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MODULATION OF CONNEXIN 43 IN ROTENONE-INDUCED MODEL OF PARKINSON'S DISEASE

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Abstract—Gap junctional communication plays an important role in various models of brain pathology, but the changes of gap junctions in Parkinsonism are still not understood. In this study, we show that a major gap junctional protein, connexin43 (Cx43), in astrocytes is enhanced both in a rat Parkinson's disease (PD) model induced with rotenone, a widely used pesticide that inhibits mitochondrial complex I, and *in vitro* in cultured astrocytes stimulated with rotenone. Enhancement of Cx43 protein levels in rotenone-treated cultured astrocytes occurred in parallel with an increase in gap junctional intercellular communication, but was not accompanied with an increase in *Cx43* mRNA levels. Furthermore, the rotenone-induced increase of Cx43 protein levels both *in vitro* and *in vivo* was associated with increased levels of phosphorylated Cx43, which is required for gap junctional intercellular communication. In our rat PD model, phosphorylated Cx43 was selectively enhanced in the basal ganglia regions, which contain DA neurons or their terminal areas. The increase of Cx43 levels was lower in the substantia nigra pars compacta and the striatum than in the substantia nigra pars reticulata and the globus pallidus. Our findings indicate that modulation of Cx43 protein, and consequently gap junctional cellular communication, in astrocytes may play an important role in PD pathology.

Key words: gap junction, connexin 43, astrocyte, Parkinson's disease, dopaminergic, basal ganglia.

Parkinson's disease (PD) is an adult-onset neurodegenerative disease that is characterized by a progressive and fatal loss of dopaminergic (DA) neurons in the substantia nigra and striatum. A prototypical mitochondrial complex inhibitor, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, induces Parkinsonism in humans and other mammals, and systemic administration of another mitochondrial complex I inhibitor, rotenone, also causes selective death of DA neurons and Parkinsonism in rodents, accompanied by behavioral and neurochemical changes, DA degeneration, and the appearance of eosinophilic cytoplasmic inclusions (Betarbet et al., 2000). DA neurons are known to be sensitive to extracellular ions and chemical transmitters, and extracellular K^+ and glutamate were shown to play key roles in DA neuronal cell death in an animal PD model (Obata et al., 2000; Araki et al., 2001; Ransom et al., 2003). The reason why DA neurons are particularly vulnerable to complex I inhibition is not fully understood, although their vulnerability seems to be important in the development of Parkinsonism. In addition, accumulating evidence indicates an active role for nonneuronal cells, specifically astrocytes, in DA neuronal degeneration (Cardona et al., 2006; McGeer and McGeer, 2008). Astrocytes are central to maintaining the homeostatic regulation of extracellular pH, K^+, and glutamate levels (Rouach et al., 2000). However, despite the importance of astrocyte functions, the role of astrocytes in Parkinsonism remains unknown.

We have previously reported that modulation of gap junctional intercellular communication (GJIC) and connexin43 (Cx43) affect cell viability or growth, implying that GJIC may have an important role in maintaining homeostasis in various organs (Hayashi et al., 1997; Ogawa et al., 2005). It has also been reported that GJIC in astrocytes is indispensable for the homeostatic regulation of extracellular pH, K^+, and glutamate levels in the CNS (Anderson and Swanson, 2000; Ransom et al., 2003). Astrocytes are thought to be coupled by gap junctions, which consist of Cx43 (Dermietzel et al., 2000; Nagy and Rash, 2003). Alteration of Cx43 has recently been observed in ischemia, Alzheimer's disease, and Huntington's disease (Nagy et al., 1996; Vis et al., 1998; Kielian, 2008), and an increase or loss of Cx43 and GJIC in astrocytes has been observed after brain injuries and in pathogenesis associated with reactive astrocytosis (Meme et al., 2006; Haupt et al., 2007). However, whether or not altered astrocyte GJIC is involved in the development of PD remains unanswered.

Therefore, in this study we examined the changes in astrocyte GJIC and Cx43, as well as the phosphorylation status of Cx43, in a rat model of PD induced by chronic exposure to rotenone and in cultured astrocytes stimulated with rotenone. The former model has been widely used to investigate the etiology of Parkinsonism (Betarbet et al., 2000; Alam and Schmidt, 2002); the latter is useful to study

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Abbreviations: Cx43, connexin43; DA, dopaminergic; DMSO, dimethyl sulfoxide; FRAP, fluorescence recovery after photobleaching; GJIC, gap junctional intercellular communication; PBST, PBS-0.1% Triton X-100; PD, Parkinson's disease; PVDF, polyvinylidene difluoride; RR, recovery rate; SNc, substantia nigra pars compacta; SNr, substantia nigra pars reticulata; TH, tyrosine hydroxylase.

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the molecular mechanisms of rotenone's effects on Cx43 and GJIC.

EXPERIMENTAL PROCEDURES

Drugs and chemicals

Rotenone and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Rotenone was dissolved in DMSO (100 mM) and stored at −20 °C.

Rats

The animals were acclimated and maintained at 23 °C under a 12-h light/dark cycle (lights on 08:00–20:00 h). Rats were housed in standard laboratory cages and had free access to food and water throughout the study period. All animal experiments were carried out in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the protocols were approved by the Committee for Animal Research at Hiroshima University. All efforts were put in place to minimize the number of animals used and their suffering. Lewis rats (200–250 g each) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The rats were randomly divided into a rotenone group (n=6) and a control group (n=6). The rotenone group subcutaneously received rotenone (2.5 mg/kg, diluted in Panacet); the controls received vehicle (Panacet) only.

