4,4-Diisothiocyanatostilbene Disulfonic Acid Enhanced 15-Deoxy-Δ\textsubscript{12,14}-prostaglandin J\textsubscript{2}-Induced Neuronal Apoptosis

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4,4-Diisothiocyanatostilbene disulfonic acid (DIDS), an antagonist of anion channel including voltage-dependent anion channel (VDAC), acts as both neurotoxicant and neuroprotectant, resulting in the controversy. VDAC contributes to neuronal apoptosis and is a candidate target protein of 15-deoxy-Δ\textsubscript{12,14}-prostaglandin J\textsubscript{2} (15d-PGJ\textsubscript{2}). Caspase-3 is activated during neuronal apoptosis caused by 15d-PGJ\textsubscript{2}. In the present study, we ascertained whether DIDS was neuroprotective or neurotoxic in the primary culture of rat cortical neurons. Neuronal cell viabilities were primarily evaluated by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) reduction assay. Plasma membrane integrity and apoptosis were detected by the staining of propidium iodide (PI) and Hoechst33342, respectively. Alternatively, apoptosis was also measured by caspase-3 assay kit. DIDS did not prevent neurons from undergoing the 15d-PGJ\textsubscript{2}-induced apoptosis. In contrast, DIDS caused neuronal cell death in a concentration-dependent manner by itself, confirming its neurotoxicity. The sublethal application of DIDS did not decrease MTT-reducing activity, increase caspase-3 activity, condense chromatin, allow PI to enter neuron and degenerate neuronal morphology significantly. Interestingly, DIDS enhanced the 15d-PGJ\textsubscript{2}-induced neuronal apoptosis markedly under the sublethal condition. To our knowledge, this is the first report of synergistic effects of DIDS on the neurotoxicity of 15d-PGJ\textsubscript{2}.

Key words primary cortical neuron; 15-deoxy-Δ\textsubscript{12,14}-prostaglandin J\textsubscript{2}; apoptosis; 4,4-diisothiocyanatostilbene disulfonic acid; voltage-dependent anion channel

INTRODUCTION

4,4-Diisothiocyanatostilbenedisulfonic acid (DIDS) is a commonly used nonspecific inhibitor of anion channel including voltage-dependent anion channel (VDAC). DIDS has exhibited notable prospects as a neuroprotective agent against brain ischemia.\textsuperscript{13} In contrast, it has been reported to induce apoptosis in a hippocampal neuronal cell line and not prevent neurons from ischemic degeneration.\textsuperscript{21} Since the effect of DIDS on neuronal cells is still controversial, the aim of our study was to ascertain whether DIDS exhibits neuroprotective or neurotoxic effect in primary neurons.

VDACs are small, abundant in the outer mitochondrial membrane.\textsuperscript{3} VDAC has a crucial role in apoptotic mitochondrial changes by interacting with Bax/Bak and Bel-x\textsubscript{L}.\textsuperscript{29} In neurons, VDAC is also localized in the plasma membrane,\textsuperscript{5} especially in isolated caveolae and caveolae-like domains.\textsuperscript{59} In Alzheimer’s disease (AD) brains, accumulation of VDACs is detected in dystrophic neurites of senile plaques.\textsuperscript{79} Caveolae are microstructures of the plasma membrane, in which lipid composition is particular and density of lipid-anchored proteins is high.

The level of prostaglandin D\textsubscript{2} (PGD\textsubscript{2}) was elevated in the frontal cortices of the AD patients in comparison with those of the controls.\textsuperscript{89} Amyloid β (Aβ) has been believed to be causative in AD, and triggered neuronal apoptosis via L-type voltage-dependent calcium channel in primary cortical neurons.\textsuperscript{9} During apoptosis, Aβ elevated the level of PGD\textsubscript{2} transiently.\textsuperscript{30} PGD\textsubscript{2} is also increased in the model of stroke as well as that of AD.\textsuperscript{11–13} Although PGD\textsubscript{2} caused neuronal cell death via apoptosis, its receptor blockers did not prevent neurons from the PGD\textsubscript{2}-induced apoptosis.\textsuperscript{14} PGD\textsubscript{2} is non-enzymatically metabolized to 15-deoxy-Δ\textsubscript{12,14}-PGJ\textsubscript{2} (15d-PGJ\textsubscript{2}).\textsuperscript{14–16} 15d-PGJ\textsubscript{2} showed neurotoxicity most potently among PGD\textsubscript{2} metabolites and mediated the neurotoxicity of PGD\textsubscript{2}.\textsuperscript{14}

