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15-DEOXY-$\Delta^{12,14}$-PROSTAGLANDIN J_2 MODIFIES PROFILES OF NUCLEAR TDP-43 PROTEIN THROUGH ITS DIRECT BINDING: IMPLICATION FOR THE PATHOGENESIS OF TDP-43 PROTEINOPATHY

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Abstract TDP-43 proteinopathy (amyotrophic lateral sclerosis and frontotemporal lobar degeneration with ubiquitin-positive inclusions) is a newly categorized group of neurodegenerative disorders characterized by abnormal accumulation and mislocalization of nuclear TDP-43 protein in the neuronal cytoplasm. 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) is non-enzymatically produced from PGD_2, and plays roles in inflammation and oxidative stress responses. Indeed, 15d-PGJ_2 is up-regulated in the spinal motor neurons in ALS. In this study, biochemical and fluorescent staining analyses showed that 15d-PGJ_2 modifies expression, solubility, and subcellular localization of TDP-43. This alteration was at least partly related to a cyclopentenone ring structure containing an electrophilic carbon of 15d-PGJ_2, because 15d-PGJ_2 analogue, which lacks an cyclopentenone ring structure, had almost no effect on TDP-43 protein. Finally in vitro binding experiment indicated that 15d-PGJ_2 is covalently bound to TDP-43 protein. These findings suggest that a sustained high level of 15d-PGJ_2 is involved in the pathogenesis of neurodegenerative disorders related to abnormal TDP-43 protein.

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Key words: Amyotrophic lateral sclerosis; Frontotemporal lobar degeneration; 15d-PGJ_2; Prostaglandin; TDP-43 proteinopathy.

Introduction

The 43-kDa transactive response (TAR) -DNA-binding protein (TDP-43) was recently identified as a major component of neuronal cytoplasmic inclusions in frontotemporal lobar degeneration with ubiquitin-positive inclusions (FTLD-U) and amyotrophic lateral sclerosis (ALS)[1,2]. These neurodegenerative disorders comprise a new disease concept, namely that of "TDP-43 proteinopathy". Moreover, TDP-43 is deposited in the brain of patients with Guam parkinsonism-dementia complex[3,4] as well as in a significant proportion of cases with hippocampal sclerosis[5,6], Alzheimer disease and dementia with Lewy bodies[7,8]. In addition, biochemical analyses of TDP-43 in affected brain regions in FTLD-U and ALS revealed that TDP-43 undergoes a variety of posttranslational modifications, including proteolytic cleavage (generating low-molecular weight 25 and 35 kDa species), hyperphosphorylation, and ubiquitination[1].

TDP-43 is a nuclear protein that involves its binding to single stranded DNA, RNA and nuclear proteins[9,10]. The protein is ubiquitously expressed in multiple organs including the central nervous system. TDP-43 immunohistochemistry shows diffuse nuclear staining in unaffected neurons.

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Interestingly, several investigators described a distinct cytopathological profile consisting of a cell nucleus devoid of endogenous TDP-43 staining coupled with abnormal cytoplasmic immunoreactivity in ALS and FTLD-U. The loss of nuclear TDP-43 immunoreactivity may result from reduced protein expression, relocation from the nucleus to the cytoplasm, and sequestration within ubiquitinated inclusions. However, the mechanism of loss of nuclear TDP-43 in TDP-43 proteinopathy remains unclear.

Prostaglandin D_2 (PGD_2) is the largest amount of prostanoid produced in the central nervous system of mammals[11] and its synthase is secreted into the cerebrospinal fluid as β-trace, which is the second most abundant protein after albumin[12-14]. PGD_2 is further metabolized non-enzymatically to produce the J series of PGs including PGJ_2, Δ^{12}-PGJ_2 and 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15d-PGJ_2)[15,16]. These J series of PGs are actively transported into cells and accumulated in the nuclei[11], where they modulate the expression of protein related to cell cycle such as cyclin D1 or B1, resulting in growth inhibition of the cells[17-19]. Additionally, they are characterized by the presence of a cyclopentenone ring with α,β-unsaturated carbonyl group, which are able to form Michael adducts with nucleophiles such as free sulfhydryls in cysteine residues of glutathione and various cellular proteins. Indeed, 15d-PGJ_2 covalently binds to IκB kinase, and Keap1[20,21], resulting in altered protein complex and subsequently activation of NF-κB and Nrf2-related genes that play a role in inflammation and oxidative stress responses.

