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PREVENTION OF METHYLMERCURY-INDUCED MITOCHONDRIAL DEPOLARIZATION, GLUTATHIONE DEPLETION AND CELL DEATH BY 15-DEOXY-DELTA-12,14- PROSTAGLANDIN J2

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Abstract

Methylmercury (MeHg) is an environmental toxin that causes severe neurological complications in humans and experimental animals. In addition to neurons, glia in the central nervous system are very susceptible to MeHg toxicity. Pretreatment of glia with the prostaglandin derivative, 15-deoxy-delta-12,14-prostaglandin J₂ (15d-PGJ₂), caused a significant protection against MeHg cytotoxicity. Results with the C6 glioma cells demonstrated that the protection was dependent on the duration of pretreatment, suggesting that time was required for the up-regulation of cellular defenses. Subsequent experiments indicated that 15d-PGJ₂ prevented MeHg induced mitochondrial depolarization. Similar protection against MeHg cytotoxicity was observed in primary cultures of mouse glia. Analysis of cellular glutathione (GSH) levels indicated that 15d-PGJ₂ caused an up-regulation of GSH and prevented MeHg-induced GSH depletion. Buthionine sulfoximine (BSO), a GSH synthesis inhibitor, completely inhibited the GSH induction by 15d-PGJ₂. However, BSO did not prevent the stabilization of mitochondrial potential and only partially prevented the protection caused by 15d-PGJ₂. While induction of heme oxygenase-1 was implicated in the cytoprotection by 15d-PGJ₂ under some experimental conditions, additional experiments indicated that this enzyme was not involved in the cytoprotection observed in this system. Together, these results suggested that while up-regulation of GSH by 15d-PGJ₂ might help cells to defend against MeHg toxicity, there may be other yet unidentified mechanism(s) initiated by 15d-PGJ₂ treatment that contributed to its protection against MeHg cytotoxicity.

Keywords

15d-PGJ₂; glutathione; methylmercury; mitochondrial membrane potential

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Introduction

Methylmercury (MeHg) causes severe neurological disorders in the central nervous system (CNS) in humans and experimental animals (Clarkson et al., 2003). The particularly devastating effects on development of the fetal CNS prompted the Food and Drug Administration and the Environmental Protection Agency to issue a series of Consumer Advisories alerting pregnant women and women of childbearing age of the dangers of MeHg toxicity. Other than direct neurotoxicity, the malfunction of glia when poisoned with MeHg is an important factor contributing to MeHg-induced neuronal death (Aschner, 1996, Aschner et al., 1999).

Mitochondria are believed to be a major target of MeHg. For example, Yee et al. reported that cultured CNS cells ceased respiration within 30 min of MeHg treatment, and presented evidence that MeHg inhibited the mitochondrial electron transport chain (Yee and Choi, 1996). Allen et al. also reported that MeHg had a selective effect on mitochondria of cultured astrocytes (Allen et al., 2001). Electrical distribution on the mitochondrial membrane is polarized such that it is negative inside the organelle. MeHg causes mitochondrial dysfunction and depolarization in a variety of cell types (Limke and Atchison, 2002, InSug et al., 1997, Shenker et al., 1998, Garg and Chang, 2006). This could lead to subsequent glutathione (GSH) depletion and reactive oxygen species (ROS) generation (Yee and Choi, 1996, Shenker et al., 1999), and oxidative stress may play a role in MeHg cytotoxicity.

The prostaglandin derivative 15-deoxy-delta-12,14-prostaglandin J₂ (15d-PGJ₂) has a wide variety of effects on cellular functions. This compound is normally present in tissues at low levels (<1 nM), but can reach high concentrations during infection and inflammation (Gilroy et al., 1999, Itoh et al., 2004). This agent has a potent inhibitory effect on immune cells. For example, it is a potent inhibitor of macrophage (Jiang et al., 1998, Ricote et al., 1998, Castrillo et al., 2000) and microglia (Petrova et al., 1999, Bernardo et al., 2000, Koppal et al., 2000) activation. Some of its functions are dependent on the activation of the nuclear receptor, peroxisome proliferator activated receptor-gamma (PPAR γ), but others are PPAR γ independent (see (Straus and Glass, 2001) for a review). Our previous studies showed that 15d-PGJ₂ prevented cell death caused by oxidative stress induced by H₂O₂ or *t*-butyl hydroperoxide (Garg and Chang, 2003, Garg and Chang, 2004) in a PPAR γ independent manner. Results from our studies indicate that 15d-PGJ₂ can prevent oxidant-induced mitochondrial depolarization, which should partially account for its cytoprotection (Garg and Chang, 2004). We also demonstrated that MeHg cytotoxicity in the N9 microglia cell line could be partially prevented by 15d-PGJ₂ (Garg and Chang, 2006). The protection of this agent against MeHg was not mediated through PPAR γ activation because other PPAR γ agonists did not exhibit the protective effect (Garg and Chang, 2006).

Recent studies indicate that the α,β -unsaturated carbonyl group within the cyclopentenone ring of 15d-PGJ₂ is essential for its PPAR γ -independent activity (Straus and Glass, 2001). Specifically, it enables the molecule to function as an electrophile which can bind to sulfhydryl groups or cysteine residues of proteins and change their functions. By this mechanism, 15d-PGJ₂ can activate the Keap1-Nrf2-ARE pathway in the following manner (Kensler et al., 2007): Binding of 15d-PGJ₂ to Keap-1 releases the transcription factor, Nrf-2, which is translocated to the nucleus. Together with other transcription factors and proteins, the transactivation of ARE (Antioxidant Responsive Element, also known as Electrophile Responsive Element)-responsive genes occurs. Many of the genes induced in this manner are cytoprotective.

With respect to MeHg cytotoxicity, the induction of cellular GSH synthesis by 15d-PGJ₂ through the mechanism described above appears to be very important. 15d-PGJ₂ can up-

regulate glutamylcysteine synthetase, the rate limiting enzyme for GSH synthesis (Qin et al., 2006, Saito et al., 2007). This property can prepare cells to fight against oxidative stress induced by oxidant (Qin et al., 2006) or by glutamate (Aoun et al., 2003, Saito et al., 2007). Enhanced cellular GSH levels might modulate MeHg cytotoxicity because cells with intrinsic higher GSH levels appear more resistant to MeHg cytotoxicity (Shenker et al., 1993).

Another important gene induced by 15d-PGJ₂ is heme oxygenase-1 (HO-1). Induction of HO-1 in immune cells by 15d-PGJ₂ may modulate inflammation (Gong et al., 2002, Itoh et al., 2004, Alvarez-Maqueda et al., 2004). While up-regulation of HO-1 is neuroprotective under various experimental conditions (Hegazy et al., 2000, Imuta et al., 2007, Le et al., 1999, Satoh et al., 2003), it can be detrimental to cells in other conditions (Song et al., 2007, Song et al., 2006). Whether HO-1 induction caused by 15d-PGJ₂ can modulate MeHg cytotoxicity is not clear.

