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Methamphetamine Mediated Mitochondrial Dysfunction and Microglial Apoptosis

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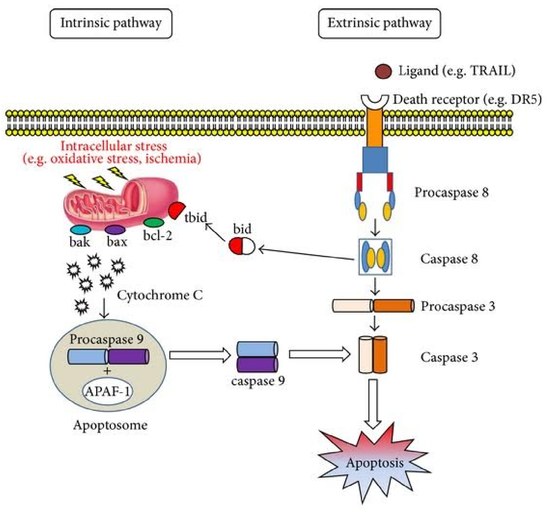
Abstract

We hypothesized that increasing concentrations of Meth will cause microglial apoptosis which induces mitochondrial dysfunction leading to neurodegeneration. Our goal was to evaluate if Meth treatment induced the activation of the mitochondrion-dependent intrinsic pathway. Gene expression of pro- and anti-apoptotic genes were quantitated with QPCR using RNA extracted from cultured microglial cell line (HTHU). Our results indicated that Meth treatment resulted in a significant increase in the gene expression levels of apoptotic proteins like APAF-1, BAX and BCL-2. Analysis of Western Blot data showed a significant modulation of the expression level of the antiapoptotic protein BCL-2 with varying Meth concentrations. Using immunofluorescence analysis we examined the expression of mitochondrial proteins, Cytochrome C, COX 4 and MCL-1, all of which were significantly activated on Meth treatment indicating the activation of the mitochondrion-dependent (intrinsic) apoptotic pathway. We conclude that Meth causes microglial apoptosis via the mitochondrion-dependent (intrinsic) apoptotic pathway contributing to neurotoxicity and subsequent neurodegeneration.

Background

According to the National Survey on Drug Use and Health (SAMHSA 2013) Meth is considered as "the new drug of choice" by the youth, far surpassing cocaine and heroin in availability and human destruction. Meth abuse results in Meth associated neurotoxicity causing a decline in cognitive functions and neurodegeneration. Meth induced damage of the nerve terminals of dopamine-producing neuronal cells triggers activation of apoptotic mechanisms resulting in the complete disintegration and death of neurons, astroglia and microglia. The exact molecular mechanisms of Meth-induced neurotoxicity remain unclear. Meth associated neuronal cell death is accompanied by endonucleosomal DNA cleavage and differential expression of pro- and anti-apoptotic proteins. In the current study we will examine an array of pro- and anti-apoptotic biomarkers that are activated as a consequence of Meth treatment. This will help us identify the specific mechanisms that underlie Meth induced apoptosis. There are two main apoptotic signaling pathways namely, a death receptor-dependent (extrinsic) pathway and a mitochondrion-dependent (intrinsic) pathway.



Methods

Cell Culture: The HTHU microglial cell line originally isolated from tissue biopsies were a generous donation from Dr. Jonathan Karn (CWRU, Cleveland, OH). Cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 100 µg/ml penicillin, and 100 µg/ml streptomycin. Cultures were maintained at 37˚C in a 5% CO2 humidified incubator.

Cell Counting Kit-8: Assay used to measure cell viability.

TUNEL Staining: TUNEL Apo-Green Detection Kit Assay measures nuclear DNA fragmentation, which is an important biochemical indicator of apoptosis.

RNA Extraction & Real time QPCR: Total RNA was extracted from microglial cells treated with Meth for 24hr using Trizol reagent (Invitrogen). 500 ng of total RNA was used for the RT reaction (25 µl total volume) by using the First-Strand cDNA synthesis kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instruction. One µl of the resultant cDNA from the RT reaction was used as the template in PCR reactions.

Immunofluorescence: HTHU cells are grown to 70% confluence in a petri dish with a glass bottom and treated with Meth (10-100 nM) for 24hr. Cells are fixed for 10 min at 37˚C in 4% formaldehyde, followed by permeabilization with ice-cold 90% methanol. Cells are then washed in 1X phosphate buffered saline (PBS) and treated with a specific primary antibodies and detected using fluorescent labelled secondary antibodies. Imaging was performed with the EVOS® FL Cell Imaging System.

Western Blot Analysis: Microglial cells treated with Meth for 48hr followed by protein extraction using the MPER reagent. The amount of protein used was 50 µg/lane. Standard Western blotting procedures were followed. 1:1000 dilution of BCL-2 antibody was used., followed by AP labelled secondary antibody and development of blot using NBT/BCIP.

Results

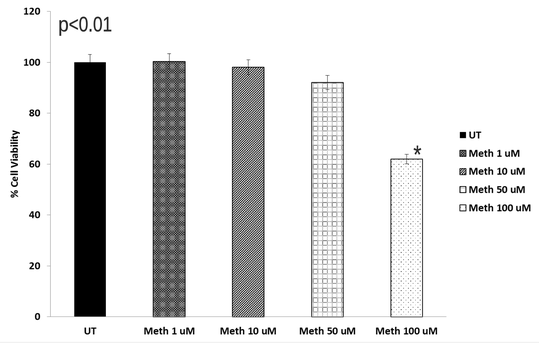


Figure 1: CCK-8 Cell viability assay. Meth treatment (0.5-50 µM) did not affect cell viability. >90 % cell viability was observed.

