Proteomic Identification of Protein Targets for 15-Deoxy-Δ<sub>12,14</sub>-Prostaglandin J<sub>2</sub> in Neuronal Plasma Membrane

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Abstract

15-deoxy-Δ<sup>12,14</sup>-prostaglandin J<sub>2</sub> (15d-PGJ<sub>2</sub>) is one factor contributing to the neurotoxicity of amyloid β (Aβ), a causative protein of Alzheimer’s disease. Type 2 receptor for prostaglandin D<sub>2</sub> (DP2) and peroxysome-proliferator activated receptor-γ (PPARγ) are identified as the membrane receptor and the nuclear receptor for 15d-PGJ<sub>2</sub>, respectively. Previously, we reported that the cytotoxicity of 15d-PGJ<sub>2</sub> was independent of DP2 and PPARγ, and suggested that 15d-PGJ<sub>2</sub> induced apoptosis through the novel specific binding sites of 15d-PGJ<sub>2</sub> different from DP2 and PPARγ. To relate the cytotoxicity of 15d-PGJ<sub>2</sub> to amyloidoses, we performed binding assay [3H]15d-PGJ<sub>2</sub> and specified targets for 15d-PGJ<sub>2</sub> associated with cytotoxicity. In the various cell lines, there was a close correlation between the susceptibilities to 15d-PGJ<sub>2</sub> and fibrillar Aβ. Specific binding sites of [3H]15d-PGJ<sub>2</sub> were detected in rat cortical neurons and human bronchial smooth muscle cells. When the binding assay was applied to subcellular fractions of neurons, the specific binding sites of [3H]15d-PGJ<sub>2</sub> were detected in plasma membrane, nucleus and cytosol, but not in microsome. A proteomic approach was used to identify protein targets for 15d-PGJ<sub>2</sub> in the plasma membrane. By using biotinylated 15d-PGJ<sub>2</sub>, eleven proteins were identified as biotin-positive spots and classified into three different functional proteins: glycolytic enzymes (Enolase2, pyruvate kinase M1 (PKM1) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)), molecular chaperones (heat shock protein 8 and T-complex protein 1 subunit α), cytoskeletal proteins (Actin β, F-actin-capping protein, Tubulin β and Internexin α), GAPDH, PKM1 and Tubulin β are Aβ-interacting proteins. Thus, the present study suggested that 15d-PGJ<sub>2</sub> plays an important role in amyloidoses not only in the central nervous system but also in the peripheral tissues.

Introduction

Eicosanoids are divided into two groups, according to their mechanism of action: the conventional eicosanoids, e.g., prostaglandin D<sub>2</sub> (PGD<sub>2</sub>) and the cyclopentanone-type PGs, e.g., 15-deoxy-Δ<sup>12,14</sup>-PGJ<sub>2</sub> (15d-PGJ<sub>2</sub>). PGD<sub>2</sub> has been considered to be a pro-inflammatory mediator in inflammatory diseases such as Alzheimer’s disease (AD) and Asthma. In AD, PGD<sub>2</sub> formation increased in the frontal cortex of the patients when compared with those of the healthy subjects [1]. AD is characterized pathologically by cortical atrophy, neurogenereneration and deposits of amyloid protein in the various regions of brain such as cerebral cortex [2]. Amyloid β (Aβ) generated PGD<sub>2</sub> from cortical neurons before inflammation [3]. However, the toxicity of PGD<sub>2</sub> via its GTP-binding protein-coupled PGD<sub>2</sub> receptors does not occur. First, the PGD<sub>2</sub> receptor blocker did not inhibit PGD<sub>2</sub>-induced neuronal cell death [4]. Second, little mRNA of the PGD<sub>2</sub> receptor is observed in the rat [5] and human [6] cerebral cortex. Third, few binding sites of [3H]PGD<sub>2</sub> were detected in the plasma membranes from rat cortices [4]. Fourth, the extent of specific [3H]PGD<sub>2</sub> in total binding is much lower (30–40%) than that of [3H]15d-PGJ<sub>2</sub> (>80%), although binding sites of PGD<sub>2</sub> have been reported in synaptosomes of rat [7] and human brains [6]. Fifth, the LD<sub>50</sub> value (8.2 μM) of PGD<sub>2</sub> is much higher than the affinity for PGD<sub>2</sub> receptor (association constant = 14 nM) [5]. Finally, PGD<sub>2</sub> required a latent time to exert toxicity. PGD<sub>2</sub> was non-enzymatically metabolized to prostaglandin J<sub>2</sub> (PGJ<sub>2</sub>), Δ<sup>12</sup>-PGJ<sub>2</sub> and 15d-PGJ<sub>2</sub> [4]. Among PGD<sub>2</sub> metabolites, 15d-PGJ<sub>2</sub> exhibited most potent inflammatory effects [4]. Taken together, PGD<sub>2</sub> appeared to mediate inflammation via 15d-PGJ<sub>2</sub> in the amyloidoses.

The surface receptors specific for 15d-PGJ<sub>2</sub> have not been identified, and 15d-PGJ<sub>2</sub> is believed to be actively transported into cells. It possesses an α, β-unsaturated carbonyl group in the cyclopentenone ring that can form covalent adducts with free thiols in proteins by Michael addition. 15d-PGJ<sub>2</sub> covalently binds to Cys<sup>203</sup> of its nuclear receptor [8], peroxysome-proliferator

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activated receptors (PPARγ) [9], [10]. Recently, 15d-PGJ2 has been implicated in the antiproliferation independently from PPARγ[11]. Moreover, 15d-PGJ2 inhibits the NF-κB-dependent gene expression through the covalent modification at Cys317 in 15d-PGJ2 exhibits the highest affinity for the specific binding sites. Other eicosanoids and PPAR agonists did not affect the specific binding sites. 15d-PGJ2 regulated cell numbers in primary cultures of rat cortical neurons. The neurotoxicity of 15d-PGJ2 was the most potent among PGD2 and its metabolites, whereas little effect of other eicosanoids and PPAR agonists was detected. In peripheral tissues, 15d-PGJ2 also exhibited toxicity independently of PPARγ. In response to basic fibroblast growth factor, bronchial smooth muscle cells (BSMC) proliferate and remodel airway in asthma [13]. 15d-PGJ2 inhibits proliferation in a PPARγ-independent manner[14]. Thus, the identification of cell surface targets for 15d-PGJ2 is required to clear how 15d-PGJ2 induces cell toxicity and involves in amyloidoses.

