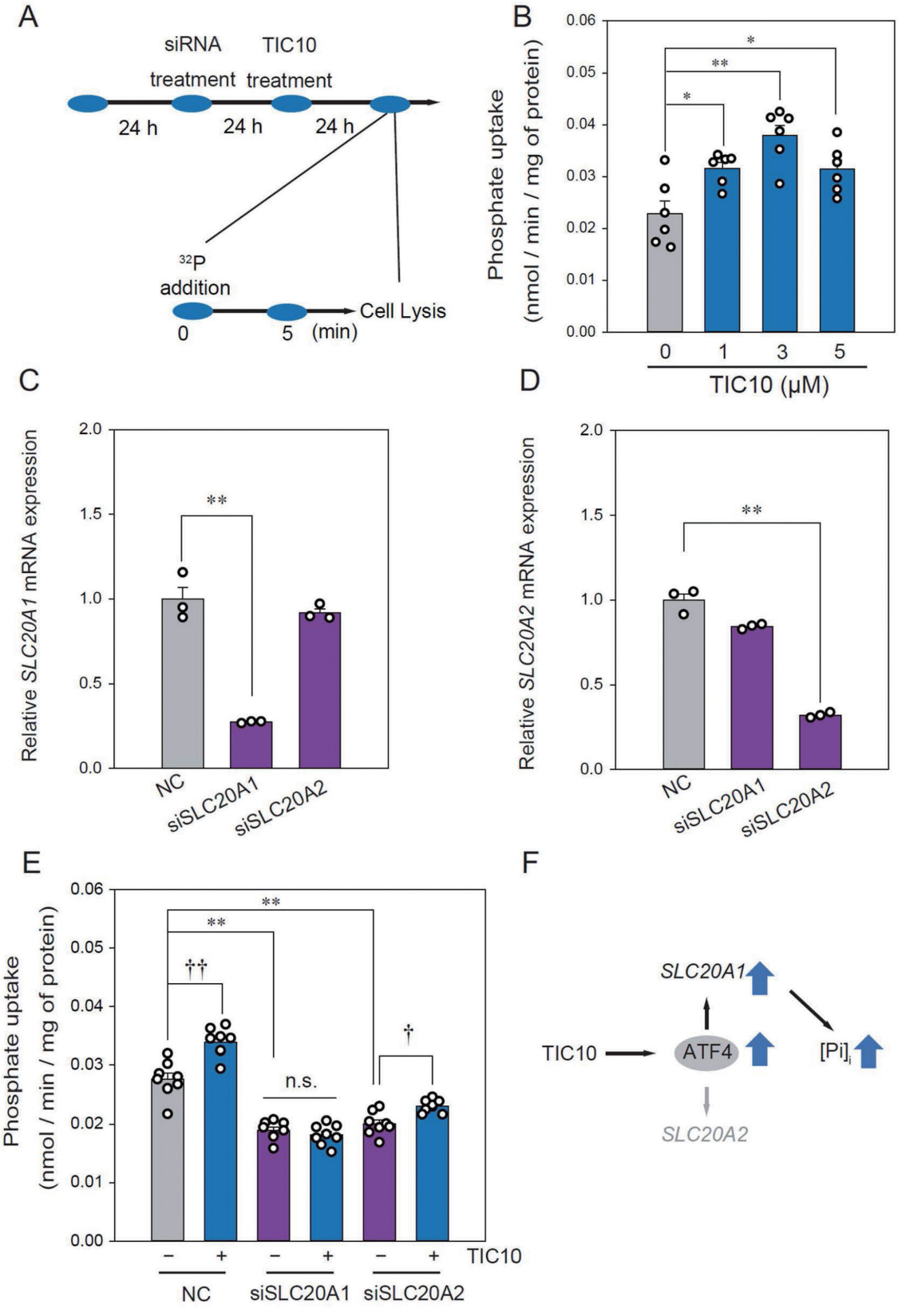

Fig. 2. TIC10 Increases Phosphate Uptake via *SLC20A1*-Mediated Transport

The scheme of the phosphate uptake experiment is shown in Figures 2B and E. (B) The quantitative analysis of phosphate uptake after treatment with 1, 3, and 5 μM TIC10. Data were expressed as the mean ± SEM (n = 6). Significance was determined by a one-way ANOVA followed by Bonferroni's post hoc test (** $p < 0.01$ vs. 0 μM TIC10). (C–D) qRT-PCR analysis of *SLC20A1* or *SLC20A2* using the respective gene-knockdowned SH-SY5Y cells. Data are expressed as the mean ± SEM (n = 3). Significance was determined by a one-way ANOVA followed by Bonferroni's post hoc test (** $p < 0.01$ vs. NC). (E) The quantitative analysis of phosphate uptake after treatment of *SLC20A1*- or *SLC20A2*-knockdowned SH-SY5Y cells with 1, 3 and 5 μM TIC10. Data are expressed as the mean ± SEM (n = 8). Significance was determined by a two-way ANOVA followed by Bonferroni's post hoc test (** $p < 0.01$ vs. NC, $^{\dagger\dagger}p < 0.01$ or $^{\dagger}p < 0.05$ vs. non-TIC10 treated SH-SY5Y cells). (F) The effect of TIC10 on the intracellular phosphate regulation. TIC10 increased intracellular phosphate ($[Pi]_i$) by upregulating SLC20A1 expression.

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Conflict of interest The authors declare no conflict of interest.

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