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Voltage-Gated Calcium Channels and Tenocyte

Response to Oxidative Stress

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Abstract

Voltage-gated channels are important for excitable cells, such as neurons or muscle cells. Surprisingly, they have been described, at the gene expression and protein levels, in non-excitable cells such as bone, kidney, and tendons. Their function is largely unknown, but the Abreu lab has found significant gene expression for these channels, specifically dihydropyridine receptor, in the tenocytes of aged mice compared to young animals. This suggests that dihydropyridine receptor (DHPR) are not only expressed in tenocytes, but expression decreases with age. To investigate the role of DHPR in tenocyte function, cells are exposed to oxidative stress by hydrogen peroxide to examine effects of an inhibitor and agonist of the channel. Cell samples were cultured in Nifedipine (calcium channel inhibitor) or Bay K 8644 (calcium channel agonist), then exposed to hydrogen peroxide at varying doses. Oxidation in each group induced either apoptosis or necrosis. When necrosis is induced, results suggested that increased DHPR sensitivity provides a protective effect to cells. When apoptosis is induced, these data suggest that DHPR doesn't play a major role in helping tenocytes respond.

Background

Intracellular calcium regulation

Calcium is a ubiquitous signaling molecule in biology, and its cellular levels are regulated by ion channels. Perhaps the most remarkable part of calcium's role is that it affects each cell differently, depending on the function of the cell that it is affecting. This versatility can lead to marvelous consequences; because it arouses different cell types in different ways, the body's organ systems have adapted to maximize efficiency. One cell type may release calcium

under a specific stress, which leads nearby organs and tissues to work together to respond quickly in unison.

There are several types of calcium channels, but they are usually placed into two basic categories: Voltage-operated and non-voltage operated. Ions, such as calcium and sodium, have a voltage when ionized, and the charge that these atoms carry make signal transduction much faster. Evolution is not a perfect mechanism, but it tends to favor structures and adaptation that increase efficiency, and discourages spending biological capital on anything that hinders an organism. Cells usually build proteins and structures that are most advantageous, so at first it stands to reason that voltage-operated calcium channels (VOCCs) would only be found on cells that rely on quick signal transduction.

Second only to neurons, muscle fibers rely heavily on quick signal transduction to carry out their function. Muscle fibers have the capacity to dramatically alter their composition based on how they are used. These changes have an effect on their speed and endurance of contraction, and are classified as either fast- or slow-twitch fibers. High amounts of calcium channels help to speed up muscle activation, and muscle fibers are capable of altering their abundance (Radzyukevich, 2004). Cav1.1, commonly referred to as dihydropyridine receptor, the main target in this study, is positively correlated with contractile speed in various muscles. Dihydropyridine receptor (DHPR) is an L-type calcium channel, and a vital player in skeletal and cardiac muscle contraction (Robin, Allard, 2012), (Komazaki, Hiruma, 1997). By working with ryanodine receptor, the two players control intracellular calcium concentrations. The cooperation of these two structures links the processes of muscle excitation and contraction (Meissner, Lu, 1995), (Seebacher, Pollard, James, 2012).

VOCCs in multiple tissue types

VOCCs are known to exist on neurons and muscle cells, which both need quick reaction times in order to function well. However, recent research has found that these channels are also made in other cell types, such as nephrons (Kurtz, 1990) and tenocytes (Magra, 2006). This finding is very perplexing, because tenocytes are seen by most biologists as relatively sedentary cells. Their main purpose is the excretion and maintenance of an extracellular collagen matrix, and these cells have few known functions outside of that. Recent findings have shown that DHPR mRNA expression is high in young mice, and expression gradually decreases with age. These results were confirmed at the protein level by Western Blot (Unpublished research from the Abreu Lab).

