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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 induced neurotoxicity via suppressing phosphoinositide 3-kinase

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ABSTRACT

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) induces neuronal cell death via apoptosis independently of its receptors. 15d-PGJ_2 inhibits growth factor-induced cell proliferation of primary astrocytes via down-regulating phosphoinositide 3-kinase (PI3K)-Akt pathway. Although 15d-PGJ_2-reduced cell viability is accompanied with attenuation of the PI3K signaling in neuroblastoma, it has not been sufficiently clarified how 15d-PGJ_2 induces cell death in primary neurons. Here, we found that 15d-PGJ_2 exhibited neurotoxicity via inhibiting the PI3K signaling in the primary culture of rat cortical neurons. A PI3K inhibitor induced neuronal cell death regardless serum throughout maturation, confirming that PI3K is required for neuronal cell survival. The inhibitor disrupted neuronal cell bodies, shortened neurites thinly, damaged plasma membranes and activated caspase-3 similarly to 15d-PGJ_2. Little additive or synergistic neurotoxicity was detected between 15d-PGJ_2 and the PI3K inhibitor. A PI3K activator prevented neurons from undergoing the 15d-PGJ_2-induced cell death in *vitro*. In *vivo*, the PI3K signaling is required for contextual memory retrieval, which was impaired by bilateral injection of 15d-PGJ_2 into hippocampus. The activator suppressed the 15d-PGJ_2-impaired memory retrieval significantly. In neurons as well as primary astrocytes and neuroblastomas, 15d-PGJ_2 exhibited cytotoxicity via suppressing the PI3K-Akt pathway in *vivo* and *in vitro*.

1. Introduction

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is a cyclopentenone-type prostaglandin (PG) with a wide spectrum of physiological activities. A precursor of 15d-PGJ_2 is a conventional PGD_2 (Fitzpatrick and Wynalda, 1983; Shibata et al., 2002; Yagami, 2006), which is major in mammalian brains (Kikawa et al., 1984). 15d-PGJ_2 possesses opposite functions as a neuroprotectant at low concentrations and a neurotoxicant at high concentrations in the brain (Hsia et al., 2014). Chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells (CRTH2) is a membrane receptor for 15d-PGJ_2 (Hata et al., 2003), whereas peroxysome-proliferator activated receptor γ (PPARγ) is a nuclear receptor for 15d-PGJ_2 (Forman et al., 1995; Kliewer et al., 1995). CRTH2 contributes to nerve growth factor-induced neurite outgrowth in PC12 cells (Hatanaka et al., 2010). PPARγ is involved in the neuroprotection in cerebellar granule cells (Heneka et al., 2000). 15d-PGJ_2 decreases middle cerebral artery occlusion-induced infarct size and improves neurological scores via PPARγ (Pereira et al., 2006). Although 15d-PGJ_2 induces neuronal cell death via apoptosis (Rohn et al., 2001; Yagami et al., 2003), neither CRTH2 nor PPARγ contributes to the neurotoxicity of 15d-PGJ_2 (Yagami et al., 2015). Other targets have been suggested to be involved in the 15d-PGJ_2-neuronal apoptosis (Arnaud et al., 2009; Yamamoto et al., 2011). In the central nervous system, 15d-PGJ_2 inhibits proliferation of primary astrocytes (Giri et al., 2004) and neuroblastoma x DRG neuron hybrid cell line N18D3 (Hsia et al., 2014) via down-regulating phosphoinositide 3-kinase (PI3K)-Akt pathway. In the present study, 15d-PGJ_2 induced neuronal cell death via suppressing the PI3K/Akt pathway in the primary neuron.

Abbreviations: 15d-PGJ_2, 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2; CRTH2, Chemoattractant receptor-homologous molecule expressed on T-helper type 2 cells; CS, conditioned stimulus; DIV, days *in vitro*; ERK, extracellular signal-regulated kinase; JNK, Jun-N-terminal kinase; MAPK, mitogen activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PIP_3, phosphatidylinositol-3,4,5-trisphosphate; PPARγ, peroxysome-proliferator activated receptor γ; ROS, reactive oxygen species; US, unconditioned stimulus.

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PI3K is a key regulator of neuronal function. PI3K signaling transduces signals from cell surface receptors to Akt pathway and is essential for enduring forms of synaptic plasticity underlying learning and memory (Sanna et al., 2002). AKT is activated by a cascade of events that is initiated by the recruitment of PI3K to the cell membrane, as occurs following activation of transmembrane receptor tyrosine kinases. PI3K phosphorylates phosphatidylinositol-4,5-disphosphate (PIP_2) to form phosphatidylinositol-3,4,5-trisphosphate (PIP_3) on the inner cell membrane, which recruits proteins with pleckstrin homology domains including AKT and PDK1 to the cell membrane. The PI3K pathway is the dominant survival mechanism for serum-dependent survival (Hetman et al., 1999). We confirmed that PI3K is required for neuron to survive in the presence of serum. Furthermore, we found that the enzyme activity was also involved in the neuronal viability in the absence of serum.

