Methylisothiazolinone Inhibits the Development of Neurites in Differentiating PC-12 Cells

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Abstract

Methylisothiazolinone, an antimicrobial agent commonly used in hygiene and cosmetic products, has been shown to cause cell death in cultured rat cortical neurons. There is insufficient data on neurotoxicity in humans at concentrations that result from household product exposure, although methylisothiazolinone exposure has been linked to dermatitis and allergic reactions. In this study, we examine its effects on PC-12 cells both alone and in combination with nerve growth factor (NGF), including its effects on the number of neurites that develop when exposed to conditions that favor differentiation. For controls, we counted neurites in cells treated with NGF, epidermal growth factor (EGF), dbcAMP (a cyclic AMP analog that can cross the plasma membrane) and combinations thereof. In addition, we measured the amount of tubulin and actin produced in the presence and absence of NGF. Our results show that differentiating PC-12 cells produce fewer neurites per cell when exposed to MIT and NGF together than when exposed to NGF alone.

Good

Should also mention SDS-PAGE data even if it is negative

Introduction

Methylisothiazolinone (MIT) is a small hydrophilic molecule with antimicrobial properties at relatively low concentrations; it is used for this purpose in many hygiene and cosmetics products. It has been shown that MIT exhibits neurotoxic effects *in vitro*, but no clinical evidence has been published for patient CNS or nerve damage, although this cannot be ruled out, considering the lack of research. However, the tendency of MIT to cause dermatitis and allergic reactions in some individuals has been documented (García-Gavín et al., 2010).

In this study, we examine the effects of MIT on PC-12 cells. PC-12 cells are derived from a rat adrenal medulla tumor, and can differentiate to become very similar to sympathetic neurons: neurites and other neuron-like processes are extended from the cell body and synapses are formed. The secondary messenger cAMP is known to promote this neurite growth alone and in combination with NGF (Richter-Landsberg, Jastorff, 1986), but the polarity of cAMP makes it difficult to cross the plasma membrane, so we use a less polar analog, dbcAMP (dibutyryl cAMP). Earlier experiments by collaborators in our lab grew PC-12 cells in a single type of medium containing fetal bovine serum both after splitting and to initiate differentiation, but it was found that different types of media (containing mixtures of fetal calf serum and horse serum in different proportions) for these two stages produced better results.

Previous work has revealed the mechanism by which methylisothiazolinone causes the death of cultured rat cortical neurons: certain soluble zinc-binding proteins rely on thiol groups for their function, and so the cytosolic Zn^{2+} concentration increases when these thiol groups are oxidized by MIT. The high concentration of Zn^{2+} leads to a signal transduction cascade ultimately resulting in cell death (Du, et al., 2002).

G-actin and tubulin are monomers of cytoskeletal components that (among other functions) allow neurites to be extended and provide their structural integrity. In this study, we use a Western blot to compare the amounts of actin and tubulin produced in the presence and absence of NGF.

Be sure to mention WHY you would do this: Differentiating cells express different proteins...

Materials and Methods

Neurite outgrowth under varying chemical conditions. PC-12 cells were grown under seven different conditions: unsupplemented differentiation media (1x penicillin/ streptomycin/ fungizone (P/S/F) and 2% horse serum in DMEM), and differentiation media supplemented with 20ng/mL EGF, 200ng/mL NGF, 2mM dbcAMP, 200ng/mL NGF + 2mM dbcAMP, 1uM MIT, or 200ng/mL NGF + 1uM MIT. Cells were supplemented only after growing for one day in normal media (1x P/S/F, 5% fetal calf serum, and 10% horse serum in DMEM) to provide time to adhere, except for the MIT

cells, which were grown in normal media for three days before treatment. Cells were observed and neurites were counted after 3 and 5 days of growth.

Cells for Western blot. In parallel with the aforementioned experiment, two plates of PC-12 cells were grown: one treated with 100ng/mL NGF, and one without treatment. After 7 days, the number of cells with neurites were counted. After 9 days, the confluence fractions for each plate were measured and cells were lysed with the corresponding proportion of lysis buffer (300uL for 60% confluent cells). Proteins were separated from cell debris by centrifugation at 10,000 rpm for 10 min., and protein content was assayed using a NanoDrop instrument.

Don't forget: In diff. medium

SDS-PAGE. The above two protein solutions were diluted with lysis buffer to equalize volume and protein concentration, and 240 ug protein in 1X loading buffer solution (total volume 40uL) were loaded in each well of a polyacrylamide stacking+resolving gel contained in a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Hercules, CA); both -NGF and +NGF were performed in duplicate to permit both Coomassie Blue staining and Western blot. Gel was run at 200V for 40 min.

