THIOL-CONTAINING COMPOUNDS ATTENUATE OXIDATIVE STRESS AND NEURONAL HYPEREXCITABILITY *IN VITRO*

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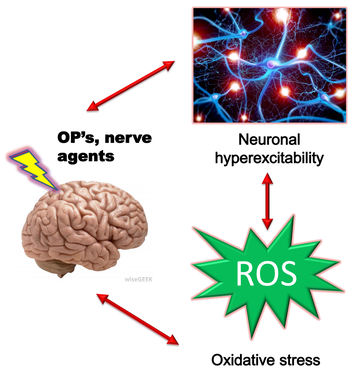
ABSTRACT

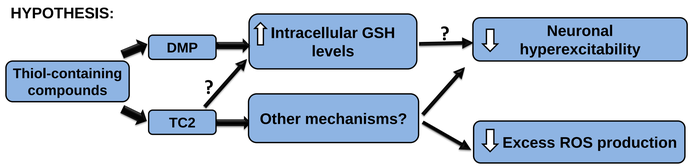
Chemical agents such as industrial chemicals, pesticides, and chemical warfare agents can induce uncontrolled **seizure activity** (neuronal hyperexcitability). **Oxidative stress** has been implicated as a pathogenic factor in the etiology of seizures and epilepsy. However, whether and how **cellular redox status** modulates **neuronal hyperexcitability** is unclear. We hypothesized that the modulation of cellular redox status with thiol-containing compounds would decrease oxidative stress, and attenuate neuronal hyperexcitability *in vitro*. 2,3-dimercapto-1-propanol (DMP), a thiol-containing compound significantly ($p<0.001$ vs vehicle control) increased intracellular glutathione levels in mixed rat primary cortical cultures at 4 and 24h. Next, we determined if DMP could dampen "seizure-like" activity *in vitro* induced by 4-Aminopyridine (4AP), a toxicant that inhibits potassium channels. In mixed rat primary neuronal-glial cultures, incubation with 100µM DMP for 4h significantly ($p<0.0001$ vs 1mM 4AP) decreased 4AP-induced neuronal hyperexcitability. We tested the ability of another thiol compound, TC2 which is FDA approved as a systemic protective agent against chemotherapy, for its ability to alter oxidative stress and neuronal hyperexcitability *in vitro*. To visualize and quantify the oxidative stress response in primary cortical cultures, we utilized a highly sensitive fluorescent probe, HKSOX-1r, that could detect endogenous superoxide levels. Co-treatment with TC2 decreased Antimycin A-induced superoxide levels to control values. In addition, 500µM TC2 significantly ($p<0.0001$ vs 1mM 4AP) attenuated 4AP induced neuronal hyperexcitability in mixed rat primary cortical cultures. Taken together, the data suggest that thiol-containing compounds decrease oxidative stress and attenuate neuronal hyperexcitability.

BACKGROUND



- Seizures are a common and debilitating manifestation of nerve agent (sarin, soman) and organophosphate (insecticides/pesticides) toxicity
- Oxidative stress has been implied as both cause and consequence of seizures
- Redox signaling has been shown to influence neurotransmission
- Previous unpublished work had identified a series of thiol-containing compounds that increase intracellular GSH levels in lung epithelial cells by increasing the formation of GCL holoenzyme by a post-translational mechanism
- FDA approved chemo-protective agent TC2 has been shown to have antioxidant and anti-inflammatory effects in organ systems such as kidneys and colon
- Thiol-containing compounds can elevate GSH, decrease oxidative stress, dampen neuronal hyperexcitability and ultimately confer neuroprotection

HYPOTHESIS:

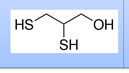


METHODS

- ❖ **HPLC measurement of intracellular GSH and GSSG levels:** Intracellular GSH and GSSG levels were measured in BV2 cell line and primary cortical culture cells by HPLC with electrochemical detection (HPLC-EC) following minor modifications to the method described previously (Lakritz et al.,1997 and Beal et al.,1990).
- ❖ **PAGE:** For the native PAGE, BV2 (murine microglial cell line) cell lysates were probed with polyclonal GCLC rabbit antisera (1:10,000). For SDS-PAGE, the lysates were probed with GCLC (1:10,000) and GCLM (1:5000) rabbit antibodies.
- ❖ **HPLC measurement of GCL activity:** GCL activity was measured in BV2 cells using HPLC-EC following the method previously described by Gregg et al., 2002.
- ❖ **Confocal imaging of primary cortical cultures**: Endogenous superoxide levels in mixed rat primary cortical cultures were imaged using a specialized probe (1r – courtesy of Prof. Dan Yang). Fluorescence intensity analyses were done using Image J.
- ❖ **Microelectrode Array (MEA) method**: Neuronal excitability measurements in mixed rat primary cortical cultures were performed using the Maestro MEA system (Axion Biosystems). Briefly, cells were plated and treated in 48-well MEA plates that can detect neuronal electrical activity.