Primary astrocyte cultures

Primary astrocytes were prepared from whole brains of neonatal Wistar rats (1–2 days of age) (Hosoi et al., 2000). In brief, the brains were digested with 0.05% trypsin–EDTA (Invitrogen, Grand Island, NY, USA) at 37 °C for 10 min, and then mechanically dissociated by gentle pipetting and passed through a 70-μm-pore nylon mesh. Cells were plated onto 75 cm^2 plastic flasks and grown in DMEM (Invitrogen) supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37 °C in a humidified 5% CO_2-containing atmosphere. The medium was changed twice a week. When cells reached confluence, at ~12 days *in vitro*, they were harvested with trypsin-EDTA (Invitrogen). Cells were then replated as a secondary culture. The purity of the primary astrocyte cultures was assessed by immunocytochemical staining, using an antibody against an astrocyte-specific marker (GFAP, dilution 1:1000; Sigma-Aldrich). At 30 days *in vitro*, 99% of the primary-cultured cells were GFAP-positive. Cultured astrocytes were treated with 0–16 nM rotenone for 48 h.

Fluorescence recovery after photobleaching (FRAP) assay for GJIC

The procedure used was a modified version of the standard method for measuring GJIC by quantitative FRAP (Wade et al., 1986; Trosko et al., 2000). Assays were performed using a Zeiss LSM 510 laser-scanning confocal microscope (Carl Zeiss International, Jena, Germany). After bleaching of randomly selected cells with a micro-laser beam, the rate of transfer of 5,6-carboxyfluorescein diacetate (Molecular Probes, Inc., Eugene, OR, USA) from adjacent labeled cells back into bleached cells was calculated. Recovery of fluorescence was examined after 0.5 min, and the recovery rate (RR) was calculated as percentage of photobleached fluorescence per min. The RR was adjusted for the loss of fluorescence measured in unbleached cells, and the results are expressed as the ratio (mean±SE) of RR to that of untreated control cells.

Extraction of Cx43 RNA

Cells were grown in 6-cm dishes and prepared as described previously (Ogawa et al., 2005). In brief, after 48 h of incubation, the cells were trypsinized and suspended in DMEM medium containing 10% FCS. Total RNA was isolated from the cells using QIAshredder and RNeasy Mini kits (Qiagen, Inc., Chatsworth, CA, USA). An initial strand of cDNA was synthesized from 500 ng of RNA extracts in a volume of 20 μl using AMV reverse transcriptase XL (TaKaRa, Otsu, Japan) priming with random 9-mers at 42 °C for 10 min. The cDNA strand was stored at −20 °C until use. Expression of *rCx43* mRNAs was evaluated by real-time RT PCR based on TaqMan methodology. In brief, PCR was performed in an ABI PRISM 7900 sequence detector (PerkinElmer/Applied Biosystems, Foster City, CA, USA) in a final volume of 20 μl. The PCR mixture contained 10 mM Tris–HCl buffer, pH 8.3 (PerkinElmer/Applied Biosystems), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP mixture, 0.5 U of "AmpliTaq Gold" (PerkinElmer/Applied Biosystems), 0.2 μM primers and probe. The primer and probe sequences for gene amplification were as follows: rCx43; 5-ATCAGCATCCT CTTCAAGTCTGTCT-3 (FP), 5-CAGGGA-TCTCTCTTGCA−GGTGTA-3 (RP) and 5-CC TGCTCATCCAGT-GGT-3 (probe). The "TaqMan" probe carried a 5-FAM reporter label and a 3-MGB and nonfluorescence quencher group, synthesized by Applied Biosystems. The determination of *rGAPDH* used the TaqMan rodent GAPDH control reagents (Applied Biosystems). The AmpliTaq gold enzyme was activated by heating for 10 min at 95 °C, and all genes were amplified by 50 cycles of heating for 15 s at 95 °C, followed by 1 min at 60 °C.

Quantification for *Cx43* mRNA

For the construction of standard curves of positive controls, the total RNA of primary astrocytes was reverse-transcribed into cDNA and serially diluted in water in five or six log steps to afford fourfold serial dilutions of cDNA from about 100 ng to 100 pg. These cDNA serial dilutions were prepared once for all examinations performed in this study and stored at −20 °C. The coefficient of linear regression for each standard curve was calculated, and then when the cycle threshold (CT) value of a sample was substituted in the formula for each standard curve, the relative concentration of *rCx43* or *rGAPDH* could be calculated. To normalize for differences in the amount of total RNA added to each reaction mixture, *GAPDH* was used as an endogenous RNA control. The data represent the average expression of target genes, relative to *GAPDH*, from three independent cultures.

Immunoblotting

Cells and rat brains were lysed in ice-cold lysis buffer containing 20 mM Tris-buffered saline (TBS), pH 7.5, 1% Triton X-100, 150 mM NaCl, and 1 mM each of EDTA, EGTA, β-glycerophosphate, Na_3VO_4, and phenylmethylsulfonyl fluoride, 2.5 mM sodium pyrophosphate, 1 μg/ml leupeptin. The lysates were then sonicated. The samples were diluted 1:4 in water, and their protein concentrations were determined using DC protein assay (Bio-Rad Corp., Richmond, CA, USA). Samples (10 μg) of protein were dissolved in Laemmli Sample Buffer, separated on 12.5% acrylamide gel, and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Then blots were incubated with anti-Cx43 monoclonal antibody (Chemicon International, Inc., Temecula, CA, USA) overnight at 4 °C, followed by PBS-0.1% Triton X-100 (PBST) washes three times for 15 min each. As an internal control to determine whether equal amounts of protein had been loaded on to the gel, the PVDF membranes were stripped and reprobed with anti-α-tubulin (T5168, Sigma-Aldrich). Blots were incubated with goat–antirabbit antibody–conjugated horseradish peroxidase or mouse–antimouse antibody–conjugated horseradish peroxidase. Immunoreactivity was determined by ECL detection using the ECL