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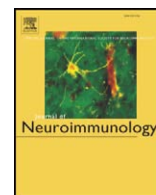

Prothrombin kringle-2-induced oxidative stress contributes to the death of cortical neurons *in vivo* and *in vitro*: Role of microglial NADPH oxidase

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ABSTRACT

In the present study, we examine whether prothrombin kringle-2 (pKr-2), a domain of prothrombin distinct from thrombin and a potent microglial activator induces reactive oxygen species (ROS) generation through stimulation of microglial NADPH oxidase activity, and whether this phenomenon contributes to oxidative damage and consequent neurodegeneration. Intracortical injection of pKr-2 caused significant loss of cortical neurons *in vivo* after seven days, as evident from Nissl staining and immunohistochemical analysis using the neuronal-specific nuclear protein (NeuN) antibody. In parallel, pKr-2-activated microglia and ROS production were observed in rat cortex displaying degeneration of cortical neurons. Reverse transcription-PCR at various time points after pKr-2 administration disclosed early and transient expression of inducible nitric oxide synthase (iNOS) and proinflammatory cytokines, such as interleukin 1β (IL-1β). Co-localization of iNOS, IL-1β, and TNF-α within microglia was evident with double-label immunohistochemistry. Additionally, pKr-2 induced upregulation of cytosolic components of NADPH oxidase ($p67^{phox}$), translocation of cytosolic $p67^{phox}$ protein to the membrane, and $p67^{phox}$ expression in microglia in the cortex *in vivo*, signifying NADPH oxidase activation. The pKr-2-induced oxidation of proteins and loss of cortical neurons were partially inhibited by DPI, an NADPH oxidase inhibitor, and trolox, an antioxidant. Consistent with our hypothesis, following treatment with pKr-2 *in vitro*, neurotoxicity was detected exclusively in co-cultures of cortical neurons and microglia, but not in microglia-free neuron-enriched cortical cultures, indicating that microglia are required for pKr-2 neurotoxicity. Our results strongly suggest that pKr-2 as an endogenous compound participates in cortical neuron death through microglial NADPH oxidase-mediated oxidative stress.

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1. Introduction

Microglia are intrinsic immune cells in the central nervous system (CNS). Microglial activation is a common phenomenon in response to brain injury (Aloisi, 2001; Nakajima and Kohsaka, 2001). Activated microglia produce NADPH oxidase-derived reactive oxygen species (ROS), which trigger or exacerbate neurotoxicity by inducing oxidative stress to neurons (Choi et al., 2005a,b). Additionally, nitric oxide (NO) synthesized by inducible nitric oxide synthase (iNOS), and proinflammatory cytokines, such as tumor necrosis factor (TNF-α) and interleukin 1β (IL-1β) are secreted by activated microglia (Choi et al., 2005b).

NADPH oxidase is a multicomponent enzyme complex with the capacity to produce the highly reactive free radical superoxide. Upon activation, cystolic components of NADPH oxidase are translocated to the membrane and assemble with membrane-associated proteins for activity. Microglial NADPH oxidase is activated in the brains of Alzheimer's disease (AD) (Sawada et al.) patients, including the cortical regions (Shimohama et al., 2000), as well as *in vivo* (Wilkinson and Landreth, 2006) and in *in vitro* (Qin et al., 2002) models of AD produced by β-amyloid (Aβ), the major component of senile plaques in AD, resulting in ROS formation. In this regard, brains of AD patients and *in vivo* and *in vitro* models of AD show evidence of oxidative damage, including oxidative stress modification of proteins, lipids, and DNA (Butterfield et al., 2001). Additionally, NADPH oxidase-derived ROS (Choi et al., 2005b) and other neurotoxic proinflammatory cytokines (such as iNOS, TNF-α and IL-1β) originating from activated microglia induce death of cortical neurons, both *in vivo* and *in vitro* (Lee da et al., 2006). Reactive microglia have been

