

Effects of Silibinin Against Prothrombin Kringle-2-Induced Neurotoxicity in the Nigrostriatal Dopaminergic System *In Vivo*

Eunju Leem,^{1,*} Yong-Seok Oh,^{2,*} Won-Ho Shin,^{3,*} Byung Kwan Jin,⁴ Jae Yeong Jeong,^{3,4} Minsang Shin,⁵ Dong Woon Kim,⁶ Jin-Hyeok Jang,² Hyung-Jun Kim,⁷ Chang Man Ha,⁷ Un Ju Jung,⁸ Gyeong Joon Moon,¹ and Sang Ryong Kim^{1,9}

¹BK21 Plus KNU Creative BioResearch Group, School of Life Sciences, Institute of Life Science and Biotechnology, Kyungpook National University, Daegu, Korea.

²Department of Brain-Cognitive Science, Daegu-Gyeongbuk Institute of Science and Technology, Daegu, Korea.

³Department of Predictive Model Research Center, Korea Institute of Toxicology, Korea Research Institute of Chemical Technology, Daejeon, Korea.

⁴Departments of Biochemistry and Molecular Biology, and Neuroscience Graduate School, School of Medicine, Kyung Hee University, Seoul, Korea.

⁵Department of Microbiology, School of Medicine, Kyungpook National University, Daegu, Korea.

⁶Department of Anatomy, Brain Research Institute, Chungnam National University School of Medicine, Daejeon, Korea.

⁷Department of Neural Development and Disease, Korea Brain Research Institute, Daegu, Korea.

⁸Department of Food Science and Nutrition, Pukyong National University, Busan, Korea.

⁹Brain Science and Engineering Institute, Kyungpook National University, Daegu, Korea.

ABSTRACT Parkinson's disease (PD) and Alzheimer's disease exhibit common features of neurodegenerative diseases and can be caused by numerous factors. A common feature of these diseases is neurotoxic inflammation by activated microglia, indicating that regulation of microglial activation is a potential mechanism for preserving neurons in the adult brain. Recently, we reported that upregulation of prothrombin kringle-2 (pKr-2), one of the domains that make up prothrombin and which is cleaved and generated by active thrombin, induces nigral dopaminergic (DA) neuronal death through neurotoxic microglial activation in the adult brain. In this study, we show that silibinin, a flavonoid found in milk thistle, can suppress the production of inducible nitric oxide synthase and neurotoxic inflammatory cytokines, such as interleukin-1 β and tumor necrosis factor- α , after pKr-2 treatment by downregulating the extracellular signal-regulated kinase signaling pathway in the mouse substantia nigra. Moreover, as demonstrated by immunohistochemical staining, measurements of the dopamine and metabolite levels, and open-field behavioral tests, silibinin treatment protected the nigrostriatal DA system resulting from the occurrence of pKr-2-triggered neurotoxic inflammation *in vivo*. Thus, we conclude that silibinin may be beneficial as a natural compound with anti-inflammatory effects against pKr-2-triggered neurotoxicity to protect the nigrostriatal DA pathway and its properties, and thus, may be applicable for PD therapy.

KEYWORDS: • microglia • neurodegeneration • nigrostriatal dopaminergic system • prothrombin kringle-2 • silibinin

INTRODUCTION

THE COMMON FEATURES of Parkinson's disease (PD) are progressive dopaminergic (DA) neuronal loss in the substantia nigra (SN) and a striatal dopamine reduction.^{1–6}

Although the main pathogenic cause of PD remains controversial, excessive inflammatory processes are important in PD initiation and progression, and the regulation of neurotoxic inflammation is considered as a therapeutic strategy against PD.^{7,8} We recently reported that upregulation of prothrombin kringle-2 (pKr-2), one of the domains of prothrombin, which is cleaved from prothrombin during proteolytic activation of thrombin, induces microglial activation, resulting in overexpression of neurotoxic inflammatory cytokines, such as interleukin-1 β (IL-1 β) and tumor necrosis factor- α (TNF- α), and degeneration of the nigrostriatal DA projection in adults; however, pKr-2 itself is not directly neurotoxic.^{1,2} Moreover, our previous studies showed that pKr-2 is upregulated in the brain of patients with

*These authors contributed equally to this work.

Manuscript received 2 June 2018. Revision accepted 28 November 2018.

Address correspondence to: Sang Ryong Kim, PhD, BK21 Plus KNU Creative BioResearch Group, School of Life Sciences, Institute of Life Science and Biotechnology, Kyungpook National University, Daegu 41566, Korea, E-mail: srk75@knu.ac.kr or Gyeong Joon Moon, PhD, BK21 Plus KNU Creative BioResearch Group, School of Life Sciences, Institute of Life Science and Biotechnology, Kyungpook National University, Daegu 41566, Korea, E-mail: neobios7@gmail.com

PD,^{1,9,10} suggesting that inhibiting pKr-2-triggered microglial activation is an effective approach for preserving DA neurons.

Silibinin, a polyphenolic flavonoid and the main biologically active compound derived from milk thistle (*Silybum marianum* L. Gaertn.), has been reported to have neuroprotective effects in ischemia and Alzheimer's disease (AD) models.^{11–15} In addition, silibinin suppressed the expression of IL-1 β and TNF- α (pro-inflammatory cytokines), resulting in the preservation of DA neurons against 1-methyl-4-phenylpyridinium (MPP⁺)-triggered toxicity in the adult rat SN.⁹ This suggests that silibinin can inhibit the occurrence of neurotoxic inflammatory events in the nigrostriatal DA system *in vivo*. In this study, we examined whether silibinin treatment decreases harmful pKr-2-triggered microglial activation and prevents neurodegeneration in the nigrostriatal DA system after pKr-2 injection in the adult mouse SN.

