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Investigating the Effects of Sex and Carvedilol on Ischemia Preconditioning Protective Effect

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INVESTIGATING THE EFFECTS OF SEX AND CARVEDILOL ON ISCHEMIC PRECONDITIONING PROTECTIVE EFFECT

by

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Abstract

Many studies have been done on ischemic preconditioning and its protective effects using the murine model with overwhelmingly consistent and promising results. However, many clinical trials have failed to demonstrate the protective effects of ischemic preconditioning. The causes of the inconsistency are unknown. The effect of sex on ischemic preconditioning protection is largely unclear, which may be a contributing factor. Additionally, in clinical trials, the recruited patients were most likely taking some medications. This project aimed to test the differences in sex on preconditioning protection and the effect of a common β-blocker medication, Carvedilol, on preconditioning protection. Tourniquet-induced unilateral hindlimb ischemia-reperfusion in mice was used as the model. Blood flow in hindlimb paws was measured. Related cytokines were examined using Western blots, including pro-inflammatory IL-6, MCP-1, and TNFα. Additionally, both the non-canonical and canonical NF-κB pathways were investigated using Western blots. Using immunofluorescence staining, monocyte marker CD11b, pro-inflammatory macrophage marker CD68, and anti-inflammatory macrophage marker CD206 were assessed. Overall, there were differences after hindlimb reperfusion in males and females. The macrophage markers were the most notably different between the preconditioning group and the Carvedilol + preconditioning group, making this an exciting topic for further study.

KEYWORDS: Ischemia-reperfusion, Preconditioning, Carvedilol, Murine, Sexual Dimorphism

1. Introduction

Ischemia-reperfusion (IR) is a leading cause of injury in the heart, brain, liver, skeletal muscle, and other tissues and organs (Hausenloy and Yellon, 2016). Ischemic preconditioning (IP) is a procedure with short bursts of ischemia followed by short bursts of reperfusion to the area of interest (Hausenloy and Yellon, 2016). Many preclinical studies have shown that IP lessens the damage caused by IR (Hausenloy and Yellon, 2016). The mechanisms underlying IP protection have been extensively studied yet have not been fully understood. Though the preclinical studies have shown that IP results in tremendous decreases in the damage caused by IR, this has not been successfully translated into the clinical setting of cardiovascular disease (Gourine and Gourine, 2014). Obviously, clinical patients are much more complex compared to controlled experimental animals. Many factors, such as age, sex, health conditions, and medications, may interfere with the protection offered by IP. These variables have not been thoroughly studied. Our research aims to address the influence of sex and one medication on IP protection.

Skeletal muscle is the largest tissue mass in the body. Skeletal muscle IR injury occurs in trauma and surgical procedures. For example, tourniquet placement and removal, a standard practice in trauma aid and orthopedic surgeries, can cause muscle IR injury. Prevention of IR injury is, therefore, clinically important yet challenging. The effectiveness and underlying mechanisms of IP protection against muscle IR injury remain to be fully understood.

β-blockers are widely used for a variety of cardiovascular diseases. Carvedilol is a common β-blocker medication prescribed to patients with hypertension, coronary arterial disease, and chronic heart failure. At low doses, it works as a β_1-selective blocker. At higher doses, Carvedilol works as a nonspecific β-blocker, blocking both β_1 and β_2 receptors. Carvedilol

has also been shown to act as an α_1-blocker (Bristow, 2000). In the clinical setting, Carvedilol is most commonly prescribed at a dose that it acts as a nonspecific β-blocker (Bristow, 2000). Since this is a pilot study, we wanted to determine if a non-specific β-blocker would influence the IP protection. Then if there did seem to be some sort of interference of Carvedilol on IP protection, further studies could investigate if this would be due to β_1 blockers, β_2 blockers, or only non-specific β-blockers.

