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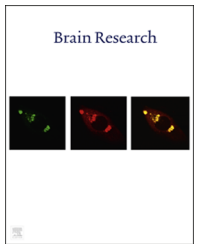

Research Report

Attenuation of rotenone toxicity in SY5Y cells by taurine and N-acetyl cysteine alone or in combination

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ABSTRACT

There is accumulating evidence that supports the involvement of reactive oxygen species (ROS), mitochondrial dysfunction and inflammation in the pathogenesis of neurodegenerative diseases. Thus, it is plausible that a multi-targeted therapeutic approach may be a more effective strategy to retard or even potentially halt the progression of the disease. Taurine is an organic acid that has a role in the regulation of oxidative stress and promoting mitochondrial normal functions, and N-Acetyl cysteine (NAC) is a well-known anti-oxidant and glutathione precursor. The main purpose of this study was to examine the cytoprotective effects of taurine alone or in combination with NAC against rotenone-induced toxicity in the SH-SY5Y neuroblastoma cell line. Taurine treatment produced a concentration-dependent reduction in rotenone-induced cell death. From this, we tested sub-effective concentrations of taurine in combination with low, sub-effective concentrations of NAC against rotenone toxicity, and found the combined treatment afforded greater cytoprotection than either treatment alone. The combined taurine/NAC treatment also attenuated rotenone-induced reductions in aconitase activity suggesting the cytoprotection afforded by the combined treatment may be associated with anti-oxidative mechanisms. Together, our data suggest that a multi-targeted approach may yield new avenues of research exploring the utility of combining therapeutic agents with different mechanisms of actions at concentrations lower than previously tested and shown to be cytoprotective.

1. Introduction

A substantial body of evidence demonstrates that mitochondrial dysfunction and oxidative damage play critical roles in the etiology of cell death associated with several neurodegenerative diseases (Albers and Beal, 2000; Uttara et al., 2009). A large number of studies exploring the therapeutic utility of agents with metabolic and/or anti-oxidative properties have been shown to be effective in providing beneficial effects in both in vitro and in vivo models of neurological diseases. However, fewer studies have explored combining agents to assess whether greater therapeutic utility could be achieved. Given

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the difficulty of achieving appropriate brain levels of such agents to achieve desired biological effects without incurring systemic toxicity makes development of new therapeutic strategies for neurological indications all the more challenging.

Emerging data suggests that taurine may be involved in a wide range of biological processes exerting its beneficial effects through a variety of mechanisms. For example, taurine has been shown to possess anti-oxidative effects by decreasing the levels of reactive oxygen species (ROS) and enhancing electron transport chain activity (Jong et al., 2012). Taurine has also been demonstrated to decrease intracellular calcium levels following an excitotoxic insult (Wu et al., 2005). Anti-inflammatory effects have been demonstrated by showing taurine interacting with myeloperoxidase–halide system of leukocytes to produce taurine halo amines resulting in inflammation reduction, or by down-regulation for of ADP ribose polymerase (PARP) and nuclear factor-kappaB (NF-κB) (Sun et al., 2012; Marcinkiewicz and Kontny, 2014). Finally, taurine's beneficial effects have been linked to activating specific signaling pathways involved with cellular osmotic processes (Schaffer et al., 2000), or that are essential to stimulating glucose utilization and/or insulin secretion (Carneiro et al., 2009; Nandhini and Thirunavukkarasu Anuradha).

Based on these putative mechanisms, we tested taurine's ability to provide cytoprotection against the toxicity caused by rotenone, a pesticide that inhibits complex I activity of the mitochondrial electron transport chain (Orth and Tabrizi, 2003). Previous reports have shown rotenone exposure to result in both metabolic and oxidative stress resulting from ATP depletion and oxidative damage to cellular macromolecules (Fiskum et al., Chinopoulos). Several compounds and mechanisms have been demonstrated to reduce the toxic effects of rotenone, many of which counter the metabolic abnormalities and/or free radical production resulting from the pesticide. In particular, a recent study by Han and colleagues (2013) showed substrates of ATP-generating biochemical pathways rescued rat retinal cells form rotenone-induced toxicity. The authors concluded that substrates, such as glucose, were able to overcome rotenone-induced decreases in ATP, which in turn, reduced oxidative damage.