MATERIALS AND METHODS

Used antibody list

The following antibodies were purchased from commercial sources: tyrosine hydroxylase (TH) antibody from rabbit was manufactured by Pel-Freez Biologicals, located in Rogers, AR (USA) and ionized calcium-binding adapter molecule 1 (Iba1) antibody from rabbit was purchased from Wako Pure Chemical Industries, located in Osaka (Japan). Extracellular signal-regulated kinase (ERK) antibody from rabbit, phosphorylated ERK (p-ERK) antibody from rabbit, and β -actin antibody from rabbit were manufactured by Cell Signaling Technology, located in Danvers, MA (USA). Induced nitric oxide synthase (iNOS) antibody from rabbit was obtained from Abcam, located in Cambridge (United Kingdom), and TNF- α antibody from mouse and IL-1 β antibody from rabbit were purchased from Santa Cruz Biotechnology, located in Dallas, TX (USA). The following secondary antibodies were also purchased from each company: biotin-linked secondary antibody and Texas Red-linked secondary antibodies against rabbit or mouse IgG were manufactured by Vector Laboratories, located in Burlingame, CA (USA), fluorescein isothiocyanate-linked secondary antibodies against rabbit or mouse IgG were obtained from Jackson ImmunoResearch Laboratories, located in West Grove, PA (USA), and horseradish peroxidase-linked secondary antibodies against rabbit or mouse IgG were manufactured by Enzo Life Sciences, located in Farmingdale, NY and Thermo Fisher Scientific, located in Waltham, MA (USA).

Approval from the Animal Care Committee for experiments

The male C57BL/6 mice (postnatal 8–10 weeks, 24 $\pm$ 1 g) were purchased from Daehan Biolink, located in Eumseong in Republic of Korea. These mice were bred in a controlled environment and mice consumed food and water *ad libitum*. All experiments using mice were performed in conformity with permitted protocols and official advice enacted by the

Animal Care Committee, organized within Kyungpook National University (Approval No. KNU 2016-42).

Administration of silibinin and surgical injection of pKr-2

Milk thistle-derived silibinin [(2R,3R)-3,5,7-trihydroxy-2-[(2R,3R)-3-(4-hydroxy-3-methoxyphenyl)-2-(hydroxymethyl)-2,3-dihydrobenzo[b][1,4]dioxin-6-yl]chroman-4-one; C₂₅H₂₂O₁₀], purchased from Sigma-Aldrich, located in St. Louis, MO (USA; Fig. 1A), was dissolved in 0.9% saline containing 0.25% carboxymethylcellulose (Sigma-Aldrich), and silibinin was assigned to be administered in doses of 50, 100, or 200 mg/kg per day. To examine the effects of silibinin against pKr-2-triggered inflammatory responses in the nigrostriatal DA neural system, mice received intraperitoneal injection of silibinin, starting a day before administration of pKr-2 and daily continued until 1 or 6 days postinjection as shown in Figure 1A.

Unlike silibinin administration, pKr-2 injection needs more steps as described.⁹ Anesthetized and shaved mice were carefully fastened on a stereotaxic frame, made by Kopf Instruments (USA), and incised to mark the injection site according to the brain atlas (relative to bregma, anterior–posterior axis: –0.32 cm, medial–lateral axis: –0.13 cm; relative to surface of brain, dorsal–ventral axis: –0.4 cm).¹⁶ After forming a cranial pore for injection, each mouse was unilaterally injected with pKr-2 [Haematologic Technologies, Inc., Essex Junction, VT, USA; injection volume: 2 μ L; dissolved in 0.1 M phosphate-buffered saline (PBS) at 12 μ g/ μ L] into the right SN at a rate of 0.5 μ L/min. After administration, the needle used for pKr-2 infusion was left in the injection site for an additional 5 min to reliably diffuse pKr-2 in the SN and then the needle was slowly retracted. The brain tissues were collected from mice for analysis according to the experimental schedule (Fig. 1A).

Locomotor activity analysis

To analyze locomotor activities of mice, the open-field test was performed as instructed in reference with slight alteration.¹⁷ One week after pKr-2 injection (Fig. 1A), the mice were individually placed in the right side of a test box comprising five white acrylic squares (40 $\times$ 40 cm). The upper side of the box was left unclogged to record the behaviors of mice with a video recording system. Animal behavior was recorded for 6 min, including an adaptation period of 1 min. Changes in locomotor activity were analyzed with SMART 3.0 video-tracking software (Panlab, Spain), and displayed as graphs depicting the total traveled distance (cm) and speed (cm/s). The test box was disinfected between each trial with 70% ethanol. Tests were performed under low-illumination conditions to minimize the stress level of mice.

Immunohistochemical staining procedures

Immunohistochemical staining was performed as described previously.^{18–20} Brain tissues were collected from