In the present study, we identified cell surface targets for 15d-PGJ2 in cortical neurons. In general, glycolytic enzymes, molecular chaperones and cytoskeleton identified as membrane targets for 15d-PGJ2 are known to localize in the cytosol, but their roles on the cell surface have not been elucidated sufficiently. Here, we propose hypothetical role of membrane targets for 15d-PGJ2 on the cell toxicity and amyloidoses.

Materials and Methods

Materials

 Dulbecco’s modified Eagle’s medium, Leibovitz’s L-15 medium, Roswell Park Memorial Institute 1640 medium, MCDB, CS-C, trypsin, deoxyribonuclease I, fetal bovine serum (FBS), horse serum (HS), penicillin, and streptomycin were obtained from Invitrogen (Carlsbad, CA). Aβ (25–35) was purchased from Bachem AG (Bubendorf, Switzerland). [3H]15d-PGJ2 (115 Ci/mmol) and human hepatocytes were purchased from Perkin Elmer Life Science Products (Boston, MA). Human BSMC and human dermal fibroblasts were purchased from Lonza (Basel, Switzerland). PGD2, PGJ2, Δ12-PGJ2, 15d-PGJ2 and biotinylated 15d-PGJ2 were obtained from Cayman Chemicals (Ann Arbor, MI; Cabru, Milan, Italy). Immobiline™ DryStrip Gels (pH 3–10), Aspermers ECL Plus™ Western Blotting Detection Reagents, were obtained from GE Healthcare Bio-Sciences Corp. (Piscataway, NJ). Iodoacetamide, dithiothreitol (DTT), ethenylglycol bis tetraacetic acid (EGTA) and ATP (dissodium salt) were from Sigma-Aldrich (Milan, Italy). Sequence grade modified trypsin was purchased from Pronova (Madison, WI; Milan, Italy), and N-(l-pyrenyl) iodoacetamide was from Molecular Probes (Eugene, OR). Horseradish peroxidase-linked antibody against biotin was obtained 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.

Cell viability

Two different methods were employed for assessment of cell viability as previously reported [15]. First, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide dye (MTT) reduction assay reflecting mitochondrial succinate dehydrogenase activity was employed. Second, residual cells were counted according to morphologic criteria; neurons with intact neurites and a smooth, round soma were considered viable, whereas those with degenerated neurites and an irregular soma were considered nonviable. BSMC with extended cell bodies and their bright phase-contrast appearance were considered viable, whereas those with shrank and round cell bodies were considered nonviable.

Cell fractionation

Cell fractionation was performed as previously reported [17]. Cerebral cortices from rat brains were homogenized in 3 volumes of ice-cold STEA solution (0.25 M sucrose, 5 mM Tris-HCl (pH 7.5), 1 mM EGTA and 50 kariylin/ml aprotinin). The homogenate was filtered through three meshes and centrifuged at 700×g for 10 min. Fractionations of nuclear and plasma membrane; The pellet was resuspended in 120 ml of STEA solution by gentle homogenization, and the resuspension was dispersed in 1080 ml of isomotic Percoll solution (15.7% Percoll, 0.25 M sucrose, 1 mM EGTA, 50 kariylin/ml aprotinin and 10 mM Tris HCl (pH 7.5)). The mixture was centrifuged at 35,000×g for 30 min. The resulting pellet was suspended in HEPES-NaOH (pH 7.4), 1 mM EGTA and 50 kIU/ml aprotinin as the nuclear fraction. On the other hand, the second band from the surface in the supernatant was collected, washed by dilution with 2–3 volumes of HEPES solution and centrifuged at 10,000×g for 30 min. The pellet was suspended in HEPES solution as the plasma membrane

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buffer with 4 mg/ml trypsin and 0.4 mg/ml deoxyribonuclease I. Cells were plated at a density of 2.5×105 cells/cm2 on poly-L-lysine-coated dishes in conditioning medium, Leibovitz’s L-15 medium supplemented with 5% FBS and 5% horse serum at 37°C in 5% CO2 and 9% O2. On day 1 after plating, cultures were treated with 0.1 μM arabinosylocytosine C. On day 4, cortical cultures were immunostained with anti-MAP2 specific for neurons, anti-GEAP for astrocytes, and anti-microglial antigen (OX-42). Cultures prepared by this method, consisted of approximately 95% neurons. Human BSMC were cultured at a density of 3.5×104 cells/cm2 on 48-well plates in Molecular, Developmental, and Cellular Biology medium supplemented with 5% FBS, 50 μg/ml gentamicin, 50 ng/ml amphotericin. Human hepatocytes were cultured at a density of 5×104 cells/cm2 on 48-well plates in CS-C medium (Applied Cell Biology Research Institute) supplemented with 10% FBS. Human dermal fibroblasts were cultured at a density of 5×104 cells/cm2 on 48-well plates in DMEM supplemented with 10% FBS, 50 units/ml penicillin, and 50 μg/ml streptomycin.

Aggregation assessment of fAβ

A stock solution of fibrillar Aβ (25–35) (fAβ) was prepared by dissolving Aβ at 1 mM in deionized water and incubating Aβ at 37°C for 2–5 days to aggregate the peptide and stored at −20°C until use [16]. The aggregation state of fAβ was assessed in two ways. First, light microscopy was used to identify the presence of precipitated peptides both in stock solutions and after their addition to tissue culture wells; the observations were confirmed by three observers. Second, the aggregation state of fAβ was assessed by migration patterns after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Samples of fAβ stock solutions were added to reducing buffer, heated at 100°C for 3 min, and electrophoresed on 15% SDS-PAGE at 70 V.
fraction and stored in liquid nitrogen until used [18]. Fractionations of cytosol and microsome: The supernatant was centrifuged at 7,000 x g for 10 min. The resulting supernatant was recentrifuged at 100,000 x g for 1 h. The pellet was used as the microsomal fraction. The supernatant was used as the cytosolic fraction.

Binding assay of \([^{3}H]15d-PGJ_{2}\)

Binding assay of \([^{3}H]15d-PGJ_{2}\) were performed as previously reported [18]. The standard reaction mixture of 10 nM \([^{3}H]15d-PGJ_{2}\) contained 50 mM Tris-HCl buffer (pH 8.0), 100 mM NaCl and plasma membranes (10 μg) in a total volume of 100 μl. Incubation was initiated by addition of the reaction mixture to plasma membranes, and was carried out at 4°C for 24 h. We determined non-specific binding by performing incubations with \([^{3}H]15d-PGJ_{2}\) in the presence of 100 μM unlabeled 15d-PGJ2. The specific binding was calculated by subtraction of the non-specific binding from the total binding. Data are expressed as means ± standard error of the mean (S.E.M.) values (n = 4).

