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Modification of ubiquitin-C-terminal hydrolase-L1 by cyclopentenone prostaglandins exacerbates hypoxic injury

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Abstract

Cyclopentenone prostaglandins (CyPGs), such as 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (15d-PGJ₂), are active prostaglandin metabolites exerting a variety of biological effects that may be important in the pathogenesis of neurological diseases. Ubiquitin-C-terminal hydrolase L1 (UCH-L1) is a brain specific deubiquitinating enzyme whose aberrant function has been linked to neurodegenerative disorders. We report that [15d-PGJ₂] detected by quadrupole mass spectrometry (MS) increases in rat brain after temporary focal ischemia, and that treatment with 15d-PGJ₂ induces accumulation of ubiquitinated proteins and exacerbates cell death in normoxic and hypoxic primary neurons. 15d-PGJ₂ covalently modifies UCH-L1 and inhibits its hydrolase activity. Pharmacologic inhibition of UCH-L1 exacerbates hypoxic neuronal death while transduction with a TAT-UCH-L1 fusion protein protects neurons from hypoxia. These studies indicate UCH-L1 function is important in hypoxic neuronal death and excessive production of CyPGs after stroke may exacerbate ischemic injury by modification and inhibition of UCH-L1.

Keywords

Ubiquitin proteasome pathway; Ubiquitin-C-terminal hydrolase L1; Cyclopentenone prostaglandins; Protein post-translational modification; Arachidonic acid; Hypoxic neuronal injury

Introduction

Expression of cyclooxygenase-2 (COX-2) is induced after cerebral ischemia and exacerbates ischemic neuronal injury through a variety of mechanisms (Nakayama *et al* 1998). PGD₂,

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the major COX-2 product synthesized in the central nervous system, is dehydrated to generate biologically active cyclopentenone prostaglandins (CyPGs) of the J₂ series, including PGJ₂, Δ^{12} -PGJ₂ and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15d-PGJ₂) (Uchida and Shibata 2008). These CyPGs are characterized by the presence of a cyclopentenone ring which can directly modify nucleophiles such as free sulfhydryls in cysteine residues of cellular proteins. While CyPGs can covalently modify cysteines in a large number of proteins, CyPGs are thought to play specific signal transduction roles by high affinity interactions with specific proteins such as PPAR γ , and regulate cell proliferation and lipid metabolism (Kliwer *et al* 1995; Shiraki *et al* 2005). However, 15d-PGJ₂ can also modify many other cellular proteins, and therefore has many PPAR γ independent effects including protein turnover inhibition, inducing cytoskeletal dysfunction and apoptosis (Ogburn and Figueiredo-Pereira 2006; Shibata *et al* 2003b; Stamatakis *et al* 2006). These PPAR γ independent effects of CyPGs may include disruption of the ubiquitin proteasome pathway (UPP) (Li *et al* 2004b).

The UPP is responsible for the degradation of mutant or misfolded proteins in cells and plays a critical role in maintaining cell homeostasis (Vernace *et al* 2007). Interruption of UPP function results in the accumulation and aggregation of ubiquitinated proteins (Ub-proteins) in cells, which are the pathological hallmark of some neurodegenerative diseases including Parkinson's disease (PD) and Alzheimer's disease (AD) (Giasson and Lee 2003; Oddo 2008). Ub-proteins also accumulate in neurons after global and focal cerebral ischemia (Ge *et al* 2007; Liu *et al* 2005), and the aggregation of Ub-proteins may contribute to cell stress following ischemia, thereby amplifying neuronal damage (Meller 2009). Ubiquitin C-terminal hydrolase L1 (UCH-L1), an important component of the neuronal UPP, is selectively expressed in brain (Setsuie and Wada 2007). Inhibition of UCH-L1 activity induces the aggregation of Ub-proteins and enhances cell death in primary neurons (Li *et al* 2004b). UCH-L1 is a major oxidative damage target in brain and that it can be modified by a variety of reagents under different pathological conditions (Choi *et al* 2004). These post-translational modifications to UCH-L1 may substantially change its structure and function; thereby disrupting the UPP function and cell survival (Choi *et al* 2004; Kabuta *et al* 2008; Liu *et al* 2009; Meray and Lansbury 2007).

While modification of UCH-L1 has been implied in the pathogenesis of some neurodegenerative diseases, its role in ischemic neuronal injury is still largely unknown. The present study aims to detect the generation of CyPGs such as 15d-PGJ₂ in brain after ischemia using highly specific MS methods. The effect of hypoxia on CyPG-protein adducts formation was determined in primary neurons using biotinylated arachidonic acid and PgD₂. Modification of UCH-L1 by 15d-PGJ₂ was studied *in vitro* and in intact primary neurons, and the effect of this modification on UCH-L1 activity and ischemic neuronal injury was assessed.

Materials and Methods

Animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Reagents and Antibodies

Free or biotinylated arachidonic acid and prostaglandins PGD₂, PGE₂, PGA₁, 15-deoxy- $\Delta^{12,14}$ -prostaglandin D₂ (15d-PGD₂), 15d-PGJ₂, and 9,10-dihydro-15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ (CAY10410) were from Cayman Chemical (Ann Arbor, MI); Anti-UCH-L1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich (St. Louis, MO); monoclonal anti-ubiquitin, anti-6-His and anti-HA antibodies were from Covance (Berkeley, CA); anti-GAPDH antibody was from Ambion (Austin, TX), while

Cy3-conjugated monoclonal mouse anti-biotin and Alexafluor 488-conjugated secondary antibodies were from Jackson ImmunoResearch Lab (West Grove, PA). Mouse monoclonal anti-mono- and poly ubiquitinated proteins antibody (clone FK2) was from Enzo Life Sciences (Plymouth Meeting, PA). LDN57444 was purchased from Calbiochem (San Diego, California) and ubiquitin AMC was from BostonBiochem (Cambridge, MA). Protein A/G beads, NeutrAvidin beads and HRP-conjugated streptavidin (streptavidin-HRP) were from Pierce (Rockford, IL). UPLC organic solvents and water were from VWR (West Chester, PA). Anti- β -actin antibody and all other chemicals were from Sigma-Aldrich.

Plasmid constructs

The DNA sequence encoding full-length rat UCH-L1 was amplified by PCR, and cloned into pET22b vector (Novagen, San Diego, CA). A UCH-L1 point mutation substituting serine for cysteine (C90S) was introduced by PCR. For recombinant transduction domain HIV-transactivator protein (TAT) tagged protein expression, rat UCH-L1 wild type (WT) and mutant UCH-L1 C90S sequences were cloned into a modified pET30a vector (provided by Drs. Jun Chen and Guodong Cao, University of Pittsburgh) containing the N-terminus TAT and HA sequences (Cao *et al* 2002). Constructs were confirmed by sequencing.