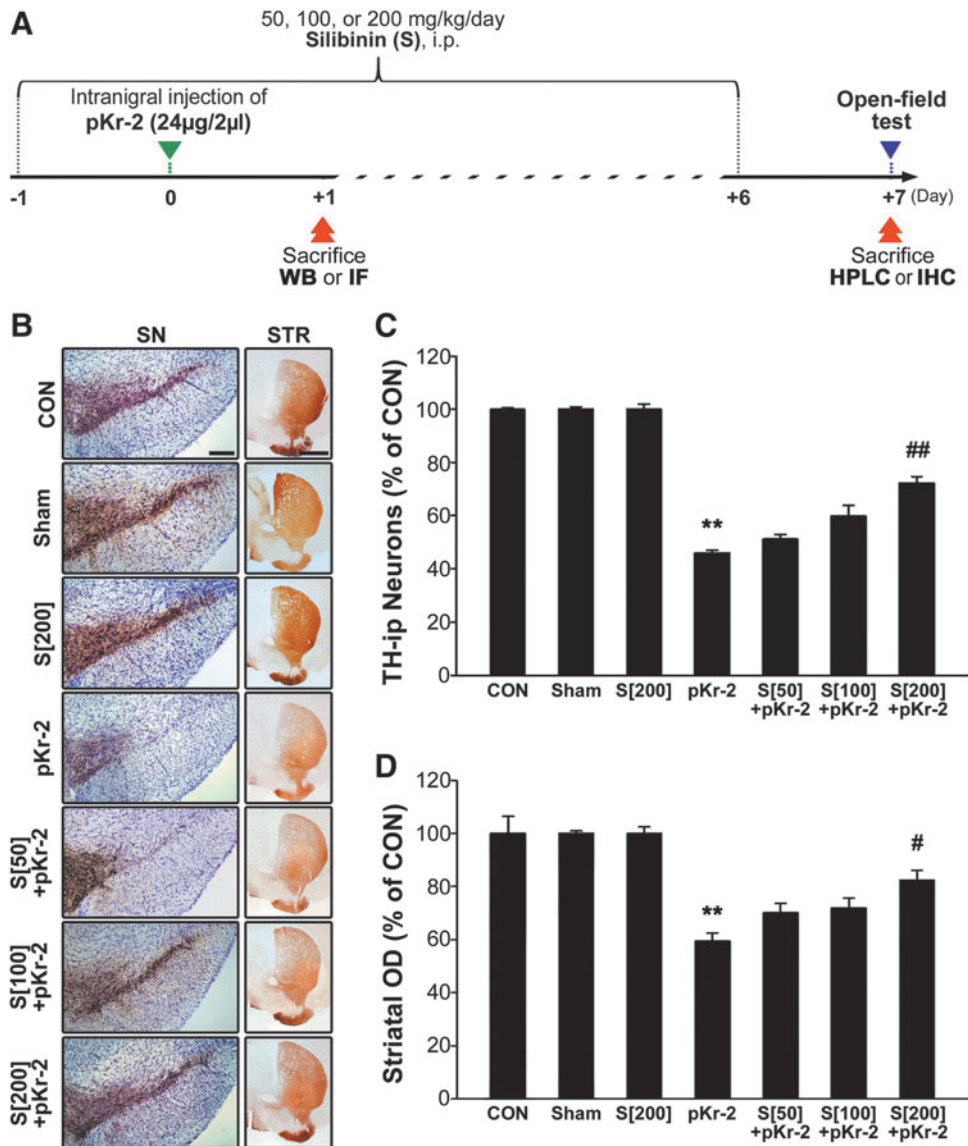


FIG. 1. Preservation of the nigrostriatal DA projections by silibinin treatment against pKr-2-triggered neurotoxicity. **(A)** Experimental schematic for effects of silibinin against pKr-2-triggered neurotoxicity in the nigrostriatal DA system. Mice received a daily intraperitoneal injection of silibinin (50, 100, or 200 mg/kg per day), starting a day before pKr-2 injection into the SN, and were sacrificed according to the experimental purpose. **(B)** Representative coronal sections of the SN and the STR stained with TH antibodies at a week after pKr-2 injection. Scale bar, 200 µm in the left panels (SN) and 1000 µm in the right panels (STR), respectively. **(C)** The number of TH-positive cells in the SN. $**P < .001$ versus CON and $##P < .01$ versus pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test, mean $\pm$ SEM; $n = 4$ for each group). **(D)** The optical densities of the TH-positive fibers. $**P < .01$ versus CON and $#P < .05$ versus pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test, mean $\pm$ SEM; $n = 4$ for each group). ANOVA, analysis of variance; CON, control; DA, dopaminergic; pKr-2, prothrombin kringle-2; S, silibinin; SEM, standard error of the mean; SN, substantia nigra; STR, striatum; TH, tyrosine hydroxylase.

mice, which transcardially perfused with preperfusion solution and fixed in 4% paraformaldehyde (PFA). The tissues were postfixed with 4% PFA dissolved in 0.1 M phosphate buffer (PB; PFA/PB) for a day, soaked in 30% sucrose dissolved in 8% PFA/PB for cryoprotection for 2 days, and prepared by cutting in 30-µm-thick slices for tissue staining. The sections were immersed in 0.1 M PBS containing 0.5% bovine serum albumin (BSA; 0.5% B/P) to block nonspecific antigens. To improve the cellular permeability of antibodies, the sections were incubated with 0.01% Triton X-100 dissolved in 0.5% B/P and incubated with rabbit-TH antibodies at 4°C for 2 days. After incubation, the sections were rinsed with 0.5% B/P, incubated at room temperature (RT) with biotin-linked anti-rabbit IgG, and then processed with an avidin-biotin complex kit (Vector Laboratories) to enhance the secondary antibody signal. Staining signals were visualized by incubating the sections in 0.5 mg/mL 3,3'-diaminobenzidine (Sigma-Aldrich) in 0.1 M PB con-

taining 0.003% H_2O_2 . Immunostained SN and striatum sections were carefully mounted on gelatin-smear slides, and then counterstained with 0.5% cresyl violet (Sigma-Aldrich), which stains the Nissl substance in neurons. The stained sections were analyzed with an Axio Imager under a bright-field microscope (Carl Zeiss, Oberkochen, Germany).

The protocol for double immunofluorescence staining of brain sections was in accordance with immunohistochemistry methods until incubation with primary antibodies as written in the first paragraph in immunohistochemical staining procedures. Sections were washed with 0.5% B/P after incubation with primary antibodies, incubated with appropriate secondary antibodies linked with fluorescence, and then rinsed with PBS. Stained sections were mounted on gelatin-smear slides, dried under dark condition, and cover-slipped with antifade reagent (Invitrogen, USA) to sustain dye fluorescence. The stained sections were carefully analyzed with an Axio Imager converted to a fluorescence microscope.

Stereological estimation

As described previously, both hemispheres of each mouse brain were stained with TH antibodies, and then it was counted that the number of TH-positive neurons in the SN. The total number of TH-positive cells, indicating DA neurons, was estimated with the optical fractionator's method by using the Olympus Computer-Assisted Stereological Toolbox system, version 2.1.4, purchased from Olympus, established in Tokyo (Japan). The entire SN collected from the stained sections was mounted on a slide. The slide was placed on a microscope, and the SN of the section was defined as the region of interest (ROI). To estimate the distribution of TH-immunopositive cells in the ROI, the area of interest (AOI) was precisely marked at low magnification. The thickness of the AOI was calculated and its height was fixed according to the mean of the section thickness. The guard region was determined to accurately measure the number of DA neurons, and the zone was established based on the distribution of TH-immunopositive signal in the ROI. The distance between sections was determined and the ROI and AOI were determined in all sections. The frame size was measured, and TH-positive neurons were sampled using the mean height of the AOI and distance between sections. After the completion of all these steps, TH-positive neurons were counted at high magnification. Finally, the counted number of TH-immunopositive cells was graphed by expressing a percentage of that in the contralateral control side.