Based on the discussion above, it is clear that while MeHg causes mitochondrial dysfunction and GSH depletion, 15d-PGJ₂ can prevent oxidant induced mitochondrial dysfunction and increase cellular GSH levels. The current study tested the protective effect of 15d-PGJ₂ in glia, and tested the hypothesis that up-regulation of GSH by 15d-PGJ₂ was the major mechanism leading to prevention of MeHg-induced mitochondrial depolarization and cell death. The C6 glioma cell line and primary mouse glia were used in this study.

Materials and Methods

JC-1 (5,5', 6,6'-tetrachloro-1, 1', 3,3'-tetraethylbenzimidazolcarbocyanine iodide) was from Molecular Probes (Eugene, OR). 15d-PGJ₂ (1 mg/ml in methyl acetate) was from Cayman (Ann Arbor, MI). Tin-mesoporphyrin was from Frontier Scientific (Logan, Utah). BSO (D, L-buthionine-(S,R)-sulfoximine) was from Acros Organics (distributed by Fisher Scientific (Pittsburgh, PA)). Hank's Balanced Salt Solution (HBSS), MeHgCl (contained ~13% Cl), monochlorobimane and other general biochemical reagents were from Sigma (St. Louis, MO) unless otherwise stated.

Cell cultures

The C6 glioma cells (from the American Type Culture Collection) were grown in 75 cm² tissue culture flasks for routine maintenance. Mixed mouse cerebral glia derived from 1–2 days old C57BL mice were plated in 75 cm² flasks, expanded once into 150 cm² flasks before plating into multi-well culture plates for experiments. Astrocytes constituted the majority of cells in these cultures because more than 90% of culture surface was covered by cells positive for glial fibrillary acidic protein (GFAP) staining. The growth medium, identical for both cell types, was composed of Minimum Essential Medium (MEM) supplemented with 5% newborn calf serum and 2.5 mM glutamine. To prepare for experiments, cells were plated into culture plates in this growth medium overnight at the following density: 20,000 cells/well in 96-well plates with 100 µl medium or 140,000 cells/well in 24-well plates with 700 µl medium. The medium was switched the following day to MEM supplemented with 1% newborn calf serum and 2.5 mM glutamine for MeHg treatment.

Cell viability

Viability was determined by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Garg and Chang, 2004, Garg and Chang, 2006). The OD of each well was measured by a plate reader (Spectra Max 190, Molecular Devices, Sunnyvale, CA) with a filter setting at 570 nm (reference filter setting was 630 nm). Our previous studies indicated that the MTT assay agreed well with results from the trypan blue exclusion assay (Garg and Chang, 2006).

Measurement of mitochondrial membrane potential (MMP, $\Delta\Psi_m$)

Cells were plated in 24-well plates overnight before the assay was performed as described previously (Garg and Chang, 2004). Each culture was treated with MeHg (700 μ l/well prepared in medium with 1% newborn calf serum) at 37°C for a period of time indicated in each experiment. MeHg-containing medium was then replaced with 5 μ M JC-1 prepared in Hank's Balanced Salt Solution, (HBSS, 300 μ l/well) for 15 minutes at 37°C. After this incubation period, cells in each well were rinsed once with 500 μ l HBSS, collected into 300 μ l HBSS, and then transferred to a 96-well tissue culture plate. The fluorescence of each well was detected by a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA) with the following settings: excitation 485 nm, emission 545 nm and 595 nm, cutoff 530 nm. In some experiments, the emission spectra between 500–620 nm were obtained. To prepare JC-1, stock solution (5 mM prepared in DMSO) was diluted (1:4) into 5% BSA, and then further diluted (1:199) into HBSS. After proper mixing, this solution was filtered through a 0.2 μ m filter, shielded from light until use.

Measurement of GSH

This was performed according to the method described by Sebastia et al. (Sebastia et al., 2003). After treatment with testing agent, cells were incubated with 40 μ M monochlorobimane (prepared in HBSS) at 37°C for 20 min. The fluorescence of each well was detected by a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices, Sunnyvale, CA) with the following settings: excitation 390 nm, emission 485 nm, cutoff 530 nm.

Statistical analysis

Unless otherwise stated, results were pooled from 12 replicate samples derived from 3 independent experiments (cell viability, GSH assay) or from 9 replicate samples derived from 3 independent experiments (mitochondrial membrane potential), and expressed as mean $\pm$ SEM ($n=3$). Statistical analyses were performed by one-way- or two-way ANOVA. When appropriate, the two-way ANOVA was followed by a one-way ANOVA analysis to determine the differences within a selected group. The Bonferroni test was used for post-hoc analysis, and $p<0.05$ was considered as significant difference. See figure legends for detailed description of statistical analysis for each particular set of experiments.

Results

15d-PGJ₂ prevented cytotoxicity of MeHg in C6 glioma cells

Our previous studies indicated that pre-incubation of the N9 microglial cells with 15d-PGJ₂ for 4 hours attenuated MeHg toxicity (Garg and Chang, 2006). The first set of experiments in the current study determined whether the protection could be observed in other cell types, and whether a shorter or longer incubation period would yield different protective effects.

C6 glioma cells were treated with 1 μ M 15d-PGJ₂ for 0.5 hrs, 4 hrs or overnight (>18 hrs) followed by various concentrations of MeHg (without 15d-PGJ₂) overnight, and then the viability of each treatment was determined. Results indicated that MeHg at 2.5 μ M, 5 μ M or 10 μ M reduced the viability to ~75%, ~33% or ~8% of control, respectively (Fig. 1A, 1st column in each set). Pre-incubation of cells with 15d-PGJ₂ for 0.5 hrs showed no protection compared to MeHg alone (Fig. 1A, 2nd column in each set). Pre-incubation for 4 hrs caused a slight protection (Fig. 1A, 3rd column in each set), similar to what we observed earlier with the N9 microglial cell line (Garg and Chang, 2006). Overnight incubation led to significant protection by this agent (Fig. 1A, 4th column in each set). These results suggested that time was required for 15d-PGJ₂ to up-regulate the cellular defense system against MeHg cytotoxicity. Unless otherwise stated, overnight (>18 hours) incubation was used in all of the

following experiments to further explore the protective effect of 15d-PGJ₂ against MeHg cytotoxicity.

Subsequent experiments indicated that 15d-PGJ₂ could prevent MeHg cytotoxicity in a concentration-dependent manner. For example, while 5 μ M MeHg reduced cellular viability to ~26% of control in this set of experiments, overnight incubation of 15d-PGJ₂ at 0.5 μ M, 1 μ M or 2 μ M raised the viability to ~42%, ~72% or ~86% of control, respectively (Fig. 1B, left-side columns). Similar protective effect by 15d-PGJ₂ could be observed when cells were treated with 10 μ M MeHg (Fig. 1B, right-side columns).