Expression and Purification of Recombinant proteins

Plasmids were introduced into Escherichia coli Rosetta (DE3) strains (Novagen, San Diego, CA) producing 6 $\times$ His-tagged full length UCH-L1 or its mutant proteins. Protein production was induced by shaking the E.Coli Rosetta cells in “overnight express autoinduction system” (Novagen, San Diego, CA) at 300 rpm for 16h, then purified with a His-select column (Sigma-Aldrich) according to the manufacturer's protocol. Protein purity was assessed by SDS-PAGE and Coomassie blue staining. Recombinant UCH-L1 protein was further confirmed by Western blot. Similar procedures were performed for expression and purification of TAT fusion proteins.

Adenovirus vector construction and generation

Adenoviruses containing mouse Wild Type COX-2 (AdV- COX-2) and enhanced green fluorescent protein (AdV-EGFP) sequences were constructed, propagated, and titered as described previously (Li *et al* 2010). Recombinant adenoviruses were generated by homologous recombination in HEK 293 cells expressing Cre recombinase (CRE8 cells) after cotransfection of DNA with an adenovirus 5-derived, E1- and E3-deleted adenoviral backbone (psi5) and pAdlox. The inserted cDNA expression was under the control of a human CMV promoter.

Cortical Primary neuronal culture

Cortical primary neuronal cultures were prepared from E17 fetal rats (Sprague-Dawley, Charles River, Wilmington, MA) as previously described (Li *et al* 2008) and used for experiments after 9 days. Cells were grown in serum-free Neurobasal medium (Invitrogen, Carlsbad, CA) supplemented with B27 and GlutaMAX (Invitrogen).

In vitro hypoxia and cell death measurements

Hypoxia was performed using a hypoxic glove box (Coy Laboratories, Grass Lake, MI) flushed with 92% argon, 5% CO₂ and 3% H₂ for 2-3 hours as described previously (Li *et al* 2008) resulting in ~40% cell death after 24h reperfusion. Staurosporin (20 μ M, a 100% cell death internal standard) and 1 μ M MK801 (a 100% cell survival internal standard) treatments were included in each cell death assay experiment. Cell death and cell viability were quantitatively assessed by measuring LDH release into the culture medium 48h after hypoxia or indirectly through the MTT cell viability assay. Cell death was also measured by

staining cells with 0.1µg/ml propidium iodide (PI) plus 0.1µg/ml Hoechst and fixed with 2% formaldehyde in PBS 24h after hypoxia. PI and Hoechst -stained cells were counted by a blinded observer from 10-12 fields of at least three cultures with a fluorescence microscope. Apoptotic cells were detected by TUNEL staining following manufacturer's directions (Apoptag Plus, Millipore, Billerica, MA). In brief, neurons were seeded on the coverslips and incubated with 20µM of 15d-PGJ₂, 15d-PGD₂, CAY10410 or vehicle together under hypoxic or normoxic conditions. Cells were fixed 24h later with 1% paraformaldehyde and ethanol:acetic acid (2:1, V:V). DNA fragments were labeled with the digoxigenin-nucleotide and detected by an fluorescein conjugated anti-digoxigenin antibody. TUNEL-positive cells undergoing apoptosis were counted as well as cells with normal rounded nuclear morphology as a measure of live cells. Data are expressed as percentage total cells.

***In vitro* binding assay**

Purified recombinant UCH-L1 protein (1 µg, ~0.4µM) was incubated with 1µM or 10µM biotinylated (b-) 15d-PGJ₂ or vehicle for 1h in 100µl of binding buffer (20mM Tris, pH7.0; 45mM NaCl; 5mM MgCl₂; 0.1mM DTT, 1% glycerol) before immunoblotting. For the competition binding assay, UCH-L1 protein was pre-incubated with 500µM of 15d-PGJ₂, CAY10410 or vehicle for 30min at room temperature (RT) before adding 5µM b-15d-PGJ₂.

Avidin pull-down assay

The avidin pull-down assay was performed as previously described (Stamatakis *et al* 2006). Primary neurons were incubated with 10µM 15d-PGJ₂ or b-15d-PGJ₂ for 2h before harvest. Equal amounts of proteins were incubated with NeutrAvidin beads for 4 h at 4 °C. Bound proteins were washed three times with lysis buffer (50mM Tris, pH7.4; 150mM NaCl; 1mM EDTA; 1% Triton X-100) before elution and detected by Western blot.

Immunoprecipitation

Primary neurons were treated with 10µM 15d-PGJ₂ or b-15d-PGJ₂ for 2h before harvesting. Cell lysates were precipitated using polyclonal anti-UCH-L1 antibody or control IgG at 4°C overnight before incubation with protein A/G beads. Immunoprecipitates were washed with lysis buffer (50mM Tris, pH7.4; 150mM NaCl; 1mM EDTA; 1% Triton X-100), eluted and immunoblotted.

Fluorescent immunocytochemistry

Fluorescent immunocytochemical staining was performed as previously described (Colgan *et al* 2007). Primary neurons were seeded on glass coverslips and incubated with 15d-PGJ₂ or methyl acetate (veh) for 24h before fixation with 4% paraformaldehyde in PBS. Cells were stained with anti-Ub-protein antibody and Alexafluor 488 conjugated secondary antibody. Coverslips were mounted on slides with Vectashield mounting medium with DAPI and confocal images were acquired with a FluoView FV1000 confocal microscope (Olympus, Tokyo, Japan). For UCH-L1 and 15d-PGJ₂ localization, primary neurons were treated with b-15d-PGJ₂ for 2h before fixation then double stained with anti-UCH-L1 and Cy3-conjugated anti-biotin antibodies.

***In vitro* hydrolase activity assay**

The UCH-L1 hydrolase activity assay was performed as previously described with slight modifications (Liu *et al* 2002). Briefly, 2 µM UCH-L1 protein was incubated with various concentrations of 15d-PGJ₂ or vehicle for 2h before dilution with UCH hydrolase buffer (50mM Tris, pH7.6; 0.5mM EDTA, 0.1mg/ml ovalbumin, 5mM DTT). Samples were then mixed with 500nM ubiquitin-AMC. The final concentration for UCH-L1 was 100nM. Free AMC fluorophore was read using a fluorescence plate reader (ex 360nm, em 460 nm).

UCH-L1 C90S mutant protein (100 nM) lacking hydrolase activity (Gong *et al* 2006) was included as a negative control.

Middle Cerebral Artery Occlusion (MCAO)

MCAO was performed as previously described (Ahmad *et al* 2009) for a duration of 90 min. A silicone coated 4.0 nylon filament was advanced 17mm into the internal carotid artery from the bifurcation of internal carotid artery and external carotid artery. Sham surgeries were performed identically without insertion of suture. 24 h after reperfusion, rats were sacrificed and their brains were processed for CyPG measurement.