Quantitative determination of striatal TH immunoperoxidase staining

The whole striatum in the stained sections was identified as previously described.²⁰ In brief, the optical density of the TH-positive signal was measured using Multi Gauge version 3.0, manufactured by Fujifilm in Japan. The density of the corpus callosum was subtracted from the density of the striatum for each section to deduct variations in background brightness. The striatal TH-positive signal, indicating the DA fiber, was shown as a graph expressing the density ratio on the ipsilateral lesion side versus the percentage of the density on the contralateral control side.

Western blot analysis

To extract protein samples from the SN, the SN tissues were prepared from the mice in the previously described manner at 1 day postlesion.^{9,19,20} In brief, the SN tissues were sliced using a brain matrix (Roboz Surgical Instrument Co., USA), and the sliced tissues were homogenized and centrifuged at 4°C for 15 min at 14,000 g. The supernatant was gathered and put in an autoclaved tube, and the concentration of extracted protein was measured using a DC Protein assay kit, manufactured by Bio-Rad, located in Hercules, CA (USA). The quantified samples were denatured by boiling at 100°C for 5 min before gel loading. The proteins were equally loaded into each lane in the gel with the loading buffer, proteins were separated by polyacrylamide gel electrophoresis (Bio-Rad), and the separated

proteins were transferred to polyvinylidene difluoride membranes, manufactured by Millipore (Billerica, MA, USA). After transferring the separated proteins, the membranes were blocked with 5% BSA dissolved in Tris-buffered saline with 1% Tween-20 (a nonionic detergent; 5% BSA/TBST) and incubated overnight at 4°C with primary antibodies. On the following day, the membranes were rinsed with 5% BSA/TBST and incubated with secondary antibodies for an hour at RT. Finally, the membranes were exposed at the enhanced chemiluminescence (GE Healthcare Life Sciences, located in Little Chalfont (United Kingdom), that is western blot detection reagents. The signal was measured with a LAS-500 image analyzer (GE Healthcare Life Sciences) and analyzed using Multi Gauge software, version 3.0.

Measurement of striatal dopamine and its metabolites

Striatal dopamine and its metabolites were measured with using an electrochemical detector in reversed-phase high-performance liquid chromatography (HPLC).⁹ In brief, mouse striatum tissues were sliced using a brain matrix. The supernatants gathered from the homogenized and centrifuged striatum tissues were put into a 717 plus autosampler (Waters Corporation, USA) at 4°C and dopamine and its metabolites, 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), were eluted through a Sunfire C18 column (4.6 × 100 mm × 5 μm) that filled with a catecholamine analysis mobile phase (Chromosystems, Germany). The peaks of dopamine, DOPAC, and HVA were analyzed using an ESA Coulochem III electrochemical detector and integrated using ChemStation software, manufactured by Agilent Technologies located in the United States. All samples were normalized for protein content as spectrophotometrically determined using the Pierce BCA protein assay kit (Thermo Scientific).

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's *post hoc* tests was conducted to analyze the statistical differences between more than two groups, and *t*-test was performed to compare two groups. All values in the graphs are the mean ± standard error of the mean (SEM), and analyses were executed using SigmaStat software, programmed by Systat Software, located in San Jose, CA, USA.

RESULTS

Protection of DA neurons by silibinin treatment against pKr-2-triggered neurotoxicity

To examine the protective effects of silibinin on the nigrostriatal DA projections against pKr-2-triggered neuronal loss, pKr-2 was unilaterally injected into the SN at 1 day after intraperitoneal injection of silibinin (50, 100, or 200 mg/kg per day; Fig. 1A). The dosage of silibinin was decided based on previous reports.^{11,21} Brains were harvested at a week postinjection of pKr-2, and immunostaining for TH, which indicates DA neurons, was

performed on coronal sections, including the SN or striatum. The injection of pKr-2 led to degeneration of TH-positive neurons in the SN and axonal fibers in the striatum (Fig. 1B). However, silibinin treatment prevented these losses in both SN and striatum of pKr-2-injected mice (Fig. 1B). As the quantitative results show, a significant degeneration of TH-positive neurons and axonal fibers was found due to injection of pKr-2 (Fig. 1C, D; $**P < .001$ vs. control), and silibinin treatment at 200 mg/kg significantly diminished the destruction of both TH-positive neurons and fibers compared with pKr-2 alone (Fig. 1C, D; $\#P < .05$, $\#\#P < .01$ vs. pKr-2 alone). Silibinin treatment at 50 or 100 mg/kg also showed protective patterns in both the SN and striatum against pKr-2 toxicity, but the effects were not significant (Fig. 1C). There were no neurotoxic effects in the sham or silibinin-treated mice brains without pKr-2 (Fig. 1B–D).

Preservation of dopamine, DOPAC, and HVA by silibinin treatment in the striatum of pKr-2-injected mice

To evaluate the dopamine production capacity, we examined the levels of dopamine, DOPAC, and HVA in the striatum using HPLC. Similar to our previous report,⁹ the levels of dopamine (57%, $**P < .01$), DOPAC (39%, $*P < .05$), and HVA (34%, $**P < .01$) were significantly reduced in the striatum of pKr-2-injected mice compared with control (Fig. 2A). In contrast, the amounts of dopamine (85%, $\#\#P < .01$), DOPAC (102%, $\#P < .05$) and HVA (108%, $\#\#P < .01$) were preserved in the striatum of silibinin-treated mice compared with control mice (Fig. 2A). There were no significant changes in basal levels of striatal dopamine, DOPAC, and HVA in silibinin-treated mice (Fig. 2B). To examine whether the protected DA system after silibinin treatment could contribute to the preservation