15d-PGJ₂ prevented mitochondrial depolarization caused by MeHg

MeHg is known to cause mitochondrial dysfunction, which leads to mitochondrial membrane depolarization (Limke and Atchison, 2002, InSug et al., 1997, Shenker et al., 1998, Garg and Chang, 2006). The next set of experiments characterized the alteration of mitochondrial membrane potential ($\Delta\Psi$ m) in C6 glioma cells caused by MeHg, and determined whether 15d-PGJ₂ could prevent mitochondrial depolarization. In control cells, the emission intensity of the green peak (JC-1 monomers) and the red peak (JC-1 aggregates) could be determined optimally at ~545 nm and ~595 nm, respectively (Fig. 2A). Treatment of cells with 5 μ M MeHg for 4 hours altered the spectra, such that the height of the 545 nm peak increased and that of the 595 nm peak decreased, a sign of mitochondrial depolarization (Fig. 2B).

The emission intensity ratio of the 545 nm and 595 nm peaks was used as a measure of mitochondrial depolarization; a higher ratio indicated more depolarization. MeHg caused a concentration-dependent increase of the 545/595 emission intensity ratio, such that a 4-hr treatment of cells with MeHg at 2.5 μ M, 5 μ M and 10 μ M raised the 545/595 emission intensity ratio from ~0.239 (control) to ~0.417, ~0.641 or ~0.892, respectively (Fig. 3A). At a fixed MeHg concentration (e.g., 5 μ M), there was a time-dependent increase in the 545/595 emission intensity ratio (Fig. 3B, open columns). Treatment of cells with 1 μ M 15d-PGJ₂ did not alter the 545/595 ratio (Fig. 2C). However, pretreatment of cells with this agent prevented mitochondrial depolarization induced by MeHg treatment (Fig. 2D and Fig. 3B, closed columns).

15d-PGJ₂ prevented cytotoxicity of MeHg in primary mouse glia: possible involvement of GSH

Given the protective effect of 15d-PGJ₂ in a continuous cell line, i.e., C6 glioma cells (Fig. 1), subsequent experiments tested whether a similar protection could be observed in primary cultures. Furthermore, an earlier report indicated that 15d-PGJ₂ could up-regulate glutathione (GSH), thus preventing oxidant induced cell death (Qin et al., 2006). The possible involvement of GSH in the observed protection caused by 15d-PGJ₂ was investigated.

Treatment of mouse glia with 10 μ M MeHg overnight reduced cell viability to ~22% of control. Pretreatment of cells with 0.5 μ M, 1 μ M or 2 μ M 15d-PGJ₂ overnight raised the viability to ~52%, 63% or ~85%, respectively (Fig. 4, left-side columns). MeHg at 5 μ M reduced the viability to ~71% of control, and 2 μ M 15d-PGJ₂ pretreatment raised the viability to ~97% of control (not shown).

Subsequent experiments indicated that 4-hr treatment of cells with 10 μ M MeHg reduced cellular GSH levels to ~53% of control (Fig. 5A, compare 1st column with 2nd column). Overnight pretreatment of cells with 2 μ M 15d-PGJ₂ raised cellular GSH levels to ~174% of control (Fig. 5A, 3rd column), which prevented the GSH depletion caused by MeHg treatment (Fig. 5A, 4th column).

BSO prevented GSH up-regulation caused by 15d-PGJ₂, but not the protective effect of 15d-PGJ₂

If induction of GSH was responsible for the protection provided by 15d-PGJ₂, blockage of 15d-PGJ₂-induced GSH should prevent the protection. Buthionine Sulfoximine (BSO), an inhibitor of the rate-limiting enzyme of GSH synthesis, was used to test this possibility. The effects of this agent on GSH levels, mitochondrial depolarization and viability were investigated.

Experiments were first conducted to verify that BSO could block the up-regulation of GSH caused by 15d-PGJ₂. Results indicated that overnight treatment of cells with 10 μ M BSO reduced cellular GSH levels to ~51% of control (Fig. 5B, compare 1st column with 2nd column). Furthermore, BSO completely prevented GSH up-regulation induced by 2 μ M 15d-PGJ₂ (Fig. 5B, 3rd column).

Assessment of mitochondrial depolarization indicated that 10 μ M MeHg raised the 545/595 ratio in the primary mouse glia (Fig. 6, compare 1st with 2nd column). While 15d-PGJ₂ itself had little effect on mitochondrial depolarization (Fig. 6, 3rd column), it prevented the mitochondrial depolarization caused by MeHg (Fig. 6, 4th column). These results were consistent with those observed in the C6 glioma cells (see Fig. 2 and Fig. 3).

BSO itself had little effect on mitochondrial depolarization (Fig. 6, 5th column) but it slightly increased mitochondrial depolarization caused by MeHg (Fig. 6, 6th column). The combination of BSO and 15d-PGJ₂ had little effect on mitochondrial depolarization (Fig. 6, 7th column). Cells pretreated with a combination of these two agents maintained mitochondrial potential at the control level when challenged with MeHg (Fig. 6, last column). These results indicated that 15d-PGJ₂ had a dominant effect over BSO in stabilizing mitochondrial potential in cells challenged with MeHg.

Assessment of cell viability indicated that BSO at 10 μ M caused no effect on cell viability (not shown), but increased the cytotoxicity caused by 10 μ M MeHg. The viability decreased from ~22% (Fig. 4, 1st open column from the left) to half of this level, i.e., to ~11% (Fig. 4, 1st closed column from the left). When cells were treated with 10 μ M BSO plus 15d-PGJ₂ overnight followed by MeHg treatment (without BSO or 15d-PGJ₂), the protective effect of 15d-PGJ₂ was attenuated (Fig. 4, compare left-side columns with right-side columns). Since BSO at this concentration completely blocked the GSH induction caused by 15d-PGJ₂ (Fig. 5B) but did not completely block the protection caused by 15d-PGJ₂ (Fig. 4), these results suggested that GSH induction by 15d-PGJ₂ might help to prepare cells against a MeHg challenge, but other cellular effects were also involved in the protection caused by 15d-PGJ₂.

HO-1 was not involved in the cytoprotective effect of 15d-PGJ₂

As discussed in the Introduction, 15d-PGJ₂ can also induce HO-1, which may modulate cellular responses against toxic insults. HO-1 activity can be effectively blocked by tin-mesoporphyrin (SnMP), such that 2.5 μ M SnMP can inhibit more than 97% of its activity (Dwyer et al., 1998). A set of experiments was performed to determine whether SnMP could modulate the cytoprotective effect of 15d-PGJ₂. Results indicated that MeHg at 5 μ M or 10 μ M reduced cellular viability to 56 $\pm$ 4% or 25 $\pm$ 5%, respectively (results derived from 12 wells from 3 independent experiments). 15d-PGJ₂ at 1 μ M raised the viability to 92 $\pm$ 4% or 45 $\pm$ 6%, respectively. Co-treatment of cells with SnMP (5–20 μ M) together with 15d-PGJ₂ did not affect the cytoprotection caused by 15d-PGJ₂ one way or the other (not shown). These results suggested that HO-1 may not be involved in the cytoprotection caused by 15d-PGJ₂.

