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ORIGINAL ARTICLE

15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) protects neurons from oxidative death via an Nrf2 astrocyte-specific mechanism independent of PPARγ

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Abstract

Astrocytes are critical for the antioxidant support of neurons. Recently, we demonstrated that low level hydrogen peroxide (H_2O_2) facilitates astrocyte-dependent neuroprotection independent of the antioxidant transcription factor Nrf2, leaving the identity of the endogenous astrocytic Nrf2 activator to question. In this study, we show that an endogenous electrophile, 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2), non-cell autonomously protects neurons from death induced by depletion of the major antioxidant glutathione. Nrf2 knockdown in astrocytes abrogated 15d-PGJ2's neuroprotective effect as well as 15d-PGJ2 facilitated Nrf2-target gene induction. In contrast, knockdown of the transcription factor peroxisome proliferator activated-receptor gamma (PPARγ), a well-characterized 15d-PGJ2 target, did not alter 15d-PGJ2 non-cell autonomous neuroprotection. In addition, several PPARγ agonists of the thiazolidinedione (TZD) family failed to induce neuroprotection. Unexpectedly, however, the TZD troglitazone (which contains a chromanol moiety found on vitamin E) induced astrocyte-mediated neuroprotection, an effect which was mimicked by the vitamin E analogs alpha-tocopherol or alpha-tocotrienol. Our findings lead to two important conclusions: (i) 15d-PGJ2 induces astrocyte-mediated neuroprotection via an Nrf2 but not PPARγ mediated pathway, suggesting that 15d-PGJ2 is a candidate endogenous modulator of Nrf2 protective pathways in astrocytes; (ii) selective astrocyte treatment with analogs or compounds containing the chromanol moiety of vitamin E facilitates non-cell autonomous neuroprotection.

Keywords: astrocytes, neuroprotection, Nrf2, PPARγ, vitamin E.

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Astrocytes play an integral role in the CNS by regulating blood flow in response to neuronal activity, modulating synaptogenesis and synaptic activity, and providing metabolic intermediates to neurons (Dringen *et al.* 2000; Barres 2008; Sofroniew and Vinters 2010). In stroke and other CNS disorders, astrocytes contribute to neuroprotection in part by providing antioxidant support, removing excess extracellular levels of the excitotoxic neurotransmitter glutamate and providing a barrier to inflammation (Sofroniew and Vinters 2010). However, astrocytes may also play a detrimental role by contributing to cytokine and chemokine release and forming a barrier to neuronal regeneration (Sofroniew and Vinters 2010). Thus, exploring therapeutic strategies aimed

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Abbreviations used: 15d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; ARE, antioxidant response element; CNS, central nervous system; COX, cyclooxygenase; H_2O_2, hydrogen peroxide; HMOX or HO-1, heme oxygenase-1; L-PGDS, lipocalin prostaglandin D2 synthase; Nrf2, nuclear factor erythroid 2 related factor 2; PPARγ, peroxisome proliferator activated-receptor gamma; siRNA, short interfering RNA; TZD, thiazolidinedione.

at enhancing the prosurvival and diminishing the prodeath roles that astrocytes play in the CNS are of paramount importance.

Our previous study uncovered the astrocyte-specific neuroprotective properties of enzymatically generated H_2O_2 (Haskew-Layton *et al.* 2010). Although H_2O_2 is postulated to exert some of its biological actions via stabilization of the antioxidant transcription factor nuclear factor erythroid 2 related factor 2 (Nrf2) (Bell *et al.* 2011; Haskew-Layton *et al.* 2011), our results showed that the non-cell autonomous neuroprotective effect of astrocytic H_2O_2 is independent of Nrf2 (Haskew-Layton *et al.* 2010). In this study we explored additional adaptive responses in astrocytes involved in mediating neuroprotection. Specifically, we aimed to delineate the non-cell autonomous neuroprotective effect of 15-deoxy-Δ12,14-Prostaglandin J2 (15d-PGJ2), the levels of which are augmented in response to inflammatory stress (Gilroy *et al.* 1999).

15d-PGJ2 is an electrophilic cyclopentene prostaglandin produced downstream from cyclooxygenase (COX) and prostaglandin D2 synthase activity (PGD2), which yields 15d-PGJ2 following a series of non-enzymatic dehydration reactions (Gilroy *et al.* 1999; Scher and Pillinger 2005). Through its direct or indirect interaction with a host of cellular targets, 15d-PGJ2 exerts multiple biological effects on numerous biological systems, culminating in protection against vascular damage, pulmonary damage, gastrointestinal injury, myocardial ischemia, pancreatitis, rheumatoid arthritis, and numerous other inflammatory injuries (Surh *et al.* 2011). In the CNS, 15d-PGJ2 also exerts a multitude of biological effects including anti-inflammatory responses, neurite outgrowth, growth factor secretion, reduction in infarct in ischemia models, and neurological improvement in multiple sclerosis (MS) and spinal cord injury (SCI) (Park *et al.* 2003; Giri *et al.* 2004; Toyomoto *et al.* 2004; Pereira *et al.* 2006; Zhao *et al.* 2006; Hatanaka *et al.* 2010). 15d-PGJ2 has been extensively characterized as an activator of peroxisome proliferator-activated receptor gamma (PPARγ) (Forman *et al.* 1995; Kliewer *et al.* 1995). In addition to PPARγ, 15d-PGJ2 forms covalent adducts with numerous other cellular targets via Michael addition including Keap1 (Nrf2 repressor protein), IκB kinase, thioredoxin, HDACs, UCH-L1, and Ras (Rossi *et al.* 2000; Shibata *et al.* 2003; Renedo *et al.* 2007; Oh *et al.* 2008; Doyle and Fitzpatrick 2010; Koharudin *et al.* 2010).

To explore the mechanism responsible for 15d-PGJ2's astrocyte-dependent neuroprotective effect, we focused on two transcription factors, PPARγ and Nrf2 (Forman *et al.* 1995; Kliewer *et al.* 1995; Oh *et al.* 2008; Kobayashi *et al.* 2009). Nrf2 is a potent mediator of astrocyte-dependent neuroprotection in *in vitro* models of oxidative stress and several *in vivo* models including amyotropic lateral sclerosis (ALS), Parkinson's disease (PD), and Huntington disease (HD) (Shih *et al.* 2003; Vargas and Johnson 2009). Nrf2 binds to genes containing the antioxidant response element (ARE) in their promoter regions and enhances the transcription of antioxidant genes (i.e. heme oxygenase-1, GSH synthetase) and phase II detoxification genes (i.e. glutathione s-transferase) (Shih *et al.* 2003). Although important for astrocytic neuroprotection, endogenous activators of Nrf2 remain elusive. As 15d-PGJ2 is an electrophile that stabilizes Nrf2 via its interaction with Keap1 (Kaspar *et al.* 2009), 15d-PGJ2 is a likely endogenous signal responsible for activating Nrf2 in brain cells.