15d-PGJ\textsubscript{2} induces neuronal differentiation\textsuperscript{71} and neuroprotection\textsuperscript{18} through its nuclear receptor, peroxisome proliferator-activated receptor γ (PPAR γ) at low concentrations. In contrast, 15d-PGJ\textsubscript{2} induces neuronal cell death at high concentrations\textsuperscript{10,19} independently of PPAR γ or its membrane receptor chemoattractant receptor-homologous molecule expressed on T-helper 2 (Th2) cells.\textsuperscript{20} VDACs have been reported as one of candidates targeted for 15d-PGJ\textsubscript{2}.\textsuperscript{21} Here, we confirmed DIDS as neurotoxicant rather than neuroprotectant. Furthermore, we reported for the first time that DIDS enhanced the 15d-PGJ\textsubscript{2}-induced neuronal apoptosis markedly.

MATERIALS AND METHODS

Materials We purchased Leibovitz’s L-15 medium, trypsin, deoxyribonucleic acid, horse serum (FBS), penicillin, and streptomycin from Invitrogen (Carlsbad, CA). We obtained 15d-PGJ\textsubscript{2} from Cayman Chemicals (Ann Arbor, MI, U.S.A.). We purchased propidium iodide (PI) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) from DOJINDO (Kumamoto, Japan). We measured the protein concentration by the BCA protein assay reagent (Thermo Fisher Scientific, Rockford, IL, U.S.A.). We purchased propidium iodide (PI) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) from DOJINDO (Kumamoto, Japan). We measured the protein concentration by the BCA protein assay reagent (Thermo Fisher Scientific, Rockford, IL, U.S.A.). We purchased Hoechst 33342 from Nacalai Tesque (Kyoto, Japan). We purchased DIDS and Caspase-3 Assay Kit (Fluorimetric) from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals were of reagent grade as previously reported.\textsuperscript{22}

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Animals In macrolon cages maintained at 25°C on a 12 h light/dark cycle, pregnant female Wistar rats were housed individually with free access to food and water. Procedures using animals were approved by the committee of HDU concerning the Care and Use of Laboratory Animals, which are accordant with NIH.23)

Tissue Cultures We cultured neurons primarily from cerebral cortices of day-19 Slc: Wistar rat embryos as previously reported.23) Trypsin (4 mg/mL) and deoxyribonuclease I dissociated cortical neurons. We seeded at a density of 2.5 × 10⁵ cells/cm² on 24 × well plates coated with poly-L-lysine. Neurons were cultured in 5% FBS and 5% HS-supplemented L-15 medium at 37°C under 5% CO₂. Primary cultures of cortical neurons on day 2 or 3 in vitro (DIV 2 or 3) were used as immature neurons, whereas those on day 7 in vitro (DIV 7) as mature neurons. The compositions of neurons and astrocytes in cortical cultures were determined by use of antibodies for microtubule associated proteins 2 (MAP2) and glial fibrillary acidic protein (GFAP), which are specific for neurons and astrocytes, respectively.22) On DIV 2 or 3, approximately 95% of the cells were stained by anti-MAP2 antibody, whereas there were few anti-GFAP-positive cells. Thus, neurons were rich in the immature neurons (DIV 2 or 3). On the other hand, astrocytes were proliferated and co-cultured with neurons in the mature neurons (DIV 7).

