The mechanism in which lysosomes are damaged by free radicals during neuronal ischemia is examined in rat hippocampus. There are two hypotheses. The free radicals can degrade the lysosome membrane through liquefaction; all of the intralysosomal proteins would be able to penetrate the membrane, albeit at slower rates for larger proteins. The second mechanism is that pores are created; the proteins within the lysosome that are smaller than the pores can escape while those larger than the pores would not due to the robust nature of the undamaged membrane. Acid Phosphatase (67kda) and β-Galactosidase (540 kDa) has been identified as rat intralysosomal neuronal proteins that can be detected as proxies for leakage. Our initial results indicate that the damage undergoes the pore mechanism of damage based on preferential acid phosphatase leakage.

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COVER SHEET

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Introduction

Just as the physical effects of Agent Orange defined the 1970's, or radiation induced sickness for the post WWII era, traumatic brain injuries in the Iraq war have come to define our modern generation's wounds (McCracken, 2005). Traumatic brain injuries (TBI), which include a spectrum of blunt force injuries ranging from sporting accidents to motor vehicle accidents to IED explosions, begin soon after a physical force to the head. Much of the damage from TBI occur through a secondary mechanism that is simply mediated by this initial blunt trauma—oxidative stress mechanisms have been implicated as a possible explanation (Okie, 2005).

Furthermore, strokes, which are the third leading cause of deaths in both the United States and the world, affect 5.5 million people worldwide, according to the World Health Organization (Mackay and Mensah, 2004). It is becoming more common for medical personnel to induce artificial hypothermia in patients with ischemic brain damage, such as strokes. High profile media stories of induced hypothermia following traumatic brain injury such as professional football player Keith Everett have increased the level of public interest in the mechanism of ischemic damage and ways to prevent damage at the cellular level (Krieger et al. 2001). Furthermore, the studies done on laboratory animals have shown powerful protective advantages for cooling during ischemic conditions (Corbett, et al. 1997).

Between traumatic brain injuries and strokes, oxidative stress is the convergent mechanism, and many aspects are still unclear about how the oxidative stress creates damage on a cellular level. A precise understanding of these mechanisms allows for researchers to refine the cooling process for optimal temperatures and duration of therapy, or to develop new drug targets following the onset of a stroke or traumatic brain injury. There are a wide variety of biochemical pathways that are potentially temperature sensitive or therapeutic, and thus a greater understanding of these fundamental pathways and the mechanism in which they function is imperative.

Oxidative Stress

Windelborn and Lipton (2008) noted that neuronal death is mediated by the release of lysosomal proteases within neurons initiated by NMDA mediated calcium entry, extracellular signal regulated and kinase initiated Arachidonic Acid metabolism leading to free radical oxygen species (ROS) product. Arachidonic Acid is a critical actor within this pathway, as it increases from 30uM to 300uM immediately following ischemia (Abe et al. 1987). Lipton (1999) states that oxidative metabolism of the newly freed Arachidonic acid leads to a superoxide or hydroxide radical formation. This causes a positive feedback loop to form, whereby the increase in reactive oxygen species (ROS) increases the release of Arachidonic acid, inducing more release of ROS into the surrounding neuronal tissue. This damage then releases the protease Cathepsin B to mediate neuronal death.

Differentiating the Dynamics of Lysosome Permeabilization

While the steps in the release of these proteases are understood, the mechanism of several steps, most notably how the free radicals mediate membrane damage in the lysosome is unknown, and will be examined in this study. There are two hypotheses proposed (figure 1). The first implicates the free radicals as a dissolving agent—the integrity of the lysosome membrane is degraded through a process analogous to liquefaction. This would mean that while the lysosome membrane still has some of its principle components, it would not be able to contain the internal constituents of the lysosome due to the uniformly disordered nature of the remaining membrane. Another possible mechanism of free radical damage is that large fissures are created through a more destructive method. This would mean that large pores are created with no components of the membrane in location of the damage. Thus, the proteins within the lysosome that are smaller than the fissures created by the ROS would be able to escape into the intracellular space, while those larger than the diameter of these pores would not due to the robust nature of the undamaged membrane. The variant characteristics of these two ROS damage pathways allows for an opportunity to shed light on the chaotic events of ischemia in neurons.