Capillary LC ESI-TOF MS analysis

UCH-L1 and 15d-PGJ₂ modified UCH-L1 was analyzed using capillary LC ESI-TOF MS. UCH-L1 protein was incubated with 15d-PGJ₂, arachidonic acid, PGD₂ or methyl acetate (vehicle) in 1XPBS at RT for 2h. Samples were diluted in 25mM ammonium bicarbonate before loading on the C4 precolumn (Acclaim PepMap300 C4, 5μm, 300A, 300μm i.d. × 5 mm, Dionex) of the LC system (Ultimate 3000, Dionex, Sunnyvale, CA). The LC system was directly coupled to an electrospray ionization time-of-flight mass spectrometer (micrOTOF, BrukerDaltonics, Billerica, MA). Desalting was performed with 2.5% acetonitrile, 0.1% formic acid followed by elution with 80% acetonitrile, 0.1% formic acid. Mass spectra were acquired in positive ion mode over the mass range *m/z* 500 to 3000. ESI spectra were deconvoluted to obtain molecular ion masses with DataAnalysis 3.3 (Bruker Daltonics, Billerica, MA).

Measurement of 15d-PGJ₂ in rat brain

15d-PGJ₂ was measured by quadrupole liquid MS as previously described (Miller *et al* 2009). Samples were homogenized in 0.12 M potassium phosphate buffer (5mM MgCl₂ and 0.113 mM BHT) and centrifuged. The supernatant was removed and 7.5 ng of deuterated 15d-PGD₂ was used as the internal standard. Samples were loaded onto Oasis HLB solid phase extraction cartridges (Waters, Acquity, Milford, MA) and washed with 1 ml volumes of 5% methanol before elution with 100% methanol. Extracts were dried under nitrogen and reconstituted in 125 μL of 80:20 methanol:de-ionized water. 15d-PGJ₂ was separated via reverse phase UPLC with a Acquity UPLC BEH C18 1.7μm, 2.1 × 100 mm column. MS analysis was performed via a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (ThermoFisher Scientific). MRM transition of *m/z* was 316.1 to 272 with a collision energy of 13 eV and a scan time 0.01 seconds. Parameters were optimized to obtain the highest [M-H]⁺ ion abundance. Data was acquired using Xcalibur Software version 2.0.6.

Statistical analysis

15d-PGJ₂ concentrations were analyzed using Student's T test; all other data were analyzed using one-way ANOVA to calculate differences between groups. Results were considered to be significant when *p* < 0.05.

Results

15d- PGJ₂ concentration is increased in rat brain after temporary focal ischemia

The triple quadrupole MS/MS daughter ions of 15d-PGJ₂ were identified and used to quantify 15d-PGJ₂ concentration in rat brain via selected reaction monitoring. 15d-PGJ₂ produced a unique MS/MS fragmentation pattern that includes an abundant parent ion at *m/z* 316 (Fig 1A) and a major daughter ion at *m/z* 271. 15d-PGJ₂ was quantified by selective reaction monitoring of the *m/z* 271 fragment of the 316 *m/z* parent 15d-PGJ₂ ion. Additional

selectivity was afforded by ultra performance liquid chromatography (UPLC) separation of 15d-PGJ₂, eluting at 3.1 minutes as determined from reference standards. Brain tissue extracts from sham surgery and MCAO animals demonstrated that free endogenous 15d-PGJ₂ was below detection limits in sham surgery brain but was quantifiable in ischemic hemisphere 24 h after 90 min of middle cerebral artery occlusion (MCAO), at concentrations of 23.72 ± 6.48 nM (Fig 1B).

15d-PGJ₂ increases ubiquitinated protein accumulation and induces neuronal cell death

Treatment of primary neurons with 15d-PGJ₂ resulted in a dose dependent increase in the accumulation of Ub-proteins compared to vehicle controls (Fig 2A, left). The distribution of Ub-proteins in the neuron was altered 24h after incubation with 15d-PGJ₂ as detected by fluorescent immunocytochemistry: Ub-proteins were distributed evenly throughout the cytoplasm and were seen as small discrete punctate structures in control neurons, while large perinuclear aggregates containing Ub-proteins were frequently observed in neurons treated with 20 μ M 15d-PGJ₂ (Fig 2A, right). Thus, 15d-PGJ₂ treatment interferes with the UPP's function in primary neurons.

To test whether 15d-PGJ₂ treatment exacerbates hypoxic neuronal cell death, primary neurons were pre-incubated with 15d-PGJ₂ for 1h before hypoxia. Cell death was measured 48h after hypoxia. 15d-PGJ₂ exacerbated hypoxic neuronal cell death (Fig. 2B) in a dose dependent manner with 20 μ M 15d-PGJ₂ treatment significantly increasing cell death. 15d-PGJ₂ treatment also induced primary neuronal cell death under normoxic conditions. There was a significant increase in the percentage of PI stained cells in 15d-PGJ₂ treated cells as compared to vehicle controls (Fig 2D) further supporting that incubation with 20 μ M 15d-PGJ₂ increases cell death under both hypoxic and normoxic conditions.

The above data indicate that 15d-PGJ₂ impairs the UPP's function and exacerbates cell death in primary neurons. To test whether these effects are mediated through a PPAR γ dependent mechanism, two analogs of 15d-PGJ₂, CAY10410 (CAY) and 15-deoxy- $\Delta^{12,14}$ -prostaglandin D₂ (15d-PGD₂), were used to treat primary neurons. CAY10410 has a similar structure to 15d-PGJ₂ but lacks the reactive cyclopentenone ring, retaining PPAR γ ligand activity (Shibata *et al* 2003a); 15d-PGD₂ does not have the cyclopentenone ring structure nor can it activate PPAR γ . Primary neurons were incubated with CAY10410 or 15d-PGD₂ at the indicated concentrations for 24h, Ub-proteins were detected (Fig 2C, left) and cell death was measured (Fig 2C, right). In contrast to 15d-PGJ₂, neither CAY10410 nor 15d-PGD₂ treatment increased Ub-protein accumulation or exacerbated hypoxic neuronal cell death. Additionally, there was no significant difference in the percentage of PI stained cells in 20 μ M CAY10410 treated cells compared to vehicle control under hypoxic or normoxic conditions (Fig 2D, a-b). 15d-PGJ₂-treated, but not 15d-PGD₂- or CAY10410- treated cultures had a significantly lower percentage of cells with normal nuclear morphology as determined by DAPI staining compared to vehicle controls (Fig 2D,c). In the TUNEL assay, 15d-PGJ₂ – treated normoxic cultures had significantly more TUNEL-positive cells compared to vehicle controls ($22.7 \pm 2.2\%$ vs $12.7 \pm 1.0\%$, $P < .05$); there was no increase in TUNEL-positive cell percentage when treated with CAY10410 or 15d-PGD₂. No significant difference in TUNEL staining was observed in hypoxic cells between any of the treatment groups. 15d-PGJ₂ induces predominantly PI- rather than TUNEL staining in neurons, particularly after hypoxia, consistent with a primarily necrotic mechanism of cell death. These results demonstrate that 15d-PGJ₂ impairs the neuronal UPP and exacerbates neuronal death through a PPAR γ independent and cyclopentenone ring dependent mechanism.