Discussion

Results from this study and those from our previous report (Garg and Chang, 2006) indicate that 15d-PGJ₂ can prevent MeHg cytotoxicity in various glial cell types including cell lines and primary cultures. It appears that time is required for the cells to up-regulate their cellular defense against MeHg. For example, 30 min pre-incubation of cells with 15d-PGJ₂ had no protective effect at all, but overnight incubation led to significant protection (Fig. 1A). This observation rules out the possibility that chemical interactions between 15d-PGJ₂ and MeHg somehow neutralize MeHg cytotoxicity, because one would expect that 30 min pre-incubation should cause some protection if this is the case.

The concentrations of 15d-PGJ₂ used in this study is 2 μ M or below. This agent in this concentration range exhibits cytoprotective effects in several experimental systems. For example, our previous studies showed that 15d-PGJ₂ prevented cell death caused by oxidative stress induced by H₂O₂ or *t*-butyl hydroperoxide (Garg and Chang, 2003, Garg and Chang, 2004) or by toxic compounds generated by oxidative stress (e.g. oxysterols) (Chang and Liu, 2001). At higher concentrations (10 μ M or above), 15d-PGJ₂ can cause cell death in a variety of cell types. For example, treatment of C6 glioma cells with 10 μ M 15d-PGJ₂ causes a reduction in cell viability to less than 10% of control after one day, and apoptosis is the mode of cell death (Zander et al., 2002). This agent at 10–100 μ M also causes death of cultured rat cerebellar granule neurons (Smith et al., 2003). This biphasic activity (cytoprotective versus cytotoxic) of 15d-PGJ₂ is a subject of study in several reports (Levonen et al., 2001, Na and Surh, 2003, Wang et al., 2006).

While MeHg caused mitochondrial depolarization, pretreatment of cells with 15d-PGJ₂ greatly prevented this from happening (Fig. 2, Fig. 3 and Fig. 6). This is significant because mitochondrial dysfunction can impair cellular energy supply, cause the generation of reactive oxygen species, and even initiate the apoptosis pathway (Belletti et al., 2002, Shenker et al., 1999, Yee and Choi, 1996). Prevention of mitochondrial dysfunction can thus prevent all of these downstream events.

The mechanism by which 15d-PGJ₂ stabilizes mitochondrial membrane potential is not clear. As described in the Introduction, one possibility is that 15d-PGJ₂ can bind Keap1, which subsequently activate the transcription factor Nrf2, resulting in an up-regulation of cellular mechanism(s) against oxidative stress. Through this mechanism, 15d-PGJ₂ can up-regulate glutamylcysteine synthetase, the key enzyme for cellular GSH synthesis, as further discussed below.

Qin et al. reported that 15d-PGJ₂ caused a dose-dependent increase in GSH levels in the ARPE-19 cells, with a maximal induction at ~2 μ M 15d-PGJ₂. With 1 μ M 15d-PGJ₂, the induction occurred maximally with 18–24 hrs treatment (Qin et al., 2006). This concentration and time reported in their study regarding GSH induction fits very well with the protection caused by 15d-PGJ₂ observed in the current study (Fig. 1).

MeHg treatment can lead to cellular GSH depletion, a phenomenon also observed in the current study (Fig. 5A). There is convincing evidence that higher cellular GSH levels may reduce MeHg-induced cytotoxicity. For example, by using monochlorobimane staining and cell sorting, Shenker et al. reported that lymphocytes with higher intrinsic GSH levels were more resistant to mercury treatment (Shenker et al., 1993). Subsequent studies by this group indicated that depletion of cellular GSH with 50 mM BSO promoted MeHg-induced apoptosis (Shenker et al., 1999). Kaur et al. reported that a reduction of GSH led to a slight increase in MeHg accumulation and ROS generation in glia. This also caused a slight increase in MeHg toxicity in glia (Kaur et al., 2006). The involvement of GSH depletion in MeHg cytotoxicity was also supported by Gatti et al. (Gatti et al., 2004).

In contrast to those reports above, other studies suggested that the alteration of cellular GSH levels alone did not account for MeHg toxicity. For example, Lee et al. reported that 0.2 mM BSO reduced GSH in glioma to approximately 44% of control levels, but did not cause cell death. In comparison, 10 μ M HgCl₂ reduced GSH to 66% of control but caused significant cell death. Based on these observations, it was concluded that mercury-induced depletion of GSH alone did not cause cell death (Lee et al., 2001). Ou et al. reported that significant up- or down-regulation of cellular GSH levels had little effect on MeHg cytotoxicity in embryonic neuronal or embryonic limb bud cells. They thus concluded that cellular GSH did not affect MeHg toxicity in their experimental system (Ou et al., 1999).

Results from this study indicated that overnight treatment of mouse glia with 10 μ M BSO led to a decrease in GSH levels to ~51% of control (Fig. 5B), but had no negative effect on cell viability. This supports the hypothesis that depletion of GSH levels itself should not be responsible for cell death. This treatment, however, increased cell death caused by 10 μ M MeHg (Fig. 4, compare the 1st column on the left-side set and that on the right-side set). This suggests that a reduction of cellular GSH makes cells less defensive against MeHg.

Up-regulation of GSH by 15d-PGJ₂ was the mechanism proposed that accounted for the protection of ARPE-19 cells against H₂O₂ insult (Qin et al., 2006), cortical neurons against glutamate toxicity (Saito et al., 2007) as well as PC12 cells against nitrosative insults (Lim et al., 2004). The current study showed that 15d-PGJ₂ caused up-regulation of GSH, which helped to prevent GSH decrease caused by MeHg (Fig. 5A). It is possible that the induced GSH can greatly increase cellular defense against oxidative stress caused by MeHg. Furthermore, the induced GSH may bind to MeHg directly and minimize its toxicity toward cellular target proteins. In addition to GSH levels, 15d-PGJ₂ also induces the expression of glutathione S-transferase, thus should enhance cellular cytoprotection (Kawamoto et al., 2000).

While concurrent addition of BSO completely blocked the GSH increase caused by 15d-PGJ₂ (Fig. 5B), this did not reverse the stabilization of mitochondrial potential caused by 15d-PGJ₂ (Fig. 6). Nor did this completely block the protection of 15d-PGJ₂ against MeHg cytotoxicity (Fig. 4, compare left-side columns with those on the right-side). In other words, 15d-PGJ₂ still exhibited significant protection against MeHg cytotoxicity in the absence of GSH induction. Thus, induction of GSH might be helpful, but it did not completely account for the protection caused by 15d-PGJ₂.

