

Homocysteine acts via nitric oxide synthase to sensitize the crayfish neuromuscular junction to hydrogen-peroxide-induced oxidative damage.

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ABSTRACT

Oxidative stress may be a significant source of damage in many neurodegenerative disorders, including amyotrophic lateral sclerosis. Reactive oxygen species such as hydrogen peroxide cause oxidative stress. We hypothesized that: (1) hydrogen-peroxide-induced oxidative damage would decrease excitatory junction potential amplitude at the crayfish neuromuscular junction, (2) incubation in homocysteine would enhance the amplitude decrease caused by hydrogen peroxide, and (3) inhibiting nitric oxide synthase would negate the effect of homocysteine. We used intracellular recording to measure excitatory junction potential amplitudes in each of these conditions before and after the neuromuscular junction was exposed to hydrogen peroxide for twenty minutes. Our findings support these hypotheses, suggesting that homocysteine sensitizes neurons to oxidative damage via stimulation of nitric oxide synthase.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease. Some researchers theorize that oxidative damage to motor neurons, or an increased sensitivity to oxidative stress, might be involved in the progression of ALS (Bukharaeva et al., 2015). If this is the case, increased understanding of the process by which the motor nervous system becomes sensitized to oxidative damage could lead to the identification of more potential targets for ALS treatment.

Previous research has shown a connection between homocysteine, oxidative stress, and ALS. Zoccolella et al. (2008) found that the plasma level of homocysteine (HCY) in ALS patients is significantly higher than that in normal people. Bukharaeva et al. (2015) investigated the effect of HCY on peripheral impairment in ALS. They found that HCY facilitates neurons' susceptibility to oxidative damage caused by reactive oxygen species (ROS).

Many studies have investigated links between HCY and inducible nitric oxide synthase (iNOS), an enzyme that catalyzes the production of nitric oxide. Woo et al. (2002) suggested that HCY stimulates iNOS-mediated nitric oxide (NO) production in macrophages. Almer et al. (1999) also studied NOS, finding that the apparent loss of neuronal nitric oxide synthase (nNOS) immunoreactivity in ALS is due to the loss of neurons. Additionally, neurons with a higher density of nNOS are considered to be more susceptible to neurodegeneration than those with a lower density of nNOS. Moreover, the experiments of Chen et al.

(2009) show a clear link between NOS upregulation and ALS disease progression in ALS mice. Phul et al. (2000) revealed a higher density of NOS in the spinal cord of ALS mice, indicating a higher NO concentration in the tissue. This increase in NOS expression may facilitate neurodegeneration.

Previous research has established a link between HCY and increased neuron sensitivity to oxidative stress, as well as a link between NO, oxidative damage, and ALS progression. While Bukharaeva et al. (2015) proposed that NO could play an important part in the process of sensitization, they did not specifically test NO, so this involvement still requires further investigations. This gap led us to examine whether HCY does in fact act via NOS to sensitize neurons to H₂O₂-induced oxidative damage by studying whether HCY still exhibits its sensitizing effect if the production of NO is inhibited. We predicted that H₂O₂ exposure would decrease excitatory junction potential (EJP) amplitude at the crayfish neuromuscular junction. We also predicted that exposure to HCY would increase the effect of H₂O₂. Finally, we predicted that inhibiting NOS would negate the effect of HCY, showing that HCY acts via stimulation of NOS. The results of our experiment support these hypotheses.

MATERIALS AND METHODS

Organism and Dissection

We stored the live crayfish in ice water to anaesthetize it, alleviating its pain during the dissection. Between 2 to 3 hours before measurement began, we took out the anaesthetized crayfish from the ice water, cutting off its tail. We followed the dissection procedures from

previous research and dissected the crayfish tail, exposing the superficial extensor muscles of the tail (Wytenbach et al., 1999). We pinned the dissected crayfish tail in the petri dish and covered it with solutions required based on different experimental conditions.

Solutions

We used normal Ringer's solution (5.4 mM KCl, 196 mM NaCl, 2.6 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 10 mM Hepes buffer, 13.5 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$; pH of 7.4) to maintain normal function of all cells in the isolated tail. We also used solutions of homocysteine (500 μM), hydrogen peroxide (332 μM), and the nitric oxide synthase inhibitor L-NAME (300 μM) added to normal crayfish Ringer's solution to manipulate our experimental conditions.