15d-PGJ₂ covalently modifies UCH-L1 *in vitro* and in intact primary neurons

UCH-L1 is a brain specific protein that plays an important role in the neuronal UPP as a deubiquitinating enzyme (Setsuie and Wada 2007). Its modification and malfunction is associated with the accumulation of Ub-proteins and cell death in neurons. Because CyPGs modify protein cysteine residues through Michael additions, and UCH-L1 contains 6 cysteine residues, it is possible UCH-L1 could be directly modified by 15d-PGJ₂. To test this hypothesis, we first performed an *in vitro* binding assay using recombinant UCH-L1 protein and biotinylated 15d-PGJ₂ (b-15d-PGJ₂). Recombinant UCH-L1 protein (1 µg) was incubated with 1 µM or 10 µM b-15d-PGJ₂ or vehicle (Veh) for 1 hour. Biotin signals were detected with immunoblotting and streptavidin-HRP. A band representing the b-15d-PGJ₂-UCH-L1 adduct was detected and increased in a dose-dependent manner (Fig. 3A, top). Binding specificity was further assessed using a competition assay. UCH-L1 was incubated with 100 fold excess unlabeled 15d-PGJ₂ or its analog, CAY10410, for 30 min before b-15d-PGJ₂ was added. As shown in Fig 3A (bottom), the b-15d-PGJ₂-UCH-L1 band was greatly attenuated after incubation with excess unlabeled 15d-PGJ₂ but not CAY10410. Taken together, these results indicate that 15d-PGJ₂ directly modifies UCH-L1, and the reactive α,β -unsaturated carbon in the cyclopentenone ring is required for this protein modification.

Next, ESI-MS analysis was performed on recombinant UCH-L1 incubated with 15d-PGJ₂. As shown in Fig 3B, the mass detected for free UCH-L1 is 25903 Da, whereas incubation of UCH-L1 with 15d-PGJ₂ at a 1:1 ratio results in a second peak at 26220 Da, corresponding to the incorporation of one 15d-PGJ₂ molecule into the UCH-L1 protein. However, incubation of UCH-L1 with arachidonic acid or other prostaglandins lacking a cyclopentenone ring structure, including PGD₂ and 15d-PGD₂ (data not shown), only resulted in a single peak of 25903 Da, the same as free UCH-L1. These results further support that UCH-L1 is directly modified by 15d-PGJ₂.

To test whether endogenous UCH-L1 in intact neurons is modified by 15d-PGJ₂ in the presence of other cellular proteins, we incubated primary neurons with either 15d-PGJ₂ or b-15d-PGJ₂ for 2 hours followed by avidin pull-down and immunoprecipitation assays. Cell lysates were incubated with NeutrAvidin beads, and biotinylated proteins bound to NeutrAvidin beads were then eluted and immunoblotted. As shown in Fig 3C (top), UCH-L1 was detected in the pull-down eluent only of the b-15d-PGJ₂ treated group, whereas it could be detected in the cell lysates of both groups (Fig 3C, center). Using an immunoprecipitation assay, the b-15d-PGJ₂-UCH-L1 complex was precipitated by an anti-UCH-L1 antibody (U) and protein A/G beads using normal rabbit IgG (I) as a negative control. The complex was eluted and detected with streptavidin-HRP by Western blot. A band with the expected molecular weight was detected only in the b-15d-PGJ₂ treated group (bottom, Fig 3C), confirming that direct interaction between b-15d-PGJ₂ and endogenous UCH-L1 occurs within the intact neuron.

Colocalization of 15d-PGJ₂ and endogenous UCH-L1 within the cell is shown in Fig 3D. Both UCH-L1 (a) and 15d-PGJ₂ (b) were primarily located in the cytoplasm and neurites, and to a lesser extent, in the peri-nuclear region in b-15d-PGJ₂-incubated neurons. 15d-PGJ₂ extensively co-localized with UCH-L1 (d).

15d-PGJ₂ modification inhibits UCH-L1 hydrolase activity

As a deubiquitinating enzyme, UCH-L1 has hydrolase activity which cleaves ubiquitin from ubiquitinated peptides for later reuse. To test whether 15d-PGJ₂ modification interferes with UCH-L1 hydrolase activity, an *in vitro* hydrolase activity assay was performed using ubiquitin-AMC, a substrate that fluoresces when hydrolyzed by UCH-L1. Recombinant

UCH-L1 was incubated with 15d-PGJ₂, CAY10410, or vehicle and fluorogenic AMC was detected by spectrofluorometry after cleavage from the ubiquitin-AMC molecule. Incubation with 0.5 μ M 15d-PGJ₂ significantly decreased the rate of UCH-L1 hydrolysis of ubiquitin-AMC compared to vehicle control (Fig 4); furthermore, incubation with 12.5 μ M 15d-PGJ₂ reduced hydrolase activity to that of inactive mutant UCH-L1 C90S protein. However, incubation with CAY10410 had no inhibitory effect on UCH-L1 hydrolase activity. Incubation of ubiquitin-AMC with free 15d-PGJ₂ (without UCH-L1) as a negative control resulted in no detectable change in fluorescence (data not shown). Taken together, these data indicate that covalent modification of UCH-L1 by 15d-PGJ₂ leads to significant loss of the enzyme's hydrolase activity.

Impaired UCH-L1 hydrolase activity exacerbates hypoxic neuronal death, while delivery of UCH-L1 fusion protein protects against hypoxic injury

UCH-L1 dysfunction has been associated with the accumulation of Ub-proteins and cell death in neurons. However, its role in hypoxic neuronal injury is unclear. LDN 57444 (LDN), a specific and competitive ubiquitin C-terminal hydrolase inhibitor with an IC₅₀ of 0.88 μ M for UCH-L1 and 25 μ M for UCH-L3 (Liu *et al* 2003), was used to test the role of UCH-L1 activity in hypoxic injury. Primary neurons were incubated with 0.5–4 μ M LDN or vehicle prior to hypoxia and cell death was determined. Incubation with LDN significantly exacerbated hypoxic neuronal death compared to vehicle control in a dose dependent manner (Fig 5A, right). However, incubation of primary neurons with 20 μ M LDN alone under normoxic conditions had no effect upon cell death (Fig 5A, left). Thus inhibition of endogenous UCH-L1 activity with LDN exacerbates hypoxic neuronal cell death suggesting that UCH-L1 hydrolase activity is important for cell survival under conditions such as hypoxia. Consistent with the cell death effect, treatment with LDN also increased accumulation of Ub-proteins in primary neurons after hypoxia, whereas the same treatment failed to show any significant change under normoxic conditions (Fig 5B). Thus, inhibition of UCH-L1 hydrolase activity with LDN impairs the UPP resulting in accumulation of Ub-proteins and exacerbates cell death in hypoxic neuronal cells.

