Increased osteopontin expression and mitochondrial swelling in 3-nitropropionic acid-injured rat brains

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Abstract
Osteopontin (OPN) is involved in the regulation of calcium precipitation in the brain pathology including ischemia. A 3-Nitropropionic acid (3NP) irreversibly inhibits mitochondrial complex II in the electron transport chain, with subsequent loss of transmembrane potential and calcium entry into the mitochondria. The present study examined the 3NP-induced calcium elevation in mitochondria and OPN expression in the 3NP-lesioned striatum. Rats were subcutaneously injected 3NP (15 mg/kg) every other day for six weeks. Histological analysis, including the Hematoxylin–Eosin, Nissl, and Alizarin Red S stainings, was performed to examine the neurotoxic effects of 3NP. The expression of OPN in the striatum of 3NP-treated rats was investigated with immunohistochemistry and immunoelectron microscopy. In the striatal lesions, extensive loss of neurons and white matter bundles was detected. OPN was mainly detected in the penumbra region of the 3NP lesion. Scattered OPN expression was colocalized in the striatal neurons. After Alizarin Red S staining, the increase of calcium deposition was detected in the striatal lesions. In the electron microscopic analysis, the localization of OPN was clearly observed in the ultrastructure of mitochondria by immunoperoxidase and immunogold-silver staining techniques. Taken together, present findings suggest that calcium-induced mitochondrial swelling is highly associated with OPN expression. Thus, striatal calcium accumulation may be derived from 3NP-induced alteration in mitochondrial calcium homeostasis and pathologically associated with the induction of OPN protein.

Keywords: calcium, mitochondrial swelling, 3-nitropropionic acid, osteopontin.

Introduction
Osteopontin (OPN) is a calcium-binding multifunctional protein containing an arginine-glycine-aspartate-binding integrin motif [1]. It is involved in numerous pathophysiological processes, including the inflammatory response [1], cell adhesion [2], wound repair [3], apoptosis [4], and tumor metastasis [5]. OPN is expressed in a variety of tissues, including bone, vascular tissue, kidney, and brain [6]. Recent studies have described the functions and characteristics of OPN in pathological brain conditions. Especially, OPN expression is associated with calcium precipitation in the ischemic lesion [7, 8]. Although OPN has been correlated with the calcium dyshomeostasis-induced cell degeneration, little is known about the underlying mechanisms of calcium accumulation in lesioned areas.

3-Nitropropionic acid (3NP), an irreversible inhibitor of mitochondrial complex II (succinate dehydrogenase), impairs energy metabolism which leads to subsequent neuronal cell death [9]. 3NP has been used to model Huntington’s disease (HD) for its neurodegenerative effects on striatum replicating anatomical, histopathological, and neurochemical features of HD [10–12]. Specifically, the formation of nonselective mitochondrial permeability transition (MPT) pores is related with 3NP-induced neurodegeneration [13, 14]. Excessive calcium in mitochondria allows opening of MPT pores with subsequent swelling, loss of membrane potential, and cytochrome c release [3, 15–17].

In our previous study, we reported that induction of OPN is associated with the mitochondrial dysfunction in 3NP-treated striatal neurons [18]. In the present study, we investigated the OPN expression, mitochondrial morphological changes, and calcium (Ca2+) deposits in the 3NP-treated model.

Materials and Methods
Male Sprague–Dawley rats (n=10) weighing 300–320 g (9–10-week-old) were used. 3NP (Sigma-Aldrich Co., St. Louis, MO, USA) was dissolved in saline (pH 7.4). The animals were administered subcutaneous injections of 3NP (15 mg/kg) every other day for six weeks. Control rats (n=5) were subcutaneously administered the equivalent volume of saline. The animals were anesthetized with Chloral hydrate (400 mg/kg, intraperitoneal injection) and sacrificed two days after the final 3NP administration. All of the experimental procedures were conducted according to the guidelines of the Institutional Animal Care and Use Committee of Konkuk University (Seoul, Korea).