In our current study, we assessed the cytoprotective potential of taurine, a semi-essential amino acid that has been shown previously to enhance glucose utilization, alone or in combination with N-acetyl cysteine (NAC), a well-known free radical scavenger, against the cellular toxicity induced by rotenone. Specifically, lactate dehydrogenase (LDH) activity was measured to assess cellular toxicity while aconitase activity was measured as marker of oxidative stress (Lobner, 2000). Aconitase is an iron-sulfur containing enzyme catalyzing the isomerization of citrate within the tricarboxylic acid cycle. The activity of aconitase is particularly sensitive to oxidative damage and has been used previously as an indirect marker for oxidative stress (Gardner et al., 1995).

2. Results

2.1. Assessment of rotenone cytotoxicity

A concentration-response toxicity profile of rotenone in SH-SY5Y cell line is shown in Fig. 1. We observed a statistically significant increase in LDH activity in cells treated with rotenone at 3 (403±55), 6 (611±38), and 9 (674±49) uM as compared to control (232±38). Based on this data, we selected 6 μM for all subsequent studies, which provided a large enough toxicity window relative to control to capture any potential cytoprotective effects of taurine alone or in combination with NAC.

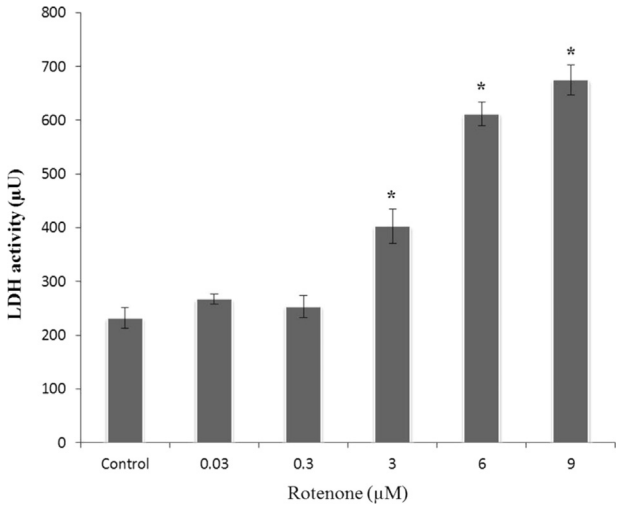

Fig. 1 – Rotenone cellular toxicity in SH-SY5Y cells as determined by LDH release and activity: LDH assay was performed to evaluate the induction of cell death in normal SH-SY5Y cell line using different concentrations of rotenone (0.03 μM, 0.3 μM, 3.0 μM, 6.0 μM and 9.0 μM) after 48 h exposure. Data are presented as mean±SEM, $n=4$. $^{*}p<0.05$ compared to control group.

2.2. Cytoprotective effect of taurine and NAC

We observed a concentration-dependent reduction in rotenone-induced toxicity by increasing concentrations of taurine (Fig. 2a). Cells concurrently treated with rotenone and taurine at 5 or 10 mM, produced statistically significant decreases in LDH activity as compared to cells treated with rotenone alone, while taurine at 2.5 mM did not have any effect on modulating rotenone toxicity. All concentrations of taurine tested by itself did not produce any cell damage as measured by changes in LDH activity (data not shown).

As expected, NAC produced concentration-dependent decreases in rotenone-induced toxicity (Fig. 2b), which is consistent with previous reports (Sun et al., 2012; Molina-Jimenez et al., 2003). NAC, at either 2.5 or 5 mM, produced significant differences as compared to rotenone alone whereas NAC at 0.5 mM produced no cytoprotective effect. Similar to taurine, all concentrations of NAC tested by itself did not produce any cell damage as measured by changes in LDH activity (data not shown).