To further confirm that UCH-L1 activity protects against hypoxic neuronal injury, we constructed TAT-UCH-L1 wild type (WT) and mutant TAT-UCH-L1 C90S fusion proteins to transduce primary neurons. Both proteins contain a 6 $\times$ His tag for purification and an HA tag for detection (Fig 5C, top). Construction and expression of the fusion proteins were confirmed by immunoblotting using both anti-UCH-L1 and anti-HA antibodies. A hydrolase activity assay performed using both TAT fusion proteins resulted in similar hydrolase activities to those of native recombinant UCH-L1 or UCH-L1-C90S proteins (data not shown). The ability of TAT fusion proteins to transduce primary neuronal cells was evaluated by immunoblotting and immunostaining primary neurons incubated with TAT-UCH-L1 WT and TAT-UCH-L1 C90S fusion proteins. TAT fusion proteins were detectable in cell lysates by immunoblotting (Fig 5C, bottom left). Immunostaining of primary neurons with an anti-HA antibody shows that TAT-UCH-L1 enters neurons at a transduction efficiency of about 80% (Fig 5C, bottom right). These data indicate that these TAT fusion proteins are biologically active and are able to transduce primary neurons.

To test the effect of overexpression UCH-L1 on hypoxic neuronal cell death, primary neurons were incubated with TAT-UCH-L1 WT or TAT-UCH-L1 C90S fusion proteins for 1h prior to hypoxia. Cell death was determined 24h post reperfusion. Incubation with TAT-UCH-L1 WT fusion protein significantly decreased hypoxic neuronal death in a dose dependent manner, whereas incubation with the inactive mutant, TAT-UCH-L1 C90S fusion protein had no effect on cell death as compared to vehicle control (Fig 5D). These data further support the hypothesis that UCH-L1 hydrolase activity is a significant determinant of hypoxic neuronal cell death.

Modification of UCH-L1 by arachidonic acid and PGD₂ metabolites is increased after hypoxia in the primary neurons

To test whether arachidonic acid or its metabolites covalently modify endogenous proteins including UCH-L1 in primary neurons, primary neurons were incubated with biotinylated arachidonic acid or biotinylated prostaglandins PGD₂, PGE₂, 15d-PGJ₂ or PGA₁ under normoxic conditions for 2h. Biotinylated proteins in cell lysates were then detected by Western blot and were detected only in those neurons treated with biotinylated CyPGs 15d-PGJ₂ and PGA₁, but not in cells treated with biotinylated arachidonic acid or biotinylated prostaglandins lacking the cyclopentenone moiety such as PGD₂ and PGE₂ (Fig 6A). These data further support the necessity of the cyclopentenone ring for prostaglandin-protein modifications.

Our *in vivo* data has shown that the concentration of 15d-PGJ₂ significantly increased after brain ischemia (Fig1), indicating that 15d-PGJ₂-UCH-L1 modification as well as other CyPG-protein modifications in neuronal cells might also increase after hypoxia. To test whether arachidonic acid metabolites increase protein modifications in hypoxic neurons, primary neurons were incubated with 20μM biotinylated arachidonic acid and subjected to hypoxic or normoxic conditions for 3h. Cell lysate immunoblots show a progressive increase in biotinylated proteins at 3h and 6h after hypoxia (Fig 6B, center), while UCH-L1 protein levels remain constant. Cell lysates were also incubated with NeutrAvidin beads and bound proteins were eluted and immunoblotted. A band corresponding to biotinylated UCH-L1 was detected in increasing intensity at 3 and 6 h after hypoxia, but not in normoxic samples (Fig 6B, top). Since arachidonic acid itself as well as other prostaglandins without a cyclopentenone ring can't modify UCH-L1 or other endogenous proteins directly, this increased formation of biotinylated UCH-L1 is likely due to the enhanced generation of CyPGs after hypoxia. Because the conversion of arachidonic acid to prostaglandins and CyPGs is COX-2 activity related, the amount of neuronal proteins modified by arachidonic acid metabolites in hypoxic neuronal cells should also be COX-2 activity related. To test this hypothesis, adenoviral vector containing the wild-type COX-2 gene sequence (AdV-COX-2) was used to overexpress the COX-2 protein in primary neurons. A similar adenoviral vector containing EGFP (AdV-EGFP) was used as control. 72h after infection, the neurons were incubated with 20μM biotinylated arachidonic acid and subjected to hypoxia or normoxia. Cells were harvested 3h after hypoxia and biotin incorporated proteins were detected (Fig. 5C). Consistent with the previous experiment, hypoxia enhanced biotin-protein adduct formation compared to normoxic neurons. Furthermore, higher levels of biotinylated protein accumulation were detected in the AdV-COX-2 infected neurons as compared to AdV-EGFP infected neurons or vehicle treated controls, suggesting that increased COX-2 activity enhances the formation of arachidonic acid metabolite-modified proteins in primary neurons. Additionally, the selective COX-2 inhibitor, rofecoxib, was applied to primary neurons prior to incubation with biotinylated arachidonic acid and hypoxia. Rofecoxib significantly reduced biotinylated protein formation in neurons harvested 3h after hypoxia (Fig 6D). These experiments show that arachidonic acid metabolites modify endogenous proteins including UCH-L1 in primary neurons and that the extent of this modification is increased after hypoxia in a COX-2 activity related manner.

PGD₂ is the immediate precursor to CyPGs of the J₂ series including 15d-PGJ₂. Primary neurons were incubated with 10 μM biotinylated PGD₂ under hypoxic or normoxic conditions (Fig 6E). Cells were harvested 0 and 24h after hypoxia and biotinylated proteins were detected. Biotinylated proteins were not detected at 0h in either group; however, they were detected 24h post treatment and more so in hypoxic neurons. Similarly, biotinylated UCH-L1, detected by anti-UCH-L1 antibody after pull down with NeutrAvidin beads, was not found in normoxic controls but was detected in cells harvested 24h after hypoxia (Fig 6E, bottom). These results suggest that UCH-L1 as well as other endogenous neuronal

proteins are modified by PGD₂ metabolites such as CyPGs of the J₂ series, and that this modification is increased by hypoxia. Because hypoxia did not alter the expression of UCH-L1, the increased biotinylated UCH-L1 formation after hypoxia most likely is due to increased CyPGs metabolized from PGD₂.

Discussion

A number of studies have demonstrated that CyPGs such as 15d-PGJ₂ may modify important cellular proteins, including a number of proteins that regulate neuronal cell death and survival (Kondo *et al* 2002a; Kondo *et al* 2002b; Satoh and Lipton 2007; Uchida and Shibata 2008). Thus it has been proposed that CyPGs may play an important role in the pathogenesis of neurological disorders such as stroke, PD and AD (Milne *et al* 2005; Musiek *et al* 2006). However, the physiological relevance of CyPGs is uncertain since brain concentrations of CyPGs such as 15d-PGJ₂ under these pathological conditions have not previously been determined.