PPARγ is a nuclear receptor transcription factor, which in addition to exerting antioxidant and anti-inflammatory responses, is involved in facilitating adipogenesis, glucose homeostasis, cell differentiation, and apoptosis (Tontonoz and Spiegelman 2008). Similar to Nrf2, PPARγ facilitates neuroprotection in models of stroke, Alzheimer disease (AD), HD, PD, MS, and SCI, via anti-inflammatory or antioxidant-dependent mechanisms (Kapadia *et al.* 2008; Kiaei 2008). In astrocyte cultures, PPARγ dampens inflammation in astrocytes and protects neurons from OGD via an astrocyte-dependent mechanism (Tjalkens *et al.* 2008). Many studies exploring the biological role of PPARγ have utilized compounds from the thiazolidinedione (TZD) family, yet the specificity of these compounds for PPARγ is questioned by several studies (Davies *et al.* 2001; Feinstein *et al.* 2005; Phulwani *et al.* 2006). In addition to determining the role of PPARγ in mediating the astrocyte-dependent 15d-PGJ2 neuroprotective effect, we also sought to establish if PPARγ agonists from the TZD family induce astrocyte-dependent neuroprotection and if so, is their effect dependent on PPARγ.

In this study we show that 15d-PGJ2 or the TZD troglitazone potently protect neurons via astrocyte-specific mechanisms independent of PPARγ. Our results show that 15d-PGJ2 works entirely via Nrf2, and troglitazone likely via a mechanism involving its vitamin E moiety.

Methods

Additional information regarding the materials and methods can be found in the on-line supplemental material.

Cell culture

All animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University. Primary astrocyte and neuronal cultures were derived from rats obtained from Harlan Laboratories and were prepared as described in (Haskew-Layton *et al.* 2010).

Glutathione measurements

Intracellular GSH measurements of astrocytes were determined using the GSH-Glo™ Glutathione Assay kit from Promega (Madison, WI) according to the manufacturer's protocol. Dithiothreitol was used to convert GSSG to GSH for total glutathione measurements.

Neuronal viability

Neuronal viability was quantified using the MAP2 quantification method developed by Carrier *et al.* (Carrier *et al.* 2006), as modified in Haskew-Layton *et al.* (Haskew-Layton *et al.* 2010).

Phase contrast imaging

Phase contrast images were taken with a Nikon Eclipse TS 100 (Nikon Instruments Inc, Melville, NY) using a 40x objective.

15d-PGJ2 quantitation

Total lipids were extracted using a modified Bligh and Dyer method (Ryan *et al.* 2009). 15d-PGJ_2 levels were analyzed using a 15-deoxy-Δ12,14-PGJ_2 EIA kit (Enzo Life Sciences, Lörrach, Germany) as per manufacturer's protocol with modifications.

Quantitative PCR

Total RNA was isolated from primary rat astrocytes using the NucleoSpin® RNA II kit (Clontech, Mountain View, CA). A TaqMan® RNA-to-C_T™ *1-Step* Kit (Applied Biosystems, Carlsbad, CA) was used for both the reverse transcription reaction and the real-time polymerase chain reaction (PCR), according to the manufacturer's protocol.

Luciferase reporter assays

PPRE-luciferase: astrocytes were cotransfected with the PPRE X3-TK-luc plasmid upstream of a luciferase reporter [Addgene Plasmid 1015 (Kim *et al.* 1998)] and TK renilla plasmid. Luciferase activity was measured using a Dual-Luciferase assay kit (Promega). ARE-luciferase: astrocytes were transduced as described in Haskew-Layton *et al.* (Haskew-Layton *et al.* 2010) with an adenovirus containing the ARE derived from the human NADPH quinone oxidoreductase gene, upstream of a luciferase reporter (Moehlenkamp and Johnson 1999). Luciferase activity was measured using a luciferase assay substrate reaction buffer (Promega) containing luciferin.

Western blots

Following standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transfer procedures, proteins were incubated with primary antibodies [1 : 1000 anti-HMOX (Enzo Life Sciences, Farmingdale, NY); anti-actin 1 : 5000 (Sigma, St. Louis, MO)], followed by secondary antibody incubation (1 : 20 000). The immunoblot bands were detected using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). Images are shown in gray scale for clarity.

siRNA transfections

Knockdown of PPARγ and Nrf2 was achieved using short interfering RNA (siRNA). Commercially available rat PPARγ siRNA duplexes were from SABiosciences (Valencia, CA). Rat Nrf2 siRNAs were custom designed according to the coding sequences described in Villacorta *et al.* (2007) and Haskew-Layton *et al.*, (2010) and synthesized by Qiagen (Valencia, CA).

Statistics

Statistics were performed with Statistica software, StatSoft, Tulsa, OK; or Prism v.6.0 (GraphPad Software, La Jolla, CA); data are the averages ± SEM for three to five experiments per group. Data were analyzed using one-way ANOVA followed by *post hoc* Dunnett's (for multiple comparisons) or unpaired *t*-tests (when comparing two groups).

Results

In our previous study, we characterized a glutathione depletion-induced model of oxidative stress in a neuron-astrocyte coculture model (Haskew-Layton *et al.* 2010). In this model the glutamate analog homocysteic acid (HCA) inhibits the x_C^- transporter complex to block cystine uptake, subsequently depleting the glutathione precursor cysteine and leading to substantial glutathione depletion in both astrocytes and immature neurons, but only death in the immature neurons (Haskew-Layton *et al.* 2010) (Fig. 1a and b). As immature neurons lack NMDA receptors, we verified in our previous study that that HCA-induced death of neurons in the cocultures is mediated by oxidative stress-independent of excitotoxicity (Haskew-Layton *et al.* 2010). To test the role of 15d-PGJ2 in protecting neurons in an astrocyte-neuron coculture, immature neurons were plated in the presence of 15d-PGJ2 on top of a monolayer of confluent astrocytes followed by HCA treatment. Indeed, 15d-PGJ2 potently protected the cocultured neurons from HCA-induced death (Fig. 1c and e). To determine if the mechanism is astrocyte-specific, astrocytes were pre-treated with 15d-PGJ2 prior to neuronal plating in the presence of HCA. In support of an astrocyte-specific role, astrocytic 15d-PGJ2 pre-treatment protected the cocultured neurons despite the fact that the neurons were not directly exposed to 15d-PGJ2 (Fig. 1d and f).