In addition to the 15d-PGJ_2 has a wide variety of biological actions including both neurotoxic and neuroprotective properties[22-24], 15d-PGJ_2 immunoreactivity is significantly enhanced in spinal cord affected with ALS[22].

Based on these findings, we hypothesized 15d-PGJ_2 has any impact on nuclear TDP-43 protein. To test this possibility, we examined the effect of 15d-PGJ_2 on TDP-43 regarding expression level, distribution and solubility in cultured cells. We here revealed that 15d-PGJ_2 modified biochemical and cytopathological profiles of TDP-43, which resembling findings observed in TDP-43 proteinopathy.

Materials and Methods

Reagents and antibodies

PGJ_2, 15d-PGJ_2, PGD_2, PGE_2, PGA_2 and 9,10-dihydro-15d-PGJ_2 were obtained from Cayman Chemicals (Ann Arbor, MI). Rabbit polyclonal and mouse monoclonal antibodies against TDP-43 (ProteinTec Group, Inc., Chicago, IL) (Abnova Corporation, Taipei, Taiwan) were used.

Immunohistochemistry

We examined the spinal cord from five patients with ALS, aged 53-76 years (mean 65.2 years) and five neurologically normal individuals, aged 51-81 years (mean 66.4 years). Serial 4-μm-thick paraffin-embedded sections were cut from the spinal cord (cervical, thoracic and lumbar segments). The sections were pretreated with heat retrieval using a microwave oven for 15 min in 10 mM citrate buffer (pH 6.0). The sections were then subjected to immunohistochemical processing using the avidin-biotin-peroxidase complex method with diaminobenzidine as the chromogen. Mouse monoclonal or rabbit polyclonal antibodies against 15d-PGJ_2 (diluted 1:100)[16] was used as a primary antibody. The sections were counterstained with haematoxylin.

Cell cultures and treatment

SK-N-SH human neuroblastoma, human embryonic kidney (HEK293), HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. SK-N-SH or HeLa cells were treated with 20 μM of various PGs in dimethyl sulfoxide (DMSO). At the indicated

times, the cultures were washed twice with phosphate-buffered saline (pH 7.4, PBS), and the cells were harvested and used as samples for further studies.

Plasmids construct and transfection

Human TDP-43 was amplified from a human fetal brain cDNA library using PCR. Following DNA sequencing, it was inserted into the pcDNA3 vector (Invitrogen) tagged with RH or Flag at the C terminus. TDP-43 without all cysteine residues was made using mutagenesis kit (Takara, Ostu, Japan), following by confirmation by sequence. Plasmids were transfected into mammalian cells using FuGENE 6 (Roche Molecular Biochemicals, Mannheim, Germany).

Western blot analysis

After SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blot analysis was performed as previously described[25]. Transfer and detection were carried out according to the protocol provided with the ECL detection system (Amersham Pharmacia Biotech). Horseradish peroxidase (HRP)- conjugated anti-mouse IgG or anti-rabbit IgG antibody (Santa Cruz Biotechnology) was used as a secondary antibody.

Immunofluorescence and confocal microscopy

SK-N-SH, HEK293 or HeLa cells grown on coverglass were treated with vehicle and various PGs. After 24 h, the cells were fixed with 4% paraformaldehyde solution for 20 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. The cells were incubated with polyclonal antibodies against TDP-43 (1:1000). After washing, the cells were labeled with anti-rabbit IgG tagged with Alexa fluora 488 (1:1000) (Invitrogen, Carlsbad, CA). The cells were then analyzed under a confocal microscope (TCS-SP5, Leica, Wetzlar, Germany).