Cell Viability Neuronal cell viabilities were primarily evaluated by the MTT reduction assay based on mitochondrial succinate dehydrogenase activity as previously reported.24) Following various treatments, mediums were exchanged by phosphate buffered saline containing 10 µM MTT and incubated at 37°C for 2–3 h. The resulting intracellular purple formazan was solubilized with dimethyl sulfoxide (DMSO) and quantified by a CytoFluor® Plate Reader (absorbed wave length = 570 nm). According to morphologic criteria, cell viabilities were secondary evaluated. Neuronal cell with intact neurites and a smooth, round soma was defined as viable one. On the other hand, neuronal cell with degenerated neurites and an irregular soma was defined as nonviable one.22) By blinded investigations, the morphological assay was confirmed to be correlated with the MTT reduction assay.22)

Fluorimicroscopic Analysis Plasma membrane integrity and chromatin condensation were detected by the staining of Hoechst33342 and PI.22) Following the treatment with various drugs, neurons were incubated with 10 µM Hoechst33342 for 10 min at 37°C in the dark and washed with PBS. Stained nuclei were categorized as follows: (i) nuclei with homogeneously stained chromatin; (ii) nuclei with condensed chromatin, crescent-shaped areas of condensed chromatin often located near the periphery of the nucleus; and (iii) fragmented nuclei, more than two condensed micronuclei within the area of a neuron. Hoechst33342-stained nuclei and condensed chromatin were counted blindly. Nuclei stained with Hoechst33342 were counted from nine fields with data expressed as number/field or percentage of control. Condensed chromatin was counted from nine fields with data expressed as number/field or percentage of Hoechst33342-positive nuclei. Following treatments with various drugs, neurons were incubated with 0.1 mg/mL PI at 37°C for 5 min. The investigators were blinded for the treatments. PI-stained nuclei were counted from nine fields with data expressed as number/field or percentage of total cells. PI-stained cells normalized to the untreated group.22)

Fluorimetric Caspase-3 Assay We measured caspases-3 activity fluorimetrically by a Caspase-3 Assay Kit (Sigma-Aldrich) as described previously.22) Following incubation with antibodies for 24 h on day 2 and aspiration of supernatants, neurons were harvested with lysis buffer (N-(2-hydroxyethyl)-piperazine-N’-2-ethanesulfonic acid (HEPES) (50 mM, pH 7.4), CHAPS (5 mM) and dithiothreitol (DTT) (5 mM)). The reaction was initiated by the addition of caspase-3 specific substrate, Acetyl-Asp-Glu-Val-Asp-7-amido-4-methyl coumarin (Ac-DEVD-AMC). Sequentially, the production of AMC was excited at 360 nm and detected at 460 nm with CytoFluor® Plate Reader. The initial velocities (nmol AMC/min/mL) corrected with the quantity of protein in each well detected by BCA protein assays (Thermo Fisher Scientific, Waltham, MA, U.S.A.) was defined as the enzyme activity.

Statistical Analysis Data are given as means ± standard error of the mean (S.E.M.) (n = number of observations). At least, we performed three independent experiments on different days and confirmed their reproducibility. Data were analyzed statistically by use of Student’s non-paired t-test for comparison with the control group, and data on various inhibitors and blocker groups were analyzed statistically by use of two-way ANOVA followed by Dunnett’s test for comparison with 15d-PGJ₂ alone.
RESULTS

DIDS Exhibited Little Protective Effect against the Neurotoxicity of 15d-PGJ2

DIDS has been reported to protect against neuronal injury in organotypic hippocampal slice cultures.1 Here, we ascertained whether DIDS protected against the neurotoxicity of 15d-PGJ2 or not in the primary culture of cortical neurons. Neurons were treated with DIDS at the concentrations (0–100 µM) in the absence or presence of 5 µM 15d-PGJ2 for 48 h. DIDS did not affect neuronal cell survival at the concentration lower than 10 µM in the immature neurons. Contrary to our expectations, it alone induced neuronal cell death at 100 µM (Fig. 1A). In the mature neurons, DIDS did not also alter neuronal cell viability at the concentration lower than 20 µM, whereas it induced neuronal cell death at 100 µM (Fig. 1B). We confirmed that 15d-PGJ2 decreased the MTT-reducing activity at 5 µM (Fig. 1A) in the immature cortical neurons and at 7 µM (Fig. 1B) in the mature one. However, DIDS did not prevent neurons from undergoing the 15d-PGJ2-induced neuronal cell death independently of maturation. Rather than neuroprotective effect, it exhibited the degenerative effect on the neurotoxicity of 15d-PGJ2 at 100 µM. Thus, we have not succeeded in detecting the neuroprotective effect of DIDS on the neurotoxicity of 15d-PGJ2 during neuronal maturation.

