PTEN induced putative kinase 1 (PINK1) is a familial PD-related gene whose mutation causes autosomal recessive and early-onset PD [1]. PINK1-knock down and -knockout (KO) cells, including neurons, are more vulnerable to various insults than wild-type (WT) cells [2, 3]. However, animal models that carry a PINK1 mutation do not develop PD-like symptoms such as degeneration of dopaminergic neurons and Lewy body formation [4]. Therefore, the emerging concept of the onset and progression of dopaminergic neuronal degeneration in vivo is that certain environmental factors must cooperate with genetic factors in the development of PD [5, 6]. As environmental factors, toxins including pesticides and herbicides have been considered [7]. However, the most important environmental factor that regulates...
neuronal function and survival is glia (astrocytes and microglia). Accordingly, glia have recently been suggested as a turning point in the therapeutic strategy for PD [6].

In response to brain injury, microglia as well as neurons die in injury sites [8-10], and microglia in the penumbra region rapidly isolate injury sites and produce cytokines such as interleukin-1β (IL-1β), which are not harmful to brain cells [9, 10]. However, it is not known how PINK1 deficiency changes microglial inflammatory response. It has been reported that expression of pro-inflammatory cytokines increases in cerebrospinal fluid and brain parenchyma of patients with PD [11]. Inflammatory responses including microglia activation and expression of inflammatory cytokines increase in animal models of PD [12, 13]. Furthermore, brain inflammation is a risk factor for neurodegenerative diseases including PD [14, 15], and anti-inflammatory drugs such as dexamethasone, ibuprofen, and rofecoxib show neuroprotective effects against MPTP toxicity [16, 17]. A recent study reported that abnormal expression of innate immunity genes precedes dopaminergic neuronal death in PINK1-deficient mice [18].

In this study, we hypothesized that a PINK1 mutation alters brain inflammation, which, in turn, affects the onset and progression of PD. We found that a PINK1 deficiency enhanced brain inflammation using acutely prepared organotypic brain slices from PINK1 KO and WT mice.

**MATERIALS AND METHODS**

**Animals**

PINK1-KO mice were a gift from Dr. UJ Kang in Chicago University. PNIK1-KO mice were generated by replacing a 5.6-kb genomic region of the PINK1 locus, including exons 4-7 and the coding portion of exon 8, with a PGK-neo-polyA selection cassette flanked by FRT sequences [19, 20].

**Organotypic cortical slice cultures**

Cortical slices were prepared using a modified Stoppini method [21]. Briefly, postnatal day 7 (P7) WT and PINK1 KO mice were decapitated. Their brains were removed, and coronal slices (400-μm thick) were prepared using a McIlwain tissue chopper (Mickle Laboratory Engineering, Goose Green, UK). Slices were placed into 24-well plates and each well was filled with 500 μl culture medium (MEM containing 25% v/v Hank’s balanced salt solution, 25% v/v heat-inactivated horse serum [Hyclone, Logan, UT, USA], 6.5 mg/ml glucose, 1 mM L-glutamine, 10 U/ml penicillin-G, and 10 mg/ml streptomycin).

**Reverse transcriptase-polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR)**

Total RNA was isolated using an easy-BLUE RNA Extraction kit (iNTRON, Sungnam, Korea), and cDNA was prepared using Reverse Transcription Master Premix (ELPIS Bio, Taejeon, Korea). The primers (Bioneerne, Deajeon, Korea) used for the RT-PCR were: tumor necrosis factor-α (TNF-α) (5'-GTAGCCCACTGCTAGCAGAAA 3'-CCCTTCTCCAGCAGGAGAC), IL-1β (5'-TGTGTTCCCAATTAGACAGC 3'-GAGGTGCTGATGTACCAGTT), IL-6 (5'-AAAATCTGCTCTGGTCTTCTGG 3'-GGTTTGGCCAGTAGACCTCA), and GAPDH (5'-TCCCTCAAGATTTGT CACGAA 3'-AGATCCAACGCGGATACATT). The amplified products were verified by electrophoresis on 1.5% agarose gels with GelRed (Biotium, Hayward, CA, USA). Band intensities were analyzed using Quantity One 1-D analysis software, v. 4.6.5 (BioRad Laboratories, Inc., Hercules, CA). cDNA was analyzed using a KAPA SYBR FAST qPCR kit (KAPA Biosystem, Woburn, MA, USA). qPCR was performed using the RG-6000 real-time amplification instrument (Corbett Research, Sydney, Australia). The qPCR conditions were 40 cycles of 95°C for 3 sec, 55°C for 20 sec, and 72°C for 3 sec. The threshold cycle number of each gene was calculated and normalized compared to that of GAPDH.