Based on these data sets, we combined the two sub-effective concentrations of taurine (2.5 mM) and NAC (0.5 mM) and tested this mixture against rotenone-induced cytotoxicity (Fig. 3). We observed a statistically significant decrease in rotenone-induced toxicity by the combination treatment as compared to cells treated with rotenone alone. By itself, the combined

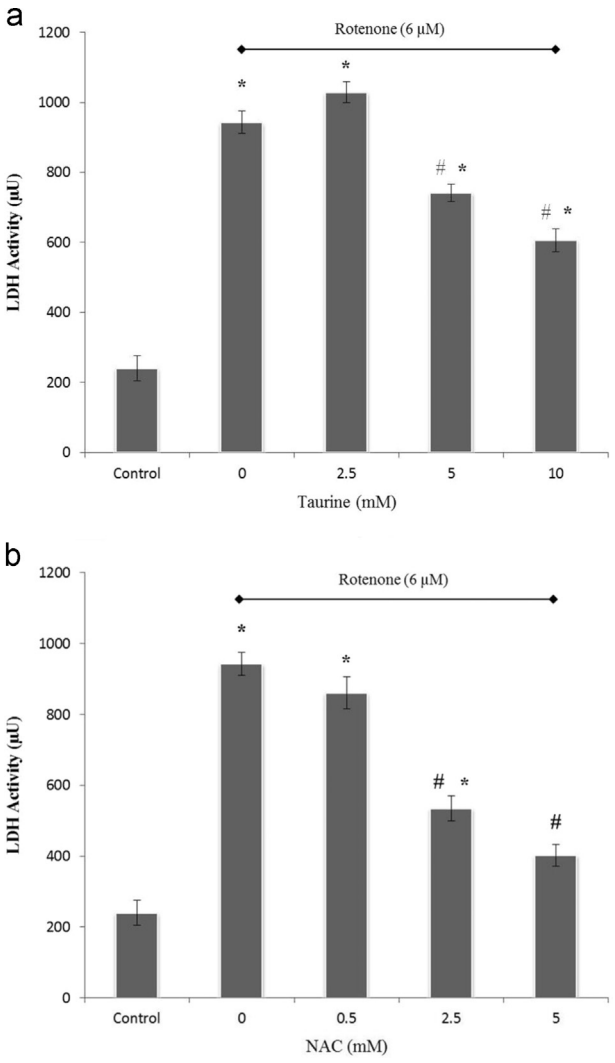

Fig. 2 – Cytoprotective effect of taurine and NAC: SH-SY5Y cells treated with taurine (a) or NAC (b) were concurrently treated with 6 μM rotenone for 48 h. Data are presented as mean ± SEM, $n=3$. $^{*}p<0.05$ compared to control group, $^{\#}p<0.05$ compared to rotenone treatment alone.

taurine/NAC treatment did not induce any significant LDH release from the cells as compared to control wells.

2.3. *Aconitase activity in cells treated with NAC and/or taurine*

It is well-established that defects in energy metabolism will lead towards increased oxidative damage to cellular macromolecules. Enzymes such as aconitase, an iron-sulfur protein that catalyzes the isomerization of citrate to isocitrate in the Krebs cycle, is particularly sensitive to oxidative attack particularly from superoxide anions (for review, see Gardner et al., 1995; Andersson et al., 1998; Villafranca, 1974). Consequently, we measured aconitase activity in rotenone-treated cells to assess whether taurine and/or NAC might influence these modulations and thus provide insight into it cytoprotective mechanism.

