Post-translational Activation of Glutamate Cysteine Ligase with Dimercaprol

A NOVEL MECHANISM OF INHIBITING NEUROINFLAMMATION IN VITRO*

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Neuroinflammation and oxidative stress are hallmarks of various neurological diseases. However, whether and how the redox processes control neuroinflammation is incompletely understood. We hypothesized that increasing cellular glutathione (GSH) levels would inhibit neuroinflammation. A series of thiol compounds were identified to elevate cellular GSH levels by a novel approach (i.e., post-translational activation of glutamate cysteine ligase (GCL), the rate-limiting enzyme in GSH biosynthesis). These small thiol-containing compounds were examined for their ability to increase intracellular GSH levels in a murine microglial cell line (BV2), of which dimercaprol (2,3-dimercapto-1-propanol (DMP)) was found to be the most effective compound. DMP increased GCL activity and decreased LPS-induced production of pro-inflammatory cytokines and inducible nitric-oxide synthase induction in BV2 cells in a concentration-dependent manner. The ability of DMP to elevate GSH levels and attenuate LPS-induced pro-inflammatory cytokine production was inhibited by buthionine sulfoximine, an inhibitor of GCL. DMP increased the expression of GCL holoenzyme without altering the expression of its subunits or Nrf2 target proteins (NQO1 and HO-1), suggesting a post-translational mechanism. DMP attenuated LPS-induced MAPK activation in BV2 cells, suggesting the MAPK pathway as the signaling mechanism underlying the effect of DMP. Finally, the ability of DMP to increase GSH via GCL activation was observed in mixed cerebrocortical cultures and N27 dopaminergic cells. Together, the data demonstrate a novel mechanism of GSH elevation by post-translational activation of GCL. Post-translational activation of GCL offers a novel targeted approach to control inflammation in chronic neuronal disorders associated with impaired adaptive responses.

Oxidative stress and inflammation are two prominent processes linked to the etiology of acute and chronic neurological disorders, such as stroke, traumatic brain injury, epilepsy, Alzheimer’s disease (AD), and Parkinson’s disease (PD) (1–5). A cross-talk between these processes has been noted in their temporal and spatial occurrence following neurotoxic insults (2). It is not currently known whether the two processes are mechanistically linked or how the cellular redox status controls neuroinflammation. Perturbed redox status has been most notably observed by measurement of the interconvertible major cellular redox couple, glutathione (GSH) and its disulfide (GSSG) (6). GSH is a tripeptide comprising glutamate, cysteine, and glycine and is the most abundant non-protein thiol within cells. GSH plays an important role in countering oxidative stress by scavenging reactive oxygen species (ROS). GSH is utilized as a substrate for glutathione peroxidases that are responsible for the detoxification of hydrogen peroxide (H2O2) and glutathione S-transferases involved in the removal of xenobiotics (7, 8). Additionally, GSH keeps critical thiol groups on proteins in a reduced state, stores and transports cysteine, and protects against reactive electrophiles. GSH depletion is a common occurrence in neuronal disorders. For example, redox cycling neurotoxicants linked to neurodegenerative diseases, such as PD, like the herbicide paraquat (PQ), have been demonstrated to deplete brain GSH via generation of H2O2 (9). In fact, GSH depletion has been suggested to play a primary pathogenic role in the etiology of environmental and idiopathic causes of PD (8, 10, 11). GSH depletion has been observed in experimental models of stroke (12), multiple sclerosis (MS) (13), AD (14) and epilepsy (15–17). Therefore, elevating and preserving the intracellular stores of GSH to maintain the redox homeostasis is a logical therapeutic target for neuroprotection.

Neuroinflammation is an important host defense mechanism that facilitates tissue recovery after an insult, but prolonged inflammation can induce injury. Key features of neuroinflammation are the activation of microglia and production of cytokines and chemokines (18). Recent studies suggest that

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‡ The abbreviations used are: AD, Alzheimer’s disease; GCL, glutamate cysteine ligase; DMP, 2,3-dimercapto-1-propanol; PD, Parkinson’s disease; ROS, reactive oxygen species; PQ, paraquat; MS, multiple sclerosis; iNOS, inducible nitric-oxide synthase; NAC, N-acetylcysteine; BSO, L-buthionine-(S,R)-sulfoximine; γ-GC, γ-glutamylcysteine; LDH, lactate dehydrogenase; HO-1, heme oxygenase-1; NQO1, NADPH dehydrogenase (quinone 1); KC/GRO, keratinocyte chemoattractant; BAL, British anti-Lewisite; NF-κB, nuclear factor-κB; MPP+1, 1-methyl-4-phenylpyridinium; ANOVA, analysis of variance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; p-, phospho.
neuroinflammation plays a causative rather than an ancillary role in the etiology of neurological diseases such as PD, AD, MS, and epilepsy (19–21). This suggests that targeting inflammation may be an important therapeutic avenue for disease modification. Interestingly, there is a close temporal and spatial occurrence of neuroinflammation and oxidative stress in neurodegenerative diseases (22). Activation of microglia can also produce ROS and reactive nitrogen species by up-regulation of NADPH oxidase 2 (22) and inducible nitric-oxide synthase (iNOS), respectively (23, 24).