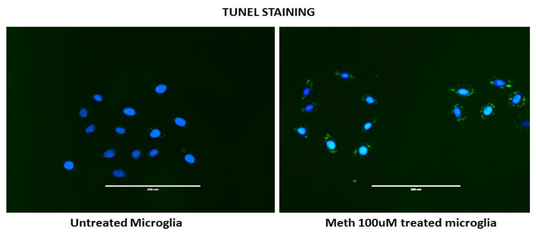


Figure 2: TUNEL staining shows microglial apoptosis. A 46% increase in mean pixel units was observed.

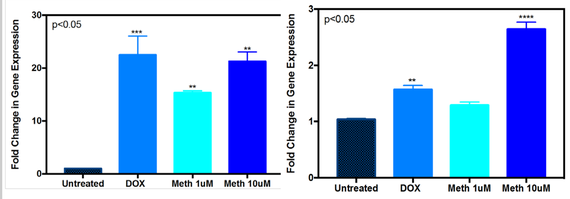


Figure 3: Significant expression of APAF-1 (left) and BAX (right) based on qPCR data.

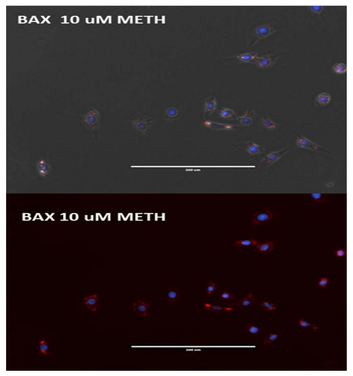


Figure 4: Immunofluorescence confirms apoptosis. Image J software shows 49% increase in mean pixel units of cells treated with BAX antibodies.

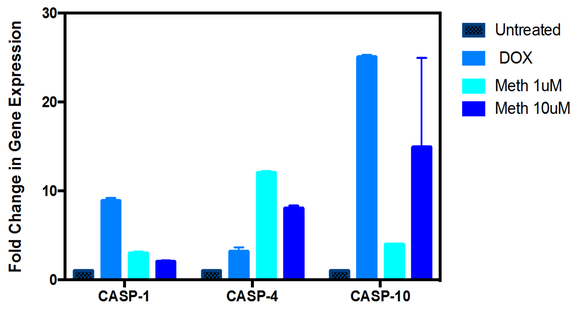


Figure 5: Caspases show varying expression based on qPCR data.

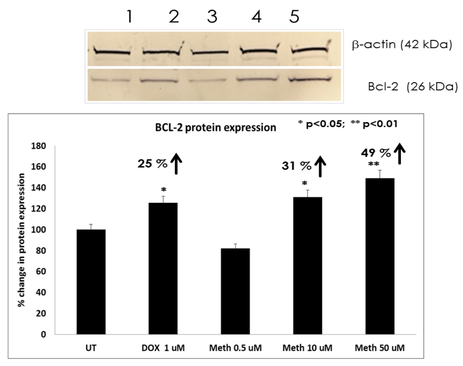


Figure 6: Effect of Meth on BCL-2 protein expression by Western Blot analysis

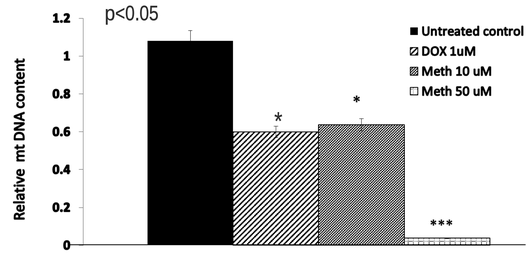


Figure 7: Significant decreases in mitochondrial DNA based on qPCR data which shows a drastic decrease from 40% in the DOX samples, to 36%, then to 96% in the 50 uM Meth treated samples.

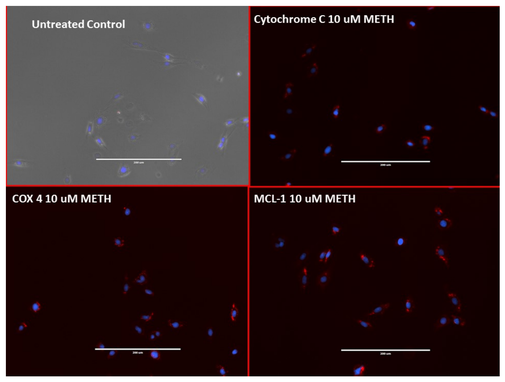


Figure 8: Immunofluorescence staining of mitochondrial proteins Cytochrome C, COX-4, and MCL-1 . Which show increases in mean pixel units of 22%, 43% and 62%, respectively.

Discussion

Our results show that the balance between pro- and antiapoptotic proteins in Meth treated microglia depends on the concentration of Meth. The ratio of pro- and antiapoptotic proteins is altered by Meth dose and may regulate mitochondrial physiology and cell apoptosis. Our next step is to evaluate Meth induced changes in mitochondrial morphology and function and their subsequent impact on neuronal cell apoptosis.

References

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Acknowledgements

- Supriya D. Mahajan, PhD
- Institute for Strategic Enhancement of Educational Diversity (iSEED)
- CURCA Undergraduate Research Award
- University at Buffalo's Honor College Academic Enrichment Award
- Collegiate Science and Technology Entry Program (CSTEP) Research Internship