Protein separation by two-dimensional electrophoresis

Membrane preparation and binding assay of biotinylated 15d-PGJ2 were conformed to “Binding assay of \([^{3}H]15d-PGJ_{2}\)”. The standard reaction mixture of 1 μM biotinylated 15d-PGJ2 contained 50 mM Tris-HCl buffer (pH 8.0), 100 mM NaCl and plasma membranes (400 μg) in a total volume of 4 ml. Incubation was initiated by addition of the reaction mixture to plasma membranes, and was carried out at 4°C for 24 h in the presence or absence of unlabeled 15d-PGJ2. We determined non-specific binding by performing incubations with biotinylated 15d-PGJ2 in the presence of 100 μM unlabeled 15d-PGJ2. According to the method of Toda and Kimura [19], two-dimensional electrophoresis was performed with the CoolPhoreStar Horizontal Gel Electrophoresis Unit IPGIEF (Anatech: Tokyo, JP). The samples containing 400 μg of membrane lysates were dissolved in a rehydration buffer (5 M urea, 2 M thiourea, 2% w/v CHAPS, 2% w/v SB3-10, 2% Pharmalytes and 65 mM DTT) for the first dimensional isoelectric focusing (IEF). The pH range of the IEF was 3–10. Before IEF was performed, the gel strips were incubated with a swelling buffer (6 M urea, 2 M thiourea, 2% w/v) TritonX-100, 2% w/v SB3-10, 2% Pharmalytes, 2.5 mM acetic acid, 0.0025% BPB and 15 mM DTT). After IEF was performed, the gel strips were incubated with an SDS buffer (6 M urea, 32 mM DTT, 2% w/v SDS, 0.0025% BPB, 30% w/v glycerol, and 25 mM Tris-HCl pH 6.8) for 10 min, and then with...
an alkylation buffer (6 M urea, 243 mM iodoacetamide, 2%(w/v) SDS, 0.0025% BPB, 30%(v/v) glycerol, and 25 mM Tris-HCl pH 6.8) for 10 min. For the second dimensional electrophoresis, polyacrylamide gel (12% acrylamide, 0.4% bis-acrylamide, 10.6% glycerol, 0.1% SDS, 1.2% APS, 0.1%(v/v) TEMED and 369 mM Tris-HCl pH 8.8) was used. All procedures followed the manufacturer’s protocol. Separated proteins were then fixed in the gel using 1) 40% methanol and 10% acetic acid, 2) 10% methanol and 7% acetic acid, and 3) 10% methanol and 8% acetic acid. Then, they were stained with SYPRO Ruby protein gel stain, and scanned using the FluoroPhoreStar® 3000 (Anatech: Tokyo, JP). The protein spots were visualized by Progenesis Same Spots (Nonliner Dynamics Ltd: Newcastle upon Tyne, UK). For immunoblotting, gels were transferred to polyvinylidene fluoride membranes (Millipore Co., Bedford, USA). The membranes were incubated with phosphate-buffered saline containing 0.1% Tween20 (PBS/Tween) and 5% skim milk for blocking and washed with PBS/Tween. This procedure was followed by the addition of horseradish peroxidase-conjugated anti-biotin antibody and ECL reagents (GE Healthcare Biosciences). The spots were visualized by LAS-3000 (Aisin Seiki Co., Ltd., Aichi, Japan).

**Identification of 15d-PGJ2-targeted proteins**

Gel pieces were washed in 50 mM ammonium bicarbonic acid containing 50% acetonitrile for 10 min, twice. Then, they were dried in block incubator BI-516S (ASTEC Co., ltd.; Tokyo, JP) at 95°C for 10 min. Each sample was proteolyzed with 10 µl 1 mM ammonium bicarbonic acid containing 200 ng trypsin overnight at 37°C. The peptide in each gel was extracted with 50% acetonitrile containing 0.1% TFA followed by sonication for 15 min. The supernatant was collected, and peptides were further extracted with 75% acetonitrile containing 0.1% TFA followed by sonication for 15 min. Peptide extracts were concentrated to <10 µl using Speedvac concentrator. Then, they were desalted with Ziptip (Millipore Co.) and mixed with an equal volume of 5 mg 3-cyano-4hydroxycinnamic acid (Shimadzu GLC ltd.; Tokyo, JP) dissolved in 0.5 ml 50% acetonitrile containing 0.1% TFA. One micro liter samples were spotted onto a matrix assisted laser desorption/ionization (MALDI) plate. After air drying, spots were identified by MALDI time of flight mass spectrometry (MALDI-TOF MS: Shimazu, AXIMA TOF2TM). MS spectra were collected over m/z 500–3500. The acquisition parameters were Tung mode: Reflectron, Mass range: 1–3500, Max Laser Rep Rate: 10.0, CID: off, Power: 75, Profiles: 200, Shots: 5, Ion...
In the presence of unlabeled PGD2, PGJ2, respectively. Data are expressed as means in cortical neurons and BSMC were 2523 cpm and 1309 cpm, respectively. Sensitivities of various cell lines to amyloid protein were examined in the central nervous system and peripheral tissues. Cortical neurons, BSMC, hepatocytes and dermal fibroblasts were exposed to fA or vehicle (ionized water) for 48 h, and their viability was quantified by the MTT-reducing activity. In comparison with vehicle, fA significantly reduced the viability of cortical neurons and BSMC at 10 μM. On the other hand, fA did not significantly affect the viability of hepatocytes and dermal fibroblasts (Figure 1A). In neuronal cells and BSMC among tested cell lines, amyloid protein inhibited the cell viability in a concentration-dependent manner (Figure 1B).