Analysis of the interaction between 15d-PGJ_2 and TDP-43 in vitro and in vivo

The carboxyl group of 15d-PGJ_2 was modified by amidation with EZ-link 5- (biotinamido) pentylamine (Pierce, Rockford, IL) by a modification[26]. Full-length recombinant TDP-43-Flag was purified using Flag beads (Sigma, St. Louis, MO), and then incubated in 20 mM Tris-HCl (pH 7.5), 45 mM NaCl, 5 mM $MgCl_2$, 0.14mM β-mercaptoethanol, 0.01% Nonidet P-40, 1% glycerol in the presence of biotin or biotinylated 15d-PGJ_2 at a final concentration of 5 μM for 2 h at room temperature. The mixtures were analyzed by SDS-PAGE and detected incorporated biotin with HRP-conjugated streptavidin (Pierce).

Results and Discussion

Sustained high level of 15d-PGJ_2 is observed in motor neuron disease.

TDP-43 is a nuclear protein that involves its binding to single stranded DNA, RNA and nuclear proteins[9,10]. In all the examined cases, diffuse granular nuclear staining for TDP-43 was evident in normal neurons and glial cells of the spinal cord (Fig. 1A). On the other hand, TDP-43-positive, filamentous or coarse granular inclusions were found in the cytoplasm of anterior horn cells of ALS patients. Nuclei of neurons that possessed cytoplasmic TDP-43-positive inclusions were found to be negative for TDP-43. We investigated immunohistochemical localization of 15d-PGJ_2 in the control and ALS spinal cord, using anti-15d-PGJ_2 monoclonal antibodies, which specifically recognize 15d-PGJ_2 [16]. Consistent with results of previous study[22], the spinal cord in the control subjects exhibited no significant immunoreactivity for 15d-PGJ_2 (Fig. 1B). Whereas, monoclonal anti-15d-PGJ_2 antibody strongly immunolabeled the cytoplasm and processes in the anterior horn motor neurons in all the cases of ALS (Fig. 1C). Similar results were observed using polyclonal antibody. This finding indicated that the level of 15d-PGJ_2 is up-regulated in motor neuron diseases.

Based on these immunohistochemical results, we hypothesized that 15d-PGJ_2 has any impact on TDP-43 expression and distribution in certain pathological conditions. To test this hypothesis, immunoblot and immunocytofluorescent study were performed using cultured cells.

The effect of 15d-PGJ_2 on biochemical and pathological profiles of TDP-43.

After 15d-PGJ_2 was added to stimulate SK-N-SH neuroblastoma cells for indicated time, cells were harvested and analyzed with immunoblot analysis. As shown in Fig. 2A, protein level of TDP-43 was decreased in a time-dependent manner after stimulation with PGJ_2 or 15d-PGJ_2 (Fig. 2A). Three other PGs; PGD_2, PGE_2 and PGA_2, did not modify the level of TDP-43 protein. Moreover 15d-PGJ_2 induced solubility change in TDP-43 using TritonX-100 as a detergent (data not shown). Immunocytofluorescent analyses showed that anti-TDP-43 antibody reacted with the nucleus in HeLa cells (Fig. 2B). Diffuse cytoplasmic staining was also observed in only a small proportion, consistent with recent results that TDP-43 localizes to the cytoplasm during M-phase in HEK-293 cells[27]. After 15d-PGJ_2 stimulation, the immunoreactivity in the nucleus was significantly diminished and the number of cells showing cytoplasmic staining increased (Fig. 2C). Three other PGs had no effect on the cellular localization of TDP-43 (Data not shown). Similar results were also found in SK-N-SH neuroblastoma cells and HEK293 cells.

