Pioneering Neuroscience, 2017, **16**, 15-18

Nitric Oxide Increases the Damaging Effects of Hydrogen Peroxide on a Crayfish Neuromuscular Junction.

REBECCA VILLA, BRAD GEIMAN, and SAIHAM SHARIF
Department of Biology, Grinnell College, Grinnell, Iowa

ABSTRACT

Hydrogen peroxide, a reactive oxygen species, causes damage to the neuromuscular junction by inhibiting synaptic transmission. Nitric oxide is a chemical that has been tested in conjunction with hydrogen peroxide and can either react with hydrogen peroxide to form peroxynitrite, which will further harm the cell, or bind with hydrogen peroxide and prevent it from negatively affecting the neuromuscular junction. We hypothesized that the addition of nitric oxide to hydrogen peroxide will impair the crayfish neuromuscular junction and diminish the excitatory junction potential. We tested this idea by using a dull suction electrode to stimulate the motor nerve to extensor muscles in the crayfish tail with the specimen submerged in three different solutions: Ringer's saline (control), hydrogen peroxide, and hydrogen peroxide with nitric oxide. We found that the excitatory junction potential decreases significantly compared to the control when the crayfish is submerged in hydrogen peroxide, and the excitatory junction potential decreases further when nitric oxide is added.

INTRODUCTION

The excitatory junction potential (EJP) of a neuromuscular junction (NMJ) represents the amount of neurotransmitters released from a synapse. It has been established that reactive oxygen species (ROS), chemicals that contain oxygen with free radicals, can cause cell damage at the neuromuscular junction and decrease the amount of neurotransmitters released. The damage induced by ROS may contribute to the neuronal degeneration that causes amyotrophic lateral sclerosis (ALS). Currently, researchers are seeking to curtail ALS by preventing ROS damage.

Hydrogen peroxide (H_2O_2) is an ROS that has been tested and found to have damaging effects on EJP (Colton, et al.; 1986) . While it is already known that H_2O_2 is damaging to the NMJ, research has provided conflicting information on the effects of H_2O_2 in combination with nitric oxide (NO) on the NMJ. Some research indicates that NO offsets the effects of H_2O_2 (Liu, Martin; 2001), but other research using different organisms and different types of neurons demonstrate that NO may compound the effects of H_2O_2 by forming peroxynitrite ($ONOO^-$) which causes drastic damage to nerve cells (Wink, et al., 1993). Our research aims to understand the effects of NO together with H_2O_2 on the crayfish neuromuscular junction. Our results can contribute to the growing discussion on the topic and potentially find treatments for ALS.

In previous studies, ALS specimens of different brain tissues showed signs of oxidative stress more prominently than control specimens. Specific oxidative damage markers were found in excessive amounts, supporting the claim oxidative stress plays a key role in ALS (Ferrante et al. 2002). Another study supports that excess exposure of H_2O_2 negatively affects excitatory junction potentials. (Colton et al. 1986).

Current scientific literature remains divided about the effects of NO in conjunction with ROS. One study found that NO with ROS caused concentration dependent cell death of microglia (Wink et al.,1993). The researchers examined the effects of NO and ROS on hamster lung fibroblast cells. They used hypoxanthine/xanthine oxidase, a reaction system that produces superoxide/H_2O_2, and H_2O_2 itself. They also used "NONOates", a class of compounds that release NO. The researchers found that NO decreases the cytotoxic effects of H_2O_2, effecting a higher cell survival rate compared to the cells only in H_2O_2. Furthermore, the researchers saw a dose-dependent effect in NO. Similarly, another study concluded that a sublethal dose of NO protects against ROS-caused damage, but a lethal dose of NO does the opposite by exacerbating the effects of ROS (Wang, Lee, Wang; 2014).

Research has also found that NO aggravates H_2O_2 damage by combining to form $ONOO^-$. One study shows that NO and H_2O_2 harm cell DNA and can cause cell death (Liu, Martin; 2001). In this experiment, the researchers isolated motor neurons from adult rat spinal cords for individual study. They used a form of electrophoresis to perform a comet assay to show that H_2O_2, NO, and $ONOO^-$ induce DNA damage early during their degeneration by sciatic nerve avulsion-induced apoptosis. This conclusion led the researchers to discover that NO causes DNA damage. NO has also been linked to cell death. Nakajima & Shirasawa (2004) found that preventing the production of NO and or its interaction

with ions can stop cell death. Their research suggests that NO promotes the deadly effects of ROS.