In addition to GSH, 15d-PGJ₂ can also induce HO-1 in various cell types (Landar et al., 2006, Qin et al., 2006). There is evidence that HO-1 is cytoprotective in a number of experimental settings (reviewed by (Otterbein et al., 2003, Ryter et al., 2006)). Results from this study indicated that inhibition of HO-1 by SnMP (5–20 μ M) had no effect on the protection caused by 15d-PGJ₂. Induction of HO-1, thus, did not account for the protective effect of 15d-PGJ₂.

MeHg is known to cause an increase in cytoplasmic and mitochondrial calcium levels. The increase in cytoplasmic calcium may be caused by release of intracellular stores or by calcium influx from extracellular space (Dreiem and Seegal, 2007, Mundy and Freudenrich, 2000, Marty and Atchison, 1997), and there are interactions between different intracellular calcium stores (Limke et al., 2003, Limke et al., 2004). This calcium increase can lead to cell death by activation of the calpain (Sakaue et al., 2005) or the phospholipase C system (Kang et al., 2006). At low concentrations (nanomolar range), 15d-PGJ₂ can either modulate (Kobayashi et al., 2005) or initiate (Monneret et al., 2002) calcium flux in eosinophils. At high concentrations (10–100 μ M), this agent can activate calcium flux in nociceptive neurons (Taylor-Clark et al., 2008). Whether 15d-PGJ₂ can modulate MeHg-induced calcium flux in glia, and whether this can modulate MeHg cytotoxicity in glia remains to be determined.

In summary, pretreatment of glia with 15d-PGJ₂ prevents MeHg-induced mitochondrial depolarization, glutathione depletion and cell death in mouse glia. Results from this study do not support the original hypothesis that up-regulation of GSH is the main mechanism responsible for the protection caused by 15d-PGJ₂. Furthermore, there is no evidence that HO-1 is involved in the cytoprotection. The mechanism(s) by which this agent protects glia against MeHg cytotoxicity remains to be determined.

Abbreviations

15d-PGJ₂, 15-deoxy-delta-12,14-prostaglandin J₂
 BSO, D, L-buthionine-(S,R)-sulfoximine
 HBSS, Hank's Balanced Salt Solution
 HO-1, heme oxygenase-1
 JC-1, 5,5', 6,6'-tetrachloro-1, 1', 3,3'- tetraethylbenzimidazolcarbocyanine iodide
 MeHg, methylmercury
 MEM, Minimum Essential Medium
 MMP, mitochondrial membrane potential ($\Delta\Psi_m$)
 MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
 OD, optical density
 PBS, phosphate buffered saline
 ROS, reactive oxygen species
 SnMP, tin-mesoporphyrin

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References

- Allen JW, El-Oqayli H, Aschner M, Syversen T, Sonnewald U. Methylmercury has a selective effect on mitochondria in cultured astrocytes in the presence of [U-(13C)]glutamate. *Brain Res* 2001;908:149–154. [PubMed: 11454325]
- Alvarez-Maqueda M, El Bekay R, Alba G, et al. 15-deoxy-delta 12,14-prostaglandin J2 induces heme oxygenase-1 gene expression in a reactive oxygen species-dependent manner in human lymphocytes. *J Biol Chem* 2004;279:21929–21937. [PubMed: 15024026]
- Aoun P, Simpkins JW, Agarwal N. Role of PPAR-gamma ligands in neuroprotection against glutamate-induced cytotoxicity in retinal ganglion cells. *Invest Ophthalmol Vis Sci* 2003;44:2999–3004. [PubMed: 12824244]
- Aschner M. Methylmercury in astrocytes--what possible significance? *Neurotoxicology* 1996;17:93–106. [PubMed: 8784821]
- Aschner M, Allen JW, Kimelberg HK, LoPachin RM, Streit WJ. Glial cells in neurotoxicity development. *Annu Rev Pharmacol Toxicol* 1999;39:151–173. [PubMed: 10331080]
- Belletti S, Orlandini G, Vettori MV, Mutti A, Uggeri J, Scandroglio R, Alinovi R, Gatti R. Time course assessment of methylmercury effects on C6 glioma cells: submicromolar concentrations induce oxidative DNA damage and apoptosis. *J Neurosci Res* 2002;70:703–711. [PubMed: 12424738]
- Bernardo A, Levi G, Minghetti L. Role of the peroxisome proliferator-activated receptor-gamma (PPAR-gamma) and its natural ligand 15-deoxy-Delta12, 14-prostaglandin J2 in the regulation of microglial functions. *Eur J Neurosci* 2000;12:2215–2223. [PubMed: 10947800]
- Castrillo A, Diaz-Guerra MJ, Hortelano S, Martin-Sanz P, Bosca L. Inhibition of IkappaB kinase and IkappaB phosphorylation by 15-deoxy-Delta(12,14)-prostaglandin J(2) in activated murine macrophages. *Mol Cell Biol* 2000;20:1692–1698. [PubMed: 10669746]
- Chang JY, Liu L. Peroxisome proliferator-activated receptor agonists prevent 25-OH-cholesterol induced c-jun activation and cell death. *BMC Pharmacol* 2001;1:10. [PubMed: 11737865]