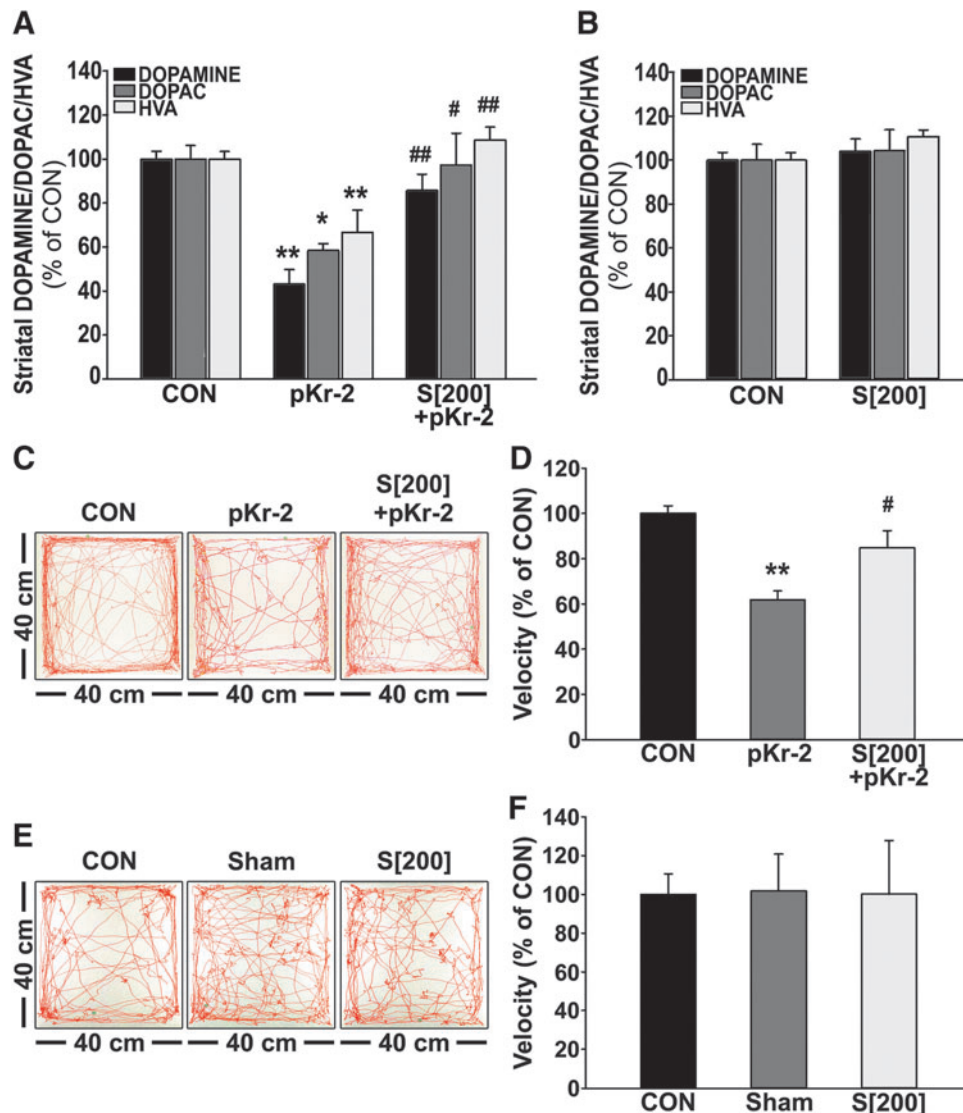


FIG. 2. Effects of silibinin on the functional protection of the nigrostriatal DA system *in vivo*. (A, B) Amounts of striatal dopamine and its metabolites, which were measured by HPLC. $*P < .05$ and $**P < .01$ versus CON; $\#P < .05$ and $\#\#P < .01$ versus pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test, mean $\pm$ SEM; $n = 4$ for each group). (C–F) The results of open-field test for measuring the locomotor activities were expressed as a histogram showing the ratio of the value of each experimental group normalized to the average of the control group in units of cm/s. $**P < .01$ versus CON and $\#P < .05$ versus pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test, mean $\pm$ SEM; $n = 4$ for each group). DOPAC, 3,4-dihydroxyphenylacetic acid; HPLC, high-performance liquid chromatography; HVA, homovanillic acid.