**Results**

**Membrane Targets for 15d-PGJ2**

**Western blotting**

The standard reaction mixture of 1 mM biotinylated 15d-PGJ2 contained 50 mM Tris-HCl buffer (pH 8.0), 100 mM NaCl and plasma membranes (400 μg) in a total volume of 4 ml. Incubation was initiated by addition of the reaction mixture to plasma membranes, and was carried out at 4°C for 24 h. Membrane lysates were incubated with Streptavidin Agarose beads (Invitrogen, Carlsbad, CA) at room temperature for 30 min. The beads were rinsed three times with lysis buffer. The proteins were eluted by boiling the beads in Laemmli sample buffer and analysed by SDS-PAGE followed by immunodetection with antibodies to GAPDH (rabbit polyclonal, abcam [ab9485]), Cambridge, UK), PKM1 (goat polyclonal, abcam [ab6191]), Enolase 2 (goat polyclonal, Santa Cruz [sc-31859], Santa Cruz, CA), Tubulin β (rabbit polyclonal, Santa Cruz [sc-9104]), TCP12 (mouse monoclonal, Enzo Life Sciences [ADI-CTA-191-D], New York, NY), Internexin α (mouse monoclonal, Millipore [MAB5224], Billerica, MA) and Actin β (mouse monoclonal, abcam [ab8226]).

This procedure was followed by the addition of horseradish peroxidase-conjugated secondary antibody and ECL reagents.

**Statistical analysis**

Data were given as means ± S.E.M. (n = number of observations). 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 the PG group (15d-PGJ2, Δ12-PGJ2, PGJ2, PGD2 and 15d-PGD2). The half maximal inhibitory concentration (IC₅₀), the half maximal lethal dose (LD₅₀) and the half maximal lethal time (LT₅₀) were calculated by Microsoft Excel Fit.
**Table 1.** Comparison of the specific binding sites for [3H]15d-PGJ2 in plasma membranes to authentic receptors, DP1 and DP2.

| Ligand     | Apoptosis LD50 (µM) | Apoptosis LT50 (h) | SBJ IC50 (µM) | DP1 Ki (nM) | DP2 Ki (nM) |
|------------|---------------------|--------------------|---------------|-------------|-------------|
| 15d-PGJ2   | 1                   | 4                  | 1.6           | 280         | 3.2         |
| Δ12-PGJ2   | 1                   | 6                  | 7             | 100         | 6.8         |
| PGJ2       | 2                   | 10                 | 11            | 0.9         | 6.6         |
| PGJ2       | 5                   | >10                | 67            | n.d.        | 23000       |
| PGA2       | >10                 | >10                | >100          | 1.7         | 2.4         |
| 15d-PGD2   | >10                 | >10                | n.d.          | 6374        | 2.9         |

Apoptotic effects of 15d-PGJ2–related compounds were correlated to their affinities for DP1, DP2 and the specific binding sites for [3H]15d-PGJ2 (SBJ). LD50: The concentration of 15d-PGJ2–related compounds required to induce apoptosis in the half of neurons which were cultured for 24h in the absence of serum. LT50: The time of 10 µM 15d-PGJ2–related compounds required to induce apoptosis in the half of neurons which were cultured in the absence of serum. IC50: The concentration of 15d-PGJ2–related compounds required to inhibit half of the specific binding of [3H]15d-PGJ2 to SBJ. LD50, LT50 and IC50 were calculated from Yagami et al.[4]. These data on Ki: The Ki values of 15d-PGJ2–related compounds to DP1 and DP2 were referred from Sawyer et al.[53].

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**Figure 6. Biotinylated 15d-PGJ2 induced neuronal cell death.** (A) Cortical neurons were treated with 15d-PGJ2 (open circles) or biotinylated 15d-PGJ2 (closed circles) at the indicated concentrations in the serum-free medium. MTT-reducing activities were determined 18 h later. Data are expressed as means ± S.E.M. (n = 4). **P<0.01, compared with control by ANOVA followed by Dunnett’s test.** (B) Cortical neurons were treated with vehicle (control), 3 µM 15d-PGJ2 or 3 µM biotinylated 15d-PGJ2 in the serum-free medium. Vehicle was 0.1% ethanol. Cortical neurons were photographed by phase-contrast microscopy 18 h later.

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Sensitivities of various cell lines to 15d-PGJ₂

We examined susceptibilities to 15d-PGJ₂ in cortical neurons, BSMC, hepatocytes and dermal fibroblasts. These cell lines were exposed to 15d-PGJ₂ or vehicle (0.1% ethanol), and their viability was quantified by the MTT-reducing activity. In comparison with vehicle, 15d-PGJ₂ significantly reduced the viability of cortical neurons and BSMC at 10 μM. On the other hand, 15d-PGJ₂ did not significantly affect cell viability of hepatocytes and dermal fibroblasts (Figure 2A). As well as amyloid protein, 15d-PGJ₂ also not significantly affect cell viability of hepatocytes and dermal fibroblasts (Figure 2A). As well as amyloid protein, 15d-PGJ₂ also did not significantly affect cell viability of neuronal cells and BSMC, but neither hepatocytes nor dermal fibroblasts in a concentration-dependent manner (Figure 2B).

In control cultures, neurons had extended neurites and smooth, round cell bodies (Figure 3A). On the other hand, some cell bodies shrank and lost their bright phase-contrast appearance in 15d-PGJ₂-treated cultures. By 24 h, there were markedly fewer cells, and extensive debris was seen attached to the substratum (Figure 3B). In BSMC, 15d-PGJ₂ extended cell bodies and exhibited their bright phase-contrast appearance (Figure 3C). When BSMC were cultured, we confirmed that the cell density was increased (data not shown). This increment was significantly prevented by 10 μM 15d-PGJ₂ (Figure 3B and 3D). In 15d-PGJ₂-treated cultures, some cell bodies shrank and became round (Figure 3D). Thus, there was a close correlation between susceptibilities to 15d-PGJ₂ and amyloid protein, suggesting an involvement of 15d-PGJ₂ in the amyloid protein-induced inflammation.

Effects of PGD₂ and Its metabolites on the viability of cortical neurons and BSMC

MTT assay is a colorimetric assay for measuring the activity of enzymes that reduce MTT or close dyes to formazan dyes. These reductions take place only when reductase enzymes in mitochondria are active, and therefore conversion is often used as a measure of viable (living) cells. Previously, we have reported that there was a linear relationship between cell density and MTT-reducing activities in cortical neurons [15]. As well as the MTT-reducing activity, the cell density was reduced by 10 μM 15d-PGJ₂ in cortical neurons and BSMC (Figure 4A). MTT-reduction assay is also established for various cell types other than neurons to enable accurate, straightforward quantification of changes in their cell densities.