To verify the role of endogenously produced 15d-PGJ2 in mediating astrocytic neuroprotection, astrocyte-neuron co-cultures were treated with the lipocalin prostaglandin D2 synthase (L-PGDS) inhibitor, AT-56. L-PGDS catalyzes the conversion of PGH2 to PGD2, which through a series of non-enzymatic dehydration reactions yields 15d-PGJ2 (Surh *et al.* 2011). Indeed, cocultures treated with AT-56 showed enhanced neuronal death in response to AT-56 (Fig. 1g), which correlated with attenuated 15d-PGJ2 levels in astrocytes (Fig. 1h). In support of an astrocyte-dependent mechanism in mediating endogenous 15d-PGJ2's neuroprotection, neuronal enriched cultures treated with AT-56 were protected from HCA-induced death (Figure S1), which is in contrast with the accelerated neuronal death observed in the AT-56 treated cocultures.

To determine if PPARγ is important for the 15d-PGJ2 response, astrocytes were pre-treated with PPARγ agonists of the TZD family prior to neuronal plating with HCA. Although the TZD troglitazone mimicked the astrocyte-dependent neuroprotective response of 15d-PGJ2 (Fig. 2a–c, and g), the other TZDs cigltazone, rosiglitazone or pioglitazone failed to induce astrocytic neuroprotection (Fig. 2d–f, and h–j), casting doubt on the involvement of PPARγ.

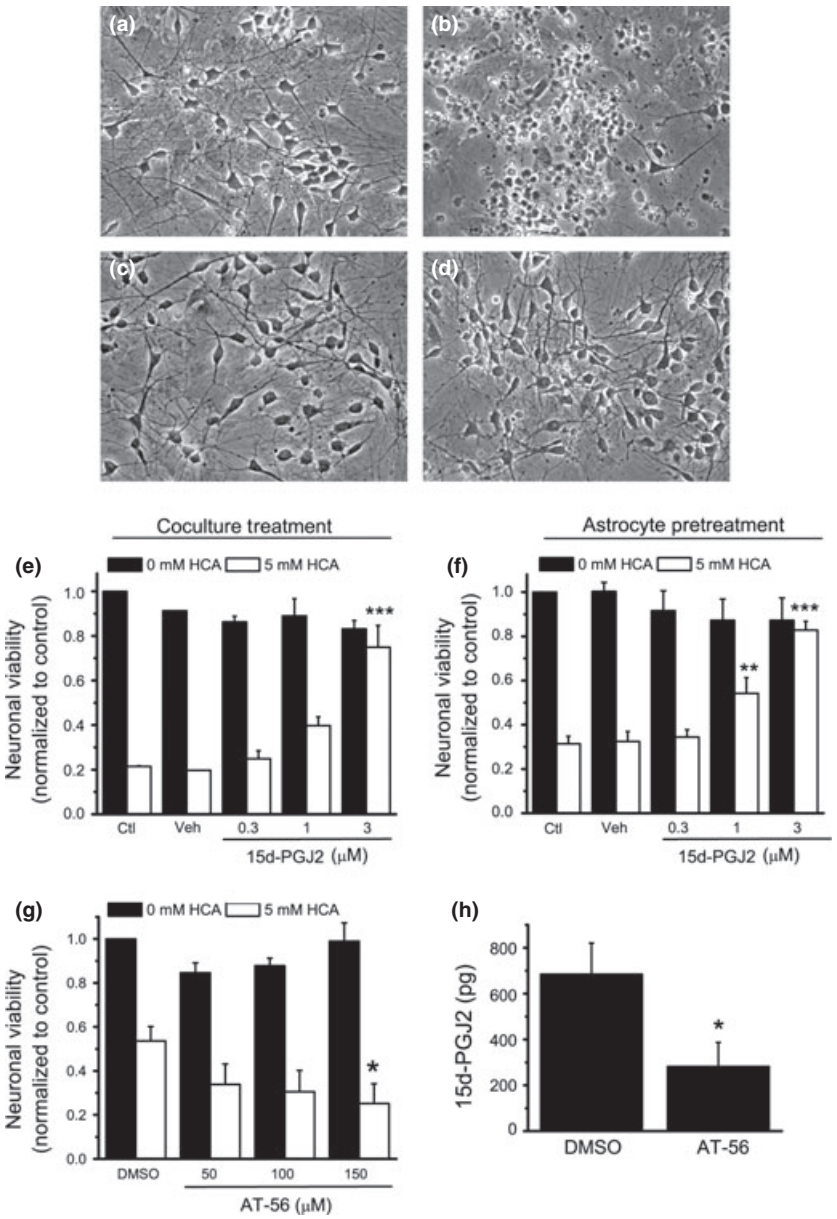

Fig. 1 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) protects neurons from oxidative stress via an astrocyte-specific mechanism. (a and b) Immature neurons were plated in the absence (a) or presence of (b) the glutamate analog, 5 mM homocysteic acid (HCA), on top of a confluent layer of astrocytes, and imaged 48 h later. (c) Neurons were plated in the presence of 3 μM 15d-PGJ2 + 5 mM HCA on top of a monolayer of astrocytes for 48 h. (d) Astrocytes were pre-treated with 3 μM 15d-PGJ2 for 18 h followed by wash-off and the plating of neurons on top of the astrocytes with 5 mM HCA for 48 h. (e) Concentration response curve for 15d-PGJ2 treatment, added with neurons (+/− HCA) when plated on astrocyte monolayer for 48 h [as seen in (c)]. ***$p < 0.001$, versus HCA control. (f) Concentration response curve for 15d-PGJ2 pre-treatment in astrocytes (18 h) followed by neuronal plating ± 5 mM HCA for 48 h [as seen in (d)]. **$p < 0.01$ or ***$p < 0.001$, versus HCA control. (g) Astrocytes were pre-treated with ± AT-56 for 18 h followed by the plating of neurons (on top of the confluent layer of astrocytes) in the presence of ± AT-56 and ± HCA for 48 h. *$p < 0.05$, versus HCA control (h) Astrocytes were treated with ± 150 μM AT-56 for 18 h. *$p < 0.05$, versus DMSO control.

To completely rule out the possible involvement of PPARγ in mediating the astrocyte-dependent neuroprotective effect of 15d-PGJ2, PPARγ was knocked down via siRNA (Fig. 3a). To verify the functionality of the knockdown, PPARγ activity following siRNA knockdown was measured using a peroxisome proliferator response element (PPRE) promoter cloned upstream of a luciferase reporter. Indeed, the PPARγ siRNAs abrogated 15d-PGJ2 and ciglitazone-induced PPRE-luciferase activity (Fig. 3b). Of note, troglitazone itself failed to induce a significant increase in PPRE-luciferase activity, providing further support that troglitazone does not protect via a PPARγ-dependent mechanism (Fig. 3b). As expected from the lack of effectiveness of the structurally diverse TZDs on neuroprotection in our coculture oxidative stress model, PPARγ knockdown failed to abrogate 15d-PGJ2's astrocyte-dependent neuroprotective effect (Fig. 3c).