RESULTS

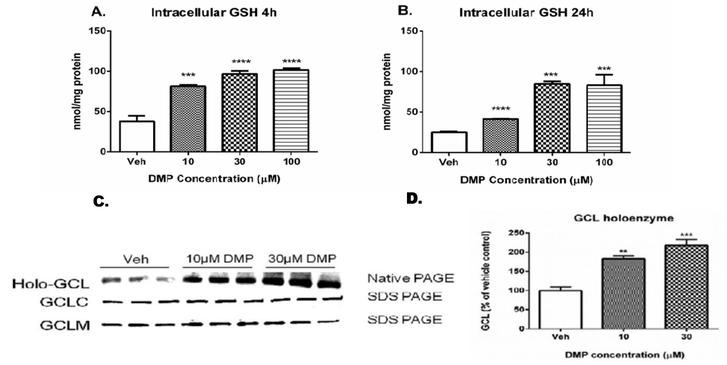
Induction of GSH (% of vehicle control)

Thiol compound	Structure	10µM	30µM	100µM
2,3-Dimercapto-1-propanol (DMP)		147 ± 3**	162 ± 6***	150 ± 14***

Induction of intracellular GSH by DMP. BV2 cells were treated with 10, 30 and 100µM of DMP and intracellular GSH levels were measured at 4 hours. Data are represented as mean ± SEM and **p<0.01, ***p<0.001 vs vehicle control by one-way ANOVA with Dunnett's post-test. n= 3-6/grp.

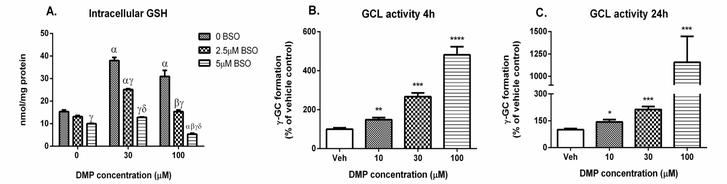
RESULTS

DMP increases intracellular GSH levels by increasing GCL holoenzyme formation



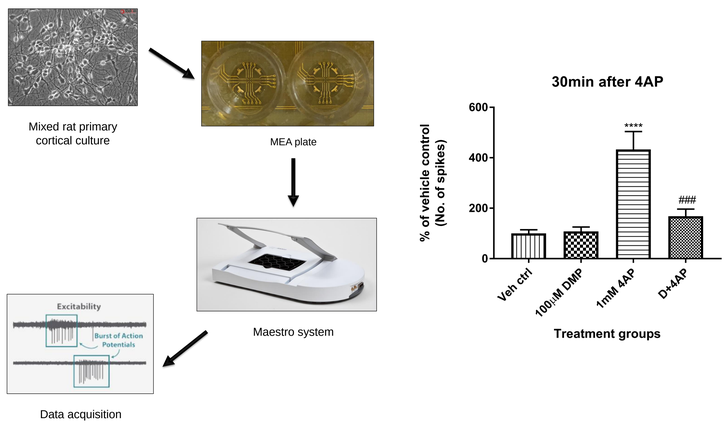
DMP increases GSH levels in mixed rat primary cortical culture cells by increasing GCL holoenzyme formation. Primary cortical culture cells were treated with varying concentrations of DMP and intracellular GSH levels were measured at 4h (A) and 24h (B). BV2 cells were treated with 10 & 30µM DMP for 1h. GCL holoenzyme was measured by native PAGE. GCLC & GCLM subunit protein levels were measured by SDS-PAGE (C). Quantification of GCL holoenzyme blot (D). Data are represented as mean ± SEM. *p<0.05,**p<0.01, ***p<0.001, ****p<0.0001 vs vehicle control by one-way ANOVA with Dunnett's post-test. n=3-6/grp.

DMP increases intracellular GSH by increasing GCL activity



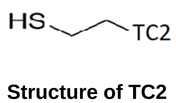
Activation of GCL by DMP. BV2 cells were treated with different concentrations of DMP and BSO and intracellular GSH levels were measured after 24h (A). Inhibiting GCL activity with BSO inhibited the increase in GSH levels by DMP. GCL activity was assessed by measuring γ-GC formation in BV2 cell lysates with 10, 30 & 100µM DMP at 4h (B) and 24h (C). DMP caused a significant increase in GCL activity at all concentrations at both timepoints. Data are represented as mean ± SEM. α=p<0.05 vs 0 DMP & β=p<0.05 vs 30µM DMP within same BSO group, γ=p<0.05 vs 0 BSO & δ=p<0.05 vs 2.5µM BSO within same DMP group by two-way ANOVA with Sidak's multiple comparisons test. *=p<0.05, **=p<0.001 and ***=p<0.0001 vs vehicle control by one-way ANOVA with Dunnett's post-test. n=3-6/grp.