For histology, brain samples were removed, immersed in 4% paraformaldehyde for 12 hours, and then paraffin embedded. The brains were cut into 5-μm sections and then stained with Cresyl Violet (Nissl), Hematoxylin–Eosin (HE), and Alizarin Red S, in order to examine the histopathological changes. Tissue sections were selected from an area between 1.68 mm anterior and 1.56 mm posterior to the bregma.

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For the immunohistochemical analysis of OPN in the striatal regions, brains were rapidly dissected into 2-mm-thick sections. The 2-mm-thick brain sections were treated with 0.3% hydrogen peroxide for quenching of endogenous peroxidase and then incubated in 1% bovine serum albumin (BSA) in 0.01 M phosphate-buffered saline (PBS) for two hours and then with a mouse monoclonal anti-OPN antibody (1:150 dilution, American Research Products, Inc., Waltham, MA, USA), at 4°C, overnight. The sections were washed three times for 10 minutes each in PBS, incubated for two hours in biotinylated goat anti-mouse IgG (1:400 dilution, Vector, Burlingame, CA, USA) and Streptavidin Peroxidase complex (1:400 dilution, Vector), at room temperature, and then washed three times for 10 minutes each with Tris buffer. The immunoreaction was visualized by using 3,3′-Diaminobenzidine (DAB) as the chromogen.

Additionally, the brain samples were cryoprotected and frozen in liquid nitrogen. For the immunofluorescence, semi-thin cryosections (1-μm thick) were cut at -80°C, on a Leica EM UC7 ultramicrotome equipped with a FC7 cryochamber. For the double-labeling studies, the brain sections were first incubated in blocking buffer (1% BSA) in PBS, in a dark humidified chamber for one hour, at room temperature and then in a mixture of the mouse monoclonal anti-OPN antibody (1:150 dilution, American Research Products, Inc., Waltham, MA, USA) and plus rabbit polyclonal antibodies to dopamine and cyclic adenosine monophosphate (cAMP)-regulated neuronal phosphoprotein (DARPP-32, 1:100 dilution, Cell Signaling Technology, Inc., Danvers, MA, USA), at 4°C, overnight. The sections were washed three times for 5 minutes each in PBS and then incubated for two hours, at room temperature, with the following secondary antibodies: Cy3-conjugated donkey anti-mouse (1:2000 dilution, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) and Alexa Fluor 488 goat anti-rabbit (1:300 dilution, Thermo Fisher Scientific, Inc., Waltham, MA, USA) for the OPN/DARPP-32 double labeling. Counterstaining of the cell nuclei was performed with 4',6-Diamidino-2-phenyindole (DAPI, 1:1000 dilution, Roche Diagnostics GmbH, Mannheim, Germany) for 10 minutes. The slides were examined and photographed with a confocal microscope (LSM 510 Meta, Carl Zeiss AG, Jena, Germany). Images were converted to TIFF files, and the contrast levels were adjusted with Adobe Photoshop ver. 7.0 (Adobe Systems, Inc., San Jose, CA, USA).

For the immunoelectron microscopic analysis, pre-embedding immunoperoxidase methods were conducted. Fifty-μm-thick vibratome sections were selected and incubated in 1% BSA in 0.01 M PBS. Subsequently, sections were incubated with the mouse monoclonal anti-OPN antibody (1:150 dilution, American Research Products, Inc., Waltham, MA, USA), at 4°C, overnight. Sections were incubated with biotinylated goat anti-mouse IgG in 0.01 M PBS, at room temperature, for two hours, and reactions were visualized by using DAB as the chromogen. Then, they were post-fixed (1% Glutaraldehyde and 1% Osmium Tetroxide solution for 30 minutes each) and embedded in Epon 812 resin. After areas of interest were selected, excised and glued onto resin blocks, ultrathin sections (70–90 nm) were produced using an ultramicrotome. Following the staining with 1% Uranyl Acetate, sections were observed with an electron microscope (JEM 1010; JEOL, Ltd., Tokyo, Japan). Additionally, vibrotome sectioning and primary antibody incubation were similarly conducted for immunogold-silver labeling. Next, sections were incubated with nanogold particles (1.4 nm) (1:100 dilution, Nanoprobes, Stony Brook, NY, USA) conjugated secondary antibody for two hours. After then, silver enhancement step was conducted using the HQ silver enhancement kit (Nanoprobes) for 3 minutes. Ultrathin sections were cut in the same condition and observed with an electron microscope.