The simultaneous production of ROS and pro-inflammatory cytokines following neurotoxic insults suggests common regulatory mechanisms. Several key studies in the literature highlight the importance of GSH levels in regulating inflammation. Antioxidants and GSH precursors, such as N-acetylcysteine (NAC) have been shown to inhibit lipopolysaccharide (LPS)-induced cytokine production (25, 26). Depletion of GSH by L-buthionine-(S,R)-sulfoximine (BSO) has been shown to inhibit the nuclear factor Nrf2 (erythroid-deoxyribonucleic acid release, where a reducing redox potential inhibits activation (27). The redox homeostasis in cells can alter inflammatory mechanisms. Several key studies in the literature highlight the importance of GSH levels in regulating inflammation. DMP was used for all subsequent experiments based on these results. To determine whether elevation of GSH was associated with cytotoxicity, lactate dehydrogenase (LDH) release was measured and 24 h after varying concentrations of DMP. There was no observed DMP toxicity with concentrations up to 100 μM at the 4 h time point (Table 2). Vehicle-treated control cells and 100 μM DMP-treated cells produced a 5.6 and 28.1% LDH release, respectively, after 24 h.

DMP increased GSH and total glutathione (GSH + GSSG) in BV2 cells at all of the concentrations tested at both time points (Fig. 1). Total GSH was elevated by 94, 124, and 113% compared with vehicle-treated controls following 4 h of incubation with 10, 30, and 100 μM DMP, respectively. Total GSH was increased by 89.4, 173, and 553% following 24 h of incubation with 10, 30, and 100 μM DMP, respectively. Therefore, we utilized 10 and 30 μM DMP for the majority of our experiments because we observed the highest magnitude of increase in GSH at these concentrations without cytotoxicity.

**DMP Increases GCL Holoenzyme Formation Post-translationally in BV2 Cells—A common pharmacological mechanism to increase GSH levels is by increased protein expression of GCL subunits, the catalytic subunit GCLC and the modifier subunit GCLM. Immunoblot analysis of the subunits of GCL revealed that neither 10 nor 30 μM DMP had any effect on the protein levels of GCLC and GCLM after 4 h of incubation (Fig. 2, A–D). A previous study showed that chalcones increased GSH levels in part through increased GCL holoenzyme formation (37). This led us to test the intriguing hypothesis that DMP could activate GCL by a post-translational mechanism, without affecting the transcription of the subunits. To address this, the effect of DMP on GCL holoenzyme formation in BV2 microglial cells was examined. An increase in the holoenzyme of GCL was observed in BV2 cells treated with 10 and 30 μM DMP for 1 h (Fig. 2, E and F). No changes in protein expression of either GCLC or GCLM were observed when the same samples were run on an SDS gel under reducing conditions (Fig. 2E). This data suggests that DMP elevates GSH levels by increasing GCL activity via a post-translational mechanism without affecting the levels of its subunits.

**Results**

**Novel Thiol Molecules Target GSH Biosynthesis by Post-translational Modification—**A library of small thiol-containing molecules was discovered to rapidly elevate cellular GSH levels. The compounds were identified and prioritized based on their ability to elevate intracellular GSH in BV2 microglial cells. The compound that produced the largest magnitude of increase in intracellular GSH 4 h after treatment compared with others was DMP. DMP elevated GSH by ~47, 62.9, and 50.7% at 10, 30, and 100 μM concentrations, respectively (Table 1). Most of the thiol compounds either did not have any effect on intracellular GSH or increased the levels. An exception was 3-mercaptopropanol, which led to a decrease in the levels of intracellular GSH at 100 μM concentration, which could be attributed to GSH being effluxed from the cells after reaching saturation levels. DMP was used for all subsequent experiments based on these results.

**TABLE 1**

| Thiol compound | Intracellular GSH (% of vehicle control) |
|---------------|-----------------------------------------|
|               | 10 μM | 30 μM | 100 μM |
| **DMP**       | 147 ± 3** | 162 ± 6*** | 150 ± 14*** |
| 3-Mercapto-1-propanol | 111 ± 3 | 135 ± 7*** | 58.2 ± 1.1*** |
| 4-Mercapto-1-butanol | 120 ± 9 | 139 ± 5** | 168 ± 5** |
| 1,3-Propanedithiol | 127 ± 3 | 136 ± 6* | 137 ± 9* |
| 2,3-Butanediol | 121 ± 7 | 93.4 ± 11.3 | 91.2 ± 12.0 |

*Data are mean ± S.E., *p < 0.05, **p < 0.01, ***p < 0.001 versus vehicle-treated controls as determined by one-way ANOVA with Dunnett’s multiple comparison test.