In most experiments, the neurotoxicity of 15d-PGJ₂ was evaluated at 10 μM for 24 h in the presence of serum. Since PGD₂ can be non-enzymatically metabolized to PGJ₂, Δ₁₂-PGJ₂ and 15d-PGJ₂ in the present culture medium [4], it is very difficult to compare their neurotoxic potencies. When serum was deprived from culture medium to decelerate the metabolism of PGD₂, we have succeeded in detecting their neurotoxic hierarchy by the treatment with each PG for 10 μM 15d-PGJ₂ in cortical neurons and BSMC (Figure 4A). MTT-reduction assay is also established for various cell types other than neurons to enable accurate, straightforward quantification of changes in their cell densities.

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Membrane Targets for 15d-PGJ₂

A

B

C

1  MERRRITSAR RSYASSETMV RGHGPTRHGLG TIPRLSLSRM TPPPLPARVDF
51  SLAGALNAGF KETRASERAE MMELNRDFAS YIEKVRFLEQ QNKALAAELN
101  QLRAKEPTKL ADVYQAELRE LRLRLDQLTT NSARLEVERD NLTQDLGTLR
151  QKLQDETNLR LEAENNLAVY RQEADEATLA RVDLERKVES LEEEIQFLRK
201  IHEEEEVRLQ EQLAQQQVHV EMDVAKPDLT AALREIRTYQ EAVATSNMQE
251  TEEWYRSDKFA DLTDVSRNA ELLRQAKHEA NDYRRLQQLAL TCDLESRLGT
301  NESLERQMRE QEERHARESA SYQEAARLE EEGQSLKEEM ARHLQELYQDL
351  LNVKLALDIE IATYRLLEG EENRITIPvQ TFSNLQIRET SLDTKSVSEG
401  HLKRNIVVKT VEMRIDGEVIK ESKQEHKDVM
The IC50 value of 15d-PGJ2 to the specific binding of [3H]15d-PGJ2 in BSMC was 31 μM, and 20-fold higher than that (1.6 μM) in neuronal cells. The binding sites of 15d-PGJ2 in cortical neurons could also be recognized by Δ12-PGJ2 and PGJ2, whereas those in BSMC could be specifically done by 15d-PGJ2 (Figure 5B). In the two cells, the MTT-reducing activities of 15d-PGJ2 and its

| Start-End | Observed Mr(expt) | Mr(calc) | Delta | Miss | Sequence               |
|-----------|-----------------|----------|-------|------|------------------------|
| 12 - 21   | 1146.37         | 1145.38  | -0.14 | 0    | R.SYASSETMV.R.G        |
|           |                 |          |       |      | Oxidation (M)          |
| 40 - 47   | 898.36          | 897.35   | -0.12 | 0    | R.MTPPLPAR.V           |
|           |                 |          |       |      | Oxidation (M)          |
| 48 - 61   | 1409.98         | 1408.97  | 0.24  | 0    | R.VDFSGLAGNLNQK.E     |
| 69 - 77   | 1140.44         | 1139.43  | -0.03 | 0    | R.AEMME.LND.R.F        |
|           |                 |          |       |      | 2 Oxidation (M)        |
| 78 - 94   | 857.25          | 856.24   | -0.19 | 0    | R.FASYIEK.V            |
| 85 - 93   | 1161.75         | 1160.74  | 0.11  | 1    | K.VRFLEQONK.A          |
| 97 - 93   | 906.35          | 905.34   | -0.12 | 0    | R.FLEQONK.A            |
| 94 - 103  | 1098.76         | 1097.75  | 0.13  | 0    | K.ALAENLQNL.R.A        |
| 110 - 119 | 1177.80         | 1176.79  | 0.18  | 0    | K.LADVYQAER.L.E        |
| 125 - 134 | 1118.72         | 1117.71  | 0.14  | 0    | R.NDLTTLNAR.L          |
| 140 - 150 | 1245.88         | 1244.87  | 0.24  | 0    | R.DNLODLGTL.R.O        |
| 153 - 160 | 998.53          | 997.52   | 0.02  | 0    | K.LQDETNL.R.L          |
| 161 - 171 | 1291.89         | 1290.88  | 0.23  | 0    | R.LEAENLAVR.Q          |
| 172 - 181 | 1103.46         | 1102.45  | 0.07  | 0    | K.VEAEDTLAR.V           |
| 188 - 199 | 1492.01         | 1491.00  | 0.24  | 0    | K.VEAEDTLAR.V           |
| 188 - 200 | 1620.05         | 1619.04  | 0.19  | 1    | K.VEAEDTLAR.V           |
| 200 - 207 | 1039.62         | 1038.61  | 0.07  | 0    | R.KHIEEVR.E             |
| 201 - 207 | 911.39          | 910.38   | 0.04  | 0    | K.IHEEVR.E              |
| 259 - 268 | 1094.65         | 1093.64  | 0.10  | 0    | K.FADLTDVSR.N           |
| 278 - 285 | 1080.56         | 1059.55  | 0.07  | 1    | K.HEANDYRR.Q            |
| 286 - 298 | 1547.00         | 1545.99  | 0.21  | 0    | R.OLOALTCDLESLR.G      |
| 318 - 328 | 1206.55         | 1205.54  | 0.02  | 0    | R.ESASYQEAQAR.L        |
|           |                 |          |       |      | Glu->pyro-Glu (N-term E)|
| 318 - 328 | 1224.78         | 1223.77  | 0.19  | 0    | R.ESASYQEAQAR.L        |
| 343 - 354 | 1499.71         | 1498.70  | -0.08 | 0    | R.HLQEYGIDLNV.K.L      |
| 355 - 365 | 1277.81         | 1276.70  | 0.10  | 0    | K.LADDIEATLYR.K        |
| 367 - 374 | 958.48          | 958.48   | 0.01  | 0    | K.LLEEGER.I             |
| 375 - 388 | 1630.11         | 1628.93  | 0.18  | 0    | K.ITIPVTGTSNLQIR.E     |
| 396 - 404 | 1012.34         | 1011.33  | -0.21 | 1    | K.SVESOHKLR.N           |

Figure 8. MALDI-TOF mass spectrum of the tryptic digest of spot 8. Spot 8 from Figure 7D was digested in gel with trypsin, and the resulting peptides were analyzed by MALDI-TOF MS as detailed in the experimental section. (A) Typical mass spectrum from a representative experiment. (B) Probability based Mowse Score. (C) Positions of matched peptides in the sequence of GFAP.
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Figure 9. Peptide matches of spot 8 with GFAP. List of the monoisotopic masses of some of the peptides identified showing their position in the sequence of GFAP.
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precursors were paralleled to the affinities of these ligands for the membrane specific binding sites of 15d-PGJ2.