**Enzyme-linked immunosorbent assay (ELISA)**

TNF-α levels in the media were measured using an ELISA kit according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA).

**Western blot analysis**

Brain slices were washed three times with cold PBS and lysed on ice in modified RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM Na3VO4, and 1 mM NaF) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 2 mM EDTA). The lysates were centrifuged at 13,000 × g for 10 min at 4°C, and the supernatant was collected. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were incubated with antibodies specific for signal transducers and activators of transcription 1 (STAT1), phospho-STAT1 (p-STAT1), p-STAT3, total STAT3 (Upstate Biotechnology, NY, USA), p-p38, total p38 (Cell Signaling Technology, Beverly, MA, USA), p-Akt, total Akt (Cell Signaling Technology), IκB (Cell Signaling Technology), and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C and then washed three times with PBS. Membranes were incubated with peroxidase-conjugated secondary antibodies (Zymed, San Francisco, CA,
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RESULTS

PINK1 deficiency increases expression of pro-inflammatory cytokines

To examine how PINK1 regulates the inflammatory response in the injured brain, we measured expression levels of TNF-α, IL-1β, and IL-6 in organotypic brain slices prepared from WT and PINK1 KO mice because the slicing process mimics brain injury [22, 23]. mRNA levels of these cytokines were measured at 3, 6, 12, and 24 h after the preparation of slices using RT-PCR (Fig. 1A) and qPCR (Fig. 1B). PINK1 KO slices expressed higher levels of all cytokines compared to those of WT. TNF-α protein production was also higher in PINK1 KO slices than that in WT slices (Fig. 1C).

Altered activation patterns of STAT3, IκB, and Akt in PINK1 KO slices

Next, we examined the signaling pathways responsible for increased expression of the pro-inflammatory cytokines in the PINK1 KO slices. In serial experiments, we examined various signaling pathways such as mitogen activated protein kinase (MAPK), STATs, and nuclear factor (NF)-κB pathways that regulate brain inflammation [24-27]. Although extracellular signal-regulated kinase (ERK) was activated within 30 min after slicing, there was no difference in the activation levels in WT and
KO slices (Fig. 2A). Phosphorylation levels of these molecules (p-p38, p-JNK, and p-STAT1) were not different in WT or KO slices (Fig. 2A, B). In contrast, activation of the STAT3 and IκB-NFκB pathways was different in WT and KO slices. Phosphorylation levels of STAT3 (p-STAT3) were attenuated in KO slices (Fig. 2B, D). IκB degradation, which represents NF-κB activation [28], was detected within 30 min in WT and KO slices but was much faster in KO slices (Fig. 2C, D).