**Post-translational Activation of GCL**
Post-translational Activation of GCL

| Table 2: Cell death in BV2 cells treated with DMP |
|-------------------------------------------|
| Cell death was measured in BV2 cells treated with varying concentrations of DMP for 4 and 24 h. There was no observed cell death at 4 h, and cell death was observed only with 100 μM DMP at 24 h (n = 22–34 wells). **** p < 0.0001 versus vehicle-treated controls as determined by one-way ANOVA with Dunnett’s multiple comparison test.

| Time of treatment | Percentage of LDH release (mean ± S.E.) |
|-------------------|----------------------------------------|
|                   | Vehicle | 10 μM | 30 μM | 100 μM |
| 4 h               | 10.3 ± 0.8 | 9.3 ± 0.9 | 11.1 ± 1.0 | 9.8 ± 0.6 |
| 24 h              | 5.6 ± 0.5 | 6.7 ± 0.9 | 9.2 ± 1.2 | 28.1 ± 2.2 **** |

DMP Increases GCL Activity in BV2 Cells—One way to determine the role of post-translational GCL activation by DMP is to use an irreversible inhibitor of GCL. BSO is a highly specific, irreversible inhibitor of GCL (38). BV2 cells were co-treated with BSO and DMP for 24 h, and intracellular GSH levels were determined. A concentration-dependent decrease in the ability of DMP to elevate GSH levels occurred with BSO treatment, indicating that the effect of DMP depends on GCL activity (Fig. 3A).

To further confirm whether DMP increased GCL activity in BV2 cells, a HPLC method was used to measure its product, γ-glutamyl cysteine (γ-GC). Incubation with DMP for 4 h significantly increased γ-GC formation by 48.9, 167, and 381% over vehicle control at concentrations of 10, 30, and 100 μM, respectively (Fig. 3B). DMP increased GCL activity by 43.3, 113, and 1056% over vehicle control at concentrations of 10, 30, and 100 μM, respectively, after 24 h of incubation (Fig. 3C).

These data indicate that DMP increases intracellular GSH by activating GCL in BV2 microglial cells.

DMP Does Not Affect Nrf2 Target Proteins—Nrf2 is a transcription factor that regulates the expression of a variety of antioxidant genes that are activated by oxidative stress (39). GCLC and GCLM are known gene targets of Nrf2. To determine whether DMP increased GSH levels in part due to Nrf2 activation, the effect of DMP on well characterized Nrf2 targets, heme oxygenase-1 (HO-1) and NADPH dehydrogenase quinone 1 (NQO1), was also assessed by their protein expression. Neither 10 nor 30 μM DMP greatly altered the expression of either HO-1 (Fig. 4, A and C) or NQO1 (Fig. 4, B and D) at 4 h, further confirming the ability of DMP to increase GSH by post-translationally activating GCL. DMP did not result in a nuclear translocation of Nrf2 in BV2 cells (Fig. 4, E and F). Together with its inability to alter GCLC or GCLM protein levels, these results suggest that DMP does not activate the Nrf2 pathway.

DMP Attenuates LPS-Induced Production of Pro-inflammatory Mediators in a GCL-dependent Manner—Elevation of GSH levels has been shown to inhibit inflammatory responses in several in vitro and in vivo models (28, 40). We determined whether increasing intracellular GSH via GCL activation using DMP could attenuate the release of pro-inflammatory cytokines and iNOS from BV2 cells stimulated with LPS. DMP differentially affected the release of pro-inflammatory cytokines from LPS-stimulated BV2 cells at two different concentrations. The levels of TNF-α, IL-1β, IL-6, KC/GRO, IFN-γ, and IL-12p70 were measured using a multiplex cytokine array (Fig. 5). DMP significantly attenuated the release of IL-1β (Fig. 5B), IL-6 (Fig. 5C), and IL-12p70 (Fig. 5F) at both 10 and 30 μM concentrations, whereas only the 30 μM concentration was able to decrease the induction of TNF-α (Fig. 5A), KC/GRO (Fig. 5D), and IFN-γ at 24 h (Fig. 5E).

iNOS is another important inflammatory mediator increased by LPS stimulation and produces nitric oxide (NO), which can have cytotoxic effects and prooxidant effects via peroxynitrite (ONOO−) formation (41). BV2 cells stimulated with LPS showed an induction of iNOS, which was significantly attenuated by 80.3% with 30 μM DMP (Fig. 6A).

Pharmacological inhibition of GCL using BSO was used to determine whether the attenuation of cytokine release by DMP was dependent on its ability to activate GCL. Treatment of BV2 cells with 30 μM DMP attenuated LPS-induced TNF-α release, which was significantly reversed by 63.6% upon GCL inactivation by BSO (Fig. 6B). This finding suggests that the effect of DMP on LPS-induced cytokine production is dependent on its ability to activate GCL.

DMP Attenuates LPS-induced MAPK Activation in BV2 Cells—We next investigated the potential signaling pathways by which increased GSH could contribute to a decrease in LPS-induced pro-inflammatory cytokine production. There are multiple redox-sensitive mechanisms by which LPS stimulates cytokine production. The MAPK pathway is one of the major signaling pathways altered by intracellular GSH levels (42–44). We investigated the effect of DMP on the activation of the three major MAPKs (p38, JNK, and ERK). LPS caused significant increases in the phosphorylation of all three MAPKs. The levels of p-JNK, p-ERK, and p-p38 were increased by 966, 54.4, and 175% with LPS stimulation, respectively. Treatment with 30 μM DMP significantly attenuated the activation of all three MAPKs by ~40% (Fig. 7). Treatment with 30 μM DMP alone had no effect on the phosphorylation status of the MAPKs (data not shown). These results are consistent with previously published literature demonstrating the anti-inflammatory effect of MAPK kinase inhibitors (45–47). Interestingly, the effect of DMP in BV2 cells was not dependent on the activation of the NF-κB pathway, which is also redox-sensitive and capable of controlling inflammatory cytokine production (Fig. 8).