Several groups have tried to detect endogenous 15d-PGJ₂ and measure its concentration *in vivo*. Using an anti-15d-PGJ₂ antibody and immunohistochemistry, Shibata reported the existence of endogenous 15d-PGJ₂ both in macrophages and in motor neurons in spinal cords from amyotrophic lateral sclerosis patients (Shibata *et al* 2002). Antibody based Enzyme Immunoassay (EIA) was used by several groups to detect changes in 15d-PGJ₂ levels under various pathological conditions. Restraint stress induces a COX-2-dependent increase of 15d-PGJ₂ in rat brain cortex (Garcia-Bueno *et al* 2005) while cortical spreading depression induced by topical KCl also significantly increased 15d-PGJ₂ levels in rat cerebrospinal fluid (Horiguchi *et al* 2006). Recently, Lin's group showed that Adv-COX1 infusion 72h before 50 min MCAO resulted in increased 15d-PGJ₂ levels in rat brain ischemic tissues; however, 15d-PGJ₂ levels in the uninfected control ischemic brain were not reported (Lin *et al* 2006). The above data all rely on antibody specificity, so the cross reactivity of the antibody for a number of structures closely related eicosanoid compounds such as PGJ₂ and Δ^{12} -PGJ₂ is always a concern.

PGD₂, the precursor of 15d-PGJ₂, is the major COX-2 produced prostaglandin in the brain (Uchida and Shibata 2008). COX-2 expression and production of PGD₂ is increased after ischemia (Li *et al* 2008; Nakayama *et al* 1998). Thus, one would expect that 15d-PGJ₂ concentrations may be increased in brain after ischemia. To test this hypothesis, quadrupole liquid chromatography mass spectrometry was used to detect and quantify free endogenous 15d-PGJ₂ in brain lysates from a focal cerebral ischemic model induced by 90 min of MCAO. Free endogenous 15d-PGJ₂ was undetectable in normal rat brain, but was detected in ischemic hemispheres 24h after MCAO. To our knowledge, this is the first report confirming the presence and the increase of the endogenous CyPG 15d-PGJ₂ in ischemic brain using a highly specific antibody-independent method. Whole hemisphere concentrations of 15d-PGJ₂ (~24 nM) detected in these experiments are significantly less than the concentrations required to produce toxicity in primary neuronal culture; however, whole hemisphere concentrations may not reflect intraneuronal concentrations in the penumbral region where COX-2 expression is selectively induced within neurons after ischemia. Additionally, 15d-PGJ₂ is highly reactive, and may readily bind to sulfhydryl-containing proteins. Thus, total hemispheric free 15d-PGJ₂ concentration may be much less than its concentration within the ischemic neuron in the penumbra.

The increase in [15d-PGJ₂] after ischemia may be due to increased expression of COX-2 resulting in increased production of PGD₂, or the increased conversion of PGD₂ to CyPGs after hypoxia/ischemia, or both. To explore these mechanisms, primary neurons were incubated with biotinylated arachidonic acid or PGD₂ and subjected to hypoxia. Binding of

biotinylated arachidonic acid metabolites to endogenous proteins significantly increased after hypoxia, concomitant with increased COX-2 activity. Incubation with biotinylated PGD₂ also produced similar changes. These results indicate that the increased generation of CyPGs in neurons after hypoxia may be the result of both increased COX-2 activity and an increased rate of dehydration of PGD₂ to CyPGs. Arachidonic acid, PGD₂ and PGE₂ don't modify proteins directly; thus, the biotinylated proteins detected here most likely represent modification from CyPGs.

CyPGs have been shown to have both protective and injurious effects in neural systems. A number of studies have shown that 15d-PGJ₂ and other CyPGs such as PGJ₂ and Δ^{12} -PGJ₂ can induce apoptosis in a range of cell systems, including neuroblastoma SH-SY5Y cells, mouse oligodendrocytes, rat mesencephalic primary cells and primary cortical neurons (Kim *et al* 1993; Kondo *et al* 2002a; Li *et al* 2004a; Rohn *et al* 2001; Saito *et al* 2003). Selective dopaminergic neuron loss in the substantia nigra and the formation of Ub-protein aggregates in spared neurons in PGJ₂ microinjected mice has been reported, further confirming the potential neurotoxic effects of CyPGs *in vivo* (Pierre *et al* 2009). Other studies suggest that 15d-PGJ₂ may be neuroprotective in some ischemia models via anti-inflammatory effects mediated by PPAR γ receptor activation (Lin *et al* 2006; Pereira *et al* 2006; Zhao *et al* 2006). To determine whether increased 15d-PGJ₂ is either protective or injurious to neuronal cells *in vitro*, primary neurons were incubated with 15d-PGJ₂ at varying concentrations under normoxic and hypoxic conditions. 15d-PGJ₂ significantly increased neuronal cell death accompanied with the accumulation of Ub-proteins in surviving cells. Significant neuroprotection was not observed at any dose. Furthermore, the present study demonstrates that the neurotoxic effect of 15d-PGJ₂ is mediated through a PPAR γ independent mechanism, since incubation with CAY10410, a structural analogue of 15d-PGJ₂ and a PPAR γ agonist lacking an electrophilic carbon, failed to induce 15d-PGJ₂ - like effects. Similarly, another 15d-PGJ₂ analog, 15d-PGD₂ which lacks the cyclopentenone ring structure and PPAR γ agonist activity, also failed to show neurotoxic effects in the same dose range. Altogether, these results indicate that the electrophilic nature of 15d-PGJ₂, which enables covalent modification of many proteins, is responsible for its neurotoxic effects.

An important intracellular target of CyPGs might be UCH-L1, one of the deubiquitinating enzymes selectively and highly expressed in brain. It has been reported that Δ^{12} -PGJ₂, another CyPG, inhibits UCH-L1 activity *in vitro* and disrupts the UPP, resulting in accumulation of Ub-proteins and cell death in neuroblastoma cells and primary mesencephalic neurons (Li *et al* 2004b). In the present study, covalent modification and inhibition of UCH-L1 by 15d-PGJ₂ has been confirmed using recombinant protein and in intact neurons through various approaches. Aberrant modification and malfunction of UCH-L1 has been implicated in the pathogenesis of several neurodegenerative diseases including PD and AD (Choi *et al* 2004; Liu *et al* 2009). However, its role in ischemic neuronal injury is still largely unknown and controversial. In the current study, two complementary approaches were applied to test the role of UCH-L1 function in ischemic neuronal death. First, primary neurons were incubated with the specific UCH inhibitor LDN, to inhibit endogenous UCH-L1 activity. Second, exogenous UCH-L1 was delivered into neurons by cell-permeable TAT-UCH-L1 fusion proteins. The protective effect of TAT-UCH-L1 WT and the exacerbating effect of LDN on hypoxic neuronal injury suggest that UCH-L1 plays an important role in maintaining cell homeostasis and viability after hypoxia. This finding is consistent with recent findings that UCH-L1 function is important for synaptic transmission and neuronal function (Gong *et al* 2006; Sakurai *et al* 2008) while down-regulation of UCH-L1 by antisense cDNA in mouse neuroblastoma cells increased the cell's sensitivity to oxygen-glucose deprivation treatment (Shan *et al* 2004). Koharudin *et al* found that covalent modification of UCH-L1 by CyPGs dramatically disrupts the structure of UCH-L1 as determined by NMR spectroscopy, resulting in unfolding and aggregation of the protein.