As our data argue against the involvement of PPARγ in 15d-PGJ2 protection, we next tested if Nrf2 is necessary using a previously validated siRNA approach (Haskew-Layton *et al.* 2010) (Fig. 4a). Confirming the functionality of the Nrf2 knockdown, the Nrf2-siRNAs completely abrogated the effects of sulforaphane (a classical Nrf2 activator) and 15d-PGJ2 on ARE-luciferase activity (Fig. 4b) and diminished 15d-PGJ2's enhancement of the Nrf2-regulated protein heme oxygenase-1 (Fig. 4c). However, although this level of Nrf2 knockdown completely abrogated the astrocytic neuroprotective effect of sulforaphane, the effect of 15d-PGJ2 was only partially abolished (Fig. 4d). Thus, to determine if an alternative mechanism also contributes to the astrocytic

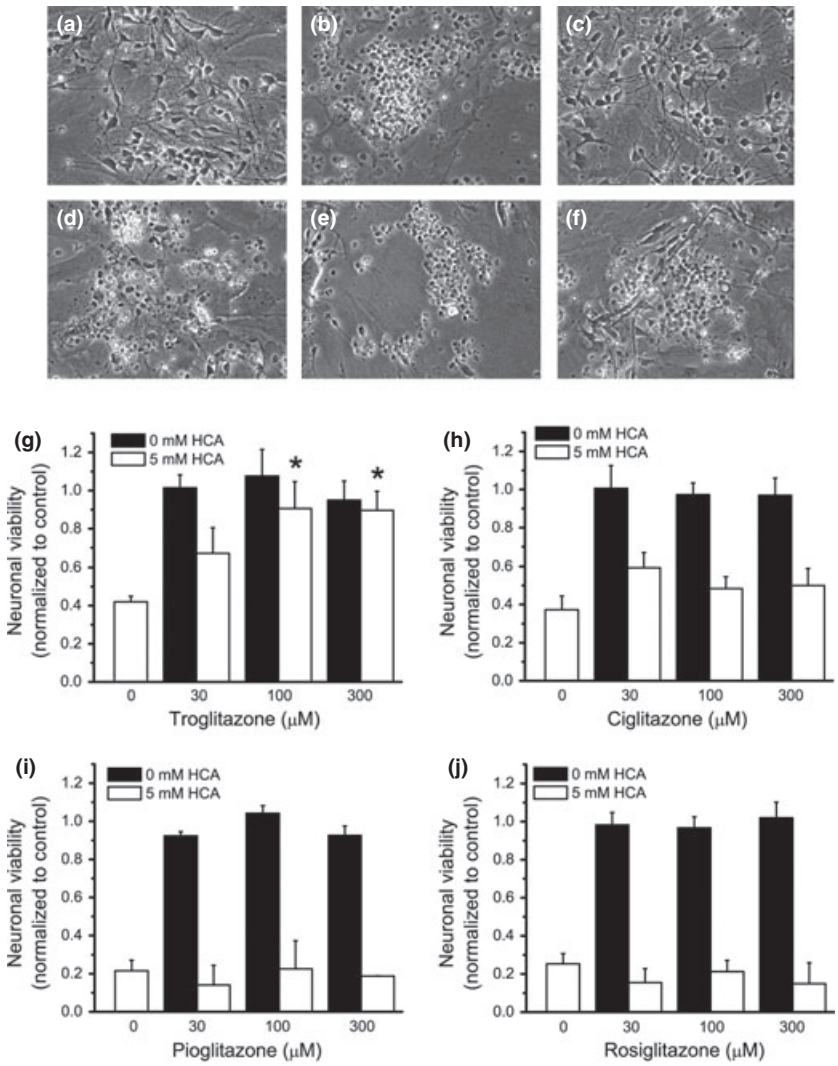

Fig. 2 The thiazolidinedione (TZD) troglitazone, but not other TZDs, protects neurons from glutathione depletion-induced oxidative stress via an astrocyte-specific mechanism. (a and b) Immature neurons were plated in the absence (a) or presence of (b) the glutamate analog, 5 mM homocysteic acid (HCA), on top of a confluent layer of astrocytes, and imaged 48 h later. (c–f) Astrocytes were pre-treated for 18 h with the TZDs troglitazone (c), ciglitazone (d), pioglitazone (e), and rosiglitazone (f). Following wash-off, immature neurons were plated in the presence of 5 mM HCA for 48 h. (g–j) Concentration response curve for troglitazone (g), ciglitazone (h), pioglitazone (i), and rosiglitazone (j). Experiments performed as described for (c–f) and show results for neurons plated in the presence or absence of 5 mM HCA. $*p < 0.05$, versus HCA control.

neuroprotective effect of 15d-PGJ2, or further molecular deletion is required to completely suppress the effect, Nrf2 levels were further depleted (Fig. 4e), leading to complete abrogation of 15d-PGJ2's effect on astrocyte-induced neuroprotection (Fig. 4f). As astrocytes can protect neurons from oxidative stress via the up-regulation of glutathione biosynthesis via an Nrf2-dependent mechanism (Shih *et al.* 2003), we provide further support for the involvement of Nrf2, by showing that 15d-PGJ2 preserves glutathione levels in astrocytes treated with HCA (Fig. 4g).

As our data point to an exclusive Nrf2-dependent astrocytic neuroprotective mechanism for 15d-PGJ2, we sought to determine if Nrf2 signaling is favored over PPARγ signaling in astrocytes. Thus, we looked at mRNA levels for heme oxygenase-1, which contains both Nrf2 and PPARγ-sensitive promoter regions (ARE and PPRE regions, respectively) (Prestera *et al.* 1995; Kronke *et al.* 2007), and found that heme oxygenase-1 (HMOX) mRNA induced by 15d-PGJ2 treatment in astrocytes is regulated by Nrf2 but not PPARγ (Fig. 5a). As well, 15d-PGJ2 enhanced the mRNA levels of NADPH quinine oxidoreducatase 1 (contains the ARE but not the PPRE promoter regions) (Li and Jaiswal 1994) but failed to alter mRNA levels of lipoprotein lipase-1 (LPL), which contains PPRE but not the ARE promoter regions (Schoonjans *et al.* 1996) (Fig. 5b and c).