Treatment with DMP alone did not affect the nuclear translocation of p65 (Fig. 8A) or the phosphorylation of p65 (data not shown).

DMP Activates GCL, Increases GSH, and Attenuates TNF-α Release from Rat Primary Cortical Cultures—We determined the ability of DMP to elevate GSH in mixed primary cerebrocortical cultures, consisting of neurons, astrocytes, and microglia. The presence of both neurons and microglia in these cultures was confirmed by performing immunocytochemistry for MAP2 and Iba1, respectively (data not shown). Treatment with 10, 30, and 100 μM DMP after 4 h resulted in significant increases in intracellular GSH levels in primary cortical cells (Fig. 9A). Treatment with DMP (10 and 30 μM) increased GSH significantly after 24 h (Fig. 9B). DMP treatment did not cause any toxicity in mixed primary cultures at 4 h but caused a 68% increase in LDH release over vehicle controls at the 100 μM concentration after 24 h (data not shown). DMP increased GCL activity in cerebrocortical cells by 79.1, 228, and 232% after 4 h.
and 74.6, 163, and 223% after 24 h at 10, 30, and 100 μM concentrations, respectively (Fig. 9, C and D). It is interesting to note that although 100 μM DMP activated GCL at 24 h, GSH levels were not elevated, which could be attributed to intracellular GSH reaching saturation levels and being effluxed from the cells into the extracellular milieu (48, 49).
We next examined whether DMP could attenuate pro-inflammatory cytokine production from primary cortical cells. LPS-induced TNF-α release was significantly attenuated by 30 μM DMP (Fig. 9E). Together, these data demonstrate that DMP activates GCL, increases intracellular GSH, and attenuates LPS-induced neuroinflammation in mixed cortical cultures, which underscores its potential utility in in vivo models of neurotoxicity.

**DMP Activates GCL, Increases GSH, and Attenuates PQ-induced Cell Death in N27 Dopaminergic Cells**—Depletion of GSH has been linked to the neurotoxic effects of the redox cycling herbicide PQ, which increases steady-state superoxide and hydrogen peroxide levels (50). We asked whether DMP could elevate GSH and inhibit PQ-induced toxicity in a dopaminergic neuronal cell line (N27). DMP did not exert any toxicity in N27 cells with concentrations up to 100 μM a t4o r2 4h (data not shown). DMP significantly increased intracellular GSH in N27 cells with 10, 30, and 100 μM by 163, 342, and 347% at 24 h, respectively, compared with vehicle controls (Fig. 10, A and B). The next step was to determine whether DMP activates GCL in N27 cells. Treatment of N27 cells for 4 h with 10, 30, and 100 μM DMP significantly increased GCL activity by 54.1 (p < 0.05), 423, and 447%, respectively (Fig. 10C). Treatment with 10 and 30 μM DMP for 24 h increased GCL activity by 93.9 and 82%, respec-

**FIGURE 3. The effect of DMP on GSH induction is dependent on GCL activation.** A, BV2 cells were treated with different concentrations of BSO and DMP, and intracellular GSH levels were measured after 24 h. B, GCL activity was assessed by measuring γ-GC formation in BV2 cell lysates with 10, 30, and 100 μM DMP at 4 h. C, GCL activity was measured in BV2 cell lysates at 24 h. Data are represented as mean ± S.E. (error bars). α, p < 0.05 versus 0 DMP within the same BSO group; β, p < 0.05 versus 30 μM DMP within the same BSO group; γ, p < 0.05 versus 0 BSO within the same DMP group; δ, p < 0.05 versus 2.5 μM BSO within the same DMP group by two-way ANOVA with Sidak’s multiple comparisons test. *, p < 0.05; ***, p < 0.001; ****, p < 0.0001 versus vehicle controls by one-way ANOVA with Dunnett’s post-test. n = 3–6/group.

**FIGURE 4. DMP does not affect the Nrf2 pathway.** A, B, and E, representative immunoblots for HO-1 (A), NQO1 (B), and nuclear Nrf2 (E) in BV2 cells treated with 10 and 30 μM DMP for 4 h. C, D, and F, quantification of the blots for HO-1 (C), NQO1 (D), and nuclear Nrf2 (F). Data are represented as mean ± S.E. (error bars). n = 5–6 for HO-1 and NQO1 and 3 for nuclear Nrf2 per group.