Materials and Methods

The nature of the lysosomal permeabilization during ischemia can be elucidated using known intralyososomal protein oligomers as a proxy for the size of a pore, if these do indeed occur. This project includes a large research component to identify lysosomal protein candidates that differ in mass to elucidate the pore size, as well as the utilization of assays to detect these proteins once the protein candidates are selected. We will compare which of the known proteins are found in the extralysosomal supernatant compared to those still retained within the lysosome. This allows us to elucidate the nature of the lysosomal damage. Thus, we should be able to answer the questions such as whether there is loss of integrity of the membrane or larger fissures in the membrane protein release as a proxy.

If the membrane undergoes a process similar to liquification of the membrane, then the proteins should be released into the extralysosomal space, albeit at variable rates. If the membrane experiences damage in the form of pores, then there should be high integrity in those areas that do not suffer damage, and thus we should hypothetically observe only a few proteins under a specific size limit to be released into the extralysosomal space, and other that are kept within the lysosome.

Neuronal Lysosome Isolation

We will induce global ischemia in the rat and isolate the lysosomes based on the protocol originally developed by Windelborn (2008). The rat is decapitated and the brain is placed in a cold high magnesium buffer. The hippocampus isolated, split into 6 parts, and placed in a Dounce homogenizer with 1mL Homogenizing buffer (HB)+ATP (250mM sucrose, 10mM HEPES, 2 mM MgCl2, 1mM Mg-ATP). The tissue is homogenized by pulling 25 times on the pestle. The lysosomes are isolated through 2 centrifugations, where the supernatant from a primary 10 minute spin at 700g is isolated and spun again for 20 minutes, at 14,000g. The pellet that is isolated from the second spin is then re-suspended in a physiologic conditions buffer (140mM KCl, 20mM NaCl, 10mM HEPES, 2mM MgCl2, pH solution to ~7.4).

Treatment

The buffers for the rat neuronal tissue described above are utilized to prevent the ischemic cascade. The effects of the reactive oxygen species on the lysosomes are studied using Xanthine and Xanthine Oxidase (XO/X), which are known to generate the ROS that occurs during ischemia. This allows us to isolate the effects of ROS on the lysosome. Olsson et al. (1989) showed that this reaction mediated impairment of lysosomal stability. The pellet that is resuspended in the physiologic conditions buffer in an eppendorf is placed through two treatments, with two duplicates each, totaling four eppendorf tubes. The two treatment options include simply the lysosomal suspension itself as a sham treatment, or a 1:1000 dilution of XO and X that is placed in the tube. These two tubes and the two duplicate tubes are placed in a 37 degree Celsius water bath for 60 minutes. Following this incubation, the tubes are spun again at 14,100g for 20 minutes to resuspend the lysosomes from the supernatant. The supernatant is removed into a separate tube from the pellet containing the lysosomes, which allows us to differentiate the proteins in the lysosomes as opposed to the soluble state proteins in the supernatant. All of the tubes are resuspended up to 250μl homogenizing buffer + .1% triton to release the entirety of the lysosomal contents so that they can be detected using our assays described below.

Acid phosphatase protein detection is applied to one tube with lysosomal suspension only, and one with lysosomal suspension + 1:1000 XO/X. The remaining two eppendorf tubes are used for β-galactosidase protein detection. These two proteins are our proxies for the lysosomal damage mechanism and the amount is measured and compared between the intralysosomal (precipitate) partition and the soluble state (supernatant) partition.

β-Galactosidase Sensitivity to XO

The first key obstacle that our project encountered was the possibility that the treatment of XO/X would create ROS that damaged the functionality of the β-galactosidase protein. This would mean that even if the β-galactosidase leaked into the soluble partition, we would not be able to detect this leakage. The β-galactosidase assay relies on the ability of a functional β-galactosidase to convert *ortho*-Nitrophenyl-β-galactoside into galactose and ortho-nitrophenol, which is detected as a yellow color. The ROS may disable or impair the β-galactosidase function so that the datum from the XO/X treated partition is not accurate. In order to determine how the

XO/X affected the β-galactosidase, we obtained synthetic β-galactosidase from the *Aspergillus oryzae*, product G5160 from the Sigma-Aldrich company, and combined it with a gradient of XO concentrations. This gives us the ability to determine the concentration of XO that would create ROS species affecting the functionality of the β-galactosidase.