Our results showing that Nrf2 but not PPARγ mediates the effect of 15d-PGJ2, coupled with the lack of effect of several PPARγ agonists of the TZD family, suggest that the TZD troglitazone leads to astrocytic neuroprotection independent of PPARγ. Consistent with this, PPARγ knockdown did not abrogate troglitazone's protective effect (Fig. 6a). In addition, because 15d-PGJ2's robust effect is mediated by Nrf2- and it has been postulated that troglitazone activates Nrf2 (Shih *et al.* 2012)- we tested for Nrf2's potential involvement, but found that Nrf2-knockdown did not alter troglitazone-induced astrocytic neuroprotection (Fig. 6b). As the effect of troglitazone was in contrast with other TZDs, we looked for structural differences as an explanation of the

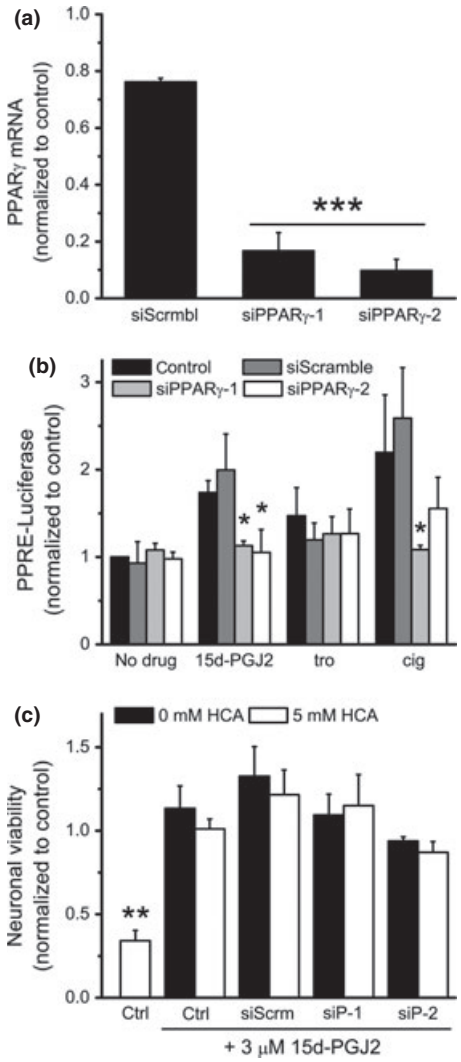

Fig. 3 Astrocytic 15-deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) treatment protects cocultured neurons from oxidative stress independent of peroxisome proliferator activated-receptor gamma (PPARγ). (a) Astrocyte cultures were treated with siRNA targeted against PPARγ for ~ 42 h prior to RNA isolation. ***$p < 0.001$, versus siScramble. (b) Astrocytes were transfected with PPRE-Luciferase plasmids for 24 h followed by 18 h treatments with 3 μM 15d-PGJ2, 300 μM troglitazone, or 300 μM ciglitazone (total siRNA treatment ~42 h). *$p < 0.05$, versus 15d-PGJ2 control or ciglitazone control. (c) Astrocytes were transfected with PPARγ siRNAs for 24 h followed by 18 h 15d-PGJ2 treatment (total siRNA treatment ~42 h). Following wash-off of the 15d-PGJ2 treatment, neurons were plated ± 5 mM homocysteic acid (HCA) for 48 h. **$p < 0.01$, versus 15d-PGJ2 control, 15d-PGJ2 + siScramble, 15d-PGJ2 + siP1, and 15d-PGJ2 + siP2.

divergent effects. Troglitazone is unique in that it contains the same chromanol ring structure as that of vitamin E (fat soluble family of α, ß, γ, and δ forms of tocopherols and tocotrienols), a structure absent from other TZDs. Thus, to determine if the vitamin E chromanol ring structure could be responsible for troglitazone's effect, we tested two biologically active forms of vitamin E, α-tocopherol and α-tocotrienol, and found that astrocyte pre-treatment with these compounds mimicked the astrocyte-dependent neuroprotective effect of troglitazone (Fig. 6c and d). Although vitamin E analogs are well-established neuroprotective agents, our data present a novel mechanism of action for vitamin E involving non-cell autonomous protection of neurons by astrocytes.

Discussion

In this study, we show that astrocytic 15d-PGJ2 provides potent non-cell autonomous neuroprotection from oxidative stress. Using siRNA-mediated knockdown, we demonstrate that 15d-PGJ2's effect is entirely mediated by Nrf2 but independent of PPARγ, suggesting that 15d-PGJ2 may be the endogenous molecule responsible for activating an adaptive antioxidant transcriptional response in astrocytes via activation of Nrf2. In addition to the potent non-cell autonomous neuroprotection seen with 15d-PGJ2, we also unexpectedly found that the PPARγ agonist of the thiazolidinedione family, troglitazone, induces astrocyte-specific neuroprotection independent of PPARγ, but likely via a mechanism involving its vitamin E chromanol ring structure.

Understanding the adaptive responses that astrocytes undergo in response to acute stress or neurodegenerative conditions will contribute to the development of novel therapies. Transcription factor signaling represents a critical aspect of adaptive cellular events that allow cells to 'sense' alterations in their environment and in response, facilitate an array of specific gene expression changes. Nrf2 is an integral transcription factor that facilitates astrocytic neuroprotection by promoting the expression of genes involved in glutathione synthesis and release in astrocytes (Shih *et al.* 2003). Several studies showed that exogenous Nrf2 activators or heterologous astrocytic expression of Nrf2 induces robust neuroprotection, while Nrf2 knockout exacerbates neuronal death (Shih *et al.* 2003; Vargas and Johnson 2009). However, because these studies relied on synthetic or nutriceutical Nrf2 activators, or forced expression of Nrf2, the specific endogenous molecule that spurs adaptive Nrf2-mediated astrocytic neuroprotection remains unknown.

Basal expression of Nrf2 in the cytoplasm is maintained at low levels via the Nrf2-repressor protein Keap1 (Kaspar *et al.* 2009). Several *in vitro* studies show that specific residues on Keap1 are targets for nucleophilic attack leading to Nrf2 stabilization, thus Keap1 is thought to be a critical sensor for intracellular redox changes (Dinkova-Kostova *et al.* 2002; Hong *et al.* 2005; Kobayashi *et al.* 2009). The reactive oxygen species H_2O_2 was considered a critical signal responsible for Nrf2 stabilization via Keap1 oxidation (Bell *et al.* 2011). However, our recent study showed that although enzymatic H_2O_2 generation in astrocytes lead to neuropro-