Western blot. The above gel was packaged with a nitrocellulose membrane into a gel box, allowed to equilibrate for 5 min. in the transfer buffer, and then run at 100V for 1 hour. Nitrocellulose membrane was soaked protein side down in blocking buffer (5% milk in PBS-Tween) for two days. A western blot was performed; primary antibodies were mouse anti-beta-tubulin and rabbit anti-actin; secondary antibodies were goat anti-mouse IgG-800nm conjugate and goat anti-rabbit IgG-680nm conjugate. The resulting nitrocellulose membrane was imaged in the infrared using a LICOR instrument.

Results

Nice subheading

Effects of MIT, NGF, EGF, and dbcAMP on neurite outgrowth. It was discovered that cells did not adhere well enough in the chambered slide we were using. Fortunately, J. Bailey was performing this experiment with plates instead of a chambered slide, and so his data will be analyzed here (Figures 1 and 2; Table 1).

Also, this is a more minor point. Start with the general trends...

The standard deviation of the dbcAMP cell groups is much larger than those of the other treatment conditions, suggesting that this maximum neurite count is not significant data (Table 1).

to say "not significant," it should be analyzed statistically

Figure 1: PC-12 cells after one day of treatment: (A) dbcAMP + NGF; (B) NGF; (C) NGF+MIT; (D) MIT; (E) dbcAMP; (F) EGF; (G) differentiation medium only; and (H) normal medium only.

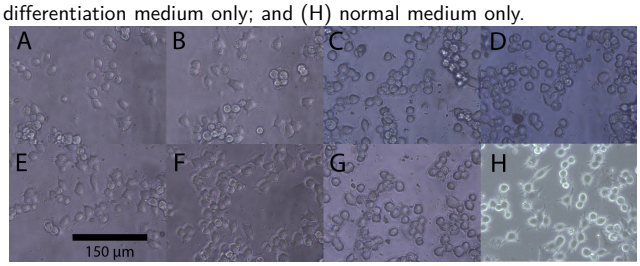


Figure 2: PC-12 cells after three days of treatment: (A) dbcAMP + NGF; (B) NGF; (C) NGF+MIT; (D) MIT; (E) dbcAMP; (F) EGF; and (G) differentiation medium only.

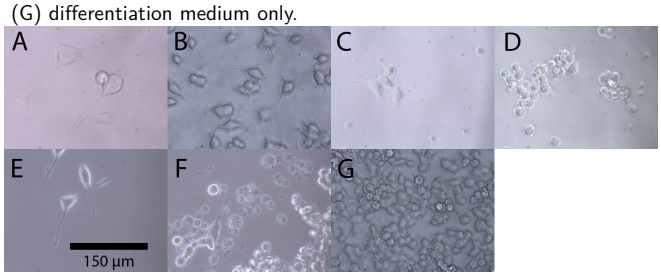


Table 1: data for neurite counts at the end of growth period, according to each treatment; N is the number of groups of cells assayed; for each group, the number of cells with neurites is divided by the total number of cells in the group.

Treatment	Mean	Std. Dev.	N
Diff. Medium	0.0111	0.0192	3
NGF	0.2444	0.1018	3
EGF	0.0444	0.0770	3
dbcAMP	0.4111	0.2502	3
dbcAMP + NGF	0.2333	0.0577	3
MIT	0.0333	0.0333	3
NGF + MIT	0.0667	0.0577	3
Total	**0.1492**	**0.1702**	**21**

Don't need the grand mean unless you're doing statistics

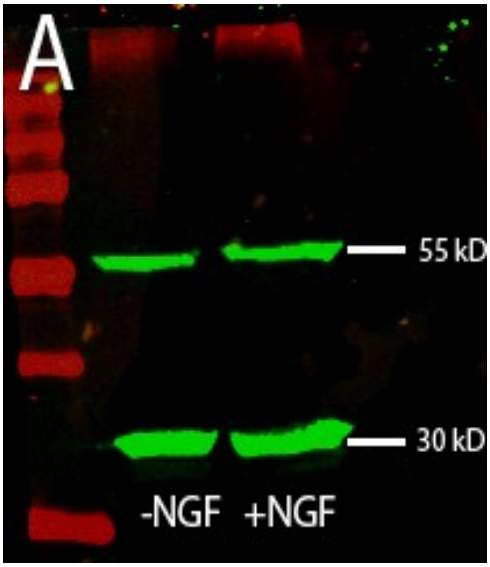
Ok, but not incredibly useful...[ ]'s were immediately equalized

Table 2: NanoDrop A280 protein assay of cell lysates for SDS-PAGE and Western blot.

+/- NGF	Protein conc.	OD_{260}/OD_{280}
-	10.889 mg/mL	1.54
+	8.037 mg/mL	1.62

Figure 3: LICOR imaging of Western blot. The secondary antibody for tubulin (conjugated to 800nm fluorophore) is visualized in green and the secondary antibody for actin (conjugated to 680nm fluorophore) is visualized in red. Actin band is not visible for unknown reasons.

speculate?