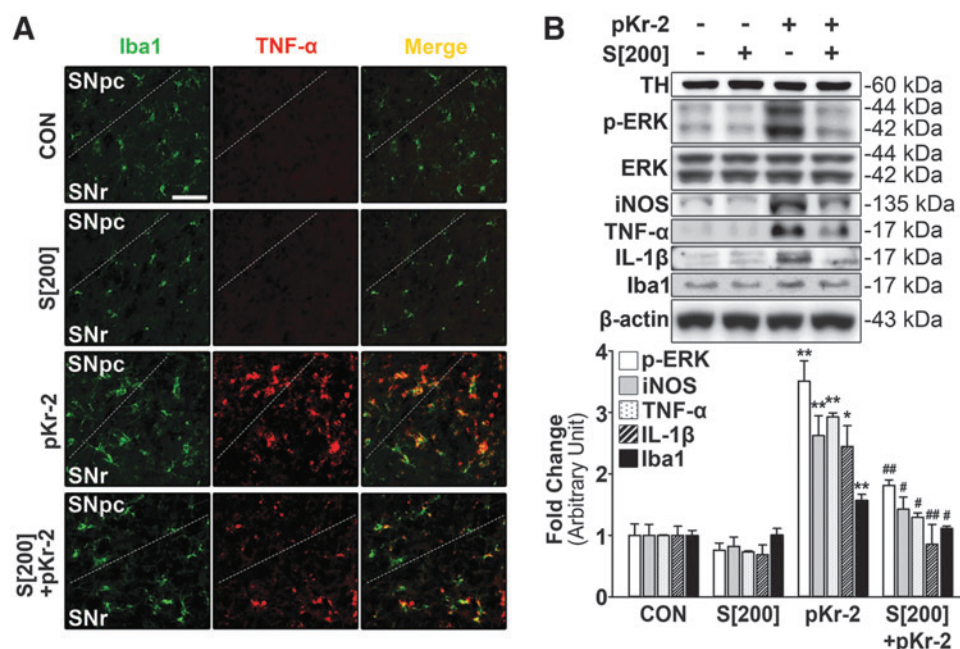


FIG. 3. Anti-inflammatory effects of silibinin on pKr-2-treated SN. (A) Representative double immunofluorescence staining for anti-Iba1 (green) and anti-TNF- α (red) in the SN of mice at 1 day after pKr-2 injection. Yellow color indicates that TNF- α expression was located in microglia. Scale bar, 20 μ m. (B) Western blotting to measure the levels of TH, iNOS, TNF- α , IL-1 β , Iba1, p-ERK, and ERK in the SN. * P < .05 and ** P < .01 versus CON; # P < .05 and ## P < .01 versus pKr-2 alone (one-way ANOVA with Tukey's *post hoc* test, mean $\pm$ SEM; n = 4 for each group). ERK, extracellular signal-regulated kinase; Iba1, ionized calcium-binding adapter molecule 1; IL-1 β , interleukin-1 beta; iNOS, inducible nitric oxide synthase; p-ERK, phosphorylated form of ERK; SNpc, SN pars compacta; SNr, SN reticulata; TNF- α , tumor necrosis factor- α .

of locomotor activity from pKr-2-triggered neurodegeneration, we measured the alteration of mouse behaviors using the open-field test *in vivo*. Abnormal locomotor activity was not observed in either the sham or silibinin-treated mice without pKr-2 (Fig. 2E, F). Consistent with the HPLC results, our observations revealed that treatment with pKr-2 alone induced a significant hypo-locomotor activity compared with the moving distance per time of control mice (Fig. 2C, D; ** P < .01). However, locomotor activity was significantly improved by silibinin at 200 mg/kg per day compared with the pKr-2-injected mice (Fig. 2C, D; # P < .05).

Anti-inflammatory effects of silibinin on pKr-2 treatment in the SN

To examine whether silibinin could inhibit microglial activation and reduce the pro-inflammatory cytokines derived from activated microglia, indicating a protective mechanism of silibinin against pKr-2-triggered neurotoxicity, 1 day after injection of pKr-2 into the SN, double immunofluorescence staining was performed to evaluate the cell type-specific expression of Iba1 (microglia marker), TNF- α and IL-1 β (pro-inflammatory cytokines), and the levels of protein expression were quantified by western blotting (Fig. 3). Immunofluorescence staining showed that silibinin treatment did not induce any change in basal TNF- α (Fig. 3A). However, silibinin administration suppressed the increase in expression of TNF- α as well as in the number of

activated microglia after pKr-2 administration (Fig. 3A). The alteration of IL-1 β expression was similar to that in TNF- α levels after silibinin or pKr-2 injection (data not shown). In addition, western blotting showed that the levels of iNOS (pro-inflammatory enzyme), Iba1 (microglia marker), IL-1 β (cytokine), and TNF- α (cytokine) were significantly increased in pKr-2-injected mice compared with control (Fig. 3B; * P < .05, ** P < .01). However, silibinin significantly downregulated iNOS, TNF- α , IL-1 β , and Iba1 expression compared with pKr-2-injected mice (Fig. 3B; # P < .05, ## P < .01).

To elucidate how silibinin could suppress neurotoxic inflammatory responses caused by pKr-2, we investigated the involvement of ERK signaling activation *in vivo*. In previous studies we demonstrated that the ERK signaling pathway could regulate the expression of iNOS (pro-inflammatory enzyme), TNF- α (cytokine), and IL-1 β (cytokine).^{1,22} The expression level of p-ERK was increased with pKr-2 injection, and attenuated with addition of silibinin treatment, similar to expression patterns of iNOS (pro-inflammatory enzyme), Iba1 (microglia marker), IL-1 β (cytokine), and TNF- α (cytokine) expression (Fig. 3B; * P < .05 and ** P < .01 vs. control; # P < .05 and ## P < .01 vs. pKr-2 alone). These results showed that pKr-2-triggered neurotoxicity and microglial activation were associated with ERK activation, and silibinin could protect the DA system through suppression of the ERK signaling pathway.

No significant changes in TH levels were found in any group at 1 day post-pKr-2 injection into the SN, suggesting

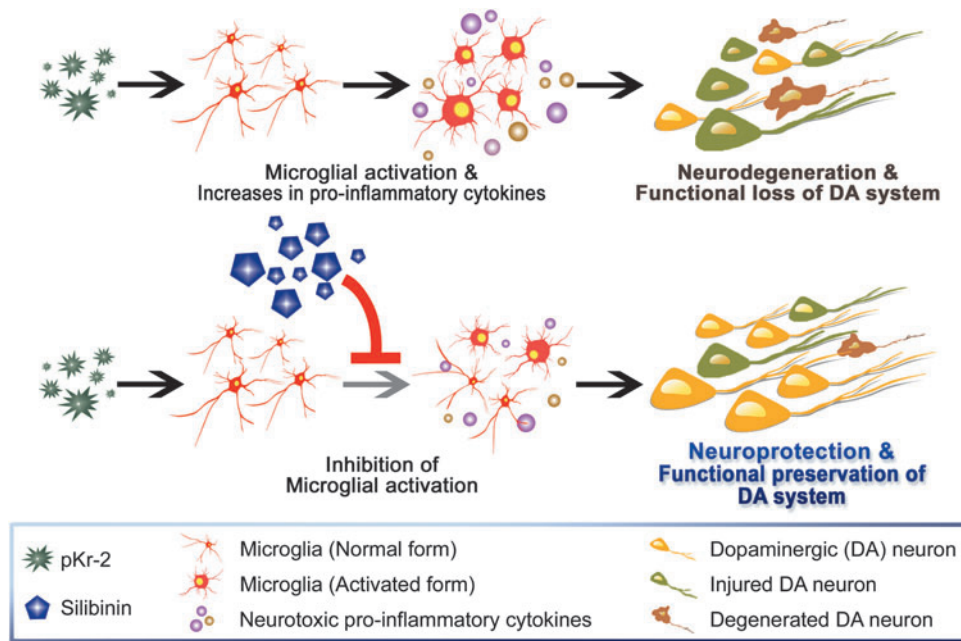


FIG. 4. Schematic representation of silibinin against pKr-2-triggered neurotoxic events. The increase in pKr-2, an endogenous microglial activator, induces microglial activation, resulting in neurodegeneration through inflammatory responses such as the production of pro-inflammatory cytokines, in the nigrostriatal DA system of the adult brain (*upper schematic*). However, its neurotoxic inflammation was significantly inhibited by silibinin administration, resulting in the preservation of nigrostriatal DA projections *in vivo* (*lower schematic*). These observations suggest that silibinin is a beneficial natural compound with anti-inflammatory effects against pKr-2-triggered neurotoxicity in the nigrostriatal DA system.

that pKr-2-triggered the microglial activation before lesions formed in DA neurons in the pKr-2-injected SN *in vivo*.