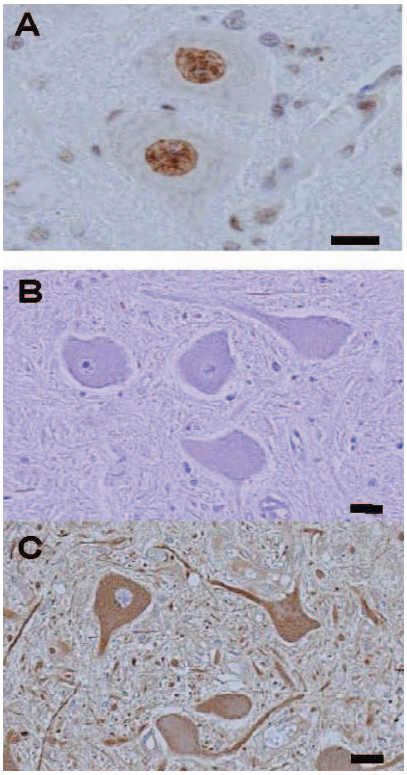

Figure 1 Immunohistochemical localization of TDP-43 (A) and 15d-PGJ_2 (B, C) in the spinal cord of control subjects (A, B) and patients with ALS (C). (A) In controls, polyclonal anti-TDP-43 antibody immunostains the neuronal and glial nuclei. (B) In controls, monoclonal anti-15d-PGJ_2 antibody immunostains the neuronal cytoplasm only weakly or unstains. (C) In ALS, the antibody strongly immunolabels the cytoplasm and processes of anterior horn cells. Bar = 10 μm.

Electrophilic portion is associated with alterations of TDP-43 profiles

To further explore the mechanism underlying changes in biochemical and cytopathological properties of TDP-43, we paid attention to the structure of 15d-PGJ_2. 15d-PGJ_2 is characterized by the presence of a cyclopentenone ring, which contains an electrophilic center that makes these PGs susceptible to undergoing additional reactions with nucleophiles such as the free sulfhydryl group of cysteine residues[28,29]. This raises the possibility that 15d-PGJ_2 can bind to TDP-43 protein directly. Therefore, 9,10-dihydro-15d-PGJ_2, which is an analogue lacking the reactive α,β-unsaturated carbonyl group, was used to explore this possibility. As shown in Fig 2D, 9,10-dihydro-15d-PGJ_2 had significantly less effects on the expression of TDP-43 protein. This result strongly suggested that the reduction of the double bond in the cyclopentenone ring of 15d-PGJ_2 participates in alterations of TDP-43. Next we made mutated TDP-43, in which all 6 cysteine residues were replaced with serine residues, because α,β-unsaturated carbonyl

group of the cyclopentenone ring can react with cysteine residues in proteins and cysteine is structurally similar to serine but contains a sulfhydryl or thiol group in place of the hydroxyl group. Biotinylated 15d-PGJ_2 was incubated with wild type or mutated TDP-43 for 2 h at room temperature in vitro, and washed with PBS. Supporting our hypothesis, positive signal was detected in samples of wild type TDP-43, whereas, no positive signal was observed in mutated TDP-43 (Fig. 2E). Collectively, these results strongly indicate that 15d-PGJ_2 directly binds to TDP-43 protein through cysteine residues.

In summary, our findings provide the evidence that 15d-PGJ_2 is a binding molecule of TDP-43 through conjugation to its cysteine residues. We further demonstrated that 15d-PGJ_2 is associated with changes in expression, solubility, and subcellular localization of TDP-43 similar to alterations found in TDP-43 proteinopathy. Thus these results suggest that sustained high level of 15d-PGJ_2 participates in the degenerative process on TDP proteinopathy, and suppression of the level of 15d-PGJ_2 could be a potential therapeutic approach for TDP-43 proteinopathy.