This CyPG effect on UCH-L1 structure is dependent upon a single highly conserved cysteine residue in UCH-L1, suggesting that CyPG binding to UCH-L1 may have important functional effects on UCH-L1 function and be an important signaling mechanism (Koharudin *et al* 2010). Thus the results from the current study and others suggest that modification and inhibition of UCH-L1 by CyPGs may have important effects on UCH-L1 function and increase the susceptibility of neurons to hypoxic and other pathogenic stresses.

15d-PGJ₂ has been reported to adduct a number of other important cellular proteins including glutathione-S-transferase P1, thioredoxin, heat-shock protein 90 and some cytoskeletal proteins such as actin, tubulin and vimentin (Aldini *et al* 2007; Ogburn and Figueiredo-Pereira 2006; Sanchez-Gomez *et al* 2007; Shibata *et al* 2003a), and our results indicate that CyPGs may have increased binding to many other proteins after hypoxia. However, it is noteworthy that not every protein containing cysteine residues can be modified by 15d-PGJ₂, and not every modification will cause inactivation of the target protein as occurs with UCH-L1 (Koharudin *et al* 2010; Renedo *et al* 2007). Furthermore, 15d-PGJ₂-induced neurotoxicity could involve mechanisms in addition to UCH-L1 modification. For instance, another CyPG, PGJ₂, was reported to affect UPP function by alteration of expression and function of proteasome components and sequestosome (Wang and Figueiredo-Pereira 2005; Wang *et al* 2006).

In summary, our results show that inhibition of UCH-L1 activity exacerbates hypoxic neuronal injury in primary neuronal culture. We found that endogenous [15d-PGJ₂] is increased in ischemic brain and that 15d-PGJ₂ binds to UCH-L1 and inhibits its activity *in vitro*. Inhibition of endogenous UCH-L1 by LDN exacerbates hypoxic cell death and results in accumulation of Ub-proteins in hypoxic neurons. 15d-PGJ₂ treatment induces apoptosis and accumulation of Ub-proteins in primary neurons. These results suggest that CyPGs produced during hypoxia or ischemia can inhibit UCH-L1 activity and exacerbate neuronal injury by disruption of the UPP resulting in accumulation of Ub-proteins. Malfunction of UCH-L1, interruption of the UPP and accumulation of Ub-proteins in brain has also been implicated in the pathogenesis of neurodegenerative disorders including Parkinson's disease. Thus, agents that compete for the CyPG binding site on UCH-L1 could have utility in the treatment of stroke and neurodegenerative diseases. To further evaluate this hypothesis, additional studies involving pharmacological inhibition of UCH-L1 in stroke models are necessary to confirm that UCH-L1 activity plays an important role in determining cell death and outcome *in vivo*. Additional studies are required to confirm that CyPGs bind to UCH-L1 *in vivo*.

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Figure 1. [15-deoxy- $\Delta^{12,14}$ -PGJ₂(15d-PGJ₂)] in rat brain after middle cerebral artery occlusion (MCAO)

A. MS/MS fragmentation pattern of 15d-PGJ₂ authentic standard with a Parent Mass (m/z) of 316. The prevalent 271m/z daughter Ion was used to quantify 15d-PGJ₂ using quadrupole MS/MS with selective reaction monitoring. Arrow (inset) indicates fragmentation point of parent ion. B. 15d-PGJ₂ concentration in rat brain hemisphere 24h after 90 minutes MCAO. Data are expressed as means $\pm$ SE. *P < .05. n=4-6.

Figure 2. Effect of treatment with 15d-PGJ₂ upon accumulation of Ub-proteins and cell death in primary neuronal culture

A. *left*: Primary neurons were incubated with 15d-PGJ₂ for 24h then harvested. Ub-proteins were detected by IB with anti-ubiquitin antibody. β-actin served as a loading control. *right*: representative confocal images (240×) of primary neurons after incubation with vehicle (veh) or 20 μM 15d-PGJ₂ for 24h prior to staining with anti-ubiquitinated protein antibody paired with FITC-conjugated secondary antibody (green) and DAPI (blue). B. Primary neurons were incubated with vehicle or 15d-PGJ₂ for 1 h prior to normoxia (a,b) or hypoxia (c,d). Cell death was measured using LDH (a,c) and MTT (b,c) methods. n = 6 wells per group. * P < 0.02. C. *left*: Primary neurons were incubated with vehicle, CAY10410 or 15d-PGD₂ for 24h prior to harvest. Ub-proteins were detected by IB with anti-ubiquitin antibody. n = 6 per group. *right*: Primary neurons were treated with CAY10410 or 15d-PGD₂ 1 h prior to hypoxia. Cell death was measured using the LDH method. D: Primary neurons were incubated with vehicle, 15d-PGJ₂ or CAY10410 (20 μM) for 24h prior to hypoxia or normoxia treatment. 24 h later, cells were stained with propidium iodide (PI) and Hoechst. a: PI-positive cells were counted and cell death was calculated as percent total cells (Hoechst). n = 10 – 12 per group. b: representative photos of Hoechst and PI-Hoechst overlay stained cells. Single arrows indicate representative cells with apoptotic morphology; double arrows are representative cells exhibiting rounded morphology. c: Percentage cells with normal morphology in normoxic (top) and hypoxic (bottom) groups treated with 20 μM of 15d-PGJ₂, 15d-PGD₂, CAY10410 or vehicle. * P < 0.001; ** < 0.01; # < .05; NS: not significant. Data are expressed as means ± SE. IB: immunoblot; MK: 1 μM MK801; SP: 20 μM staurosporin; Ub-Proteins: Ubiquitinated proteins; Veh: vehicle.