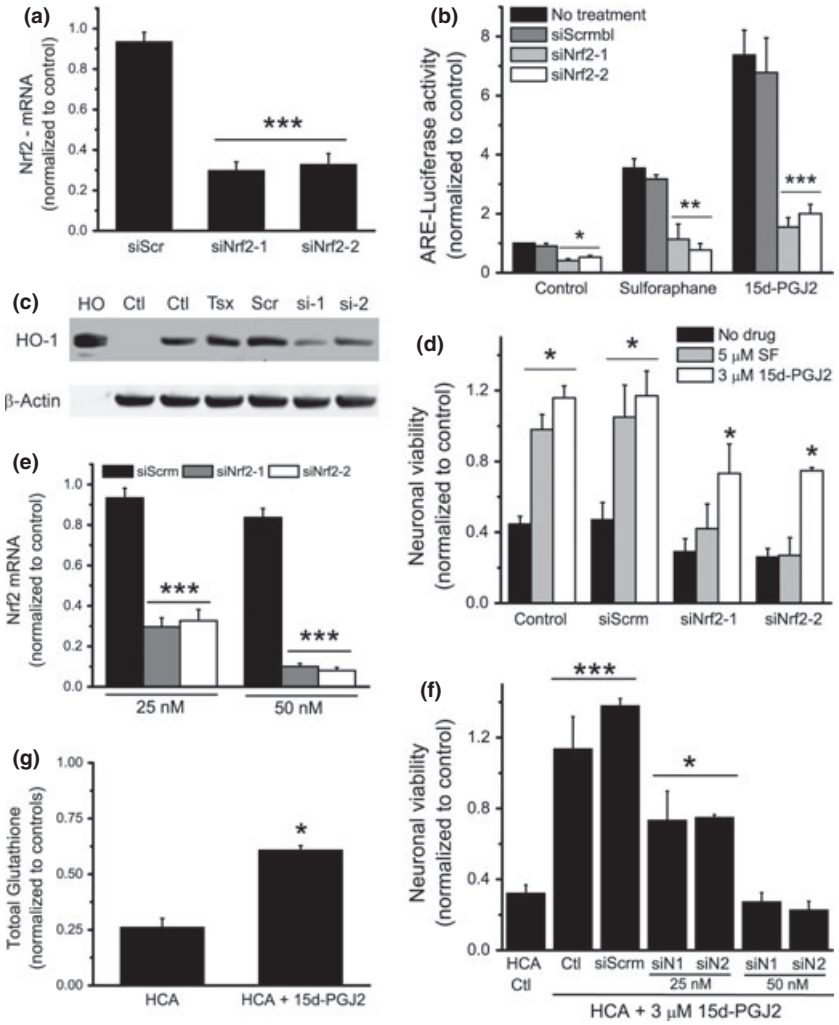

Fig. 4 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) confers neuroprotection via an astrocytic specific mechanism involving nuclear factor erythroid 2 related factor 2 (Nrf2). (a) Astrocyte cultures were treated with siRNA targeted against Nrf2 for ~42 h. ***$p < 0.001$, versus siScramble. (b) ARE-luciferase transduced astrocytes were treated with siRNA targeted against Nrf2 for 24 h followed by treatment with 3 μM 15d-PGJ2 or 5 μM sulforaphane for 18 h (total siRNA treatment ~42 h). **$p < 0.01$, versus sulforaphane no treatment, ***$p < 0.001$, versus 15d-PGJ2 no treatment. (c) Astrocytes were transfected for 24 h with siRNAs for Nrf2 followed by 18 h 3 μM 15d-PGJ2 treatment (total siRNA treatment ~42 h). Astrocyte lysates were immunblotted for heme oxygenase-1 (HO-1) and β-actin levels. Lane 1: recombinant HO-1 protein. Lane 2: control lysate. Lanes 3–7: + 15d-PGJ2. Tsx: transfection control. Scr: siScrambled. Si-1,-2: Nrf2 siRNAs. (d) Astrocytes were transfected with Nrf2 siRNAs for 24 h followed by 18 h 3 μM 15d-PGJ2 or 5 μM sulforaphane treatment (total siRNA treatment ~42 h). Following wash-off of 15d-PGJ2 or sulforaphane, neurons were plated ± 5 mM homocysteic acid (HCA) for 48 h. *$p < 0.05$, versus no drug for each respective group. (e) Astrocytes were treated with siRNA targeted against Nrf2 for ~42 h. ***$p < 0.001$, versus siScramble. (f) Astrocytes were transfected with Nrf2 siRNAs for 24 h (siN1 or siN2) followed by 18 h 3 μM 15d-PGJ2 (total siRNA treatment ~42 h). Following wash-off of 15d-PGJ2, neurons were plated ± 5 mM HCA for 48 h. ***$p < 0.001$, versus HCA control, *$p < 0.05$, versus HCA control. (g) Astrocytes were treated with 5 mM HCA ± 3 μM 15d-PGJ2 for 48 h. *$p < 0.05$, versus HCA control.

tection, this effect was independent of Nrf2 (Haskew-Layton *et al.* 2010, 2011), highlighting that both Nrf2-dependent and independent pathways in astrocytes are important for neuroprotection. Thus in our current study, we explored whether the astrocyte-dependent neuroprotective effects driven by 15d-PGJ2 involved Nrf2 as well as other 15d-PGJ2-targeted pro-survival pathways, namely PPARγ.

15d-PGJ2 is a cyclopentenone prostaglandin produced via a series of dehydration reactions downstream from COX and PGD2 synthase metabolism; as such 15d-PGJ2 levels are up-regulated in response to inflammatory stress (Gilroy *et al.* 1999; Scher and Pillinger 2005). Because of an electrophilic carbon at an α, β-unsaturated ketone within its cyclopentene ring, 15d-PGJ2 has numerous protein targets, forming

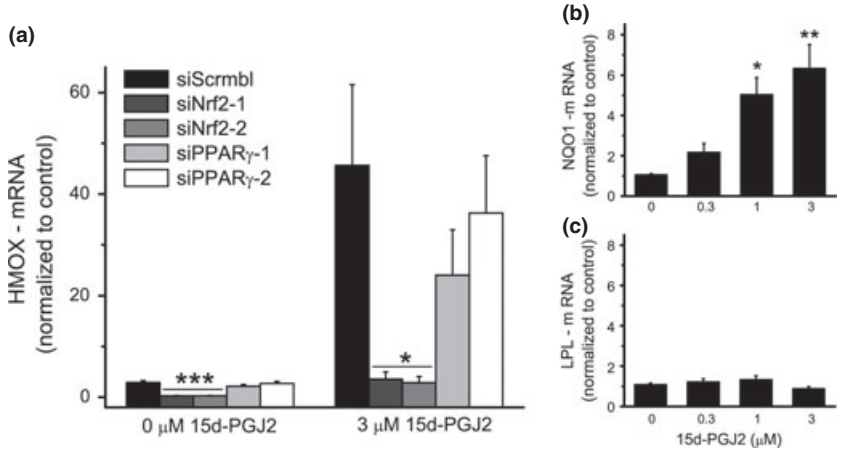

Fig. 5 15-Deoxy-Δ12,14-prostaglandin J2 (15d-PGJ2) favors nuclear factor erythroid 2 related factor 2 (Nrf2) dependent signaling over peroxisome proliferator activated-receptor gamma (PPARγ) signaling in astrocytes. (a) Astrocytes were transfected with siRNAs for Nrf2 (50 nM) or PPARγ (50 nM) for 24 h followed by treatment with 3 μM 15d-PGJ2 for 18 h. ***$p < 0.001$, versus siScramble, *$p < 0.05$, versus siScramble. (b and c) Concentration response of 15d-PGJ2 treatment in astrocytes for 18 h. *$p < 0.05$, versus 0 μM 15d-PGJ2, **$p < 0.01$, versus 0 μM 15d-PGJ2.