**Comparison of the specific binding sites for $[^{3}H]15d$-PGJ2 in plasma membranes to authentic receptors, DP1 and DP2**

In peripheral tissues including nerves, chemoattractant receptor-homologous molecule expressed on Th2 cells has been identified as a type 2 receptor for PGD$_2$ (DP2), and reported to be also a membrane receptor for 15d-PGJ$_2$ [20]. We compared characterization of specific binding sites for $[^{3}H]15d$-PGJ$_2$ (SBJ) as well as DP2. According to LD$_{50}$ and LT$_{50}$, the apoptotic effect of 15d-PGJ2–related compounds was correlated to their affinities for SBJ, but not to those for DP1 or DP2.

**Isolation of Targets for 15d-PGJ2**

To identify target proteins for 15d-PGJ2, membrane proteins were labeled with biotinylated 15d-PGJ2 under the serum-free condition to reduce non-specific binding. Under this condition, biotinylated 15d-PGJ2 induced neuronal cell death in a concentration-dependent manner as well as 15d-PGJ2. Their LD$_{50}$ values were almost 1 μM (Figure 6A). Biotinylated 15d-PGJ2 suppressed the extension of neurites and shrank cell bodies in a similar fashion to 15d-PGJ2 (Figure 6B). Next, neuronal plasma membranes were incubated with 1 μM biotinylated 15d-PGJ2 in the absence or presence of 15d-PGJ2 at the indicated concentrations. Then, membrane proteins modified with biotinylated 15d-PGJ2 were separated by two-dimensional gel electrophoresis. The patterns that were identified by Western blot analysis are shown in Figure 7. Several biotinylated 15d-PGJ$_2$-protein conjugates were detected as biotin-positive spots (Figure 7A and 7B). 15d-PGJ2 was incubated with 15d-PGJ$_2$–related compounds for 15d-PGJ$_2$–related compounds in sequence (Table 1). In the view of IC$_{50}$, the affinity of 15d-PGJ$_2$–related compounds for DP1 was PGJ$_2$ > $\Delta^{12}$-PGJ$_2$ > PGJ$_2$ > PGA$_2$ > PGD$_2$ > 15d-PGJ$_2$ in sequence (Table 1). On the other hand, the affinity of 15d-PGJ$_2$–related compounds for DP2 was PGD$_2$ > 15d-PGJ$_2$ > $\Delta^{15}$-PGJ$_2$ > PGJ$_2$ > $\Delta^{12}$-PGJ$_2$ > PGA$_2$ > PGJ$_2$ > 15d-PGJ$_2$ in sequence (Table 1). In addition, the affinity of 15d-PGJ$_2$–related compounds for DP1 was PGJ$_2$ > $\Delta^{15}$-PGJ$_2$ > 15d-PGJ$_2$ > PGD$_2$ > PGA$_2$ in sequence (Table 1). Thus, the apoptotic effect of 15d-PGJ$_2$–related compounds was correlated to their affinities for SBJ, but not to those for DP1 or DP2.

**Table 2. Membrane proteins targeted for 15d-PGJ2.**

| No | Protein | Swiss-plot | MW | PI | Score | Matches | Coverage |
|----|---------|------------|----|----|-------|---------|----------|
| 1  | Hspa8   | P63018     | 71055 | 5.37 | 72 | 25/48 | 49 |
| 2  | Hspa8   | P63018     | 71055 | 5.37 | 100 | 13/20 | 23 |
| 3  | Hspa8   | P63018     | 71055 | 5.37 | 114 | 14/20 | 24 |
| 4  | Intemixin α | P23565 | 56224 | 5.20 | 89 | 11/20 | 18 |
| 5  | Tubulin β2 | Q3KRE8 | 49931 | 4.78 | 99 | 11/20 | 21 |
| 6  | GFAP    | P47819     | 49984 | 5.35 | 97 | 14/40 | 40 |
| 7  | GFAP    | P47819     | 49984 | 5.35 | 292 | 31/40 | 51 |
| 8  | GFAP    | P47819     | 49984 | 5.35 | 267 | 28/39 | 56 |
| 9  | CK20    | P25030     | 49414 | 5.28 | 52 | 12/19 | 31 |
| 10 | TCP1α   | P28480     | 60835 | 5.86 | 51 | 10/33 | 22 |
| 11 | PKM1    | P11980     | 58331 | 6.63 | 52 | 9/29 | 26 |
| 12 | Enolase1 | P04764     | 47098 | 6.16 | 73 | 9/20 | 22 |
| 13 | Enolase2 | P04764     | 47098 | 6.16 | 62 | 8/20 | 23 |
| 14 | Enolase 2 | P07323     | 47111 | 5.03 | 50 | 7/20 | 19 |
| 15 | Actin β | P60711     | 42052 | 5.29 | 73 | 7/20 | 52 |
| 16 | Actin β | P60711     | 42052 | 5.29 | 65 | 9/37 | 28 |
| 17 | Actin β | P60711     | 42052 | 5.29 | 79 | 8/19 | 23 |
| 18 | Actin β | P60711     | 42052 | 5.29 | 58 | 9/38 | 27 |
| 19 | Actin β | P60711     | 42052 | 5.29 | 48 | 8/34 | 23 |
| 20 | Actin β | P60711     | 42052 | 5.29 | 82 | 9/24 | 26 |
| 21 | CapZa2  | Q3T1K5     | 33118 | 5.57 | 62 | 8/30 | 34 |
| 22 | GAPDH   | P04797     | 36090 | 8.14 | 73 | 10/36 | 27 |

Spots that were excised from the gel show in Figure 7E were identified by tryptic digestion and MALDI-TOF MS. Shown are the spot number, name of the identified protein, the accession number in the SwissProt database, the theoretical molecular mass and isoelectric point, the probability based MOWSE score, the number of peptides matched according to the Mascot database, the percentage of the protein sequence that is covered by the identified peptides.

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peptide matches (error ±0.02%) (Figure 9), which represents 56% sequence coverage (Figure 8C). Table 2 lists the identity of 22 protein spots, which could be identified in three independent experiments. The multiple gel spots for a single identification could be ascribed to posttranslational modification, such as phosphorylation. For example, spot 6 could contain 3 phosphorylation sites (Tyr279, Tyr283, and Tyr287), which represented the probability based MOWSE score 59, 16 peptide matches, 32% sequence coverage. The phosphorylation site of spot 8 was not detected. The identified proteins fall into several different functional classes, including glycolytic enzymes (Enolase 1, Enolase 2, GAPDH and PKM1), molecular chaperones (Hsp8a and TCP1α) and cytoskeletons (Tubulin β2b, Actin β, Internexin α, GFAP and CapZα2).