Label ladder MW

No raw data please.

Table 3: Tubulin content as measured by infrared imaging of Western blot. Actin content could not be measured, possibly because of a problem with the primary antibody to actin.

Band Measured	-NGF Lane	+NGF Lane
~55 kD	1.18	1.80
~30 kD	3.02	3.18

Bar graph of % (relative to no NGF control) or take a ratio for each

What about the results?
-talk about neurite counting
- results of the WB data??

Discussion

Actin bands were not seen in the Western blot (Figure 3), however there is signal from the secondary antibody for actin at the top of the gel. This suggests that either the primary or secondary actin antibodies are binding to high molecular weight proteins, and so too little free antibody is available to bind to G-actin and produce a visible band.

Good speculation. however, this type of saturation is unlikely based on the relatively weak actin signal

More analysis/discussion of the data.

We have demonstrated that methylisothiazolinone (MIT) exhibits neurotoxicity in rat PC-12 cells. To establish the risk of this compound to humans who use MIT-containing hygiene and cosmetic products, further experiments are needed. In particular, the ability of MIT to penetrate the epidermis needs to be assessed (previous studies have determined that MIT can cause contact dermatitis and allergic reactions, but no correlation has yet been found between central nervous system (CNS) damage or peripheral nerve damage and MIT). It would also be helpful to measure the concentration of MIT and its metabolites in the blood of mammals exposed to topical MIT; this would give a better indication of the risks to the CNS.

Good future directions

References

Du S, McLaughlin B, Pal S, Aizenman E. (2002) *In Vitro* Neurotoxicity of Methylisothiazolinone, a Commonly Used Industrial and Household Biocide, Proceeds via a Zinc and Extracellular Signal-Regulated Kinase Mitogen-Activated Protein Kinase-Dependent Pathway. *Journal of Neurosci.*, 1 September 2002, 22(17): 7408-7416.

García-Gavín J, Vansina S, Kerre S, Naert A, Goossens A. (2010) Methylisothiazolinone, an emerging allergen in cosmetics?. *Contact Dermatitis*, 63: 961-01. doi: 10.1111/j.1600-0536.2010.01754.x

Richter-Landsberg C, Jastorff B. (1986) The Role of cAMP in Nerve Growth Factor-Promoted Neurite Outgrowth in PC12 Cells. J Cell Bio, 102(3): 821-829. doi: 10.1083/jcb.102.3.821

More refs would be helpful...

MCDB 103L Module 2 Report

		Points Earned	Possible Points
Title + Abstract			
Title			
Clarifies the main hypothesis/conclusion of the paper		2	2
Abstract			
Introduces the question/hypothesis to be studied		2	2
Describes methods used and results obtained Don't forget about SDS-PAGE data		2	3
Provides clear conclusion and significance of the results Don't forget about SDS-PAGE data. Also a bit more on significance.		1	3
	Title + Abstract:	**7**	**10**
Introduction			
Appropriate description of what is known/unknown Good		3	3
Identify what you did/how it contributes to the field A bit more on unknowns in the field or potential applications		2	3
Verbal precision, logical flow, no overstatement		4	4
	Introduction:	**9**	**10**
Results			
Organized description of results There is almost no verbal description of the results…		1	6
Clarification of what the results mean Needs much more here…ex which treatments were effective? Negative data?		1	5
Verbal precision, logical flow, no overstatement Too brief to assess		1	4
	Results:	**3**	**15**
Figures			
High quality images that support the hypothesis Images: ok, but increase constrast to visualize neurites more easily. WB: good quality. Coomassie Gel: missing.		6	10
Labeling: scale bars, units, general readability of the figure Ladder MW.		4	5
Clear figure legends that allow the figure to stand alone Figure legends should also summarize the main finding for the reader.		3	5
	Figures:	**13**	**20**
Discussion			
Summary of what was shown and its significance More depth/breadth. Also, one clear summary paragraph for all the data at the beginning of the discussion is a good place to start.		3	5
Data interpretation: explain the expected/unexpected Good selective attempts, but try to address all the data at least once. More coverage…		3	6
Verbal precision, logical flow, no overstatement Make sure the paragraphs are topic-based and organized in a logical order		2	4
	Discussion:	**8**	**15**
Methods Good job here			
Clear and concise description of reagents/procedures		3	3
Appropriate level of detail is given		5	5
Organization/formatting		2	2
	Methods:	**10**	**10**
References			
All bibliographic references are cited in text		5	5
Quality/relevance of references Good quality, but more than 3 would be helpful given the amount of previous work in this area		3	5
	References:	**8**	**10**
Layout/Style/English			
Readability/English		5	5
Journal formatting			
Author listing		2	2
Affiliation (institutional affiliation?)		0	2
PDF formatted		1	1
	Layout/Style/English:	**8**	**10**
	TOTAL POINTS	**66**	**100**