With these studies providing conflicting information about the effects of H_2O_2 and NO on different organisms, it is still unknown if NO can be useful for ALS treatment. Our aim is to clarify the effects of NO on ROS by using the crayfish NMJ as our test subject. The crayfish NMJ lends itself to study this question because a simple dissection allows for easy muscle and nerve access. Also, the crayfish NMJ shares similarities with the human NMJ, including the glutamatergic synapse of the mammalian brain, so we can potentially apply our findings to ALS treatments for humans.

The purpose of our experiment was to determine the effects of NO on the effects of H_2O_2 at the crayfish NMJ. Our goal was to observe which side of the argument pertaining to the effects of NO in conjunction with H_2O_2 that the crayfish NMJ would support. We hypothesized that the EJP of the NMJ submerged in H_2O_2 with NO will decrease, caused by further damage done by the NO. We found that our hypothesis was correct; when H_2O_2 with NO was applied to the crayfish NMJ, then the EJP decreased as compared to the EJP when the specimen was submerged in H_2O_2 alone.

MATERIALS AND METHODS

Dissection

We used the crayfish extensor muscle as our specimen. Prior to exposing this muscle, we anesthetized the crayfish in ice for 10 minutes, cut the tail from the crayfish, and removed the legs. The outer muscles were gently scraped, exposing the layer of extensor muscles lining the exoskeleton. We covered the dissected crayfish tail in Ringer's solution (Wyttenbach et al.; 1999).

Preparation

Our experiment utilized Ringer's solution (5.4mM KCl, 196mM NaCl, 2.6mM $MgCl_26H_2O$, 10mM Hepes buffer, 13.5mM $CaCl_22H_2O$), 330 uM concentration of H_2O_2, and sodium nitroprusside. We inserted 1 mM of sodium nitroprusside per 1 mL of H_2O_2 solution for a final concentration of 100 uM sodium nitroprusside. We used intracellular recording to observe the voltage inside the cells. To do this procedure, two separate electrodes were used, made from glass tubing with a diameter of 1.2mm. The first was a recording electrode, which was made by using an electrode puller to pull the glass tubing apart at high temperatures. This electrode was filled with 3M KCL with a resistance of 5-20MΩ. The second was a suction electrode which was made by dulling a recording electrode with sandpaper, and it is filled with the particular solution that the crayfish is submerged in while it is being tested.

Recording

We placed the crayfish in Ringer's solution under a microscope and found a neuron to suck into the suction electrode. The recording electrode must be placed in an extensor muscle cell that is in the same section as the neuron for proper reading. When both electrodes were situated, we stimulated the neuron and recorded the resting membrane potential of the muscle cell as well as the EJP.

Data Analysis

We compared the change in EJP as an average of total EJPs recorded per solution to determine whether our hypothesis was correct or not. We also used a T-Test to check for significance.

RESULTS

The purpose of this experiment was to examine whether NO added to H_2O_2 benefited or harmed the crayfish NMJ. To test this question, we stimulated a nerve and recorded the membrane potential of muscle cells. This procedure allowed us to obtain and compare the EJPs of the dissected crayfish tail placed in H_2O_2 and H_2O_2 with NO solutions.

The average EJP of the control was 35.5mV. After stimulation, we found that the average EJP decreased after changing the control solution to H_2O_2 solution (P=0.0941). The average EJP also decreased after switching from the H_2O_2 solution to the H_2O_2 with NO solution (P=0.069). The average EJP for the H_2O_2 solution was 12.6mV which then fell to 10.6mV after adding the Sodium nitroprusside solution (Fig 1).

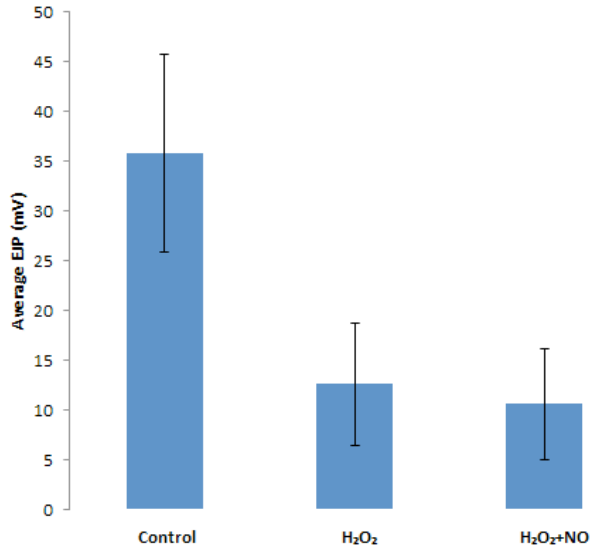

Figure 1. Average EJP of the three solutions after stimulation. The average EJP decreased after changing the control solution to H_2O_2 solution. The average EJP also decrease after switching from the H_2O_2 solution to the H_2O_2 with NO solution.