Next, we attempted to detect the 15d-PGJ2-target adducts in the plasma membranes exposed to the biotinylated 15d-PGJ2, by streptavidin agarose pull-down assays. Western blot revealed that 15d-PGJ2 interacted with Actin β, Enolase 2, GAPDH, Internexin α, PKM1, TCP1α and Tubulin β2b (Figure 10). Since plasma membranes were prepared from adult cerebral cortices including neurons and astrocytes, non-neuronal enolase 1 and GFAP appeared to be derived from astrocytes.

Regions homologous to the binding site of 15d-PGJ2 in targeted proteins

Several lines of evidences indicate the covalent binding sites of 15d-PGJ2 in previous target proteins. To ascertain whether the cysteine residue in the present target proteins responded to the covalent binding sites of 15d-PGJ2 in previous target proteins or not, homologous regions were searched (Table 3). As query sequences, we used the amino acid sequences of the previous target proteins, in which the covalent binding sites of 15d-PGJ2 are identified: Cys274 of Actin β (P60711) [21], Cys279 of c-Jun (NP_060607) [22], Cys279 of H-ras (NP_00109711) [23], Cys279 of IkB kinase β (Q8Q70)8, Cys279 of PPARγ (NP_619725) [8], Cys35 of thioredoxin (NP_446252) [24], Hspa8 contained Cys603 responded to the Cys179 of IkB-kinase β. The amino sequence of Hspa8 from Lys597 to Leu610 was homologous to that of IkB-kinase β from Lys371 to Leu406. Based on the comparison between the two sequences, the optimal score and the identity were 15, 29 and 31%, respectively. In a similar fashion, Internexin α, Tubulin β2b, GFAP, CK20, TCP1α, PKM1, Enolase 1, Enolase 2, Actin β, CapZα2 and GAPDH contained the cysteine residue responded to Cys609 of thioredoxin, Cys274 of H-ras, Cys279 of c-Jun, Cys69 of thioredoxin, Cys35 of thioredoxin, Cys279 of H-ras, Cys179 of IkB-kinase β, Cys179 of IkB-kinase β, Cys69 of thioredoxin, respectively. Thus, the present target proteins also contained the cysteine residue responded to the previous covalent binding site of 15d-PGJ2, and exhibited homologous sequences around the specific binding site.

Discussion

Cortical neurons and BSMC sensitive to amyloid protein were susceptible to 15d-PGJ2.[3H]15d-PGJ2 bound specifically to the two cells, suggesting that 15d-PGJ2 played an important role in amyloidoses not only in the central nervous system but also in the peripheral tissues. The specific binding sites of [3H]15d-PGJ2 were detected in the neuronal subcellular fractions of nuclear, cytosol and plasma membrane, but not in the microsomal fraction. 15d-PGJ2 binds to the nuclear receptor, PPARγ [9] and the cytosolic protein, Ras [23]. In peripheral tissues including nerves, chemooattractant receptor-homologous molecule expressed on Th2 cells has been identified as a type 2 receptor for PGD2 (DP2), and reported to be also a membrane receptor for 15d-PGJ2 [20]. Contrary to its mRNA, little protein of DP2 has yet been detected in the central nerve. Furthermore, we ruled out the possibility that the specific binding site of 15d-PGJ2 in the plasma membrane of cortical neurons was DP2. First, few binding sites of [3H]PGD2 are detected in plasma membranes from rat cortices [4]. Although binding sites of [3H]12-PGJ2 and [3H]PGJ2 are also detected in plasma membranes, those are displaced most potently by 15d-PGJ2 among PGD2 metabolites [4]. Second, a DP2 selective agonist, 15d-PGJ2 do not affect the cell number of neuronal cells and BSMC (Figure 3B and Table 1). Third, the LD50 value (>10 μM) of PGD2 is much higher than the affinity for PGD2 receptor (dissociation constant = 8.8 nM) [20].

In the present study, we identified membrane proteins targeted for 15d-PGJ2, including glycolytic enzymes, molecular chaperones and cytoskeletons (Table 2 and Figure 10). GAPDH, Enolase 1, Enolase 2 and PKM1 were previously believed to perform exclusively ‘house-keeping’ glycolysis. GAPDH is not only found in the cytoplasm, but also closely associated with the plasma membrane [25]. GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to D-glycerate 1,3-bisphosphate. Reduction in glycolysis precedes cognitive dysfunction and is therefore believed to be an important early event in AD development [26]. Apart from its glycolytic role, overexpression of the particular membrane-associated GAPDH has a direct role in neuronal apoptosis [27] (Figure 11). GAPDH is located in amyloid plaques [28],
GAPDH associates tightly with Enolase 2 and Hspa8, and makes (APP) [29], and co-precipitates with fA

Table 3. Regions homologous to the binding site of 15d-PGJ2 in targeted proteins.

| Protein       | Sequences | Initial Score | Optimal Score | Identity |
|---------------|-----------|---------------|---------------|----------|
| IkB-kinase β | Query 171 KELDQGSLCTSFVGTL 186 | 15 |  |  |
| Hspa8        | Sbjct 597 KELEK–VCPNI 1 T KL 610 | 15/16 (31%) |  |  |
| Thioredoxin  | Query 60 DDCCQDVAADCE 70 | 16 |  |  |
| Internexin α | Sbjct 173 EEV QRLR ARCE 183 | 4/11 (36%) |  |  |
| H-ras        | Query 183 SCKCV 187 | 14 |  |  |
| Actin b      | Sbjct 126 SCDDL 130 | 3/5 (60%) |  |  |
| c-Jun        | Query 261 RNRI AAS KCRK 274 | 16 |  |  |
| GFAP         | Sbjct 284 RRQALCT CLE 5 L 297 | 4/14 (28%) |  |  |
| Thioredoxin  | Query 59 VDDCCQDVAADCE 72 | 8 |  |  |
| CK20         | Sbjct 136 I KDAQ I ENARCVLQ 149 | 7/14 (28%) |  |  |
| Thioredoxin  | Query 34 PCKMI KPFH 43 | 17 |  |  |
| PKM1         | Sbjct 165 CKV 168 | 2/4 (50%) |  |  |
| H-ras        | Query 178 GPGMSCCKVL L 188 | 17 |  |  |
| Enolase 1    | Sbjct 331 AAGE KSCNCL L 341 | 5/11 (45%) |  |  |
| H-ras        | Query 183 SCKVL 188 | 17 |  |  |
| Enolase 2    | Sbjct 336 ACNCLL 341 | 3/6 (50%) |  |  |
| Actin β      | Query 356 WIKSEYDES GSVHRCF 375 | 17 |  |  |
| Actin β      | Sbjct 356 WIKSEYDES GSVHRCF 375 | 20/20 (100%) |  |  |
| IkB-kinase β | Query 169 YAKELDQSL CT SFVGLQ 187 | 21 |  |  |
| CapZn2       | Sbjct 131 YKEHYP RNGC TVGYKKVD 149 | 5/19 (26%) |  |  |
| Thioredoxin  | Query 35 CKMIKP 40 | 11 |  |  |
| GADPH        | Sbjct 245 CRLEKP 250 | 3/6 (50%) |  |  |