Although it is primarily activated by changing membrane potential, DHPR can be activated by mechanical tension. L-type calcium channel sensitivity is increased by shear stress on the cell membrane (Kraichely, Strege, Sarr, Kendrick, Farrugia, 2009); shear stress is the main mode of force transfer between tendon fibrils (Szczesny, Elliott, 2014). Recent research has shown that mechanical stretching leads to over activation of tenocyte calcium channels, and can eventually cause tendon calcification (Chen, Deng, Zhang, Tang, 2014). Given calcium's massive range of uses, it is difficult to pinpoint its exact function in a cell (Georgiev, Svirin, Jaimovich, Fink, 2015), (Kokoska, Smith, Miller, 1998). In bone, for instance, VOCC mRNA has also been found expressed (Gu, 1999). The skeletal system is not considered excitable at all, but as the body's reservoir for calcium it stands to reason that our bones need these channels to regulate some major aspect of its function. Even without knowing the exact purpose of each of these VOCCs, it is obvious that these channels are just as ubiquitous as the ions that they regulate (Lenz, Kleineke, 1997).

DHPR modulation

Given that one specific channel like DHPR can have an effect on a wide range of systems, it is an excellent pharmacological candidate. Nifedipine is a calcium channel blocker, and its versatility has made it an invaluable asset. It is primarily used to treat high blood pressure and angina, but is also used to treat Raynaud 's phenomenon and even prevent premature pregnancy. Blockers work by mimicking the structure of the target molecule, and remaining attached to the receptor so that the target cannot travel through the channel. An effective blocker can competitively inhibit binding of the target molecule, is specific to a particular receptor, and can bind for exactly as long as you want it to. Calcium channel blockers are divided into two types based on what they bind to: Dihydropyridine and Non-Dihydropyridine. Nifedipine belongs to the former, and is part of a massive class of drugs that can specifically inhibit the activity of DHPR. All drugs have differing characteristics that affect how they enter the body, appropriate dosage, duration of activity, etc. Having a wide range of drugs available suggests that we can block DHPR in a variety of environments, opening doors to treatments that wouldn't otherwise be possible (Savignac, Gomes, 2004).

Nifedipine can be administered orally, intravenously, sublingually, and rectally (Raemsch, 1983). Each method has its advantages, and nifedipine's versatility in application is another key asset (Henry, 1980). Oral drugs are easiest to administer, and provide a more sustained dosage. Intravenous administration ensures rapid effect, and dosage can be altered rather easily. Sublingual administration circumvents the biochemical complication of first-pass metabolism. Rectal drugs have a shorter onset, and are cheaper than intravenous options.

Hypertension is caused by a number of factors, the most important being high blood volume, cardiac output, and vascular resistance. Nifedipine works by lowering vascular resistance, which is accomplished through arterial vasodilation (Zacharias, 1969). Relaxing the walls of the arteries makes them more tolerant of expansion from the pumping of blood, bringing blood pressure back to normal. Nifedipine can lower blood pressure very quickly, leading to dizziness and faintness; the most common forms of the drug are sustained-release formulations, allowing for the long-term treatment of hypertension (Olivari et al, 1979).

Preterm birth can lead to a slew of complications for the newborn, and is one of the largest causes of death in newborns. Uterine contractions are the most common sign of incoming labor, and are a good candidate to delay birth. Similar to how it lowers pressure in arterial walls, nifedipine has been used to arrest uterine contractions and has been accepted as comparable to other tocolytics. It also has less side effects, and lower risk of consequences after halting the regimen (Conde-Agudelo, 2011).

In contrast to a calcium channel antagonist, calcium channel agonists amplify the function of their respective protein. Bay K8644 is a structural analog of nifedipine (Sanguinetti, 1986). It is most thoroughly studied in muscles, and as such is considered a positive inotrope (Thomas, Chung, Cohen, 1985). It accomplishes this by lowering the activation potential of DHPR, as well as lengthening the amount of time the channel spends open (Bechem, Hoffmann, 1993). Although it is primarily an experimental drug, it has the potential to treat a wide range of medical conditions. Bay K8644 has been used mostly in studies of neuronal pathologies, because of their abundance of L-type calcium channels (LTCCs). Damage to neurons often reduces their capacity to transport calcium, and it seems that an agonist can have a neuroprotective effect. By increasing activation of LTCCs, research suggests that Bay K8644 has the potential to extend the

intervention window for stroke victims (Hu et al., 2012). Another study performed on models suffering from FALS found that chronic treatment with Bay K8644 halted neuromuscular junction decay, and even restored muscular function (Armstrong, Drapeau, 2013).