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identified in the brains of Parkinson disease (PD) patients (Sawada et al., 2006) and SN of animal models of PD (McGeer and McGeer, 2008), stroke (Kaushal and Schlichter, 2008), multiple sclerosis (MS) (Gray et al., 2008), prion diseases (Brown, 2001), and amyotrophic lateral sclerosis (ALS) (Minghetti et al.) (Boillee and Cleveland, 2008; Minghetti et al., 1999). In combination with extensive pathological findings as well as *in vivo* and *in vitro* experimental data, these results suggest that NADPH oxidase-mediated oxidative stress, neurotoxic proinflammatory cytokines, and iNOS originating from activated microglia are involved in the pathogenesis of neurodegenerative diseases, including AD (Block et al., 2007; Brown, 2007).

Prothrombin, a precursor of thrombin, is converted to an amino terminal fragment, designated kringle-1–2, and active thrombin by the prothrombinase complex (Factor Xa) (Shikamoto and Morita, 1999), in turn, inducing blood coagulation (Davie et al., 1991). Thrombin formed during activation further cleaves kringle-1–2 into kringle-1, including the Gla domain, a kringle-1 domain and kringle-2 (Mann, 1976; Taneda et al., 1994). In rat brain treated with thrombin, infiltration of inflammatory cells, brain edema, and reactive gliosis were observed (Nishino et al., 1993). In addition, thrombin induces various biological responses in the CNS, although its effect on neurons and astrocytes is either protective or toxic, depending on the thrombin concentration. Increased thrombin has been shown to lead to the degeneration of the hippocampal neurons (Striggow et al., 2000), spinal motoneurons (Turgeon et al., 1998), and astrocytes (Donovan et al., 1997). While previous studies by our group show that prothrombin kringle-2 (pKr-2) activates cultured rat microglia (Ryu et al., 2002), limited information is available on pKr-2 functions in the CNS, particularly *in vivo*. Here, we examine whether microglial NADPH oxidase is activated by pKr-2 in the rat cortex and whether microglial NADPH oxidase-derived ROS participate in pKr-2-induced degeneration of cortical neurons *in vivo*. Additionally, we investigate the significance of microglial activation in pKr-2-induced neurotoxicity using cortical neuron and microglia co-cultures.

2. Materials and methods

2.1. Sterotaxic surgery and drug injection

All experiments were carried out in accordance with approved animal protocols and guidelines established by Ajou University. Female Sprague–Dawley (SD) rats (260–280 g) were anesthetized by injection of chloral hydrate (360 mg/kg, i.p.) and positioned in a stereotaxic apparatus (David Kopf Instrument, Tujunga, CA, USA). A midline sagittal incision was made in the scalp, and holes were drilled in the skull over the lateral ventricles and dorsal cortex using the following coordinates: 0.8 mm posterior to bregma and 1.5 mm lateral to the midline for intracerebroventricular (i.c.v) injections; and 1.4 mm posterior to bregma and 2.0 mm lateral to the midline for intracortical injections according to Paxinos and Watson (1998). The needle tip was directed vertically down to 3.6 mm beneath the surface of the brain for the ventricles and to 2.0 mm for cortex. All injections were made using a Hamilton syringe equipped with a 30 S-gauge beveled needle and attached to a syringe pump (KD Scientific, New Hope, PA, USA). Infusions were made at a rate of 0.2 μl/min for pKr-2 (48 μg/4 μl in sterile phosphate-buffered saline [PBS]; Haematologic Technologies Inc), and 0.5 μl/min for diphenylene iodonium (DPI; 100 μM in 20 μl sterile saline; Calbiochem), and for vehicle (PBS) as controls.