A quantitative detection of OPN protein in mitochondria was performed by analyzing the immunogold/silver immunocytochemistry of OPN on the electron micrographs. The analysis was done on 150 mitochondria labeled with immunogold that were chosen randomly on ultrathin sections. Digital images of mitochondria that were taken with charge-coupled device (CCD) cameras attached to the electron microscope were loaded into ImageJ ver. 1.49 software and the real area of each mitochondria were calculated by applying the scale bar length with ImageJ. The mitochondria were classified according to their area size and the number of gold particles that were obtained by determining the average value of gold particles per 0.0625 μm² of each mitochondria.

The experimental data were analyzed by one-way analysis of variance (ANOVA), which were followed by post-hoc comparisons with Dunnett’s test. P-values less than 0.05 were considered significant. The data are presented as mean ± standard error (SE).

Results

The morphology of the striata obtained from control and 3NP-treated rats were compared using Nissl and HE staining. Compared to the controls (Figure 1a), the cores of the 3NP lesions (Figure 1b) were pale in the Nissl-stained striatal sections. At higher magnification, the Nissl-stained 3NP lesions (Figure 1d) were characterized by marked neuronal loss in the striatum, whereas the controls (Figure 1c) did not show any discernible neuronal loss. The HE staining showed predominant white matter damage and cells with darkly stained nuclei, which may be involved in cell degeneration, in 3NP-lesioned striata (Figure 1f), and these were not observed in controls (Figure 1e). To investigate the calcium deposits in the 3NP-lesioned area, we performed Alizarin Red S staining. Positively stained calcium precipitates are characterized by orange-red color. In addition, the Alizarin Red S staining appeared stronger in the lesioned core than in other regions (Figure 5).

The OPN immunoperoxidase staining of the brain showed that immunoperoxidase labeling of OPN was not detected in the striatum of the control rat (Figure 2a). Whereas, the level of OPN protein was significantly elevated in the bilateral 3NP-induced striatal lesions (Figure 2b). Signal intensity was analyzed with black and white references of normal striatum and 3NP-injured striatum (Figure 2, a and b) by ImageJ program (left normal striatum: 0.138, right normal striatum: 0, left 3NP-injured striatum: 195.5, right 3NP-injured striatum: 162.28).
To identify whether the OPN protein is expressed in the striatal neurons, we conducted a double immunofluorescence staining of OPN and DARPP-32, which is a striatal neuron marker. OPN protein was not in the detectable level in the control rat striatum (Figure 3, a–c). However, OPN was expressed in the 3NP-treated striatum, double staining resulted a clear demarcation of lesion and penumbra region (Figure 3, d–f). Diffuse OPN immunolabeling, which appeared dot-like, was predominantly distributed in the lesioned core where DARPP-32 immunolabeling was absent. At a higher magnification, some OPN-immunoreactive dots in the penumbra were colocalized with DARPP-32 (Figure 3, g–i). These findings suggest that OPN expression was associated with the degeneration of the striatal neurons.

Figure 1 – Light micrographs of the Cresyl Violet (Nissl) and Hematoxylin–Eosin (HE) staining of the control brains and brains with 3-Nitropropionic acid (3NP)-injured striatum. The control section is evenly stained with Cresyl Violet (A), and the 3NP-injured lesion core is lightly stained (B). (C) Higher magnification of the boxed area in (A) shows a number of Cresyl Violet-stained neurons, whereas (D) the boxed area in (B) shows a loss of neurons. (E) The control group shows unaffected neuronal cells and normal white matter bundles on the HE-stained sections. (F) However, in the 3NP-injured lesion core, irregular and dark-stained nuclei and white matter bundle degeneration are detected. Nissl staining: (A and B) ×40; (C and D) ×200. HE staining: (E and F) ×400.
Figure 2 – Osteopontin (OPN) immunoperoxidase staining of the whole brain. Representative brain slices showing OPN immunoperoxidase immunocytochemistry in control rats that were treated with saline (A) and 3-Nitropropionic acid (3NP)-injured rats (B). OPN expression was observed in the bilateral striatum in the 3NP-injured rats, whereas no OPN expression was observed in the saline-treated control rats.