Incubation

For different experiments we carried out, we incubated the crayfish tail in different solutions for two hours before we measured EJP amplitude. In our first experiment, we incubated our dissected crayfish tail in the Ringer's solution. In our second experiment, we incubated the tail in HCY and Ringer's solution. In our third and final experiment, we incubated the tail in HCY, L-NAME, and Ringer's solution. We recorded the original, pre- H_2O_2 EJP amplitude after each incubation. Then we applied H_2O_2 and let the crayfish tail set in the solution for another 20 minutes before recording post- H_2O_2 -exposure EJP amplitudes. We ran the first experiment twice, the second experiment three times, and the third experiment once.

Electrophysiology

We used 1.2mm glass capillary tubes to make microelectrodes with a World Precision Instruments, Inc. PUL-1 electrode puller. The microelectrodes we used to measure intracellular membrane potentials had a resistance between 4-20 M Ω . We filled the microelectrodes with 3M potassium chloride solution. We used sandpaper to grind the tip of one electrode to make a suction electrode.

Recording

To investigate the effect of ROS on neuromuscular junctions of the crayfish, we used intracellular recording to measure EJP amplitude of crayfish neuromuscular fibers. We electrically stimulated the motor nerve to examine the extent of depolarization of the crayfish muscle cells in each experimental condition. We subtracted the lowest membrane voltage before depolarization, V_{m1} , from the highest membrane voltage during depolarization,

V_{m2} , to obtain the change in membrane potential (ΔV_m), or EJP amplitude. We recorded at least five measurements before applying H_2O_2 and at least five after H_2O_2 for each experimental condition.

Calculations and Statistics

For each experiment, we calculated the mean EJP amplitude before and after H_2O_2 exposure. We then calculated the ratio of these means to obtain post- H_2O_2 EJP amplitude as a percentage of pre- H_2O_2 amplitude. We calculated error bars representing range and presented our data on a bar graph.

RESULTS

To test the effects of H_2O_2 , HCY, and L-NAME on activity at the crayfish neuromuscular junction, we used intracellular recording to measure EJP amplitude in solutions of different chemicals. Under each of these conditions, we recorded EJP amplitude before and after 20 minutes of exposure to H_2O_2 to induce oxidative damage. We expressed post- H_2O_2 EJP amplitude as a percentage of pre- H_2O_2 EJP amplitude.

In our first condition, we exposed the crayfish tail to Ringer's saline for three hours before recording the control EJP amplitude. We then added H_2O_2 , waited for 20 minutes, and recorded the post- H_2O_2 EJP amplitude. Under this condition, the mean post- H_2O_2 EJP amplitude was 78.1% of control EJP amplitude. We ran this second experiment twice.

In our second condition, we exposed the tail to Ringer's saline and HCY for three hours before recording pre- and post- H_2O_2 EJP amplitudes. Under this condition, the mean post- H_2O_2 EJP amplitude was 47.9% of control EJP amplitude. We ran this second experiment three times, and recorded a wide range of results. On these three separate occasions the post- H_2O_2 EJP amplitude was 24%, 79.2%, and 40.5%. This wide range of results is a significant source of error.

In our third experimental condition, we incubated the crayfish tail in Ringer's saline, HCY, and L-NAME for three hours before recording pre- and post- H_2O_2 EJP amplitudes. Under this condition, the mean post- H_2O_2 EJP amplitude was 71.2% of control amplitude. We ran this third experiment only once. These results can be seen in Figure 1.

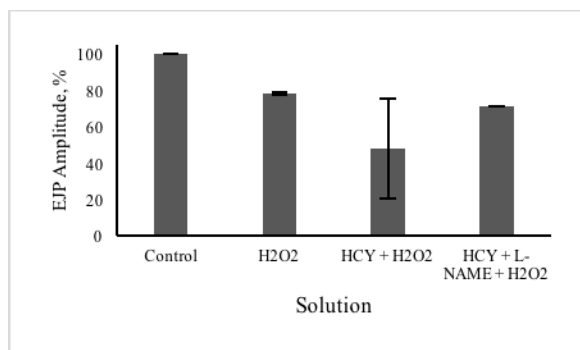


Figure 1. Post-H₂O₂ EJP amplitude as a percentage of pre-H₂O₂ EJP amplitude. H₂O₂-exposure decreased EJP amplitude to 78.1% of control. Incubation in HCY caused H₂O₂-exposure to decrease EJP amplitude to 47.9% of control. Incubation in HCY and L-NAME caused H₂O₂-exposure to decrease EJP amplitude to 71.2% of control. Bars represent the mean, and error bars represent the range. It is important to note that there is no error bar for the final experiment because we only tested once.

Our results indicate that HCY amplified the oxidative damage caused by H₂O₂, and that inhibition of NOS negated the effect of HCY.