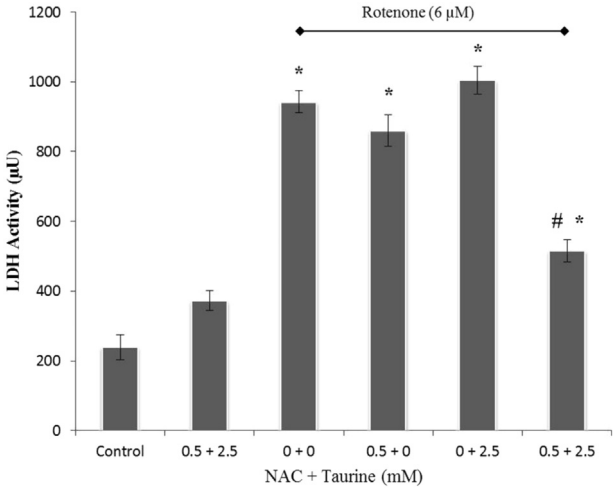

Fig. 3 – Cytoprotective effect of the combined sub-effective concentrations of taurine and NAC: SH-SY5Y cells treated with rotenone (6 μM) and either taurine (2.5 mM) or NAC (0.5 mM) alone, or in combination for 48 h. Data are presented as mean ± SEM, $n=3$. $^{*}p<0.05$ compared to the control group and $^{\#}p<0.05$ compared to cells treated with rotenone alone.

Consistent with the previously published literature, aconitase activity was decreased by rotenone treatment approximately 90% as compared to control (Table 1). Cells treated with rotenone and taurine or NAC (at 2.5 mM or 0.5 mM, respectively) did not affect rotenone-induced reductions in aconitase activity. However, the combined treatment of NAC and taurine, at these sub-effective concentrations, significantly attenuated the rotenone-induced reductions and restored the activity to 74% of control aconitase activity. This improvement in aconitase activity by the combination treatment was similar to the improvement observed in the LDH activity data when LDH release reduced from 4.2 fold, in the presence of rotenone alone, to 2 fold, with the combination treatment, as compared to control. Somewhat surprising was our observation that the combined treatment of NAC and taurine by itself produced a small, albeit significant, reduction in aconitase activity as compared to control, which cannot be explained at this time, but attribute mostly to the sensitivity of the assay. Nonetheless, we feel this anomaly should not detract from the other data showing the attenuation of the rotenone-induced decreases in aconitase activity by the combination treatment.

3. Discussion

It is well established that defects in energy metabolism and oxidative damage play crucial roles in the pathogenesis of several neurodegenerative diseases. Consequently, the utility of inhibitors of the mitochondrial electron transport chain provides meaningful insight into the mechanisms of cell death and how to halt or block the cellular loss with experimental, mechanistically-based, therapies. In this current study, we utilized rotenone, an inhibitor of complex I activity and often associated with modeling the mechanisms of

Table 1 – Aconitase activity assay in SH-SY5Y cells treated with rotenone ± taurine, NAC, or both for 48 h.

	Aconitase activity (nmoles/min/mL)
Control	6.81 ± 0.18
Rotenone	0.55 ± 0.03*
Taurine (2.5 uM)+rotenone	0.58 ± 0.08*
NAC (0.5 uM)+rotenone	0.52 ± 0.09*
Taurine (2.5 uM)+NAC (0.5 uM)+rotenone	5.04 ± 0.06*,#

Data are presented as mean ± SEM, $n=3$.
* $p<0.05$ compared to the control group and.
# $p<0.05$ compared to cells treated with rotenone.

neuronal loss characteristic of Parkinson's disease (Xie et al., 2010).

Consistent with previous published results, we observed a concentration-dependent loss of cells by rotenone as compared to control wells. This afforded the opportunity to explore the cytoprotective potential of NAC, a well-established antioxidant and glutathione precursor, and taurine, an essential organic acid, against rotenone-induced toxicity in our model. Both NAC and taurine have previously been shown to provide beneficial effects in a diverse range of models through anti-oxidative and anti-apoptotic mechanisms.

Our results with NAC were expected as it has been proven from multiple laboratories and studies that the anti-oxidative properties afforded protection against challenges that promote free radical generation, particularly via mitochondrial dysfunction. Our results with taurine, while consistent with the previous studies highlighting the beneficial effects of taurine through its anti-oxidative properties (Jong et al., 2012; Aruoma et al., 1988), are the first to show beneficial effects against toxicity resulting from inhibition of mitochondrial complex I activity. Based on the postulated mechanism(s) of taurine, the observed cytoprotection in this current study against rotenone toxicity could be attributed to any of the aforementioned mechanisms, and provides future avenues of research to explore more specifically the mechanism(s) involved.