Figure 3 – Confocal microscopy analysis of the osteopontin (OPN) expression and the striatal neuronal marker, dopamine and cyclic adenosine monophosphate (cAMP)-regulated neuronal phosphoprotein (DARPP-32). The control sections showed DARPP-32 labeling and no OPN immunolabeling (A–C). The double labeling for OPN and DARPP-32 in the 3-Nitropropionic acid (3NP)-treated rat striatum shows no detectable level of striatal DARPP-32 immunolabeling in damaged areas (asterisks) that show OPN immunolabeling (D–F). High magnifications of the boxed areas (G–I) show the co-localization of some OPN-labeled dots with DARPP-32 (arrowheads) in penumbra. These images show OPN (A, D, and G) and DARPP-32 (B, E, and H) immunoreactivity as well as merged images (C, F, and I) of double immunolabeling. Scale bars: (A–C) 50 μm; (D–F) 30 μm; (G–I) 5 μm.

We additionally conducted immunoelectron microscopy to examine the distribution of OPN in the degenerated neurons in 3NP-lesioned rats. In the control group, OPN was not detected in the immunoelectron microscopy (Figure 4a). At a higher magnification, ultrastructural examination of striatum from control showed that most mitochondria were small round or oval shape, and have clear cristae (Figure 4b). Scattered OPN-immunoreactive
profiles, which were labeled with electron-dense precipitate, appeared as dot-like shape and they were predominantly distributed in the lesioned core (Figure 4c). At a higher magnification, the labeled profiles could be identified as mitochondria that show a rounded (white arrowhead) or irregularly shaped (arrow) structures, and cristae in the electron micrograph (Figure 4d). Ultrastructural localization of OPN in mitochondria was further analyzed by immunogold/silver methods (Figure 4, e and f). At a higher magnification, a number of silver-enhanced gold particles were localized over the matrix and inner membrane of mitochondria in larger area sizes (Figure 4f). Mitochondria also showed disorganization of cristae and loss of matrix structure.

Alizarin Red S staining showed that calcium accumulation is markedly increased in the 3NP-treated striatum (Figure 5). Accumulation of calcium and OPN suggest that these parameters are positively correlated in the 3NP-treated striatum. Calcium deposition is important for mitochondrial swelling. To further investigate the correlation between OPN and the calcium precipitate, we measured the area of OPN-labeled mitochondria with immunogold/silver methods. In order to determine whether OPN protein expression was induced in the swollen mitochondria by calcium accumulation, we studied the relationship between the swelling of the mitochondria and OPN expression. The areas of the mitochondria labeled with immunogold are detected by
antibody against OPN and measured by counting the gold particles in the each mitochondrion (Figure 6). In line with the results of OPN immunoreactivity, any immunogold particles were not detected in the control group. In the control group, the average area of mitochondria was 0.124 μm². In the 3NP-treated group, counting data of the gold particles can be efficiently used for comparison of OPN expression among mitochondria with different size. There seemed to be a considerable increase in the number of gold particles that were found in the larger and swollen mitochondria compared to the smaller mitochondria (mitochondria within size range 0.1–0.2 μm² are used as a standard of comparison). In the hypothesis that the different number of gold particles in the section indicates differences in the level of OPN expression, the present quantitative analysis of gold labeling suggests that the expression level of OPN is closely associated with mitochondrial swelling.