DIDS Exhibited Neurotoxicity in the Primary Culture of Cortical Neurons

DIDS has been reported to cause neuronal cell death via apoptosis in HT22 mouse hippocampal neurons.2 In the primary culture of cortical neurons, we also detected the neurotoxicity of DIDS rather than its neuroprotective effect. Therefore, we examined the neurotoxic effect of DIDS on cortical neurons by itself. In the immature (Fig. 2A) and mature neurons (Fig. 2B), we confirmed that DIDS induced neuronal cell death in a concentration dependent manner. DIDS required the incubation time for 48 h to show the neurotoxicity at 200 µM. Thus, DIDS exhibited the neurotoxicity in both immature and mature neurons.

DIDS Enhanced the Neurotoxicity of 15d-PGJ2

At 400 µM, DIDS alone reduced the MTT-reducing activity to about 90 and 70% of control in immature (Fig. 3A) and mature neurons (Fig. 3B), respectively. Since 200 µM DIDS required a latent time for 48 h, DIDS alone showed little neurotoxicity for 28 or 21 h in immature (Fig. 3A) and mature neurons (Fig. 3B), respectively. Under the sublethal condition, we detected the synergistic effect of DIDS on the neurotoxicity of 15d-PGJ2. At the concentration higher than 200 µM, DIDS enhanced the neurotoxicity of 15d-PGJ2 synergistically (Fig. 3). Similar results were obtained in mature neurons (Fig. 3B) as well as in immature neurons (Fig. 3A). Especially, the combination of 200 µM, DIDS with 5 µM 15d-PGJ2 decreased the neuronal cell viability significantly, when each pharmaceutical agent did not decrease the neuronal cell viability.
significantly by itself. Thus, DIDS exacerbated the 15d-PGJ$_2$-induced neuronal cell death through maturational process.

In the control culture (Fig. 4A), neuronal cell bodies were smooth and round, whereas neurites were extended. Neurites were shortened and fragmented by 15d-PGJ$_2$, whereas neuronal cell bodies were shrank and disrupted. Moreover, DIDS degenerated neuronal morphology distinctively from 15d-PGJ$_2$. DIDS aggregated and reduced neurons, and appeared to decrease and stretch neurites. Combination of DIDS with 15d-PGJ$_2$ induced morphological degeneration severely. In accordance with the result obtained from Fig. 3, DIDS synergistically worsened the 15d-PGJ$_2$-degenerated neurons (Fig. 4B).

**DIDS Enhanced the 15d-PGJ$_2$-Activated Caspase-3**

Caspase-3 activation is known as a biochemical marker of apoptosis. Since DIDS-induced apoptosis is accompanied with the caspase-3 activation in hippocampal neurons, we measured caspase-3 activity to confirm whether DIDS induced neuronal apoptosis in cortical neurons (Fig. 5). Contrary to our expectation, DIDS did not affect the caspase-3 activity in both immature (Fig. 5A) and mature neurons (Fig. 5B), indicating that DIDS caused neuronal cell death in a different fashion from apoptosis.

Caspase-3 has been reported to be activated during 15d-PGJ$_2$-induced neuronal apoptosis. Next, we ascertained whether DIDS affected the 15d-PGJ$_2$-activated caspase-3 or not (Fig. 5). Under the sublethal condition, 15d-PGJ$_2$ did not activate the caspase-3 at 5 µM for 24 h in immature neurons (Fig. 5A). Under the lethal condition at concentrations higher than 5 µM for incubation time longer than 24 h, we confirmed that 15d-PGJ$_2$ significantly stimulated caspase-3 at 48 h in immature neurons (data not shown). Interestingly, DIDS enhanced the 15d-PGJ$_2$-stimulated caspase-3 activity synergistically (Fig. 5A). As shown in Fig. 5B, similar results were obtained in mature neurons.