DISCUSSION

Our results supported our hypothesis that NO, in conjunction with H_2O_2, would decrease EJP more than just H_2O_2 alone. Our data indicates that the average EJP with H_2O_2 added greatly decreased the EJP. The addition of sodium nitroprusside to the H_2O_2 solution also decreased the average EJP. These results add to the studies that show NO in conjunction with H_2O_2 worsens ROS damage (Liu & Martin, 2001; Nakajima & Shirasawa, 2004). This conclusion thereby undercuts the studies that suggest NO alleviates ROS damage (Wang, Lee, & Wang, 2014; Wink, et al., 1993). Consequently, our results indicate that NO should not be used as a potential treatment for ALS because it will further the harm at the NMJ caused by ROS.

Although our data supported our hypothesis, we do acknowledge certain limitations to our research. Throughout the experiment, the Ringer's solution may have become polluted with waste products as time progressed, which could have affected the liveliness and thus the voltage of the crayfish cells. Also, although the neuromuscular junction of a crayfish shares many similarities with that of a human, it is only a model, and our results may not exactly hold true for the glutamatergic synapses of the mammalian brain. In addition, even though our hypothesis was supported, we cannot confirm that $ONOO^-$ was formed and is the cause of the decreased EJP for the H_2O_2 with NO solution. As far as our lab setup, the concentration of NO in proportion with the H_2O_2 may have altered our results, but this question can be solved with future research on the effect of different concentrations of NO in proportion to H_2O_2 affecting the neuromuscular junction. Similarly, the time we allowed the solutions to sit before gathering our data may have also affected the scope at which the EJPs changed with each solution change, but this issue can further be researched by observing changes in EJP when the specimen is submerged in the solutions for varying amounts of time.

ACKNOWLEDGEMENTS

We thank Professor Clark Lindgren for teaching us everything we need to know about neurons and crayfish, Tim Burnette for intensely criticizing our writing, and Julia Petrusan and Ashley Wolterstorff for coming to the rescue every time we broke something.

REFERENCES

Colton, C. A., Colton, J. S., & Gilbert, D. L. (1986). Changes in synaptic transmission produced by hydrogen peroxide. *Journal of Free Radicals in Biology & Medicine*, 2(2).

Ferrante, R. J., Browne, S. E., Shinobu, L. A., Bowling, A. C., Baik, M. J., Macgarvey, U., . . . & Beal, M. F. (2002). Evidence of increased oxidative damage in both sporadic and familial amyotrophic lateral sclerosis. *Journal of Neurochemistry*, 69(5), 2064-2074.

Liu, Z., & Martin, L. J. (2001). Isolation of Mature Spinal Motor Neurons and Single-cell Analysis Using the Comet Assay of Early Low-level DNA Damage Induced In Vitro and In Vivo. *Journal of Histochemistry & Cytochemistry, 49*(8), 957-972.

Nakajima, M & Shirasawa, T. (2004). "Presenilin-1-Deficient neurons are nitric oxide-Dependently killed by hydrogen peroxide in vitro." *Neuroscience*, *125*(3), 563–568.

Wang, J., Lee, C., & Wang, J. (2014). Nitric oxide plays a dual role in the oxidative injury of cultured rat microglia but not astroglia. *Neuroscience, 281*, 164-177.

Wink, D. A., Hanbauer, I., Krishna, M. C., Degraff, W., Gamson, J., & Mitchell, J. B. (1993). Nitric oxide protects

against cellular damage and cytotoxicity from reactive oxygen species. *Proceedings of the National Academy of Sciences, 90*(21), 9813-9817.

Wyttenbach, R. A., Johnson, B. R., & Hoy, R. R. (1999). *Crawdad: A CD-ROM lab manual for neurophysiology*. Sunderland, MA: Sinauer Associates, Inc.