Oxidative stress

Oxidation alters the ionic charge of molecules within a cell, which can dramatically throw off vital biochemical pathways. It can also form free radicals, which wreak havoc on nearby molecules (Zhang et al., 2007). Their instability causes free radicals to steal electrons from more stable molecules. This reaction causes a cascade of others, and can snowball into cell and even tissue damage. In vitro, oxidation causes tenocytes to detach from their extracellular matrix and eventually lyse. In vivo, skeletal muscle contraction is a large cause of oxidation in the surrounding area. Given the proximity of skeletal muscle and tendons, it is vital that they can tolerate the stress associated with it.

The reactivity of hydrogen peroxide makes it the primary chemical used to induce oxidative stress. Research has found that exposure to hydrogen peroxide in arteries leads to activation of store-operated calcium channels (Santiago et al). Though they are found on muscle tissues, store-operated calcium channels do not play a role in muscle contraction. This is a very important distinction to make; DHPR controls intracellular calcium concentration, but in tendons it plays no role in muscle contraction. These data suggest that calcium channels play a role in protection from oxidative stress.

Research Goals

Because DHPR abundance in tenocytes decreases with age, it is likely that old and young cells would react in different ways to oxidation. Because of this, mice of 6 months and 22

months were used as young and old models, respectively. When the goal of an experiment is to damage the cells, there are two major types of cell destruction: apoptosis and necrosis. Apoptosis refers to programmed cell death, and is a highly regulated process that is specifically activated by the cell. It is brought about by large amounts of trauma, but is relatively slower than necrosis. Necrosis refers to autolysis of a cell due to acute damage. This isn't a process that is controlled internally by the cell, but rather is caused by loss of membrane integrity that results in death. Extreme or rapid distress in a cell causes necrosis, whereas apoptosis requires an extended period of cell membrane stability. This experiment also investigates DHPR's role in protecting tenocytes from each of these processes.

Methodology

Dissection/Extraction

All tenocytes were initially obtained from tails of rats and mice. Dissection was performed on animals to extract tendon fascicles. Tenocytes were extracted from fascicles via two methods: digestion and explant culture. Cells were then cultured in Complete growth medium (Dubelco's Modified Eagle's Medium + 10% Fetal Bovine Serum + 1% Penicillin/Streptomycin). Cells were grown until confluence was reached, then trypsinized into lower densities or frozen with Freezing Medium (Complete growth medium + 10% Dimethyl Sulfoxide). According to freezing protocol, vials were stored in -20 Celsius for 24 hours, then -80 Celsius for several weeks. For more long-term storage, vials were then moved into liquid nitrogen.

Oxidation

Media was aspirated and discarded from cell cultures, washed with Phosphate-Buffered Saline (PBS) twice, and a hydrogen peroxide solution was added. For trials investigating necrosis, 5 mM H_2O_2 was applied to cells for 40 minutes. For trials investigating apoptosis, cells received 0.1 μM H_2O_2 for 4 hours. Once time elapsed, media was aspirated and diluted into centrifuge tubes. Plates were then washed with PBS twice, and trypsinized. At the end of trypsinization, media was aspirated and added to their respective tubes. After centrifugation, pellets were resuspended in 2 mL of PBS, and cell viability was assessed using the Trypan Blue Exclusion Method.

N-Acetyl-L-Cysteine

The antioxidant N-Acetyl-L-Cysteine (NAC) was used as a positive control, to give an example of a protective effect on cells (Kim, Hah, Sung, Kang, Park, 2014). To examine its effect on cells without subsequent stress, cell cultures were aspirated and washed with PBS, and then an NAC solution (.1 μM NAC diluted in Complete Growth Medium) was added. Cultures were then incubated for 60 minutes. After this time, media was aspirated and cells were trypsinized and prepared for live/dead count as described above.