- Clarkson TW, Magos L, Myers GJ. The toxicology of mercury--current exposures and clinical manifestations. *N Engl J Med* 2003;349:1731–1737. [PubMed: 14585942]
- Dreiem A, Seegal RF. Methylmercury-induced changes in mitochondrial function in striatal synaptosomes are calcium-dependent and ROS-independent. *Neurotoxicology* 2007;28:720–726. [PubMed: 17442395]
- Dwyer BE, Lu SY, Laitinen JT, Nishimura RN. Protective properties of tin- and manganese-centered porphyrins against hydrogen peroxide-mediated injury in rat astroglial cells. *J Neurochem* 1998;71:2497–2504. [PubMed: 9832148]
- Garg TK, Chang JY. Oxidative stress causes ERK phosphorylation and cell death in cultured retinal pigment epithelium: Prevention of cell death by AG126 and 15-deoxy-delta 12, 14-PGJ₂. *BMC Ophthalmol* 2003;3:5. [PubMed: 12659653]
- Garg TK, Chang JY. 15-deoxy-delta 12, 14-Prostaglandin J2 prevents reactive oxygen species generation and mitochondrial membrane depolarization induced by oxidative stress. *BMC Pharmacol* 2004;4:6. [PubMed: 15149553]
- Garg TK, Chang JY. Methylmercury causes oxidative stress and cytotoxicity in microglia: Attenuation by 15-deoxy-delta 12, 14-Prostaglandin J2. *J Neuroimmunol* 2006;171:17–28. [PubMed: 16225932]
- Gatti R, Belletti S, Uggeri J, Vettori MV, Mutti A, Scandroglio R, Orlandini G. Methylmercury cytotoxicity in PC12 cells is mediated by primary glutathione depletion independent of excess reactive oxygen species generation. *Toxicology* 2004;204:175–185. [PubMed: 15388243]
- Gilroy DW, Colville-Nash PR, Willis D, Chivers J, Paul-Clark MJ, Willoughby DA. Inducible cyclooxygenase may have anti-inflammatory properties. *Nat Med* 1999;5:698–701. [PubMed: 10371510]
- Gong P, Stewart D, Hu B, Li N, Cook J, Nel A, Alam J. Activation of the mouse heme oxygenase-1 gene by 15-deoxy-Delta(12,14)-prostaglandin J(2) is mediated by the stress response elements and transcription factor Nrf2. *Antioxid Redox Signal* 2002;4:249–257. [PubMed: 12006176]
- Hegazy KA, Dunn MW, Sharma SC. Functional human heme oxygenase has a neuroprotective effect on adult rat ganglion cells after pressure-induced ischemia. *Neuroreport* 2000;11:1185–1189. [PubMed: 10817588]
- Imuta N, Hori O, Kitao Y, Tabata Y, Yoshimoto T, Matsuyama T, Ogawa S. Hypoxia-mediated induction of heme oxygenase type I and carbon monoxide release from astrocytes protects nearby cerebral neurons from hypoxia-mediated apoptosis. *Antioxid Redox Signal* 2007;9:543–552. [PubMed: 17330989]
- InSug O, Datar S, Koch CJ, Shapiro IM, Shenker BJ. Mercuric compounds inhibit human monocyte function by inducing apoptosis: evidence for formation of reactive oxygen species, development of mitochondrial membrane permeability transition and loss of reductive reserve. *Toxicology* 1997;124:211–224. [PubMed: 9482123]
- Itoh K, Mochizuki M, Ishii Y, et al. Transcription factor Nrf2 regulates inflammation by mediating the effect of 15-deoxy-Delta(12,14)-prostaglandin J(2). *Mol Cell Biol* 2004;24:36–45. [PubMed: 14673141]
- Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature* 1998;391:82–86. [PubMed: 9422509]
- Kang MS, Jeong JY, Seo JH, et al. Methylmercury-induced toxicity is mediated by enhanced intracellular calcium through activation of phosphatidylcholine-specific phospholipase C. *Toxicol Appl Pharmacol* 2006;216:206–215. [PubMed: 16854443]
- Kaur P, Aschner M, Syversen T. Glutathione modulation influences methyl mercury induced neurotoxicity in primary cell cultures of neurons and astrocytes. *Neurotoxicology* 2006;27:492–500. [PubMed: 16513172]
- Kawamoto Y, Nakamura Y, Naito Y, et al. Cyclopentenone prostaglandins as potential inducers of phase II detoxification enzymes. 15-deoxy-delta(12,14)-prostaglandin j2-induced expression of glutathione S-transferases. *J Biol Chem* 2000;275:11291–11299. [PubMed: 10753940]
- Kensler TW, Wakabayashi N, Biswal S. Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. *Annu Rev Pharmacol Toxicol* 2007;47:89–116. [PubMed: 16968214]
- Kobayashi Y, Ueki S, Mahemuti G, Chiba T, Oyamada H, Saito N, Kanda A, Kayaba H, Chihara J. Physiological levels of 15-deoxy-Delta12,14-prostaglandin J2 prime eotaxin-induced chemotaxis on

- human eosinophils through peroxisome proliferator-activated receptor-gamma ligation. *J Immunol* 2005;175:5744–5750. [PubMed: 16237065]
- Koppal T, Petrova TV, Van Eldik LJ. Cyclopentenone prostaglandin 15-deoxy-Delta(12,14)-prostaglandin J(2) acts as a general inhibitor of inflammatory responses in activated BV-2 microglial cells. *Brain Res* 2000;867:115–121. [PubMed: 10837804]
- Landar A, Zmijewski JW, Dickinson DA, et al. Interaction of electrophilic lipid oxidation products with mitochondria in endothelial cells and formation of reactive oxygen species. *Am J Physiol Heart Circ Physiol* 2006;290:H1777–H1787. [PubMed: 16387790]
- Le WD, Xie WJ, Appel SH. Protective role of heme oxygenase-1 in oxidative stress-induced neuronal injury. *J Neurosci Res* 1999;56:652–658. [PubMed: 10374820]
- Lee YW, Ha MS, Kim YK. Role of reactive oxygen species and glutathione in inorganic mercury-induced injury in human glioma cells. *Neurochem Res* 2001;26:1187–1193. [PubMed: 11874199]
- Levonen AL, Dickinson DA, Moellering DR, Mulcahy RT, Forman HJ, Darley-Usmar VM. Biphasic effects of 15-deoxy-delta(12,14)-prostaglandin J(2) on glutathione induction and apoptosis in human endothelial cells. *Arterioscler Thromb Vasc Biol* 2001;21:1846–1851. [PubMed: 11701476]
- Lim SY, Jang JH, Na HK, Lu SC, Rahman I, Surh YJ. 15-Deoxy-Delta12,14-prostaglandin J(2) protects against nitrosative PC12 cell death through up-regulation of intracellular glutathione synthesis. *J Biol Chem* 2004;279:46263–46270. [PubMed: 15319433]
- Limke TL, Atchison WD. Acute exposure to methylmercury opens the mitochondrial permeability transition pore in rat cerebellar granule cells. *Toxicol Appl Pharmacol* 2002;178:52–61. [PubMed: 11781080]
- Limke TL, Heidemann SR, Atchison WD. Disruption of intraneuronal divalent cation regulation by methylmercury: are specific targets involved in altered neuronal development and cytotoxicity in methylmercury poisoning? *Neurotoxicology* 2004;25:741–760. [PubMed: 15288506]
- Limke TL, Otero-Montanez JK, Atchison WD. Evidence for interactions between intracellular calcium stores during methylmercury-induced intracellular calcium dysregulation in rat cerebellar granule neurons. *J Pharmacol Exp Ther* 2003;304:949–958. [PubMed: 12604669]
- Marty MS, Atchison WD. Pathways mediating Ca²⁺ entry in rat cerebellar granule cells following in vitro exposure to methyl mercury. *Toxicol Appl Pharmacol* 1997;147:319–330. [PubMed: 9439727]
- Monneret G, Li H, Vasilescu J, Rokach J, Powell WS. 15-Deoxy-delta 12,14-prostaglandins D2 and J2 are potent activators of human eosinophils. *J Immunol* 2002;168:3563–3569. [PubMed: 11907120]
- Mundy WR, Freudenrich TM. Sensitivity of immature neurons in culture to metal-induced changes in reactive oxygen species and intracellular free calcium. *Neurotoxicology* 2000;21:1135–1144. [PubMed: 11233760]
- Na HK, Surh YJ. Peroxisome proliferator-activated receptor gamma (PPARgamma) ligands as bifunctional regulators of cell proliferation. *Biochem Pharmacol* 2003;66:1381–1391. [PubMed: 14555212]
- Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* 2003;24:449–455. [PubMed: 12909459]
- Ou YC, White CC, Krejsa CM, Ponce RA, Kavanagh TJ, Faustman EM. The role of intracellular glutathione in methylmercury-induced toxicity in embryonic neuronal cells. *Neurotoxicology* 1999;20:793–804. [PubMed: 10591515]
- Petrova TV, Akama KT, Van Eldik LJ. Cyclopentenone prostaglandins suppress activation of microglia: down-regulation of inducible nitric-oxide synthase by 15-deoxy-Delta12,14-prostaglandin J2. *Proc Natl Acad Sci USA* 1999;96:4668–4673. [PubMed: 10200320]
- Qin S, McLaughlin AP, De Vries GW. Protection of RPE cells from oxidative injury by 15-deoxy-delta12,14-prostaglandin J2 by augmenting GSH and activating MAPK. *Invest Ophthalmol Vis Sci* 2006;47:5098–5105. [PubMed: 17065531]
- Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature* 1998;391:79–82. [PubMed: 9422508]
- Ryter SW, Alam J, Choi AM. Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 2006;86:583–650. [PubMed: 16601269]