Our data showing the combination of sub-effective concentrations of taurine and NAC afforded statistically significant greater benefit against rotenone toxicity is most interesting, especially considering the accompanying benefit observed in attenuating the rotenone-induced decreases in aconitase activity. Previous studies have utilized aconitase activity as an indirect indicator of an oxidative stress given its sensitivity to free radical attack and damage, particularly via superoxide anion. Thus, it is tempting to speculate that NAC and taurine cooperatively scavenged free radicals and reduced further generation of free radicals to provide the observed benefits in both aconitase and LDH activity, respectively. Future studies investigating these respective mechanisms, or additional ones, will be enlightening.

In all, our results showcase the potential of taurine to modulate the toxic effects resulting from mitochondrial inhibition. Additionally, the beneficial effects of the combination treatment suggests that a multi-targeted approach utilizing lower, subtherapeutic doses of compounds, requires further evaluation. However, from such an approach, it is conceivable that a more robust and translational avenue of research and development of therapeutic strategies for neurological disorders may emerge given that higher doses of singular agents needed to achieve appropriate brain concentrations/effects also make systemic toxicities more prevalent and thus prohibitive.

4. Experimental procedures

4.1. Chemicals and reagents

Rotenone, N-acetyl-L-cysteine, taurine, fetal bovine serum (FBS) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). LDH cytotoxicity assay kit and aconitase assay kit were purchased from Cayman Chemical (Ann Arbor, Michigan, USA). Dulbecco's Modified Eagle Medium DMEM with F-12 nutrient mixture and penicillin-streptomycin (P/S) were obtained from Life Technologies (Grand island, NY, USA).

4.2. Cell culture

Human neuroblastoma SH-SY5Y cell line (ATCC, Manassas, Virginia, USA) were grown in DMEM with F-12 nutrient mixture, supplemented with 10% FBS and 1% P/S and incubated at 37 °C and 5% CO_2. Cells were either seeded in a 96-well plate at density of 5×10^4 cells/well (for LDH assay) or 12-well plate at a density of 5×10^5 cells/well (for aconitase assay), 24 h prior to drug treatment in low-serum media (DMEM with F12 supplemented with 1% FBS and 1% P/S). Rotenone was dissolved in DMSO but diluted so final concentration in wells was no greater than 0.1%. NAC and taurine were dissolved in low-serum media at 100× their final concentrations in wells. All compounds were added to concurrently to respective wells in triplicate. All experiments were independently performed three or four and the data from each experiment was combined before final statistical analysis was conducted.

4.3. LDH assay

The LDH assay was performed according to the manufacturer's instructions. In brief, supernatants were obtained 48 h after drug treatment, and the levels of LDH activity were measured at 490 nm (Synergy HT Multi-Mode Microplate Reader, BioTek Instruments, Inc. Winooski, Vermont USA). Activity levels were normalized to total protein content in μg/mL as determined by BCA total protein kit Thermo Scientific (Rockford, Illinois, USA).

4.4. Aconitase assay

The aconitase assay was performed according to the manufacturer's instructions. Briefly, cells rinsed with PBS before

being scrapped from plate 48 h after drug treatment. Cellular suspensions were collected in Eppendorf tubes before being centrifuged at 800 g for 10 min, and the supernatant discarded. Homogenization buffer was added to the pellet, and the cells were broken down by sonication for 20 s. Cellular homogenates were centrifuged at 20,000*g* for 10 min. The supernatant was collected and the total protein concentration was determined in all sample using BCA total protein kit Thermo Scientific (Rockford, Illinois, USA). The reagents were added to the supernatant and the resultant absorbance was monitored for 15 min at 340 nm, the activity of aconitase was reported as nanomoles/minute/ml.

4.5. *Statistical analysis*

Data are reported as mean ± SEM. Statistical analysis was performed with one-way analysis of variance (ANOVA) with Tukey post-hoc analysis (SigmaPlot®, San Diego, CA). Results with a $p \leq 0.05$ were considered statistically significant. Each point in the assay was done in triplicate, and each experiment was done in three or four independent experiments.

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