To examine the protective effect of NAC, cells were also exposed to NAC then H_2O_2. Cultures were exposed to NAC for 60 minutes as described above, and after that time media was aspirated and diluted in centrifuge tubes. Plates were then washed with PBS twice, and exposed to hydrogen peroxide for the amount of time as described above. Plates were then aspirated, and the media was added to their respective tubes. Plates were then trypsinized, and cells were again

added to their respective tubes. Cells were centrifuged, supernatant media was discarded, and live/dead cell counts were performed.

Nifedipine

Two concentrations of nifedipine were used: 250 μM and 5 μM. From a frozen stock solution, 25 μL of nifedipine was diluted into 5 mL CGM, and the solution was stirred in a vortex mixer. This solution is about 5 mL of 250 μM Nifedipine. To make a 5 μM solution, 100 μL of the 250 μM solution was diluted into 5 mL of CGM and vortexed. The end products of this dilution are 5 mL of 250 and 5 μM nifedipine.

Nifedipine undergoes photodegradation, meaning it is important to perform the dilution and incubation in low light. Once experimental concentrations were made, media in culture wells was aspirated and discarded. The appropriate dilution of nifedipine was then applied, and cells were incubated for 60 minutes. Media was again aspirated, then placed into a centrifugation tube. Wells were rinsed twice with PBS, and H_2O_2 was applied for the appropriate time and concentration. A trypan blue exclusion was performed as described above.

Bay K8644

Two concentrations of Bay K8644 were used: 10 μM and 1 μM. Using the refrigerated stock culture, 10 μL of 10 mM stock was diluted into 10 mL CGM. After vortex mixing, this is the 10 μM solution. Next, 1 mL of this solution was diluted into 9 mL CGM and mixed. This is 1 μM Bay K8644. After diluting 1 mL of this solution in 9 mL CGM and mixing, the end products are 9 mL 10 μM, 9 mL 1 μM, and 10 mL 0.1 μM Bay K8644. As described in above procedures, cells were incubated in their assigned drug concentration for 60 minutes, then incubated in H_2O_2 for the appropriate time and concentration, and a trypan blue exclusion was performed.

Results

Necrosis

To induce necrosis in tenocytes, cells were incubated in 5 mM H_2O_2 for 40 minutes. In experimental groups, cells were pre-incubated in nifedipine or Bay K8644 for 60 minutes. Groups that received nifedipine yielded a higher mortality rate than the control group at both 250 and 5 µM, and 250 µM had the highest percentage of nonviable cells. Groups receiving Bay K8644 in low dosages (1 and 0.1 µM) experienced a significant reduction in nonviable cells. At 10 µM Bay K8644, however, cultures had a higher percentage of nonviable cells. This suggests that outside of the drug's effective range, the drug not only loses potency but can have a damaging effect on cells. Figure 1 below displays these data.
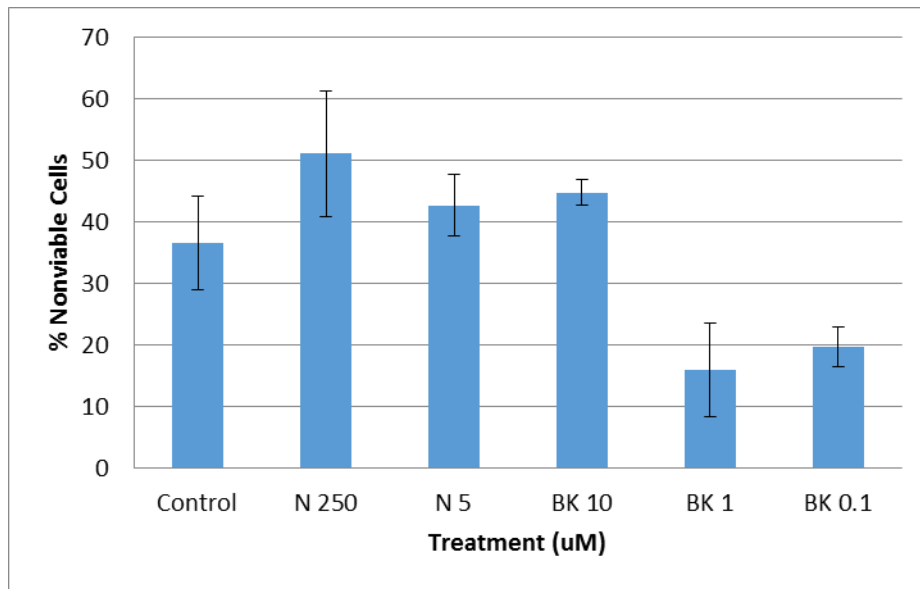

Figure 1: Tenocyte necrosis after exposure to H_2O_2 with or without pre-incubation with Nifedipine (N) or Bay K8644 (BK).