We next examined whether DMP could attenuate pro-inflammatory cytokine production from primary cortical cells. LPS-induced TNF-α release was significantly attenuated by 30 μM DMP (Fig. 9E). Together, these data demonstrate that DMP activates GCL, increases intracellular GSH, and attenuates LPS-induced neuroinflammation in mixed cortical cultures, which underscores its potential utility in in vivo models of neurotoxicity.
Exposure of N27 cells with PQ alone resulted in a 242% increase in release of LDH, a marker of neuronal injury, compared with vehicle treatment, which was significantly decreased by 44.3% after treatment with DMP (Fig. 10E). This effect was confirmed with the MTT assay, where PQ alone resulted in a 53.1% decrease in cell viability, which was significantly reversed to vehicle control levels with DMP treatment (Fig. 10F). These data indicate that GCL activation by DMP increases intracellular GSH and attenuates PQ-induced injury in N27 cells.

The observation that DMP inhibits LPS-induced MAPK activation in BV2 microglial cells coupled with the known role of the MAPK pathway in learning processes (51, 52) led us to test the ability of DMP to alter MAPK signaling in N27 neuronal cells. We assessed the effect of DMP treatment on basal and PQ-mediated alteration of MAPK signaling in N27 neuronal cells. Treatment of N27 cells with 1 mM PQ for 2 h did not significantly alter the levels of p-JNK or p-ERK but decreased the levels of p-p38 (Fig. 11). Treatment with 30 μM DMP or 1 mM PQ alone did not alter the levels of p-JNK or p-ERK but decreased the levels of p-p38. Co-treatment with DMP and PQ decreased the levels of p-JNK and p-p38, but not p-ERK. The levels of the total proteins remained unaffected by any treatment. Co-treatment with DMP and PQ led to a decrease in the p-JNK/total JNK ratio but did not alter p-p38/total p38 or p-ERK/total ERK ratios.
In this study, we report a novel mechanism of inhibiting neuroinflammation by elevation of intracellular GSH via post-translationally activating GCL, the rate-limiting enzyme for GSH biosynthesis. The small molecule thiol compound DMP elevated intracellular GSH levels and attenuated LPS-induced production of pro-inflammatory mediators from BV2 and primary cortical cells. Additionally, DMP activated GCL, elevated intracellular GSH, and attenuated PQ-induced neurotoxicity in N27 cells. These results offer a novel therapeutic approach to target neuroinflammation and neuronal death by activating GCL post-translationally independent of Nrf2 activation. The data also highlight the role of redox mechanisms in modulating neuroinflammation.

Diverse acute and chronic neurological disorders have been linked to oxidative stress. Oxidative stress is thought to be the primary mechanism contributing to the depletion of GSH. GSH is a major antioxidant that is present at a concentration of 2–3 mM in the brain (7, 53). The pathogenesis of age-related neurological disorders is associated with a decrease in brain GSH levels and could develop as a consequence of exposure to environmental agents like herbicides, pesticides, and heavy metals (8). A decrease in GSH levels has also been observed in patients with MS (54), AD (55, 56), PD (57, 58), and epilepsy (59). Alterations in the GSH redox status could contribute to the activation of deleterious inflammatory processes associated with neurodegeneration (29). Evaluation of small thiol compounds to increase intracellular GSH and alleviate neuroinflammation...
identified DMP as a potent small molecule thiol that significantly elevates GSH in microglial cells. DMP, also known as British anti-Lewisite (BAL), is already approved by the Food and Drug Administration for the treatment of heavy metal poisoning (60). The novelty of our approach lies with the finding of compounds that utilize a post-translational mechanism to increase GCL activity and intracellular GSH levels. This mechanism of action is unlikely to involve metal chelation, which would more likely inhibit rather than activate GCL based on the known requirement of Mg\(^{2+}\) in the enzymatic activity of GCL (61).

GCL is a homodimeric protein comprising a catalytic subunit, GCLC, and a modifier subunit, GCLM. GCLC catalyzes the first step in the GSH biosynthetic pathway, forming γ-GC from glutamate and cysteine, utilizing ATP and Mg\(^{2+}\) as cofactors (62). GCLM increases the \(V_{\text{max}}\) and \(K_{\text{cat}}\) of GCLC and decreases the \(K_{\text{m}}\) for glutamate and ATP. GCLM also increases the \(K_i\) for feedback inhibition of GCL by GSH (63). Post-translational modifications to both subunits have been demonstrated to affect the activity of the holoenzyme. Such mechanisms include phosphorylation, myristoylation, caspase-mediated cleavage, and oxidative stress (64). The precise mechanism by which DMP induces holoenzyme formation remains to be investigated and will be the subject of future studies. However, based on previously published studies on post-translational activation of GCL, we speculate that DMP probably induces a topological change in the subunits, such that it facilitates formation of the GCL holoenzyme (64). Post-translational activation of GCL is also what sets DMP apart from traditional thiols, such as NAC, that serve as a cysteine substrate source (65) or Nrf2 activators, such as sulforaphane, that are electrophiles and induce a mild oxidative stress response (66). Our results are consistent with previous literature showing that synthetic chalcones increase GCL holoenzyme formation, confirming that such a mechanism can be used to elevate intracellular GSH (37, 67). This approach is especially useful in instances where the tissue has decreased adaptive responses to an oxidative insult, such as in the aging brain. In this situation, no new GCL needs to be synthesized de novo, and GSH can be rapidly increased by activation of preexisting subunits.