**DIDS Enhanced the 15d-PGJ$_2$-Condensed Chromatin**

Chromatin condensation is another biochemical marker of apoptosis. To ascertain whether DIDS induced neuronal cell death via apoptosis or not, we stained nuclei by Hoechst33342 (Fig. 6). Since Hoechst33342 is permeable to not only dead cells but also living ones, we can use it to count total nuclei. Stained nuclei were categorized into three types. Type 1: chromatin was stained homogeneously in the nuclei. Type 2: chromatin was condensed in the nuclei and crescent-shaped areas of condensed chromatin were often located near the periphery of the nucleus. Type 3: nuclei are fragmented and more than two condensed micronuclei within the area of a neuron. Hoechst33342-stained nuclei and condensed chromatin were counted blindly. Neither DIDS nor 15d-PGJ$_2$ affected the Hoechst33342-staining of living and dead cells, indicating
that they did not affect the number of total nuclei (Figs. 6A, B). Although DIDS did not condense chromatin, it enhanced the 15d-PGJ2-condensed chromatin (Fig. 6C).

DIDS Exacerbated the 15d-PGJ2-Disrupted Plasma Membrane DIDS caused neuronal cell death independently of apoptosis. Loss of plasma membrane integrity allows uptake of PI. In contrast to Hoechst33342, it is excluded by intact plasma membranes. Since PI enters necrotic cells but is excluded from apoptotic cells, we used PI to detect neuronal cell death besides apoptosis. As another assessment of cell viability, nuclei of non-viable neurons were stained with PI (Fig. 7A). PI was almost not incorporated into the control neuron. We confirmed that 15d-PGJ2 increased PI-positive nuclei.22 Consistent with the result obtained from MTT assay, PI was also significantly incorporated into DIDS-treated neurons (Fig. 7B). Furthermore, combination of DIDS with 15d-PGJ2 increased the PI-positive neurons synergistically. Similar results were obtained in mature neurons as well as immature ones (data not shown).

Fig. 6. DIDS Increased the 15d-PGJ2-Condensed Chromatin (A) 200 µM DIDS was applied to neurons (DIV3) in the presence or absence of 5 µM 15d-PGJ2 for 24 h. (A) Hoechst33342-stained neurons were photographed. Bar means 50 µm. (B) The number of Hoechst33342-positive nuclei was counted in each filed photographed (magnification = 100). (C) The Hoechst33342-positive nuclei was presented % in each filed photographed (magnification = 100). (D) The number of condensed chromatin was counted in each filed photographed (magnification = 100). (E) The condensed chromatin was presented % of Hoechst33342-positive nuclei in each filed photographed (magnification = 100). Results were expressed as means ± S.E.M. (n = nine). Untreated cells were used as the control group. *p < 0.05, when compared with 15d-PGJ2 alone.
DISCUSSION

Pharmacological effects of DIDS on the neuronal cell survival remain to be controversial.\(^1,2\) On the mitochondrial outer-membrane, VDACs form the mitochondrial permeability transition pore. Cytochrome c and procaspases are released by the pore opening, resulting in apoptosis.\(^4\) Benitez-Rangel et al. reported that DIDS directly inhibits caspase activity in HeLa cell lysates.\(^28\) However, no inhibitory effect of DIDS on caspase-3 activity was detected in primary neurons. HeLa cells are proliferative, whereas neurons are non-proliferative and differentiated. Therefore, the effect of DIDS on caspase activity in non-proliferative neurons is not always same as that in proliferative cells.