DISCUSSION

Prothrombin is known to be involved in blood coagulation; it is mainly synthesized in the liver and then secreted into the bloodstream. In addition, prothrombin is a proenzyme that is cleaved to reveal the enzyme active site by producing fragments 1-2 (kringle regions) and active thrombin. Activated thrombin, which has serine proteolytic activity that transforms soluble fibrinogen into insoluble fibrin for blood coagulation, can cleave the kringle domain in pKr-1 and -2.^{1,9,10} In addition, prothrombin and thrombin may accumulate because of leakage in the blood-brain barrier in both PD and AD brains.^{1,9} We reported significantly increased pKr-2 in the postmortem brain of PD patients,^{1,9} suggesting that the relationship between pKr-2 upregulation and PD pathogenesis is clinically relevant.

Activated microglia-induced neuroinflammation is considered a major contributor to progressive neuronal loss in PD, AD, and other neurodegenerative diseases.^{1,7-9} We recently reported that upregulated pKr-2, which has been proposed as an endogenous molecule involved in microglia activation with no direct neurotoxicity, induced progressive degeneration in the nigrostriatal DA system through overexpression of activated microglia-derived inflammatory cytokines such as TNF- α and IL-1 β *in vivo*. These findings suggest that the control of pKr-2-triggered microglial activation may be helpful to reduce the loss of DA neurons under neurotoxic inflammation in the adult brain, and that the development and utilization of a potential biomolecule inhibiting pKr-2-triggered neurotoxic events can be useful to prevent neurodegeneration in the adult brain, which could be applied in PD.

Flavonoids are one of the largest families of phenolic compounds and are widely found in foods and beverages of plant origin.^{11-15,23} In general, the bioavailability of flavonoids is low due to poor absorption, rapid excretion, and the widespread biotransformation and conjugation that happens during their absorption from the intestine, in the liver, and in cells.^{11-15,23} However, despite their low bioavailability, extensive studies have reported that many flavonoids can provide numerous health benefits, including a reduction in risk of PD, due to their biologic activity, including antioxidative and anti-inflammatory properties.^{11-15,23} These reports show the intensified interest in the potential of flavonoids to protect the neurological system and regulate properties of neurons from neurotoxic conditions in the adult brain.

Milk thistle-derived silibinin can protect neurons against age-dependent neurodegenerative diseases^{11-13,24-27} and ischemic stroke,¹⁵ and its administration has been shown to induce anti-inflammatory activity through suppression of microglial activation, resulting in reduction of pro-inflammatory cytokines, in various models of inflammation *in vivo* and *in vitro*.²⁴⁻²⁷ We also recounted that silibinin treatment attenuated expression of IL-1 β , TNF- α , and iNOS resulting in neuroprotection in MPP⁺-injected rat PD model and a kainic acid-induced mouse model of epileptic seizure,^{11,21} suggesting that silibinin may have potential to inhibit neurotoxic inflammatory responses in the nigrostriatal DA system *in vivo*.

In this study, we examined whether silibinin treatment could preserve the nigrostriatal DA system against neurotoxic action triggered by pKr-2 injection in the mouse SN, and whether its administration could attenuate pKr-2-triggered microglial activation. Our results show that silibinin treatment significantly reduced degeneration of nigrostriatal DA projections after injection of pKr-2 (Fig. 1), suggesting the potential of silibinin as a protective molecule

against pKr-2-triggered neurotoxicity. Consistent with preservation of nigrostriatal DA projections, silibinin treatment apparently preserved striatal dopamine, DOPAC, and HVA as well as locomotor function against pKr-2-triggered neurotoxicity (Fig. 2). Moreover, our observations show that silibinin may attenuate pKr-2-triggered microglial activation through downregulation of the ERK signaling pathway in the SN, resulting in downregulation of iNOS, TNF- α , and IL-1 β (Fig. 3).

Altogether, our results show that silibinin can inhibit neurotoxic inflammatory responses elicited by pKr-2-triggered microglial activation, consequently resulting in the protection of the nigrostriatal DA projections *in vivo* (Fig. 4). We reported that pKr-2 upregulation is a cause of neurotoxic inflammation resulting in neurodegeneration as identified in postmortem brains of PD patients. Upregulation of pKr-2 reproduces the neuropathological features of PD, including degeneration of nigrostriatal DA projection, decreased levels of striatal dopamine, occurrence of neurotoxic inflammation, and locomotor impairment *in vivo*.^{1,9} In this study, we found that silibinin preserved the nigrostriatal DA projection, preserved striatal dopamine levels, improved behavioral phenotype, and suppressed neurotoxic inflammatory responses through inhibition of the ERK signaling pathway *in vivo*. In addition, silibinin has been reported to have anti-inflammatory and antioxidant properties that protect DA neurons against MPP⁺-triggered neurotoxicity.¹¹ In conclusion, although further clinical studies and scientific evidence may be needed to use silibinin in the clinical setting, our results suggest that silibinin, a natural product, is beneficial in protecting the nigrostriatal DA system from neurotoxic conditions induced by pKr-2 upregulation. These neuroprotective properties are attributed to its anti-inflammatory activity and may be applied to the prevention of neurodegeneration in PD.