There is ample evidence in the literature that illustrates the anti-inflammatory effect of several antioxidants in microglial cells. Some have been shown to dampen inflammation by activating the Nrf2 pathway (26, 31, 65). Our studies revealed a GCL-dependent mechanism underlying the anti-inflammatory effects of DMP against a LPS challenge. The latter is a pathogen-associated molecular pattern from Gram-negative bacteria that binds to Toll-like receptor 4 (TLR4), resulting in the release of several pro-inflammatory factors by activation of either the NF-κB and/or the AP-1 (activator protein-1) transcription factors (68). Our results indicated that the effect of increased GSH on inflammatory markers is due in part to decreased LPS-induced MAPK activation but not due to effects on the NF-κB cascade. This is an interesting finding because

**FIGURE 9.** DMP increases GSH levels, activates GCL, and attenuates TNF-α release in mixed rat primary cortical culture. Rat primary cortical cells were treated with varying concentrations of DMP, and intracellular GSH levels were measured at 4 h (A) and 24 h (B). GCL activity was also measured at 4 h (C) and 24 h (D). E, TNF-α levels in cortical cells treated with 30 μM DMP for 4 h and stimulated with 100 ng/ml LPS for an additional 20 h. Data are represented as mean ± S.E. (error bars). *, \(p < 0.05\); **, \(p < 0.01\); ***, \(p < 0.001\); ****, \(p < 0.0001\) (A–D) versus vehicle controls by one-way ANOVA with Dunnett’s post-test. *, \(p < 0.0001\) versus vehicle; #, \(p < 0.0001\) (E) versus LPS alone by one-way ANOVA with Tukey’s multiple comparison test. \(n = 6\) for GSH levels, 3 for GCL activity, and 9 for TNF-α levels per group.
most anti-inflammatory compounds typically affect the nuclear translocation of the p65 subunit of NF-κB in mediating their effects (45, 46). However, because we did not see a change in either nuclear translocation or phosphorylation status of p65, it is likely that DMP is selectively affecting the processes upstream of the MAPK cascade following TLR4 stimulation. These results are consistent with the ability of NAC, a GSH precursor, to block LPS-induced MAPK phosphorylation and of BSO to augment MAPK phosphorylation (25, 44). Studies involving selective inhibitors of NF-κB have revealed that this pathway is only partially involved in LPS-induced cytokine release, which could explain our results (25). These findings are also supported by previously published research demonstrating the suppression of AP-1 activation by overexpressing GCL (69). Collectively, this suggests that elevation of intracellular GSH in BV2 cells is affecting MAPK activation in a redox-sensitive manner and thereby attenuating inflammation.

The ability of DMP to activate GCL, increase GSH, and attenuate cytokine release from rat primary mixed cortical cells suggests that its effects are not limited to microglial cells because the mixed cultures consist of mostly neurons and astrocytes with a very low number of microglia. Therefore, having a compound that can elevate GSH in multiple cell types is an effective tool for improving the overall redox status and decreasing inflammation. Interestingly, previously published work has demonstrated the importance of GCL subunits in the survival of primary cortical cells when exposed to glutamate or NO (70), indicating that GCL activity can be increased not only to alleviate inflammation but also to decrease neurotoxicity in these cells. However, it must be noted that rodent cells behave differently from human microglial cells; therefore, different pathways might underlie the effect of DMP on neuroinflammation when using human cells.