Apoptosis

To provoke apoptosis in tenocytes, cultures were exposed to 0.1 μM H_2O_2 for 4 hours. In experimental groups, cells were pre-incubated in nifedipine or Bay K8644 for 60 minutes. In the young model, the percentage of nonviable cells was reduced in all experimental groups. In the old model, however, only the low concentration of each drug had a reduction in viability. Results are displayed in Figure 2 below.
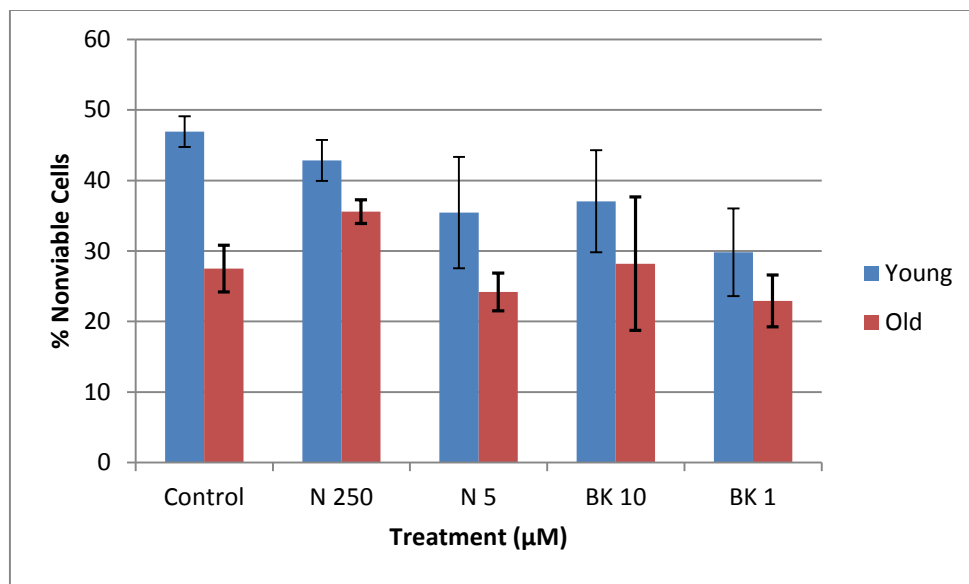

Figure 2: Tenocyte apoptosis after exposure to H_2O_2 with or without pre-incubation with Nifedipine (N) or Bay K8644 (BK).