Acid Phosphatase Protein Detection

The Lipton physiology laboratory has worked extensively with acid phosphatase protein and has developed an assay prior to this experiment. This protein is found in rat neuronal lysosomes (Sun et al. 2008). The acid phosphate protein is 67kDa in size (Braun et al. 1989) and can be readily detected using an acid phosphatase kit provided by Sigma-Aldrich, product number S0942-50TAB. The reaction is stopped with 100μl NaOH after 60 minutes at 37 degrees Celsius and read at 405nm absorbance on a SpectraMax 340PC.

β-Galactosidase Protein Detection

A second and much larger protein was needed in order to find a contrast for a hypothetical situation where the free radicals were creating fissures within the membrane. Thus, a protein larger than any pores created by the fissure mechanism should not be released—if it is, then this would be evidence that is strongly indicative of the liquification hypothesis. β-galactosidase has been identified as a viable candidate. The protein is a tetramer of 540 kDa (Fowler and Zabin 1970) and is known as a key component of rat neuronal lysosomes. A mouse model of human G_{M1}-Ganglioside disease was recreated in mice by removing lysosomal β-galactosidase, thus it is a key component in normal mammalian neuronal function (Hahn et al. 1997). Furthermore, Hahn et al. (1997) found that the β-galactosidase enzyme has an activity rate of 57 ±11 nmol/h/mg within mice that are homozygous positive for the β-galactosidase. This is enough for detection by the β-galactosidase assay kit, catalog number K1455-01 provided by the Invitrogen company. The assay is applied according to the instruction manual provided and incubated at 55 degrees Celsius and read at 420 nm absorbance on a SpectraMax 340PC.

Results

β-Galactosidase Sensitivity to XO

Our results (figure 2) show that a concentration of 1:1000 XO combined with 1μl xanthine and 1μl HCl (to neutralize the solution) in the physiologic buffer of the eppendorfs is the upper limit to the concentrations of XO that will generate ROS insufficient to impair the β-galactosidase function. The 1:1000 concentration of XO/X was already known to be sufficient to generate ROS for lysosome permeabilization from previous work in the Lipton lab (results not included).

β-Galactosidase and Acid Phosphatase Comparison

Our results (figure 3) indicate that the soluble partition from the XO/X treated (and thus ROS generated) had a higher amount of acid phosphatase in the supernatant compared to the precipitate for the XO/X treated neuronal tissue. The value of the ratio of supernatant: precipitate is .697±.094. The sham treated neuronal tissue had a lower amount of acid phosphatase in the soluble state when compared to the precipitate (.272±.004). The same effect was not seen for the β-galactosidase assay when treated with XO/X for rat neuronal tissue. The β-galactosidase had the same ratio between the supernatant and the precipitate for both the XO/X treated (.316±.025) and the sham treatments (.323±.008), indicating that the ROS generated by the XO/X combination did not affect the permeabilization of the β-galactosidase. This indicates that the β-galactosidase did not leak from the lysosome in the same manner as the acid phosphatase, giving strong evidence for the fissure mechanism of ROS damage on the lysosome.

Discussion

The cursory data that has been collected has indicated that there no leakage of the large β-galactosidase protein when lysosomes are treated with ROS that occurs during neuronal ischemia. There is, however, leakage of the smaller acid phosphatase protein. This gives evidence to the fissure hypothesis that was introduced earlier.

It should be noted that one possible dilemma to the methodology proposed above is that it may be possible that the holes in the fissure mechanism are larger than the β-galactosidase enzyme. However, at such a large size, it calls into question the nomenclature of the pore, since it would be analogous to calling the dissolution of the entire membrane as one single large pore. Thus, we expect that if a situation were to arise where there were pores larger than β-galactosidase, it would be more accurate to describe it as dissolution of the lysosome membrane in its entirety. If this is the situation, there will be a measurable difference since both smaller and larger proteins would dissolve out into the extralysosomal space at the same rate, whereas in the liquefication hypothesis, the larger protein should be released at a slower rate. Thus, we have a clear demarcation between different scenarios, and this has not been a problem with our data collection. More data needs to be collected for the project to verify our initial results, as the majority of this project to date has been to verify that the β-galactosidase assay would function to detect the intralysosomal β-galactosidase. Work will continue in the future for this goal.