**FIGURE 10.** DMP increases GSH levels, activates GCL, and attenuates PQ-induced cell death in rat dopaminergic N27 cells. N27 cells were treated with varying concentrations of DMP and intracellular GSH levels were measured at 4 h (A) and 24 h (B). GCL activity was also measured at 4 h (C) and 24 h (D). LDH release (E) and cell viability by MTT assay (F) were measured in N27 cells treated with 30 μM DMP and 1000 μM PQ for 24 h. Data are represented as mean ± S.E. (error bars). *, p < 0.05; **, p < 0.01; ****, p < 0.0001 (A–D) versus vehicle controls by one-way ANOVA with Dunnett’s post-test. *, p < 0.0001 versus vehicle alone; #, p < 0.01 versus PQ alone (E and F) by two-way ANOVA with Sidak’s multiple comparison test. n = 7–8 for GSH levels, 3–4 for GCL activity, 19–20 for LDH release, and 22–26 for MTT assay per group.
Another validation of the effect of DMP in different cell types was demonstrated by its ability to activate GCL and increase intracellular GSH in rat dopaminergic N27 cells. We chose this cell line because of its utility as a model cell system to study parkinsonian agents (71). Exposure of N27 cells to 1-methyl-4-phenylpyridinium (MPP⁺/H11001) and PQ results in depletion of intracellular GSH stores, which is a major contributor to the neurotoxic effects of these agents (72, 73). PQ has been used as a model agent to induce injury by increased ROS production (74). The ability of DMP to elevate intracellular GSH in N27, BV2, and primary cortical cells suggests that its effect is independent of cell type. Increased GSH levels, but not GCL activity, by 10 μM DMP may be due to its ability to act as a direct reductant and recycle GSH from GSSG or protein disulfides (75). The observation that PQ-induced cell death was attenuated with DMP treatment underscores the importance of maintaining GSH levels to confer neuroprotection against oxidative stress. Our results are consistent with previous observations showing that DMP delays cell death in PC12 cells in the absence of trophic support (76). Previous research has shown that exposure of N27 cells to MPP⁺ results in a decrease in GCL activity (73). γ-Glutamylcysteine ethyl ester that feeds into the GSH biosynthetic pathway downstream of GCL was illustrated to provide neuroprotection against MPP⁺ toxicity (77). Therefore, utilizing thiols such as DMP to elevate GCL activity is a novel therapeutic approach for neuroprotection. It should be clarified that DMP is currently approved for short term use due to pain associated with administration rather than brain penetration or pharmacodynamics issues. The next steps will be to develop lead candidates from this in vitro platform that overcome current therapeutic issues and are more suitable for chronic use. The investigation into the potential role of MAPKs in neuroprotection conferred by DMP against PQ-induced cell death revealed some interesting findings. Co-treatment with DMP and PQ led to a decrease in p-JNK, p-p38, and p-JNK/total JNK levels. Our study design in N27 dopaminergic cells using a single neurotoxic concentration of PQ (i.e. 1 mM at a single time point (i.e. 2 h)) did not reveal any induction of MAPK proteins as reported previously in the literature with lower concentrations of PQ (78, 79). The mechanism by which PQ decreased basal levels of p-p38 is unclear and remains to be determined. However, decreased levels of p-p38 by DMP and DMP + PQ could be due to transient activation of basal p-38 by ROS generated constitutively and PQ, respectively. Likewise, decreased p-JNK/total JNK by PQ + DMP may be due to DMP inhibiting an early, transient JNK activation by PQ, as observed previously (80). The slight but statistically insignificant increase in p-ERK/total ERK in the PQ + DMP group may be due to the critical role of ERK in neuronal survival (81). Because this study only assessed the effects of a single concentration of PQ in N27 neuronal cells at a single time point (2 h), we cannot rule out the effects of DMP on MAPK signaling in neurons. In fact, PQ has been shown to modulate MAPKs in neurons (78, 79). Under neuroinflammatory conditions that involve MAPK hyperactivation, DMP would probably exert neuroprotection via inhibition of MAPKs similar to microglial cells.

In summary, DMP provides a novel approach to post-translationally activate GCL to increase intracellular GSH levels and attenuate neuroinflammation and neurotoxicity. The resultant increase in GSH led to an attenuation of pro-inflammatory cytokines by acting on the MAPK cascade. The finding that DMP attenuates PQ-induced neurotoxicity also highlights the importance of GSH in neuroprotection and provides a thera-

![FIGURE 11. Effect of DMP on MAPK signaling in N27 cells.](image-url)
Post-translational Activation of GCL

Experimental Procedures

Chemical Reagents—All chemicals and reagents were obtained from Sigma-Aldrich unless otherwise noted.

Cell Culture—BV2 immortalized murine microglial cells were a kind gift from Dr. Nancy Lee (California Pacific Medical Center). They were maintained in Dulbecco’s minimum essential medium (DMEM) + GlutaMAX + 4.5 g/liter glucose containing 10% fetal bovine serum (FBS) and 1% penicillin (10 units/ml) and streptomycin (10 units/ml) at 37 °C in a 5% humidified atmosphere. N27 immortalized rat dopaminergic cell line was a kind gift from Drs. Curt Freed and Kedar Prasad (University of Colorado Anschutz Medical Campus) (82). Cells were grown in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin/streptomycin at 37 °C, similar to the BV2 cells. All cell culture reagents were purchased from Invitrogen.

Mixed primary cortical cultures were prepared using the protocol as described previously (83). Briefly, cerebral cortices from embryonic day 18 rat pups were dissected and enzymatically dissociated by incubation in Ca2+- and Mg2+-free Hanks’ balanced saline solution supplemented with 10 mM HEPES and 0.25% trypsin for 20 min at 37 °C. The tissue was rinsed and dispersed into a single-cell suspension using a fire-polished Pasteur pipette. The cell suspension was centrifuged and resuspended in minimum essential medium containing Earle’s salts supplemented with 3 g/liter glucose, 5% horse serum, and 5% FBS. The cells were plated at an initial density of 1.0 × 10^6 cells/well in poly-o-lysine-coated 12-well plates and maintained at 37 °C in a humidified incubator with 5% CO2, 95% air in growth medium. Mature cells were used at 14–15 days in vitro for all experiments.

HPLC Measurement of GSH and GSSG Levels—GSH and GSSG were measured with an ESA 5600 CoulArray HPLC system (Chelmsford, MA) on two coulometric array cell modules, each containing four electrochemical sensors attached in series as described previously (15). The potentials of the electrochemical cells were set at 150/300/450/580/700/820 mV versus pal-ladium. Frozen cell samples were sonicated in 0.1N perchloric acid before thawing. The homogenates were then centrifuged and aliquots of the samples were injected into the HPLC, and analytes were separated on a 4.6-mm C-18 reverse phase YMC ODS-A column (Waters Co., Milford, MA) (5-μm particle size). The mobile phase was composed of 100 mM NaH2PO4 and 1% methanol, pH 3.0, with a flow rate of 0.6 ml/min. Values were normalized to protein amounts, as determined by the Bradford assay.