covalent adducts via Michael addition with specific cysteine residues on proteins (Rossi *et al.* 2000; Shibata *et al.* 2003; Renedo *et al.* 2007; Doyle and Fitzpatrick 2010; Koharudin *et al.* 2010). As such, 15d-PGJ2 covalently binds with Keap1 leading to Nrf2 stabilization (Yamamoto *et al.* 2008; Kansanen *et al.* 2009). In line with this, 15d-PGJ2 has been found to exert many of its biological effects via Nrf2 activation, such as HMOX gene induction, anti-atherosclerotic gene induction, protection against acute lung injury, and suppression of inflammatory pathways (Syed *et al.* 2010; Surh *et al.* 2011). In the CNS, the protective effects of 15d-PGJ2 in models of stroke, MS, and SCI have largely been attributed to the inactivation of NFkB or activation of PPARγ and the subsequent dampening of pro-inflammatory responses (Diab *et al.* 2002; Pereira *et al.* 2006; Zhao *et al.* 2006; Genovese *et al.* 2008; Kerr *et al.* 2008). In astrocytes specifically, in addition to an anti-inflammatory role reported for 15d-PGJ2 (Park *et al.* 2003; Giri *et al.* 2004; Phulwani *et al.* 2006; Zhao *et al.* 2006), other reported biological effects of 15d-PGJ2 include enhanced growth factor secretion, increased glucose utilization and diminished glutamate transporter expression (Toyomoto *et al.* 2004; Garcia-Bueno *et al.* 2007); which have also been largely attributed to blocking NFkB or enhancing PPARγ. Other studies have also suggested the involvement of Nrf2 in mediating the protective effects of 15d-PGJ2 in nervous system cells, including enteric glia (Kim *et al.* 2008; Abdo *et al.* 2012). In this study, we provide strong evidence for the involvement of Nrf2 by using siRNA-mediated knockdown, demonstrating a critical role of Nrf2 in mediating 15d-PGJ2-dependent non-cell autonomous neuroprotection.

In vivo levels of 15d-PGJ2 have been reported in the ng/mL range (Rajakariar *et al.* 2007), leaving to question whether the levels of 15d-PGJ2 required to induce a biological response are relevant. However, because only ~1–4% of 15d-PGJ2 added to serum containing medium remains 'free' to enter cells (Rajakariar *et al.* 2007; Oh *et al.* 2008), the lowest 'effective' concentration of 15d-PGJ2 required to induce astrocytic-neuroprotection in this study may be as low as 10 nM (1% of 1 μM, lowest concentration shown to induce protection), suggesting a true biological effect of 15d-PGJ2 in astrocytes. Finally, in support of a role of endogenous 15d-PGJ2 in mediating the astrocytic neuroprotection, inhibition of L-PGDS, an upstream enzyme which converts PGH2 to PGD2 (a precursor to 15d-PGJ2), exacerbates glutathione depletion-induced neuronal death in the astrocyte-neuron coculture model. In contrast however, L-PGDS inhibition in neurons cultured alone yields protection from glutathione depletion-induced death. These data suggest that endogenous 15d-PGJ2 is important for astrocytic neuroprotection, but may hinder intrinsic neuroprotective pathways, an area for future investigation.

We initially speculated that in addition to Nrf2, PPARγ played a role in 15d-PGJ2's astrocyte-dependent neuroprotective effect because of its established role in neuroprotection. PPARγ is a transcription factor which enhances the transcription of antioxidant genes containing the peroxisome proliferator response element (PPRE) within their enhancer/promoter region and represses pro-inflammatory genes through a transrepression mechanism (Tontonoz and Spiegelman 2008). As such, others have shown that PPARγ agonist treatment in astrocytes induces increased glucose metabolism, increased glutamate transporter expression, repression of cytokine production and decreased iNOS levels, and enhanced neuronal survival (Kapadia *et al.* 2008; Kiaei 2008; Tontonoz and Spiegelman 2008). In *in vivo* models,