Tables and Figures

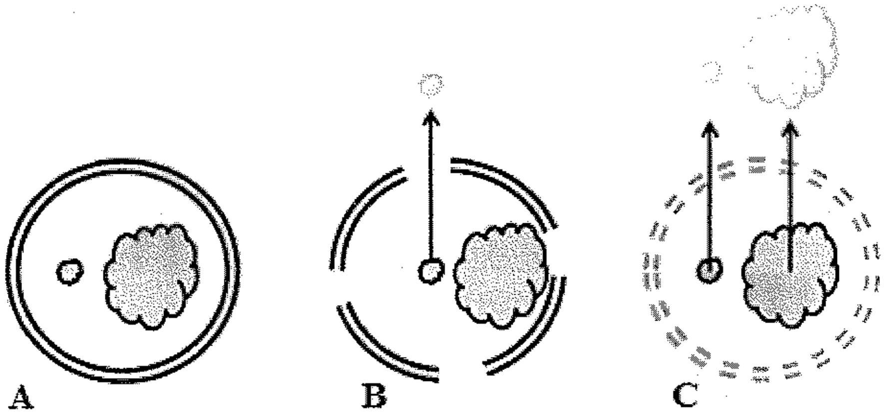

Figure 1: The hypothetical mechanisms for protein release in lysosomal damage scenarios. **A:** normal cellular conditions with no release of intralysosomal proteins. **B:** Fissure mechanism of reactive oxygen species (ROS) damage to lysosomes during ischemia, where there are large holes in the lipid membrane but other regions are left with full integrity. **C:** Liquefaction mechanism of ROS damage, whereby the entire membrane is left intact albeit with loss of capacity to partition the intralysosomal contents from the cytosol.

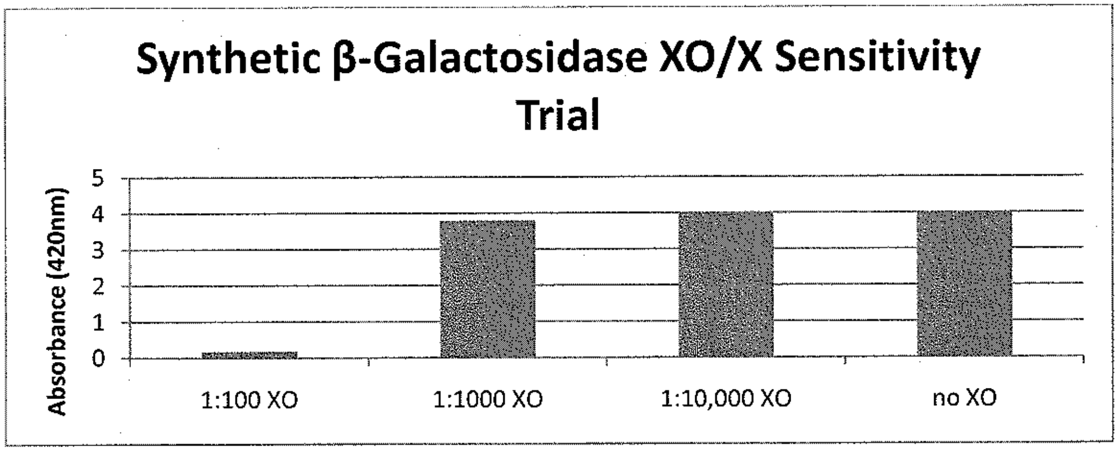

Figure 2: β-Galactosidase detection with Xanthine and variable concentrations of Xanthine Oxidase. This trial was done to ensure that the ROS generated from the XO/X reaction did not inhibit the β-Galactosidase enzyme activity, thus inhibiting the ability to assay for the protein. Our results indicate that there was inhibition of β-Galatosidase at concentrations higher than 1:1000 dilution of XO, thus we have ensured that only a 1:1000 or lower concentration of XO was used.