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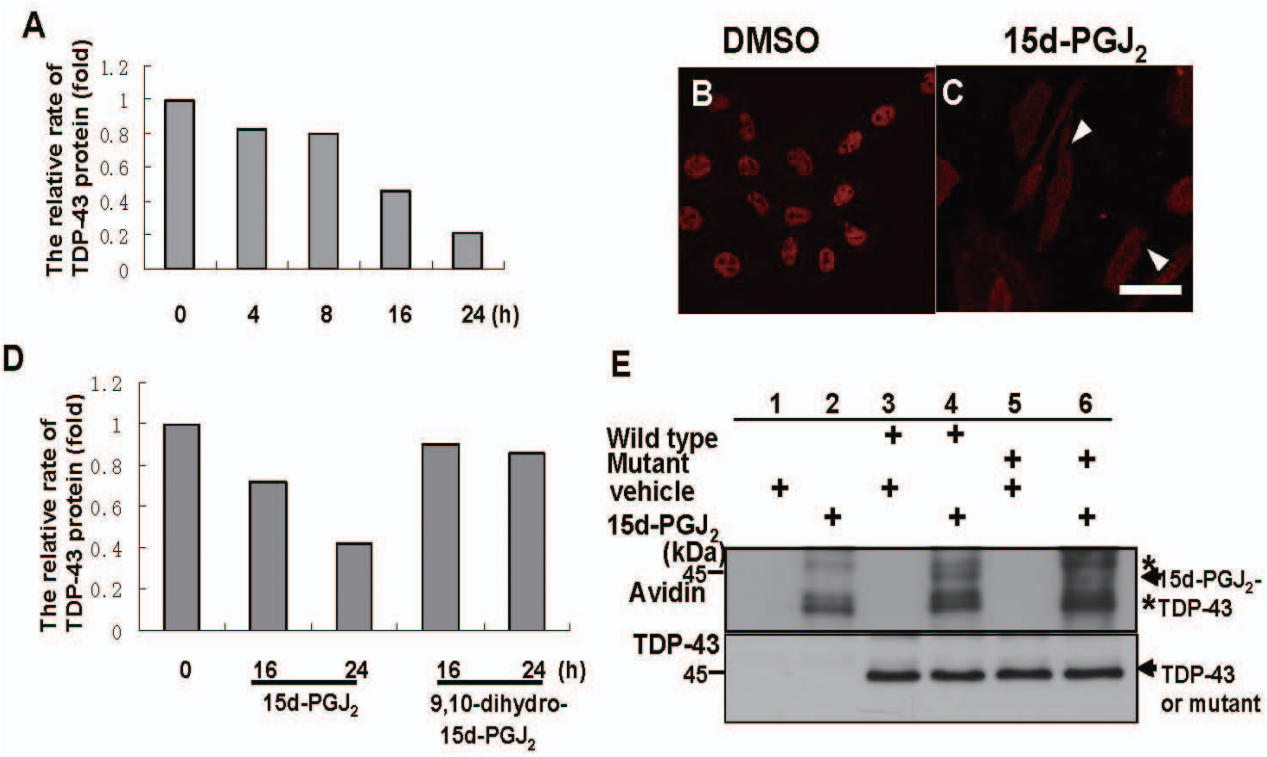

Figure 2 The effect of PGJ_2 or 15d-PGJ_2 on biochemical and pathological profiles of TDP-43. (A) Quantitative analysis shows that 15d-PGJ_2 down-regulates the amount of TDP-43 protein by about 20% at 24 h following stimulus. Similar results were obtained in duplicate experiments. (B, C) Immunocytofluorescent staining demonstrates that TDP-43 is predominantly localized in nucleus of HeLa cells, whereas 15d-PGJ_2 induces TDP-43-positive signal in nucleus as well as cytoplasm. Bar=20 μm. (C) Quantitative analysis shows that 15d-PGJ_2 suppress the protein level of TDP-43 by 40%, whereas 9,10-dihydro-15d-PGJ_2 has less effect on protein level of TDP-43 after stimulation for 24 h. (E) *In vitro* binding of TDP43-Flag with 15d-PGJ_2. TDP43-Flag protein was immunoprecipitated using Flag beads and incubated with 5 μM biotin or biotinylated 15d-PGJ_2 for 2 h. Incubation mixtures were analyzed by immunoblotting using HRP-conjugated streptavidin or anti-TDP-43 antibody. Immunoblot analysis shows that a band corresponding to 43 kDa is detected in treatment with biotinylated 15d-PGJ_2 (lane 4), but not biotin alone as stimulus (lane 2). The total amount of TDP-43 is assessed using anti-TDP-43 antibody. Asterisks indicate non-specific bands.

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