Previously, we found that phosphorylation levels of Akt (p-AKT), a downstream regulator of phosphatidylinositol 3-kinase (PI3K), decreased in astrocytes cultured from KO mice [20]. Since PI3K negatively regulates expression of pro-inflammatory cytokines [29], we examined p-Akt levels in WT and KO slices. p-Akt levels time-dependently decreased after slicing in WT and KO but more rapidly in KO (Fig. 3A). Next, we examined whether reduced p-Akt levels were related to enhanced expression of pro-inflammatory cytokines in KO slices. WT slices were treated with a PI3K inhibitor, LY294002, and TNF-α and IL-1β mRNA levels were assayed. In both RT-PCR and qPCR, LY29402 significantly increased TNF-α and IL-1β mRNA levels (Fig. 3B, C). We further examined the effect of LY204002 on p-STAT3 levels and IκB degradation. However, LY294002 had no effect on p-STAT3 levels and IκB degradation (Fig. 3D), suggesting that Akt negatively regulates the inflammatory responses in slices independent of the STAT3 and NFκB degradation pathways. Taken together, these results suggest that PINK1 deficiency increases inflammatory responses in the injured brain through decreased Akt activation and enhanced NF-κB activation. Although p-STAT3 levels decreased in PINK1 KO slices, we could not determine whether reduced STAT3 activation contributed to enhance the inflammatory response in KO slices.

**DISCUSSION**

The major findings in this study were that PINK1 deficiency increases expression of pro-inflammatory cytokines and that attenuated Akt activation and enhanced IκB-NFκB pathways may be involved in increased production of pro-inflammatory cytokines in PINK KO mice.

We used organotypic cortical slice cultures prepared from the early postnatal period (P7) to mimic brain injury since organotypic slice cultures have been used in many studies to investigate mechanisms and treatment strategies for neurodegenerative diseases, such as stroke, Alzheimer’s disease, PD, and Huntington’s disease [30-33]. We found that the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 was more highly induced in PINK1 KO mice than those in WT mice using slices (Fig. 3). These results agree with the results of a recent study in which PINK1 KO mice showed higher striatal levels of IL-1β, IL-12, and IL-10 in response to lipopolysaccharide [18].

It has been reported that PINK1-deficient cells are vulnerable to apoptosis compared to that in WT cells [34, 35]. In some experiments, we also found that more cells died in KO slices and that cytokine release rather decreased in KO slices (data not shown). However, in cases where WT and KO slices did not show differences in the extent of cell death confirmed by Live/Dead and lactate dehydrogenase assays (data not shown), we found increased inflammatory cytokine expression in KO slices compared to that in WT slices (Fig. 1).

The most important signaling pathways regulating brain inflammation are MAPKs and STATs [24-27]. However, activation patterns of STAT1 and MAPKs including ERK, p38, and JNK were not different in WT and KO slices (Fig. 2A, B).
Instead, degradation of IκB, and levels of p-STAT3 and p-Akt were different in WT and KO slices (Figs. 2B-D, 3A). It has been reported that STAT3 blocks NF-κB activation by preventing IκB phosphorylation and degradation [36]. Therefore, we speculate that IκB degradation may be related to STAT3 activation in KO slices, which results in increased pro-inflammatory cytokines in PINK1 KO slices. However, we do not have any evidence to support this speculation. The PI3K/Akt pathway may be another factor regulating the inflammatory responses in KO animals. The importance of the PI3K/Akt pathways in the inflammation has been reported in rheumatoid arthritis, multiple sclerosis, and asthma [37-39]. Inhibiting PI3K increases TNF-α and IL-6 expression in macrophages [29]. In this study, we found that p-Akt levels were attenuated in PINK1 KO slices (Fig. 3A). Furthermore, LY294002, which indirectly inhibits Akt activation by inhibiting PI3K, enhanced expression of TNF-α and IL-1β in WT slices (Fig. 3B, C). However, treatment with LY294002 did not affect p-STAT3 levels or IκB degradation (Fig. 3D). These results suggest that activating Akt increases pro-inflammatory cytokines in PINK1 KO slices independently of the STAT3 or IκB pathway.

The results of this study could shed a light on the unsolved question of why none of the PD-like symptoms occur in animal models that carry mutant PD-related genes and/or whose PD-related genes are knocked out. Because the PINK1 defect enhances brain inflammation in response to injury, the function of PINK1 may be more important in injury states rather than in normal physiological states. Thus, a defect in PINK1 could exaggerate brain inflammation in the injured brain, which increases brain damage and results in dopaminergic neuronal death.

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