- Saito Y, Nishio K, Numakawa Y, Ogawa Y, Yoshida Y, Noguchi N, Niki E. Protective effects of 15-deoxy-Delta12,14-prostaglandin J2 against glutamate-induced cell death in primary cortical neuron cultures: induction of adaptive response and enhancement of cell tolerance primarily through up-regulation of cellular glutathione. *J Neurochem* 2007;102:1625–1634. [PubMed: 17697048]
- Sakaue M, Okazaki M, Hara S. Very low levels of methylmercury induce cell death of cultured rat cerebellar neurons via calpain activation. *Toxicology* 2005;213:97–106. [PubMed: 15982794]
- Satoh T, Baba M, Nakatsuka D, et al. Role of heme oxygenase-1 protein in the neuroprotective effects of cyclopentenone prostaglandin derivatives under oxidative stress. *Eur J Neurosci* 2003;17:2249–2255. [PubMed: 12814358]
- Sebastia J, Cristofol R, Martin M, Rodriguez-Farre E, Sanfeliu C. Evaluation of fluorescent dyes for measuring intracellular glutathione content in primary cultures of human neurons and neuroblastoma SH-SY5Y. *Cytometry* 2003;51:16–25. [PubMed: 12500301]
- Shenker BJ, Guo TL, O I, Shapiro IM. Induction of apoptosis in human T-cells by methyl mercury: temporal relationship between mitochondrial dysfunction and loss of reductive reserve. *Toxicol Appl Pharmacol* 1999;157:23–35. [PubMed: 10329504]
- Shenker BJ, Guo TL, Shapiro IM. Low-level methylmercury exposure causes human T-cells to undergo apoptosis: evidence of mitochondrial dysfunction. *Environ Res* 1998;77:149–159. [PubMed: 9600808]
- Shenker BJ, Mayro JS, Rooney C, Vitale L, Shapiro IM. Immunotoxic effects of mercuric compounds on human lymphocytes and monocytes. IV. Alterations in cellular glutathione content. *Immunopharmacol Immunotoxicol* 1993;15:273–290. [PubMed: 8349953]
- Smith SA, Monteith GR, Holman NA, Robinson JA, May FJ, Roberts-Thomson SJ. Effects of peroxisome proliferator-activated receptor gamma ligands ciglitazone and 15-deoxy-delta 12,14-prostaglandin J2 on rat cultured cerebellar granule neuronal viability. *J Neurosci Res* 2003;72:747–755. [PubMed: 12774315]
- Song L, Song W, Schipper HM. Astroglia overexpressing heme oxygenase-1 predispose co-cultured PC12 cells to oxidative injury. *J Neurosci Res* 2007;85:2186–2195. [PubMed: 17526019]
- Song W, Su H, Song S, Paudel HK, Schipper HM. Over-expression of heme oxygenase-1 promotes oxidative mitochondrial damage in rat astroglia. *J Cell Physiol* 2006;206:655–663. [PubMed: 16222706]
- Straus DS, Glass CK. Cyclopentenone prostaglandins: new insights on biological activities and cellular targets. *Med Res Rev* 2001;21:185–210. [PubMed: 11301410]
- Taylor-Clark TE, Undem BJ, Macglashan DW Jr, Ghatta S, Carr MJ, McAlexander MA. Prostaglandin-induced activation of nociceptive neurons via direct interaction with transient receptor potential A1 (TRPA1). *Mol Pharmacol* 2008;73:274–281. [PubMed: 18000030]
- Wang Z, Aris VM, Ogburn KD, Soteropoulos P, Figueiredo-Pereira ME. Prostaglandin J2 alters pro-survival and pro-death gene expression patterns and 26 S proteasome assembly in human neuroblastoma cells. *J Biol Chem* 2006;281:21377–21386. [PubMed: 16737963]
- Yee S, Choi BH. Oxidative stress in neurotoxic effects of methylmercury poisoning. *Neurotoxicology* 1996;17:17–26. [PubMed: 8784815]
- Zander T, Kraus JA, Grommes C, Schlegel U, Feinstein D, Klockgether T, Landreth G, Koenigsknecht J, Heneka MT. Induction of apoptosis in human and rat glioma by agonists of the nuclear receptor PPARgamma. *J Neurochem* 2002;81:1052–1060. [PubMed: 12065618]

Fig. 1A

Fig. 1B

Fig. 1. Prevention of MeHg cytotoxicity by 15d-PGJ₂

Fig. 1A: C6 glioma cells were pretreated with nothing (1st column of each set), 1 μM 15d-PGJ₂ for 0.5 hrs (2nd column of each set), 4 hrs (3rd column of each set) or overnight (>18 hrs, 4th column of each set), followed by various concentrations of MeHg (without 15d-PGJ₂) overnight, and then the viability of each treatment was determined. Viabilities were compared by two-way ANOVA with MeHg concentrations and pretreatment duration as between group factors. The analysis revealed significant main effects of MeHg concentrations and pretreatment duration on the viability. There was no interaction between these two factors. These results indicated that MeHg caused concentration-dependent decrease in cell viability. At each MeHg concentration used, longer pretreatment with 15d-PGJ₂ led to a better protection against MeHg cytotoxicity. Three sets of one-way ANOVA and the Bonferroni post-hoc analyses were performed to determine within group differences at each MeHg concentration. Compared to controls in each set, the analysis indicated significant protection was achieved by overnight 15d-PGJ₂ pretreatment.