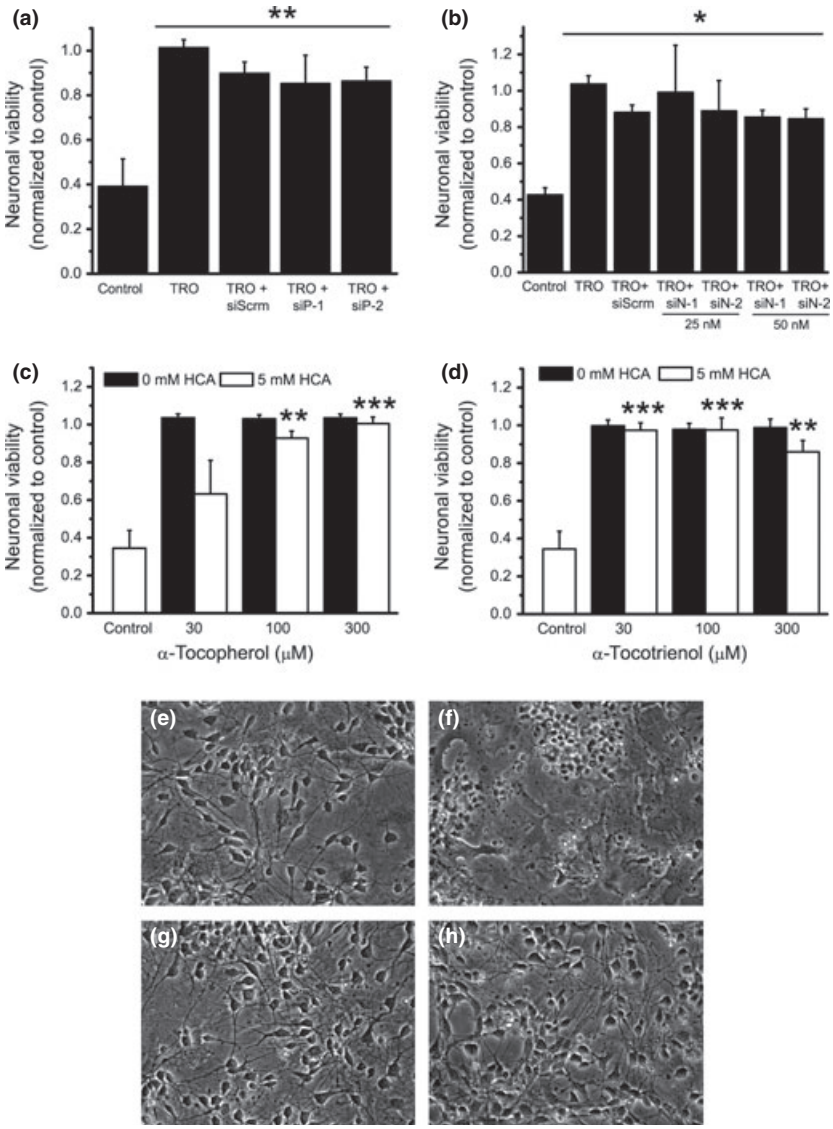

Fig. 6 The troglitazone effect is independent of peroxisome proliferator activated-receptor gamma (PPARγ) and nuclear factor erythroid 2 related factor 2 (Nrf2), but is likely mediated via a 'vitamin E' moiety; analogous astrocyte-dependent neuroprotection triggered by α-tocopherol and α-tocotrienol. (a and b) Astrocyte cultures were treated with siRNAs targeted against PPARγ (siP-1,-2; 50 nM – a) or Nrf2 (siN-1,-2–b) for 24 h. Astrocytes were then treated with 300 μM troglitazone for 18 h followed by wash-off and neuronal plating ± 5 mM homocysteic acid (HCA) for 48 h (total siRNA treatment ~42 h). $^{**}p < 0.01$, versus HCA control, $^{*}p < 0.05$, versus HCA control. (c and d) Astrocytes were pre-treated with alpha-tocopherol (c) or alpha-tocotrienol (d) for 18 h followed by wash-off and the plating of neurons ± 5 mM HCA for 48 h. $^{**}p < 0.01$, versus HCA control, $^{***}p < 0.001$, versus HCA control. (e) Control immature neurons plated over non-treated astrocytes for 48 h. (f) Immature neurons were plated with 5 mM HCA on top of confluent astrocytes for 48 h. (g and h) Astrocytes were pre-treated with 300 μM alpha-tocopherol (e) or 300 μM alpha-tocotrienol (f) for 18 h followed by wash-off and the plating of neurons + 5 mM HCA for 48 h.

PPARγ enhances neuroprotection in stroke models, models of MS, ALS, AD, and HD via both antioxidant and anti-inflammatory mechanisms (Kapadia *et al.* 2008; Kiaei 2008; Zhao *et al.* 2009). Because glutathione depletion both induces oxidative stress in cocultures (as characterized in our previous study) (Haskew-Layton *et al.* 2010) and promotes the release of proinflammatory cytokines from astrocytes (Lee *et al.* 2010), we speculated that PPARγ activation in astrocytes protects neurons from glutathione depletion via both antioxidant and anti-inflammatory actions. However, we found that 15d-PGJ2 in astrocytes protected neurons independent of PPARγ and that several PPARγ agonists failed to induce astrocytic-neuroprotection. In addition, although others have found that Nrf2 regulates the expression of PPARγ (Cho *et al.* 2010; Shih *et al.* 2012), and thus Nrf2 and PPARγ mediate prosurvival pathways in a cooperative fashion, our data suggests that in astrocytes PPARγ is not critical for Nrf2-depdendent protection. In addition to showing that PPARγ is not important for astrocytic neuroprotection from oxidative stress, we also show that despite having both ARE and PPRE consensus promoter/enhancer sequences, HMOX gene transcription is not highly regulated by PPARγ in astrocytes, but is highly regulated by Nrf2.

While exploring the potential involvement of PPARγ in mediating the effect of 15d-PGJ2, we unexpectedly found that the PPARγ agonist troglitazone, but not other compounds from the same TZD family, induced potent astrocyte-specific neuroprotection. We confirmed that troglitazone likely worked via an off-target effect because PPARγ knockdown did not abolish troglitazone's astrocyte-dependent neuroprotection. As well, we found that Nrf2 knockdown also failed to abolish troglitazone's astrocytic neuroprotective effect, highlighting a unique Nrf2-independent pathway for astrocytic neuroprotection.

TZDs are anti-diabetic drugs thought to work via a PPARγ-dependent mechanism to affect lipid metabolism (Tontonoz and Spiegelman 2008). However, as in this study, the involvement of PPARγ in mediating the biological effects of TZDs has been called into question by several studies (Davies *et al.* 2001; Feinstein *et al.* 2005; Phulwani *et al.* 2006). As troglitazone contains the chromanol ring structure found in vitamin E analogs, a moiety absent from the other TZDs, we speculated and confirmed that the vitamin E analogs, namely α-tocopherol and α-tocotrienol, would mimic the effect of troglitazone.

Our unique results with troglitazone lead to the novel finding that the vitamin E analogs α-tocopherol or α-tocotrienol can exert neuroprotection via non-cell autonomous mechanisms involving astrocytes. Vitamin E, which is composed of α, β, γ, and δ forms of tocopherols and tocotrienols, acts as an antioxidant by scavenging lipid hydroperoxyl radicals (Kaileh and Sen 2010; Sen *et al.* 2010). In addition to direct antioxidant properties, in the CNS vitamin E modulates the activity of several enzymes including protein kinase C, src kinases, 5-lipoxygenase, phospholipase A2, protein phosphatase 2A, and diacylglycerol kinase, as well as NFkB activation (Gohil *et al.* 2004; Muller 2010). Thus, not surprisingly neurological deficits are characteristic of vitamin E deficiency related disorders such as abetalipoproteinemia and ataxia with vitamin E deficiency (AVED) (Muller 2010). Studies from α-tocopherol transfer protein knockout mice, show that α-tocopherol regulates expression of the transcription factor retinoic acid orphan receptor α (ROR-α) and ROR-α downstream target genes, including the astrocyte specific gene GFAP (Gohil *et al.* 2004). In our model, astrocytes are pre-treated with the vitamin E analogs prior to both neuronal plating and glutamate depletion-induced oxidative stress; thus it seems plausible that, rather than exerting neuroprotection via direct antioxidant actions, the vitamin E analogs may be promoting neuroprotection via an astrocyte-specific cell signaling mechanism.

In conclusion, our findings show that 15d-PGJ2, troglitazone, and vitamin E analogs protect neurons from oxidative stress non-cell autonomously via astrocyte-specific mechanisms. We found that, although characterized as PPARγ agonists, 15d-PGJ2 and troglitazone work independent of PPARγ to induce astrocytic neuroprotection. In fact, we show that astrocytic Nrf2 entirely mediates the neuroprotective effect of 15d-PGJ2. On the other hand, troglitazone works independent of Nrf2 but may exert its astrocyte-dependent neurprotection via its vitamin E chromanol ring structure. Identifying the mechanism responsible for troglitazone and vitamin E's astrocyte-dependent effects should yield novel therapeutic strategies aimed at enhancing astrocytic neuroprotection. Altogether our findings point to several potent astrocyte-dependent neuroprotective pathways. We suggest that 15d-PGJ2 represents an adaptive pathway in astrocytes whereby increases in 15d-PGJ2 levels, in response to cell stress, may produce a feedback mechanism to counteract oxidative stress via activation of the antioxidant transcription factor Nrf2. We also suggest that our findings with troglitazone highlight important Nrf2-independent astrocytic neuroprotective pathways and that the potent neuroprotective actions of vitamin E *in vivo* may occur through both cell autonomous and non-cell autonomous mechanisms.

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Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Figure S1. Enriched immature neuronal cultures were plated ± 5 mM HCA for 18 h in the presence or absence of the L-PGDS inhibitor AT-56.

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