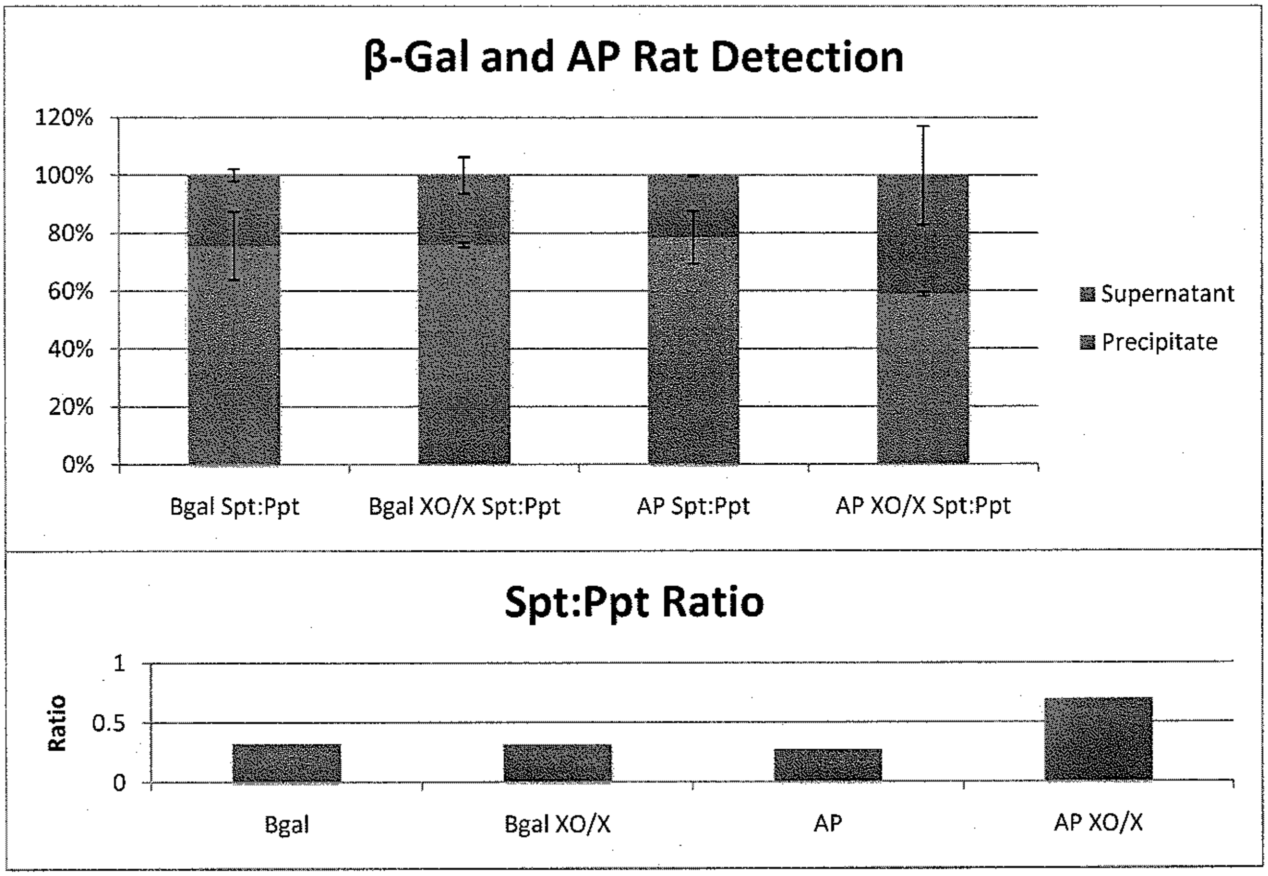

Figure 3: β-Galactosidase and Acid phosphatase protein comparison, from a single rat hippocampus. The percentage of acid phosphatase (67kDa) seems to have significantly increased in the Xanthine oxidase and Xanthine treatments, as compared to the non ROS treated hippocampal tissue. This same effect is not seen for the β-Galactosidase (540kDa), indicating that the ROS may be mediating a fissure pore mechanism as opposed to the liquification hypothesis since only the smaller protein is released.

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