Memory can be experimentally divided into several stages, including acquisition, consolidation, retrieval, reconsolidation and extinction (Abel and Lattal, 2001). Memory retrieval is an essential component of memory processing; without retrieval, memories are not expressed. Although there is controversy concerning the molecular events required for memory retrieval (Abel and Lattal, 2001; Nader, 2003; Suzuki et al., 2004), several regulatory pathways including extracellular signal-regulated kinase (ERK)/mitogen activated protein kinase (MAPK) and cAMP signaling are strongly implicated (Szapiro et al., 2003). In contrast to memory acquisition and consolidation, memory retrieval does not, however, depend on activation of N-methyl-D-aspartic acid (NMDA) receptors or Ca^{2+}/calmodulin-dependent protein kinase II (Szapiro et al., 2003). Another activity-stimulated signaling pathway implicated in memory formation and synaptic plasticity is PI3K. The PI3K signaling is required for long-term potentiation in different areas of brain, including the Schaffer collateral/commissural fiber CA1 synapses (Sanna et al., 2002), dentate gyrus (Kelly and Lynch, 2000) and amygdala (Lin et al., 2001). PI3K is activated in the hippocampus during contextual memory retrieval, and its activity is required for memory retrieval and contextual fear extinction. The blockade of memory retrieval is readily reversible and does not retroactively affect initially formed memories. PI3K activation in the hippocampus is critical for memory retrieval and is required for activation of ERK/MAPK during retrieval (Chen et al., 2005). Here, we found that 15d-PGJ_2 impaired memory retrieval via suppressing PI3K signaling. Thus, we provided the first evidence that 15d-PGJ_2 exerted neurotoxicity through inhibiting the PI3K-Akt pathway in *vitro* and *in vivo*.

2. Materials and methods

2.1. Materials

15d-PGJ_2 was obtained from Cayman Chemicals (Ann Arbor, MI; Cabru, Milan, Italy). Leibovitz's L-15 medium, trypsin, fetal bovine serum, horse serum, penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). SP600125 was purchased from Wako (Osaka, Japan). SB202190 was purchased from Merck (Darmstadt, Germany). U0126 was purchased from Promega (Fitchburg, WI). The PI3K activator was purchased from Santa Cruz Biotech (Delaware, CA, USA) and is a peptide with the sequence KKHTDDGYMPMSPGVA and a molecular weight of 1732.8 Da. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (MTT) and propidium iodide (PI) were purchased from Dojindo (Kumamoto, Japan). LY294002 was purchased from Cell Signaling Technology (Boston, MA). The protein concentration was measured using the BCA protein assay reagent obtained from Thermo Fisher Scientific. (Rockford, IL). All other chemicals were of reagent grade.

2.2. Animals

All procedures were conducted in accordance with NIH guidelines concerning the Care and Use of Laboratory Animals and with the approval of the Animal Care Committee of the Himeji Dokkyo University. Wistar rats were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Pregnant female rats were used for primary culture of cortical neurons. Young, mature male rats were used for behavioral test (contextual fear conditioning). Rats were housed one per cage, and females were housed individually in square plastic cages (width 47 cm, length 31 cm, height 20 cm) with wire lids in a temperature-controlled room (25 ± 1 °C) in a 12 h light-dark cycle (light period 0800–2000 h). The cage contained approximately 50 g of bedding. Standard pellet laboratory diet (Oriental Yeast Co., Ltd.. Tokyo, Japan) and water were provided ad libitum, and cages were cleaned on a weekly basis.

2.3. Tissue cultures

All procedures were conducted in accordance with NIH guidelines concerning the Care and Use of Laboratory Animals and with the approval of the Animal Care Committee of the Himeji Dokkyo University. Rat cortical neurons were cultured as previously reported (Yagami et al., 2013a). Cerebral cortices of day-19 Slc:Wistar rat embryos were dissociated in isotonic buffer with 4 mg/ml trypsin. Cells were plated at a density of 2.5×10^5 cells/cm^2 on poly-L-lysine-coated dishes in conditioning medium, Leibovitz's L-15 medium supplemented with 5% inactivated FBS and 5% inactivated horse serum at 37 °C in 5% CO_2 and 9% O_2. Cultures prepared by this method, consisted of approximately 95% neurons.

2.4. Cell viability

15d-PGJ_2 was dissolved in culture medium after evaporation of vehicle. Two different methods were employed for assessment of cell viability as previously reported (Yagami et al., 2013a). First, the MTT reduction assay reflecting mitochondrial succinate dehydrogenase activity was employed. Second, cell death was also measured by manually counting the percentage of neurons that stained with PI (0.1 μg/ml). Although PI is membrane impermeant and generally excluded from viable cells, damaged membrane of dead cell is permeable for PI. Nuclei stained with PI were counted from 12 fields with data expressed as percentage PI-stained cells normalized to the vehicle-treated group.

2.5. Fluorimetric assay of caspase-3 activity

Caspases-3 activity was assessed using a Caspase-3 Assay Kit, Fluorimetric (SIGMA, St. Louis, MO) as described previously (Yamamoto et al., 2011). After exposure to LY294002 for 24 h on day 2, the supernatants were aspirated and cells were harvested with lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS and 5 mM DTT). The reaction buffer, including Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC), caspase-3 specific substrates, was added to wells, and the production of AMC was sequentially detected in a CytoFluor® Plate Reader at an excitation wavelength of 360 nm/emission 460 nm. Enzyme activities were determined as initial velocities expressed as nmol AMC/min/mL. They were then corrected with the quantity of protein in each well detected by BCA protein assays (Thermo Fisher Scientific, Waltham, MA).

2.6. Surgery

Male rats were subjected to deep anesthesia with sodium pentobarbital (30–50 mg/kg, i. p.), mounted in a small-animal