Apoptosis in young model

Figure 3 below showcases the effect of each experimental group on culture viability in young cells. The effects are in relation to the control group, and are normalized such that the control group is the baseline for damage. All experimental groups had a reduction in nonviable cells. This is perplexing, given the opposing effects that the two experimental drugs have.
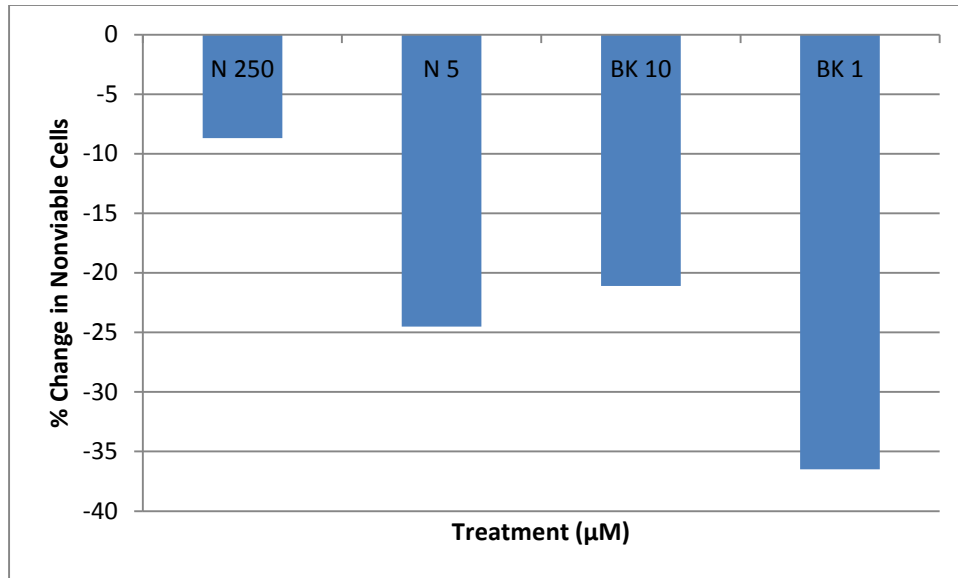
Figure 3: Young tenocyte apoptosis after exposure to H_2O_2 with or without pre-incubation with Nifedipine (N) or Bay K8644 (BK). Effects are normalized to the control group.

Apoptosis in old model

Figure 4 below displays the effects of both experimental drugs in relation to the baseline. This baseline was set by the control group. At the low concentration of each drug, a decrease in nonviable cells was seen. Bay K8644 at 10 μM had an increase in nonviable cells, but it was minimal. Treatment with 250 μM nifedipine had a statistically significant difference from the control (p = 0.005).
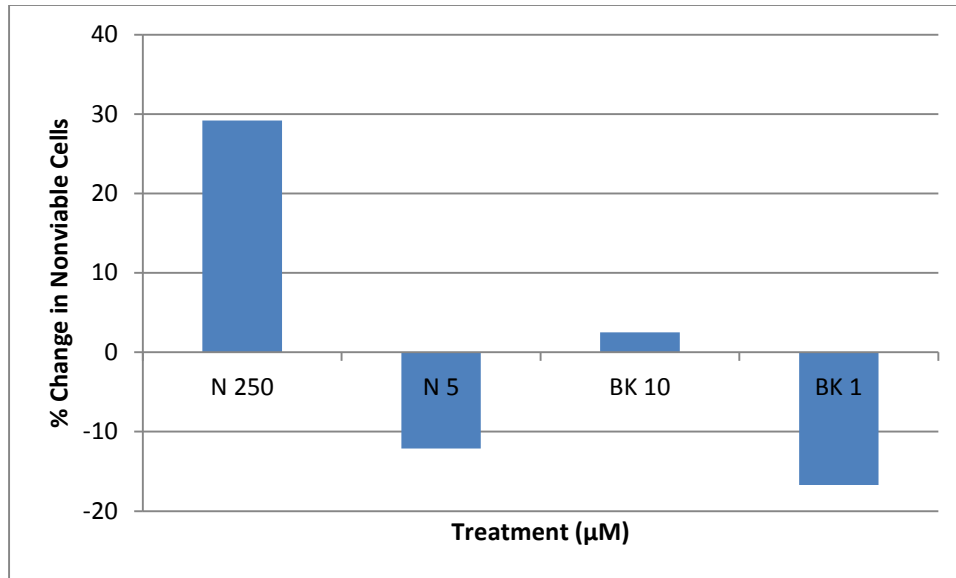
Figure 4: Old tenocyte apoptosis after exposure to H_2O_2 with or without pre-incubation with Nifedipine (N) or Bay K8644 (BK). Effects are normalized to the control group.

Discussion

Necrosis vs. Apoptosis

Both cellular destruction pathways are incredibly varied, and it seems that intracellular calcium regulation is just one of the ways that they differ. Necrosis data seemed to show a correlation between DHPR activation and retention of viability. For nifedipine, the antagonist, cell viability decreased as drug concentration increased. Bay K8644, the agonist, seemed to minimize cell destruction at low levels. At 10 µM, the drug actually had a higher mortality rate. This suggests that the drug has a defined effective range, and can quickly become hazardous at a high concentration. While necrosis trials seemed to have clear trends, both young and old cultures behaved in completely different ways when undergoing apoptosis. There were no clear

trends that related DHPR sensitivity to culture viability, which seems to suggest that DHPR doesn't play a large role in tenocytes undergoing apoptosis.