Figure 3. Covalent modification of UCH-L1 by 15d-PGJ₂ *in vitro* and in primary neurons

A. upper: Recombinant UCH-L1 protein (1 µg) was incubated with biotinylated (b-) 15d-PGJ₂ or vehicle (ethanol, Veh) for 2h. b-15d-PGJ₂-UCH-L1 adducts were detected by immunoblotting with streptavidin-HRP (Str-H). **lower:** Recombinant UCH-L1 protein (1 µg) was preincubated with methyl acetate (Veh), 500 µM 15d-PGJ₂ or CAY10410 for 30 min before incubation with 5 µM b-15d-PGJ₂ and b-15d-PGJ₂-UCH-L1 adduct in each reaction were detected. **B.** Capillary LC ESI-TOF MS analysis of UCH-L1 incubated with Vehicle (Veh), arachidonic acid (AA), PGD₂ and 15d-PGJ₂. Charge state distribution (left) and deconvoluted molecular ion mass spectra (right) are shown. **C.** Primary neurons were incubated with 10 µM 15d-PGJ₂ or b-15d-PGJ₂ for 2h before harvest. The b-15d-PGJ₂-UCH-L1 adducts in cell lysates were detected after Avidin pull-down or immunoprecipitation assays. **Top:** Avidin pull-down assay: Proteins bound to NeutrAvidin beads were separated by SDS-PAGE and blotted with anti-UCH-L1 antibody. **Center:** Immunoblots of cell lysates probed with Streptavidin-HRP (Str-H) and anti-UCH-L1 antibody. **Bottom:** IP assay: Primary neuron cell lysates were immunoprecipitated with anti-UCH-L1 antibody (U) or control IgG (I) and protein A/G beads. Immunoprecipitates were separated and probed with Streptavidin-HRP to detect b-15d-PGJ₂-UCH-L1 adducts. **D.** Immunocytochemical staining of primary neurons after 2h incubation with 10 µM b-15d-PGJ₂. Representative photos of: a) endogenous UCHL-1 distribution using FITC conjugated anti-UCHL-1 antibody. b) b-15d-PGJ₂ distribution using Cy3 conjugated anti-biotin antibody. c) DAPI staining of nuclei. d) Merged picture. Photos at 60×. IB: Immunoblot; IP: immunoprecipitation.

Figure 4. Effect of 15d-PGJ₂ on UCH-L1 hydrolase activity

1 μ g of recombinant UCH-L1 was incubated with 15d-PGJ₂, CAY10410 or vehicle (Veh, methyl acetate) at indicated concentrations. Hydrolase activity was measured using the fluorescent substrate ubiquitin-AMC. Fluorescence intensity (arbitrary units, λ_{ex} =360nm and λ_{em} =460nm) produced by cleavage of ubiquitin-AMC was recorded at indicated time points. Mutant UCH-L1 C90S lacking hydrolase activity was included as a negative control. Data shown is the mean of the experiment with two replicates. Veh: vehicle; C90S: recombinant UCH-L1 C90S protein.

Figure 5. Effect of UCH-L1 activity on hypoxic neuronal cell death and Ub-protein accumulation in primary neurons

A. Primary neurons were treated with vehicle (DMSO), LDN 57444 or untreated for 1 hour, then underwent normoxia (left) or 3h hypoxia (right) then returned to normoxic conditions for 24h. Cell death was measured by LDH assay. N= 6 wells per group. Data are expressed as means $\pm$ SE. * P < 0.01; ** < 0.05 vs vehicle treated group. B. Representative immunoblots of primary neuronal cell lysates (treated as in A) probed with anti-ubiquitin and anti-actin (loading control) antibodies. *right*: graphic illustration of western blot ubiquitinated protein lane densities expressed as % vehicle. n=3. * P < 0.05. C. *top*: Structure diagrams of HA-tagged wild type TAT-UCH-L1 WT and mutant TAT-UCH-L1 C90S fusion proteins. *bottom left*: TAT-UCH-L1 WT or mutant TAT-UCH-L1 C90S fusion proteins detected with immunoblots probed with anti-UCH-L1 antibody after incubation in primary neurons. (a-c, *bottom right*): Representative photos of immunocytochemical staining of fusion protein transduction in primary neurons after 1h incubation with 0.08 μ M of recombinant TAT-UCH-L1 WT, mutant TAT-UCH-L1 C90S proteins or PBS (UN) using anti-HA antibody (green) and DAPI nuclear stain (blue). D: Primary neurons were incubated with TAT-UCH-L1 WT, TAT-UCH-L1 C90S fusion proteins or vehicle for 1h prior to hypoxia. After hypoxia, cells were returned to normoxic conditions for 24h. Cell death was measured by assaying LDH released into the medium. N=6 per group. Data are expressed as means $\pm$ SE. * P < 0.01 versus vehicle-treated group. LDN: UCH-L1 inhibitor LDN 57444; Veh: vehicle; IB: immunoblot; WT: wild type TAT-UCH-L1; C90S: mutant TAT-UCH-L1 C90S; MK: MK801 1 μ M; SP: staurosporin 20 μ M; UN: untreated.

Figure 6. Modification of UCH-L1 and other proteins by arachidonic acid, prostaglandins and their metabolites in primary neurons

A. Primary neurons were incubated with biotinylated arachidonic acid (AA), prostaglandins (PGD₂ and PGE₂), or CyPGs (15d-PGJ₂ and PGA₁) at indicated concentrations for 2h under normal culture conditions. Modified protein in the cell lysates were detected by streptavidin-HRP. B. Avidin pull-down assay detecting AA metabolite-modified UCH-L1 in primary neurons. Neurons were incubated with 20μM biotinylated AA and subjected to hypoxia (+) or normoxia (-) before harvest at the indicated time points. *Top*: NeutrAvidin bead bound UCH-L1 was detected by IB with anti-UCH-L1 antibody. *Bottom*: Biotinylated proteins and endogenous UCHL-L1 in neuronal lysates were detected by IB with streptavidin-HRP and anti-UCH-L1 antibody respectively. C. The effect of COX-2 overexpression on AA metabolite-modified protein accumulation in primary neurons. Neurons infected with AdV-COX-2, AdV-EGFP or vehicle (Veh) were incubated with 20μM biotinylated AA and subjected to hypoxia or normoxia. 3h after hypoxia/normoxia, cells were harvested and biotinylated proteins were detected by IB with streptavidin-HRP. D. The effect of COX-2 inhibition on AA metabolite-modified protein accumulation in primary neurons. Neurons were treated with rofecoxib (1μM) or vehicle (-) for 1h prior to incubation with 20μM biotinylated AA. Cells were harvested after hypoxia (+) or normoxia (-). Biotinylated proteins were detected by IB with streptavidin-HRP. E. Avidin pull-down assay to detect PGD₂ metabolite-modified UCH-L1 in primary neurons. Neurons were incubated with 10μM biotinylated PGD₂ and subjected to hypoxia (+) or normoxia (-). Cells were harvested 0 and 24h after hypoxia/normoxia. *Upper three panels*: Biotinylated proteins and endogenous UCH-L1 proteins in neuronal lysates were detected by IB with streptavidin-HRP (arrow at band indicating MW of UCH-L1) and anti-UCH-L1 antibody respectively. *Bottom panel*: NeutrAvidin bead-bound UCH-L1 was detected by IB with anti-UCH-L1 antibody. M: marker; AA: Arachidonic acid; UN: untreated cells; IB: immunoblot; AdV-COX-2: adenovirus containing mouse WT COX-2; AdV-EGFP: adenovirus containing EGFP.