2.2. Tissue preparation and immunohistochemistry

Animals were anesthetized with chloral hydrate (360 mg/kg, i.p.) at the indicated time points after injection and transcardially perfused with saline solution containing 0.5% sodium nitrate and heparin (10 U/ml), followed by fixation with 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (PB). Brains were removed from the skull, postfixed overnight at 4 °C in buffered 4% paraformaldehyde, and stored at 4 °C in 30% sucrose solution until they sank. Brains were frozen sectioned using a sliding microtome into 40 μm coronal sections and collected in six separate series. Immunohistochemistry was performed using the avidin–biotin staining technique as described previously (Choi et al., 2003a,b). Briefly, free-floating serial sections were rinsed three times for 10 min in PBS and then pretreated for 5 min at room temperature in PBS containing 1% H_2O_2. Sections were then rinsed in PBS containing 0.3% Triton X-100 and 0.5% BSA and then preincubated for 1 h at room temperature in PBS containing 0.5% BSA. Next, the sections were incubated overnight with gentle shaking at room temperature with PBS containing 0.5% BSA and the following monoclonal primary antibodies: anti-OX-42 (specific for complement receptor type 3 (CR3), 1:200; Serotec), anti-OX-6 (specific for major histocompatibility complex class II antigens, 1:200; Pharmingen), anti-ED1 (specific for glycosylated lysosomal antigen, 1:200; Serotec) for microglia, anti-neuron-specific nuclear protein (NeuN, 1:200; Chemicon) for neurons. Sections were then rinsed in PBS and incubated for 1 h at room temperature in 1:200 biotin-conjugated anti-mouse antibody in PBS containing 0.5% BSA. Sections were rinsed again and incubated for 1 h at room temperature in avidin–biotin complex solution (Vector Laboratories, Burlingame, CA). After rinsing three times in PBS, the signal was detected by incubating sections in 0.5 mg/ml 3,3 diaminobenzidine in PB containing 0.003%H_2O_2. Sections were then rinsed in PBS, mounted on gelatin-coated slides, and viewed under a bright-field microscope (Olympus Optical, Tokyo, Japan). For Nissl staining, some of the cortex tissue was mounted on gelatin-coated slides, dried for 1 h at room temperature, stained with 0.5% cresyl violet (Sigma), dehydrated, coverslipped, and then analyzed under a bright-field microscope (Olympus Optical).

2.3. Double-immunofluorescence staining

For double-immunofluorescence staining, sections were processed as described previously (Choi et al., 2005b). Briefly, sections were mounted on gelatin-coated slides, dried for 6 h at room temperature, and washed twice in PBS. Slides were incubated overnight at 4 °C in a combination of a goat anti-TNF-α (1:100; R&D systems), a goat anti-IL-1β (1:200; R&D system), or a rabbit anti-iNOS (1:200; Upstate biotechnology, Lake Placid, NY), and then exposed for 1 h at room temperature to fluorescein-conjugated *lycopersicon esculentum* (tomato lectin) (1:200; Vector Laboratories) and Texas-Red-conjugated anti-rabbit IgG (Vector Laboratories) or Texas-Red-conjugated anti-goat IgG (Vector Laboratories). Slides were also incubated overnight at 4 °C with anti-p67^{phox} antibody (1:200; BD Biosciences) and OX-42 (1:100; Serotec) or GFAP (1:100; sigma). After washing in PBS, the sections were incubated simultaneously with a mixture of FITC-conjugated goat anti-mouse IgG (1:100; Vector Laboratories) and Texas Red-conjugated goat anti-rabbit IgG (1:100; Vector Laboratories) for 1 h at room temperature. Slides were coverslipped with Vectashield medium, and viewed using an IX71 confocal laser scanning microscope (Olympus). To analyze the localization of different antigens in double-stained samples, images were obtained from the same area and merged using interactive software.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Brain tissues from the ipsilateral cortex were dissected at the indicated time points after injection, and total RNA was extracted in a single step using RNAzol B (Tel-Test, Friendswood, TX) following the instructions of the manufacturer. Total RNA was reverse transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen, Rockville, MD) and random primers (Promega, Madison, WI). The primer sequences used in this study were as follows: 5′-TGA TGT TCC

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