Young vs. Old

Young and old trials were completed for only apoptosis experiments. When examining the differences between the two, the first factor to consider is that DHPR is much more abundant in young cells. When analyzing the young cultures, it is obvious that the cultures were more sensitive to oxidation across the board. With higher amounts of nonviable cells, it is possible that the higher amount of calcium channels makes the cell more sensitive to environmental fluctuations. However, data also shows that every experimental group had a decrease in percentage of nonviable cells. The protective effect was much stronger for each of the weaker dilutions. There are many ways to interpret this, but this variation suggests that although DHPR abundance makes cells more susceptible to oxidation, this isn't simply because of the sensitivity to calcium fluctuation. Perhaps there are some biochemical pathways inside the tenocytes that involve attachment to DHPR within the cell, but isn't affected by the activity level of the protein.

In the old cultures, the results were much more perplexing. At the higher concentration of each drug, there were more nonviable cells compared to the control. For nifedipine, there was a significant increase in nonviable cells. But at the lower concentration of each drug, there were less. A possible explanation for this is that the drug concentrations selected were not all within the effective dosage. Treating a cell culture with a drug outside of its effective dose can yield unpredictable results.

Overcoming issues in method development

Research builds nothing if not character. Protocols are never perfect, and experimental trials are only improved through trial and error. There were many hurdles to overcome in this pursuit, and flexibility is key when finally applying an experimental design. Sometimes a common protocol is not feasible during an experiment, or the testing candidate reacts in unforeseen ways to treatment. The bulk of the time spent working on this research was dedicated to addressing these issues and finding a solution.

Unlike most other tissue models, there is no tenocyte cell line. The advantage of cell lines is that cellular phenotypes remain consistent over many passages. However, to acquire tenocytes a researcher must extract them directly from the animal model, making them a primary culture. Primary cell cultures are useful because different animals allow a researcher to compare many different groups. The downside to this variation is that the accumulation of differing phenotypes can snowball in unpredictable ways.

After extracting tenocytes from the tendon fibrils, many of the cultures simply didn't grow, or went senescent. This eliminated many of our animals before trials could even begin. In many of the preliminary dose-response trials, cultures obtained from similar but different animals had very disparate responses. Some cultures were damaged much more heavily than others when receiving the same treatment, which causes high statistical variance within experimental groups. To address this, the measurements of viability were normalized for each trial. Every experimental group was compared to the control in its trial, and damage done was represented as a percentage of that control.

While trypsinization is a common practice for moving fixed cells, it is difficult to perform with enough accuracy for data analysis. Trypsin is a nonspecific enzyme, exposing cells for too long eventually results in cell membrane damage and possible compromise. This balancing act can lead to two procedural issues. First, it is difficult to lift all of the cells in a well for counting. When the cells in a culture have had days or even weeks to grow, some cells will become more fixed to the plate than others. Whether they produce more collagen than other cells, or have other cells grow over them when the plate gets crowded, some amount of cells will require more time to lift than the rest.

Given the corrosive nature of trypsin, the procedure must be halted before these stubborn cells have been lifted, otherwise the other cells will die. By leaving live cells fixed to the culture plate, a trypan blue exclusion would yield lower viability results than is accurate. Second, cells that are damaged from hydrogen peroxide may not be able to withstand the additional stress of trypsin. Oxidative stress is designed to encourage cell death, either by necrosis or apoptosis. After cells have been incubated in the damaging chemical, many of the surviving cells are in a delicate state. It's possible that exposure to trypsin is simply too much for these cells to take, and lifting the cells would kill a higher percentage than the usual. Again, this would yield a lower viability percentage than is true.