GCL Activity Assay—GCL activity was also measured using a CoulArray HPLC system, similar to the GSH/GSSG assay, following modifications to the method described previously (84). The activity was determined by measuring the formation of glutathione (GSH) and GSSG, the product of the first enzymatic step in the GSH biosynthesis pathway, catalyzed by GCL. The buffer mixture consisted of 0.1 M Tris-HCl, 100 mM KCl (pH 8.2), 20 mM MgCl2, 2 mM EDTA, 10 mM ATP, 10 mM L-cysteine, 40 mM L-glutamate, and 220 μM acivicin. Frozen samples were sonicated in 200 μl of the assay buffer, and the reaction was started by placing them in a water bath at 37 °C for 15 min. The reaction was terminated by the addition of perchloric acid, samples were centrifuged, and a 40-μl aliquot was injected into the HPLC. Protein concentrations were determined by the Bradford assay, and values were normalized to them.

PAGE and Immunoblotting—For the native PAGE, BV2 cells were lysed in 1× PBS with protease inhibitors, and 15 μg of protein was mixed with 2× Laemmli buffer (Bio-Rad) without β-mercaptoethanol or boiling. Samples were loaded on an 8% polyacrylamide gel without stacking gel or SDS and run at 80 V at 4 °C for 3 h. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) overnight at 30 V at 4 °C, blocked with a buffer containing 5% milk in TBS-T (10 mM Tris-HCl, 150 mM NaCl, and 0.5% Tween 20) for 1 h, and probed with polyclonal GCLC rabbit antiserum (1:10,000 in TBS-T with 1% milk), a kind gift from Dr. Terrance Kavanagh (University of Washington). For denaturing PAGE, samples were mixed with 2× sample buffer with β-mercaptoethanol, boiled at 95 °C for 10 min, and then run on 4–20% gradient SDS gels (Bio-Rad) at 200 V for 35 min. Proteins were transferred onto PVDF membranes using a Bio-Rad Trans-Blot Turbo transfer system; blocked; and probed with the GCLC antibody (1:10,000), GCLM rabbit antibody (1:5000) (also a gift from Dr. Terrance Kavanagh), HO-1 (1:1000; Enzo Lifesciences, Farmingdale, NY), NQO1 (1:1000; Sigma), iNOS (1:1000; Abcam, Cambridge, MA), and phosphorylated and total forms (1:1000) of JNK, ERK1/2, and p38 (Cell Signaling Technology, Danvers, MA). Membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Abcam) at a dilution of 1:10,000 in TBS-T. Membranes were developed using ECL reagent (Thermo Fisher Scientific, Waltham, MA), and protein levels were visualized and quantified using the Gel-Doc system and Image Lab version 5.0 software (Bio-Rad). iNOS immunoblot was normalized to total protein quantified using the Image Lab version 5.0 software.

Multiplex Cytokine Measurement—BV2 cells were seeded on 12-well plates at a density of 5 × 10^5 cells/well and allowed to grow overnight in 1% serum medium. Levels of pro-inflammatory cytokines were measured in cell culture supernatant using a multiplex cytokine array from Meso Scale Discovery (Rockville, MD) according to the manufacturer’s instructions. Briefly, 25 μl of samples were loaded in duplicate, and levels of TNF-α, IL-1β, IL-6, KC/GRO, IFN-γ, and IL-12p70 were measured by an electrochemiluminescence detection method using the Sector Imager 2400.

ELISA for TNF-α Levels—BV2 and mixed primary cortical cells were plated on 12-well plates as described above, and TNF-α levels were measured in cell culture supernatant using mouse and rat ELISA DuoSet kits from R&D Systems (Minneapolis, MN), according to the manufacturer’s protocol.

Cell Injury Assessment—BV2 or N27 cells were plated in 96-well plates at 2.0 × 10^4 cells/well. LDH activity was measured in media and cell lysates collected in a Tris/NaCl lysis buffer containing 0.1% Triton (v/v), as described previously (85). Activity was measured spectrophotometrically at 30 °C as the amount of pyruvate consumed, by monitoring the decrease in absorbance because of NADH oxidation at 340 nm. Cell via-
bility was also determined using the MTT assay. Briefly, cells were incubated with 1 mg/ml MTT for 2 h following treatment. The formazan crystals were solubilized by the addition of DMSO, and absorbance was measured at 550 nm.

Statistical Methods—Data were analyzed using GraphPad Prism software version 6.0 (GraphPad Software, La Jolla, CA). For comparison between controls and treatment groups, one-way ANOVA was utilized with Dunnett’s multiple comparisons post-test. For comparison between all groups, Tukey’s or Sidak’s multiple comparison post-tests were utilized.

Author Contributions—M. P., B. J. D., and P. B. M. conceived the idea of the project. P. B. M. and A. S. conducted all of the experiments and analyzed the results. P. B. M., M. P., and B. J. D. wrote the paper.

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