DISCUSSION

We hypothesized that exposure to H₂O₂ would decrease EJP amplitude. We also predicted that incubation in HCY would enhance the decrease caused by H₂O₂. Further, we expected that the presence of L-NAME would negate the effect of HCY. To test our hypothesis, we used an intracellular recording computer program to measure the EJP amplitude of crayfish NMJ after incubating in different solutions. The results of our experiment support these hypotheses. HCY sensitized the crayfish NMJ to oxidative stress induced by H₂O₂. Inhibiting NOS with L-NAME removed the sensitizing effect of HCY, as H₂O₂ had the same effect with both L-NAME and HCY as it did in normal Ringer's crayfish saline.

Our experiments support the theory proposed by Bukharaeva et al. (2015) and fill the gaps in their research. Our results suggest that homocysteine stimulates NOS to increase the neuron's susceptibility to oxidative stress caused by hydrogen peroxide. When stimulated, NOS may produce a larger quantity of nitric oxide than under normal conditions, which may be the chemical responsible for sensitizing the crayfish neuromuscular junction to oxidative damage caused by hydrogen peroxide. This sensitization to oxidative damage caused by ROS could then lead to the progressive neurodegeneration seen in ALS and other similar disorders. Our deduction is further supported

by our third condition, in which the NOS inhibitor, L-NAME, was applied with homocysteine while we incubated the crayfish tail. When combined with L-NAME, the sensitizing effect of homocysteine vanished, and post-H₂O₂ EJP amplitude went back to 71.2%, which is very close to the result seen without homocysteine sensitization. These results indicate that excessive stimulation of nitric oxide synthase may accelerate the progression of neuron death in neurodegenerative diseases including ALS.

In our repeated experiments of the second condition, we encountered various difficulties in properly recording the post hydrogen peroxide exposure of EJP, including technological problems with the computer program and uncontrollable muscle twitching of the dissected crayfish tail while applying electrical stimulus. Therefore, the range of EJPs we recorded is wide, which also explains the large error bar in our graph. However, since we were given limited time to carry out our investigations, we were unable to reduce our error bar with more experimental data. This source of error could be remedied by conducting an experiment involving HCY sensitization of the crayfish NMJ to oxidative stress with a larger sample size. In this way, the extent of HCY sensitization at the crayfish NMJ would be more fully understood.

Overall, our results supported our hypothesis. Although the mechanism by which nitric oxide promotes the progression of neurodegeneration is not specifically examined in this research, we can conclude from our data that homocysteine stimulates NOS to sensitize the neurons' susceptibility to ROS-induced oxidative damage. Future experiments may examine the effects of directly applying varying dosages of nitric oxide to the crayfish neuromuscular junction before ROS-exposure to determine whether NO itself can sensitize neurons to oxidative damage. Investigation into nitric oxide's specific mechanism of promoting neurodegeneration are vital for deeper understandings of the role of this signaling molecule in the progression of ALS and other similar neurodegenerative diseases.

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REFERENCES

Almer, G., Vukosavic, S., Romero, N., & Przedborski, S. (1999). Inducible nitric oxide synthase up-regulation in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Journal of Neurochemistry*, 72(6), 2415-2425.

Bukharaeva, E., Shakirzyanova, A., Khuzakhmetova, V., Sitdikova, G., & Giniatullin, R. (2015). Homocysteine aggravates ROS-induced depression of transmitter release from motor nerve terminals: potential mechanism of peripheral impairment in motor neuron diseases associated with hyperhomocysteinemia. *Frontiers in Cellular Neuroscience*, 9, 391.

Chen, K., Northington, F., & Martin, L. (2009). Inducible nitric oxide synthase is present in motor neuron mitochondria and Schwann cells and contributes to disease mechanisms in ALS mice. *Brain Structure & Function*, 214, 219-234.

Phul, R. K., Shaw, P. J., Ince, P. G., & Smith, M. E. (2000). Expression of nitric oxide synthase isoforms in spinal cord in amyotrophic lateral sclerosis. *Amyotrophic Lateral Sclerosis*, 1(4), 259-267.

Woo, C., Cheung, F., Chan, V., Siow, Y., & Karmin, O. (2002). Homocysteine stimulates inducible nitric oxide synthase expression in macrophages: Antagonizing effect of ginkgolides and bilobalide. *Molecular and Cellular Biochemistry*, 243, 37-47.

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

Zoccolella, S., Simone, I., Lamberti, P., Samarelli, V., & Tortelli, R. (2008). Elevated plasma homocysteine levels in patients with amyotrophic lateral sclerosis. *Neurology*, 70(3), 222-225.