The Trypan Blue Exclusion Method is a protocol designed to assess culture viability. If a cell's phospholipid bilayer has been compromised, trypan blue dye flows into the cell, which is visible to the researcher. Though sound in theory, this method is not perfect in practice. Cellular debris often collects so much dye that it can appear to be a dead cell. While most cells are clearly live or dead, it is sometimes difficult to confidently declare one way or another. Two researchers

counting the same batch of cells can have largely different viability counts, because at times experience is the only thing that ensures the researcher makes an accurate count.

Paradigm shift in tendon research

Tendon biology is a relatively unexplored area of human physiology. Exploring this new territory is exciting to say the least, but it also brings its own set of problems. Calcium channels are considered a vital structure in cell function, so it is surprising that they are still so understudied. Lack of interest has led to a lack of knowledge. One can compare the state of tendon research to that of skeletal research in the recent past: tissue function was perceived to be solely structural, and researchers devoted their attention to more interesting structures. This oversimplification has arguably set medicine back by decades, simply because the medical world did not conduct basic screening or testing. Logic suggests that these cells would not possess excitable channels, because these are perceived as needed for quick communication only. However, Western Blotting and PCR have proven their existence, and this opens the door for questions that never would have been asked in the first place.

When attempting to identify the role of a protein in a cell's function, investigation is little more than educated guessing. While one can hypothesize the probable function of a protein, a large part of it is still guesswork. Crystallography can help identify the polypeptide's shape, which is indicative of the class of protein that it belongs to. Just as one cannot assume that tendons don't express VOCCs, it is unwise to assume that a protein has one particular function, or works exactly like other proteins in its class.

Future research

No possibilities can be dismissed when investigating biological function. Evolution pushes for efficiency, and many proteins or hormones perform a wide range of functions on different tissues. Oxidative stress is one of the most common hazards in the human body, so antioxidation is a reasonable place to start. However, this experiment remains just a starting point for VOCCs in tenocytes, and it is unlikely that they hold only one function. To fully examine all possible functions of DHPR, this procedure can be improved to increase the similarity to normal physiological conditions.

As a vital component of our system of locomotion, tendons in vivo are under constant tension. Even at rest, tenocytes in truly physiological conditions are under a non-negligible amount of traction. Just like pH or oxygen concentration, tension is an environmental condition that every organ and tissue is affected by. Given the large changes in tension that tendons are likely to undergo, it would be advantageous for their cell organelles to alter their function based on the degree of force they are under.

Stretch is measured in vitro by examining a cell's aspect ratio. This describes the typical dimensions of a cell, relating length and width. Under no stress, a tenocyte has an aspect ratio of 1:1, or perfectly round. Tension causes the cells to elongate, stretching their length to a ratio as high as 5:1. This was not a factor that was modified in this experiment, so cell aspect ratios were 1:1 throughout.

Future investigations into DHPR function should examine its activity under varying levels of cell stretching. Cytomechanical culture plates are designed to allow for a cell culture to attach to the plate surface then expand so fixed cells stretch to a desired tautness. Muscles and

bones behave very differently during high activity, releasing a mix of anti-inflammatory hormones to assist during exertion. Tenocytes probably undergo something similar; DHPR activity may be proportional to aspect ratio, so that it can cope with heightened calcium oscillations.

Further reducing the differences between in vitro and in vivo experimentation can only lead to more accurate and dependable results. The largest difference between a cell culture and natural tendons is that tenocytes are usually surrounded in a thick wall of collagen. This extracellular matrix has many functions in a tendon, and one of its largest effects is that it slows diffusion. While reduced oxygen diffusion is a factor that cells have very much accounted for, this slowed release of drugs on their target area could have major consequences for the drug's true effectiveness. By clamping each end of a tendon fascicle, one can even adjust the tension at which the tenocytes are treated.

While acute effects of a drug are often studied first, prolonged exposure can often yield surprising consequences. Culturing tenocytes in a lower concentration of nifedipine or Bay K for several days may result in a number of changes to cell membrane concentrations of DHPR. Depending on its function, DHPR abundance may change to maintain a constant level of calcium oscillations. Extended culture in nifedipine may cause the cells to increase their total amount of DHPR, and Bay K may have the opposite effect.

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