ACKNOWLEDGMENTS

This study was supported by grants from the National Research Foundation of Korea (NRF-2017R1A2B4002675), the Korea Health Technology R&D Project of the Ministry of Health & Welfare (HI14C1135), and the KBRI basic research program through Korea Brain Research Institute funded by Ministry of Science & ICT (18-BR-02-06, 18-BR-03-01, and **18-BR-03-03**).

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

- Kim SR, Chung ES, Bok E, *et al.*: Prothrombin kringle-2 induces death of mesencephalic dopaminergic neurons *in vivo* and *in vitro* via microglial activation. *J Neurosci Res* 2010;88:1537–1548.
- Leem E, Jeong KH, Won SY, Shin WH, Kim SR: Prothrombin kringle-2: A potential inflammatory pathogen in the parkinsonian dopaminergic system. *Exp Neurobiol* 2016;25:147–155.
- Braak H, Ghebremedhin E, Rub U, Bratzke H, Del Tredici K: Stages in the development of Parkinson's disease-related pathology. *Cell Tissue Res* 2004;318:121–134.
- Jankovic J: Parkinson's disease: Clinical features and diagnosis. *J Neurol Neurosurg Psychiatry* 2008;79:368–376.
- Antony PM, Diederich NJ, Kruger R, Balling R: The hallmarks of Parkinson's disease. *FEBS J* 2013;280:5981–5993.
- Saiki S, Sato S, Hattori N: Molecular pathogenesis of Parkinson's disease: Update. *J Neurol Neurosurg Psychiatry* 2012;83:430–436.
- Dheen ST, Kaur C, Ling EA: Microglial activation and its implications in the brain diseases. *Curr Med Chem* 2007;14:1189–1197.
- Wang Q, Liu Y, Zhou J: Neuroinflammation in Parkinson's disease and its potential as therapeutic target. *Transl Neurodegener* 2015;4:19.
- Shin WH, Jeon MT, Leem E, *et al.*: Induction of microglial toll-like receptor 4 by prothrombin kringle-2: A potential pathogenic mechanism in Parkinson's disease. *Sci Rep* 2015;5:14764.
- Nam JH, Leem E, Jeon MT, *et al.*: Inhibition of prothrombin kringle-2-induced inflammation by minocycline protects dopaminergic neurons in the substantia nigra *in vivo*. *Neuroreport* 2014;25:489–495.
- Jung UJ, Jeon MT, Choi MS, Kim SR: Silibinin attenuates MPP⁺-induced neurotoxicity in the substantia nigra *in vivo*. *J Med Food* 2014;17:599–605.
- Lee Y, Park HR, Chun HJ, Lee J: Silibinin prevents dopaminergic neuronal loss in a mouse model of Parkinson's disease via mitochondrial stabilization. *J Neurosci Res* 2015;93:755–765.
- Lu P, Mamiya T, Lu LL, *et al.*: Silibinin prevents amyloid beta peptide-induced memory impairment and oxidative stress in mice. *Br J Pharmacol* 2009;157:1270–1277.
- Marrazzo G, Bosco P, La Delia F, *et al.*: Neuroprotective effect of silibinin in diabetic mice. *Neurosci Lett* 2011;504:252–256.
- Wang M, Li YJ, Ding Y, *et al.*: Silibinin prevents autophagic cell death upon oxidative stress in cortical neurons and cerebral ischemia-reperfusion injury. *Mol Neurobiol* 2016;53:932–943.
- Paxinos G, Franklin KBJ: *The Mouse Brain in Stereotaxic Coordinates*. (Compact 2nd ed.) Elsevier Academic Press, Boston, MA, USA, 2004.
- Kim BW, Jeong KH, Kim JH, *et al.*: Pathogenic upregulation of glial lipocalin-2 in the parkinsonian dopaminergic system. *J Neurosci* 2016;36:5608–5622.
- Kim SR, Kareva T, Yarygina O, Kholodilov N, Burke RE: AAV transduction of dopamine neurons with constitutively active Rheb protects from neurodegeneration and mediates axon regrowth. *Mol Ther* 2012;20:275–286.
- Nam JH, Leem E, Jeon MT, *et al.*: Induction of GDNF and BDNF by hRheb(S16H) transduction of SNpc neurons: Neuroprotective mechanisms of hRheb(S16H) in a model of Parkinson's disease. *Mol Neurobiol* 2015;51:487–499.
- Kim HD, Jeong KH, Jung UJ, Kim SR: Naringin treatment induces neuroprotective effects in a mouse model of Parkinson's disease *in vivo*, but not enough to restore the lesioned dopaminergic system. *J Nutr Biochem* 2016;28:140–146.
- Kim S, Jung UJ, Oh YS, *et al.*: Beneficial effects of silibinin against kainic acid-induced neurotoxicity in the hippocampus *in vivo*. *Exp Neurobiol* 2017;26:266–277.

22. Sun Y, Liu W, Liu T, *et al.*: Signaling pathway of MAPK/ERK in cell proliferation, differentiation, migration, senescence and apoptosis. *J Recept Signal Transduct Res* 2015;35:600–604.
23. Kim SR: Control of granule cell dispersion by natural materials such as eugenol and naringin: A potential therapeutic strategy against temporal lobe epilepsy. *J Med Food* 2016;19:1–7.
24. Song X, Zhou B, Cui L, *et al.*: Silibinin ameliorates Abeta25-35-induced memory deficits in rats by modulating autophagy and attenuating neuroinflammation as well as oxidative stress. *Neurochem Res* 2017;42:1073–1083.
25. Fernandes V, Sharma D, Kalia K, Tiwari V: Neuroprotective effects of silibinin: An in-silico and in-vitro study. *Int J Neurosci* 2018;128:935–945.
26. Wang MJ, Lin WW, Chen HL, *et al.*: Silymarin protects dopaminergic neurons against lipopolysaccharide-induced neurotoxicity by inhibiting microglia activation. *Eur J Neurosci* 2002;16:2103–2112.
27. Song JH, Kim KJ, Lee BY: Silibinin suppresses mediators of inflammation through the inhibition of TLR4-TAK1 pathway in LPS-induced RAW264.7 cells. *J Food Nutr Res* 2016;4:515–521.