**Discussion**

In the present study, we demonstrated that systemic 3NP injection is efficient in modeling striatal degeneration with OPN elevation. The OPN immunoreactivity in the mitochondria of striatal neurons suggests that the mitochondria are important in OPN induction in the 3NP-lesioned core. The mitochondria of striatal neurons are particularly vulnerable to calcium overload-induced MPT [19, 20], and this intracellular calcification disrupts the structural and functional integrity of the organelles [21, 22]. After Alizarin Red S staining, we observed the accumulation of calcium in the 3NP-lesioned core of striatum. MPT, which is the nonselective permeabilization of the inner mitochondrial membrane, is produced by a variety of conditions or compounds. Mirandola et al. reported that 3NP treatment was effective on calcium-induced MPT, especially in the mitochondria isolated from heart and brain [20]. Previously, Hamilton & Gould also suggested that glutamate excitotoxicity might play an influential role in the mechanisms of 3NP toxicity [23]. In the recent in vitro study, Liot et al. demonstrated that 3NP caused mitochondrial fragmentation and neuronal death via glutamatergic excitotoxicity [9]. The striatum receives massive excitatory synaptic inputs that are affected by various neurotransmitters such as glutamate and dopamine [24–26]. Therefore, striatal neurons are highly susceptible to excitotoxicity that is induced by increased N-methyl-D-aspartate (NMDA) receptor-mediated calcium influx, which results in a rise in cytosolic calcium concentrations [9, 27]. Cytosolic accumulation of calcium, which is associated with a large amount of calcium uptake and oxidative stress of mitochondria, results in the formation of MPT [28, 29]. The swelling of the mitochondria is the most important morphological change resulting from the MPT opening [30, 31]. Synaptic mitochondria, which are spread around the synapse, are more easily exposed to extensive calcium flow and accumulation than non-synaptic mitochondria [32, 33]. Yarana et al. reported that synaptic mitochondria isolated from synaptosome showed increase of mitochondrial dysfunction as a result of calcium overload than non-synaptic mitochondria do [34]. Synaptic mitochondria is suggested as associated with a different distribution of the calcium movement.
portal pathways, such as the mitochondrial calcium uni-
porter, mitochondrial ryanodine receptor, and the rapid
mode of calcium uptake between synaptic mitochondria
and non-synaptic mitochondria [34]. Accordingly, we
found more swollen mitochondria with OPN immuno-
reactivity in dendritic regions than in the cell bodies.
Our previous studies also found a correlation between
calcium and OPN in ischemic lesions [7, 8].

In our previous studies, we tried to detect calcium
in the OPN-immunogold-labeled mitochondria with an
electron probe microanalysis. However, we were unable
to detect calcium signals. The calcium signals might have
been below the threshold for their detection because of
alterations in the ionic content and distribution due to
chemical fixation and Epon infiltration [35–37]. Even
though, still we could not demonstrate the direct link
between calcium and OPN change in the 3NP-treated
stromium. In the present study, we detected a positive
relation between calcium deposition and OPN-induction
by observing the increased pattern of change in the 3NP-
lesioned core of striatum. Therefore, in this study, we
employed quantifying immunogold labeling that allowed
comparison study about concentrations of antigen to
determine the relationship between OPN and calcium [38].
As previously mentioned, the swelling of the mitochondria
was caused by the excessive levels of calcium that entered
the mitochondria space. If OPN was involved in the
calcium accumulation, then one would predict an increase
in the area of the OPN-stained mitochondria compared to
the OPN-unstained mitochondria. Several studies have
measured the area of swollen mitochondrial to reveal the
effects of diseases and gene mutations on mitochondria
[39, 40]. The area of the OPN-labeled mitochondria in the
present study was considerably larger than that of the
unlabeled mitochondria. Based on this observation, it is
conceivable that the abnormal calcium accumulation
in the mitochondria is highly correlated with the OPN
expression.

Conclusions

Our data demonstrated a correlation between the OPN
expression and calcium in the mitochondria of damaged
striatal neurons. Although we were not able to directly
show the presence of calcium in OPN-stained mitochondria,
we were able to produce data that will help us to better
understand the mechanisms of OPN expression by
measuring the swelling of mitochondria and staining of
deposited calcium.

Conflict of interests

The authors declare that they have no conflict of interests.

Author contribution

Hong-Lim Kim and Byung-Joon Chang equally contributed
to this manuscript.

HLK, BJC, SMN, SSN, and JHL conceived and
directed experiments, and collected the data. HLK, BJC,
SMN, SSN, and JHL analyzed the data. HLK, BJC,
and JHL wrote the manuscript. SMN and SSN
participated in designing of the study, discussing of results,
and revising the manuscript. All authors approved the final
version of the manuscript.

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