Homologies were determined with Lipman-Pearson searching algorithms using the Swiss-plot database. As query sequences, we used the amino acid sequences of the previous target proteins, in which the covalent binding sites of 15d-PGJ2 are identified: Cys374 of Actin b (P60771) [50], Cys35 and Cys69 of thioredoxin (NP_619725) [8], Cys269 of c-Jun (P60771) [50], Cys269 of c-Jun (NP_068607) [51], Cys35 of H-ras (NP_001091711) [20], Cys79 of IkB-kinase β (QSTQ78) [12], Cys205 of PKM1 (NP_0619725) [8], Cys38 and Cys25 of thioredoxin (NP_446252) [52]. As subject sequences, we used the amino acid sequences of our target proteins. The listed sequences exhibited the highest score in the initial score, the optimal score and the identity.

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Interacts with the C-terminal region of amyloid precursor protein (APP) [29], and co-precipitates with fAβ[30]. Furthermore, GADPH associates tightly with Enolase 2 and Hspa8, and makes up trans-plasma-membrane oxidoreductases (PMOs), the extracellular redox sensor for signaling external oxidative stress to the cell [31].

Enolase 1 and Enolase 2 belong to a superfamily of abundantly expressed carbon-oxygen lyases known for the catalysis of 2-phosphoglycerate to phosphoenolpyruvate. Ubiquitous enolase 1 and neuron specific enolase 2 exist as monomers and also as dimmers on the neuronal membrane surface [32]. Recent studies have demonstrated that enolases possess different regulatory properties from glycolysis in the brain [33]. Enolase 1 is one of the most consistently up-regulated and oxidatively modified proteins in brain of subjects of early-onset AD [34]. Enolase 1 and enolase 2 are autoantigen targets in post-streptococcal autoimmune disease of central nervous system (Figure 11). The anti-enolase antibodies induce neuronal apoptosis [35]. Enolase 2 is part of neuronal PMOs, and the anti-enolase2 antibody can inhibit PMA activity on the plasma membrane [31].

Pyrurate kinase transfers a phosphate from phosphoenolpyruvate to ADP. Pyruvate kinase is also defined as the autoantigen, and its antibodies induce neuronal apoptosis [35] (Figure 10). The significant increase in pyruvate kinase activity is found in frontal and temporal cortex of AD brains [36]. Pyruvate kinase is elevated in the cortical neurons undergoing Aβ-mediated apoptosis [37]. Pyruvate kinase is co-precipitated with fAβ[30]. Biotinylated 15d-PGJ2 binds to PKM1 in mesangial cells [31], supporting our results.

Hsp8α is dnaK-type molecular chaperone heat shock protein 72-ps1 in the PMO complex [31]. It is located in the cytoplasm [39], but nuclear localization and accumulation near or at the plasma membrane in stressed cells and in synaptosomal membranes has been observed [40]. Hsp8α binds to the cytoplasmic domain near the post-transmembrane region of APP (Figure 11). TCP1α is a selective molecular chaperone in tubulin biogenesis, by that nascent tubulin subunits are bound to TCP1α and released in assembly competent forms. Cytoskeletal proteins are deficient in AD. When TCP1α is related to its natural and
specific substrate tubulin β, the ratio is significantly decreased in
the temporal, frontal, parietal cortex and in thalamus of AD
patients [41]. Relatively decreased molecular chaperoning of
tubulin β by TCP1α is suggested to lead to misfolded tubulin
aggregating and accumulating in plaques and tangles, a hallmark
of AD (Figure 11).
Tubulin has been identified as a membrane component of
synaptosomes and various plasma membranes. Both tubulin α and
β have been shown to associate with the amyloid deposits of
familial amyloidosis [42] and to bind to the Aβ sequence of APP
[43]. Moreover, tubulin β is retained by a monomeric Aβ column
[44], and co-precipitated with αβ [30] (Figure 11). The tau
protein interacts with tubulin to stabilize microtubules and
promote tubulin assembly into microtubules. PGJ₂ induces
caspase-mediated cleavage of tau, generating Δtau, an aggregation
prone form known to seed tau aggregation prior to neurofibrillary
tangle formation [45]. Hyperphosphorylation of the tau protein
(tau inclusions) can result in the self-assembly of tangles of paired
helical filaments and straight filaments, which are involved in the
pathogenesis of AD [46]. Biotinylated 15d-PGJ₂ binds to tubulin β
in mesangial cells [38], supporting our results.

AD-linked human Aβ synergistically enhances the ability of
wild-type tau to promote alterations in the actin cytoskeleton
(Figure 11) and neurodegeneration [47]. The ability of globular
actin to rapidly assemble and disassemble into filaments is critical
to many cell behaviors. F-actin-capping protein subunit α-2
(CapZα2) regulates growth of the actin filament by capping the
barbed end of growing actin filaments (Figure 11). Members of the
actin-depolymerizing factor (ADF)/cofilin family are important
regulators of actin dynamics. ADF and cofilin’s ability to increase
actin filament dynamics is inhibited by their phosphorylation on
Ser³ by LIM kinase 1 and other kinases [48] Aβ dystrophy
requires LIM kinase 1-mediated phosphorylation of ADF/cofilin
and the remodeling of the actin cytoskeleton [49]. Biotinylated
15d-PGJ₂ covalently binds to actin β in various cells [38] other
than neurons, supporting our results in neurons.

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