Fig. 1B C6 glioma cells were treated with 0.5, 1 or 2 μM 15d-PGJ₂ 2 hrs after plating. After overnight incubation, the cultures were treated with 5 μM (left-side columns) or 10 μM (right-side columns) MeHg for 1 day (without 15d-PGJ₂) before the viability of each treatment was determined. Viabilities were compared by two-way ANOVA with MeHg concentrations and 15d-PGJ₂ concentrations as between group factors. This analysis revealed significant main effects of MeHg concentrations and 15d-PGJ₂ concentrations on the viability. There was no interaction between these two factors. These results indicated that MeHg caused concentration dependent decrease in cell viability. At each MeHg concentration used, 15d-PGJ₂ pretreatment

of cells caused a concentration-dependent protection against MeHg cytotoxicity. Two sets of one-way ANOVA and the Bonferroni post-hoc analyses were performed to determine within group differences at each MeHg concentration. Compared to appropriate controls, results indicated that 15d-PGJ₂ at 1 μ M or above caused significant protection in 5 μ M MeHg treated cultures, and this agent at 2 μ M caused significant protection in 10 μ M MeHg treated cultures.

Fig. 2. MeHg caused mitochondrial depolarization

C6 glioma cells were treated with (A) nothing or (B) 5 μ M MeHg for 4 hrs or (C) 1 μ M 15d-PGJ₂ overnight or (D) 1 μ M 15d-PGJ₂ overnight followed by 5 μ M MeHg for 4 hrs, and then the alteration of mitochondrial membrane potential ($\Delta\Psi_m$) was determined by the JC-1 staining. Results indicated that MeHg caused an increase in the JC-1 monomers (545 nm peak) and a decrease in the JC-1 aggregates (595 nm peak), a sign of mitochondrial depolarization (compare Fig. 2A with Fig. 2B). While 15d-PGJ₂ itself had no effect on the spectrum (Fig. 2C), it prevented the spectrum shift caused by MeHg (Fig. 2D).

Fig. 3A

Fig. 3B

Fig. 3. Quantification of relative mitochondrial depolarization caused by MeHg

(A) Concentration-response: C6 glioma cells were treated with various concentrations of MeHg for 4 hrs followed by the JC-1 dye, and then the emission intensity ratio between the 545 nm and the 595 nm peaks was used as an indication of mitochondrial membrane potential. Results were analyzed by one-way ANOVA and the Bonferroni post-hoc analysis. Compared to control levels, MeHg at 10 μ M caused a significant increase in the 545/595 emission intensity ratio, indicating an increase of mitochondrial depolarization. (B) Time-response: Concurrent experiments determined the time-dependent mitochondrial depolarization caused by 5 μ M and whether this could be affected by 1 μ M 15d-PGJ₂ pretreatment. Results were compared by two-way ANOVA with the duration of MeHg treatment and the presence of 15d-PGJ₂ as between group factors. This analysis revealed a marginal effect of MeHg treatment duration on the 545/595 emission intensity ratio ($p=0.07$). However, there was a significant main effect of 15d-PGJ₂ in reducing the 545/595 emission intensity ratio. There was no interaction between these two factors. Open columns: MeHg alone for 1, 2 or 4 hours. Solid columns: MeHg plus 1 μ M 15d-PGJ₂.

Fig. 4. Protection against MeHg cytotoxicity by 15d-PGJ₂: Attenuation by BSO

Primary mouse glia were treated with various concentrations of 15d-PGJ₂ (left-side columns) or 15d-PGJ₂ plus 10 μM BSO (right-side columns) overnight followed by 10 μM MeHg (without 15d-PGJ₂ or BSO) for a day, and then the viability of each treatment was determined. One-way ANOVA and the Bonferroni post-hoc analysis was performed with the data presented in the left-side columns. Compared to cells without 15d-PGJ₂ pretreatment (1st column), 15d-PGJ₂ at 1 μM or above caused significant protection against MeHg cytotoxicity. In a separate set of analysis, two-way ANOVA was used to compare the left-side and right-side columns with 15d-PGJ₂ concentrations and the presence of BSO as between group factors. This was followed by the Bonferroni post-hoc analysis to determine within group differences at each 15d-PGJ₂ concentration. Results revealed significant main effects of 15d-PGJ₂ concentrations and the presence of BSO on the viability. There was no interaction between these two factors. While 15d-PGJ₂ caused a concentration-dependent protection of mouse glia against MeHg cytotoxicity (left-side columns), the protection was attenuated if BSO was present (right-side columns). The degree of attenuation was significant at 0.5 μM 15d-PGJ₂ but not at higher concentrations (1 μM or 2 μM) of 15d-PGJ₂.

Fig. 5A

Fig. 5. Determination of cellular GSH levels

(A) Prevention of MeHg-induced GSH depletion by 15d-PGJ₂ pretreatment. Primary mouse glia were pretreated with nothing or with 2 μ M 15d-PGJ₂ overnight followed by fresh medium or 10 μ M MeHg (without 15d-PGJ₂) for 4 hrs, and then the cellular GSH levels were determined. Results from left to right were: control (1st column), MeHg-treated (2nd column), 15d-PGJ₂ overnight treatment (3rd column), 15d-PGJ₂ overnight treatment followed by MeHg (4th column). Two-way ANOVA indicated that both MeHg and 15d-PGJ₂ had significant main effects on the relative GSH levels, and there was a significant interaction between these two factors. A separate set of analysis was performed to compare GSH levels in each condition. Compared to control levels, one-way ANOVA with the Bonferroni post-hoc test indicated that MeHg treatment significantly decreased the cellular GSH levels, and 15d-PGJ₂ significantly increased the cellular GSH levels. However, there was no difference between controls and cells treated with 15d-PGJ₂ overnight followed by MeHg. These results indicated that pretreatment of cultures with 15d-PGJ₂ protected cells from MeHg-induced GSH depletion. (B) BSO inhibited 15d-PGJ₂-induced GSH up-regulation. Primary mouse glia were treated with nothing (1st column), 10 μ M BSO (2nd column) or 10 μ M BSO plus 2 μ M 15d-PGJ₂ (3rd column) overnight, and then cellular levels of GSH were determined. One-way ANOVA with the Bonferroni post-hoc test indicated that, compared to controls, BSO significantly reduced cellular GSH levels. While 15d-PGJ₂ could increase cellular GSH levels (Fig. 5A), this did not happen in the presence of BSO.

Fig. 6. Effect of 15d-PGJ₂ and BSO on MeHg-induced mitochondrial depolarization in mouse glia
 Primary mouse glia were treated with nothing (control), 2 μ M 15d-PGJ₂, 10 μ M BSO or 2 μ M 15d-PGJ₂ plus 10 μ M BSO overnight as indicated. Cells were then treated with medium (open columns) or 10 μ M MeHg (closed columns), without 15d-PGJ₂ or BSO, for 4 hrs before the degree of mitochondrial depolarization in each culture was determined. Results were compared by two-way ANOVA with the presence of MeHg and testing agents as between group factors. This was followed by the Bonferroni post-hoc analysis to determine within group differences (with or without MeHg). This analysis revealed significant main effects of MeHg on the 545/595 emission intensity ratio. Without MeHg, none of the testing agents significantly altered the 545/595 emission intensity ratio. There was no interaction between these two factors. While MeHg could raised the 545/595 emission intensity ratio, this property was modified in the presence of various testing agents. See text for detailed discussion of the results.