The VDAC inhibitor, DIDS, has been reported to protect cortical neurons against the oxygen-glucose deprivation.\(^29\) However, we detected the neurotoxicity of DIDS, but not its neuroprotective effect, in the primary cortical neurons. In the immature culture, we focused direct effects of DIDS on neurons. In the brain, there are glial cells such as astrocytes, suggesting that DIDS might exhibit neuroprotective effects indirectly through non-neuronal cells. However, little neuroprotective effect of DIDS was detected in the mature culture including astrocytes as well as the immature one. Although DIDS acts as a neuroprotectant in the in vivo model,\(^1\) it does as a neurotoxicant in the in vitro model of ischemia.\(^3\) A critical reduction of blood flow to a region of the brain such as an acute obstruction of an artery causes ischemia, which elevates levels of free radicals, extracellular glutamate and intracellular calcium, resulting in inflammation, neurologic malfunctions and neuronal cell death.\(^30\) DIDS significantly downgraded the level of reactive oxygen species in chronic cerebral ischemia-hypoxia rat models.\(^30\) Even if DIDS itself upregulates neuronal cell death locally, it is likely to inhibit the expansion of death signals between cells in the ischemic penumbra broadly, leading to the putative benefit.

VDAC has been reported as one of candidate proteins targeted for 15d-PGJ\(_2\) by the proteomic approach.\(^21\) 15d-PGJ\(_2\) condensed chromatin, activated caspase-3 and induced neuronal apoptosis. In addition, 15d-PGJ\(_2\) incorporated PI into neurons, indicating disruption of plasma membrane. PI is excluded from apoptotic cells and enters necrotic cells. According to the intensity of the insult and the proximity to the initial injury, neuronal cell death can occur as necrosis, apoptosis, and so on. Although necrosis has been defined by the absence of morphological features of apoptosis, the two deaths are both parts of a continuum.\(^20\) 15d-PGJ\(_2\) appears to exhibit not only apoptotic but also necrotic features. Apoptosis might be caused by the treatment of neurons with 15d-PGJ\(_2\) at moderate concentration for a short time, whereas necrosis might be by the exposure of neurons to a high concentration for a long period of time.

In the present study, we have succeeded in detecting the
synergistic effect of DIDS on the 15d-PGJ2-induced neuronal apoptosis. DIDS enhanced the neurotoxicity of 15d-PGJ2 under the sublethal condition that it did neither decrease MTT-reducing activity, increase caspase-3 activity, condense chromatin, allow PI to enter neuron nor degenerate neuronal morphology significantly. Since DIDS produced more than a simple additive effect, it amplified the neurotoxicity of 15d-PGJ2 synergistically. In spite of solo non-apoptotic feature, DIDS promoted the activity of caspase-3, pyknosis and the disruption of plasma membrane in the 15d-PGJ2-treated neurons markedly.

Recently, we have reported that 15d-PGJ2 inhibited the phosphoinositide 3-kinase (PI3K)–Akt pathway, resulting in the neuronal apoptosis. 32,33) 15d-PGJ2 is not only one of neurodegenerative mediators 30) but also one of endogenous anticancer agents. 34) The cytotoxicity of 15d-PGJ2 was potentiated by the combination with other pharmacological agents in renal cell carcinoma. 34–36) Although the PI3K–Akt pathway acted to mediate the cytotoxicity of 15d-PGJ2, we have not yet succeeded in detecting the plausible involvement of 15d-PGJ2 in its synergistic effects with other pharmacological agents. 30) DIDS has been reported to potentiate anti-cancer drug activity via sustained and selective reduction of intracellular pH in tumor. 37) Although neurons are different from proliferative carcinoma cells, we cannot rule out the possibility that DIDS enhance the neurotoxicity of 15d-PGJ2 by the reduction of intracellular pH. Alternatively, DIDS induces an apoptotic phenotype in a hippocampal neuronal cell line HT22, 32) suggesting that DIDS might promote the proapoptotic state of 15d-PGJ2-treated neurons under the sublethal condition. Thus, DIDS enhanced the 15d-PGJ2-induced neuronal apoptosis as the neurotoxicant in the cortical primary neurons.

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Conflict of Interest The authors declare no conflict of interest.

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