DMP attenuates 4-Aminopyridine (4AP)-induced neuronal hyperexcitability

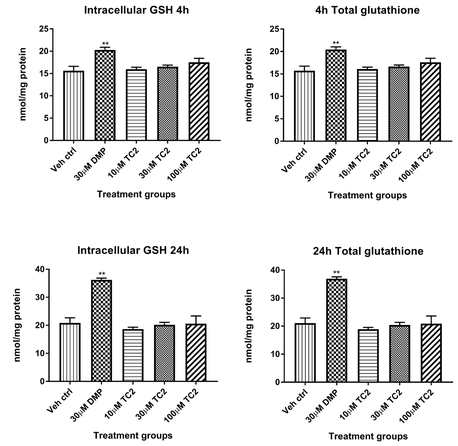


Attenuation of 4AP-induced hyperexcitability in mixed rat primary cortical cultures. Primary cortical cultures were pre-treated with 100µM of DMP for 4h followed by stimulation with 1mM 4AP for 1h. Number of spikes were recorded as a measure of neuronal excitability. Data are represented as mean ± SEM. ****p<0.0001 vs vehicle control, ###p<0.001 vs 1mM 4AP by one-way ANOVA with Tukey's post-test. n=7-8/grp.

TC2 does not increase GSH levels in primary cortical cultures

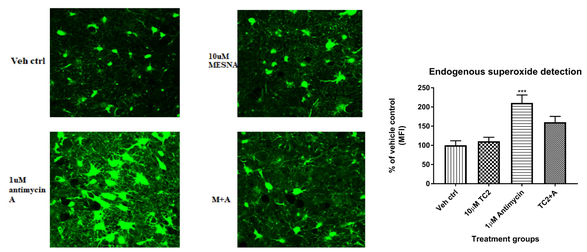


Structure of TC2



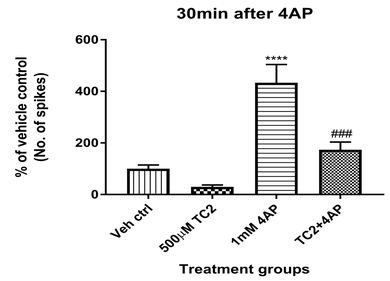
GSH levels post 4h and 24h of TC2 treatment. Primary cortical culture cells were treated with different TC2 concentrations for 4h (A) and 24h (B). DMP was used as positive control. Data are represented as mean ± SEM. *p<0.05, **p<0.01 vs vehicle control by one-way ANOVA with Dunnett's post-test. n=3-6/grp

TC2 inhibits superoxide production in mixed rat primary cortical culture



Decreased Antimycin A induced superoxide levels in primary cortical culture cells co-treated with TC2. Primary cortical culture cells were pre-treated with 10µM TC2 for 4h followed by 30min stimulation with 1µM Antimycin A. Endogenous superoxide was measured/detected by 1r probe. Data are represented as mean ± SEM. ***p<0.001 vs vehicle control by one-way ANOVA with Tukey's post-test. n=3/grp.

TC2 decreases 4-Aminopyridine (4AP)-induced neuronal hyperexcitability



TC2 decreases convulsant activity induced by 4AP in mixed rat primary cortical cultures. Primary cortical culture cells were pre-treated with 500µM TC2 for 4h followed by stimulation with 1mM 4AP for 1h. Number of spikes were recorded as a measure of neuronal excitability. Data are represented as mean ± SEM. ****p<0.0001 vs vehicle control, ###p<0.001 vs 1mM 4AP by one-way ANOVA with Tukey's post-test. n=7-8/grp.

SUMMARY AND CONCLUSIONS

- DMP (2,3-dimercapto-1-propanol), a potent thiol-containing compound, can elevate intracellular GSH levels at 4h and 24h in mixed rat primary cortical cultures
- DMP increases GSH levels by increasing GCL (glutamate cysteine ligase) holoenzyme formation, the rate limiting enzyme in GSH biosynthesis
- TC2, an FDA approved chemoprotectant does not increase intracellular GSH levels in primary cortical cultures at 4h and 24h
- TC2 decreases Antimycin A induced generation of endogenous superoxide levels in mixed rat primary cortical cultures
- DMP and TC2 attenuate neuronal hyperexcitability induced by 4AP in mixed rat primary cortical cultures
- In summary, thiol-containing compounds can exert antioxidant and neuroprotective effects either through GSH dependent or independent mechanisms

FUTURE DIRECTIONS:

❖ The exact mechanism(s) by which TC2 decreases ROS production and neuronal hyperexcitability needs to be delineated

ACKNOWLEDGEMENT:

This work was supported by RO1 NS45748 (M.P)