α_1 receptors are mainly found in vascular smooth muscle, so commonly in the muscle layer of arteries. When these receptors are blocked, it induces vasodilation (Reid, 1986). β_1 receptors are typically found in the heart, kidneys, and adipocytes. When blocked, β_1 receptors could reduce cardiac output and therefore blood flow out of the heart (Alhayek and Preuss, 2022). Finally, β_2 receptors are primarily found in the smooth muscle of the arteries, airways, and cardiomyocytes. Blocking β_2 receptors can induce vasoconstriction (Abosamak and Shahin, 2023).

Our lab established a mouse model of tourniquet-induced hindlimb skeletal muscle IR injury. Our data showed that tourniquet (an orthodontic rubber band) placement and removal on a hindlimb caused acute perfusion alteration and inflammation. Using this IR model, this Honors Thesis research project was designed to test (1) IP protection in male and female mice and (2) the effect of Carvedilol on IP protection. Our hypothesis was that sex and Carvedilol treatment may negatively affect IP protection. This is due to the reasoning that previous studies have had somewhat inconclusive results about sex in regard to IP protection (Paradis-Deschênes et al., 2017 & Sárközy et al., 2021). Additionally, we think that Carvedilol may have some impact on IP protection because it is an antagonist medication to receptors that are influencing blood flow.

2. Materials and Methods

2.1 Mouse hindlimb IP and IR

All experimental protocols and use of animals in this study were reviewed and approved by the University of South Dakota Institutional Animal Care and Use Committee (IACUC) and followed the NIH guidelines of animal use in research. Male C57BL/6 mice at the age of 3 to 4 months were anesthetized using an intraperitoneal injection with a cocktail of Ketamine (100mg/kg), Xylazine (15mg/kg), and Acepromazine (5 mg/kg). An orthodontic rubber band (ORB) was placed on the right hindlimb at the level of the hip joint using a McGivney ligator to induce ischemia. The complete blockage of blood flow was confirmed by Laser Doppler Imaging System (Moor Instruments). For IP, ORB was placed on the right hindlimb for 5 minutes, inducing ischemia, and then removed for reperfusion for 5 minutes. The regimen was completed three times, directly followed by IR. For IR, ORB was placed for 1.5 hours and removed for reperfusion for up to 14 days. A new ORB was used to transition from IP to IR to ensure proper injury. For the mice without IP, IR was directly applied. In both groups, the contralateral hindlimb served as the control (Crawford et al., 2007).

2.2 Laser Doppler blood flow imaging

Under isoflurane anesthesia, a mouse was placed in the prone position on a warming pad with hind limb paws facing up. Blood flow in the paws was scanned using Moor High-Resolution Laser Doppler Imaging System on days 0, 1, 3, 7, 11, and 13 following IR. Mice were sacrificed on day 14, and muscles were collected and processed for Western blot and immunofluorescent staining.

2.3 Western blot

Soleus and extensor digitorum longus (EDL) muscles were homogenized. An equal amount of protein was subjected to standard procedures for Western blot, as described in our previous work (Aby, et al., 2021; Antony, et al., 2022). Primary antibodies for Western blot included p65 and phosphor-p65, p100 and p52, JNK and phosphor-JNK, TNFα, MCP-1, IL-6, eNOS, and OXPHOS. Only the soleus samples were run Western blot, and the EDL samples were saved for future reference.

2.4 Tissue staining

Tibialis anterior (TA) muscles were snap frozen, sectioned, fixed, incubated with primary antibodies, fluorescent conjugated secondary antibodies, and imaged as described in our previous work (Aby et al., 2021; Antony et al., 2022). The primary antibodies were CD11b for monocytes, CD68 for pro-inflammatory macrophages, and CD206 for anti-inflammatory macrophages (Abbas, 2020).

2.5 Statistical analysis

Laser doppler images were quantified using the Moor Laser quantification program to determine blood flux through the limb. The data were then standardized by dividing each total flux by the area of the sample to find the average flux of each hindlimb. Then to form time plots of reperfusion to the IR hindlimb, the IR side was divided by the control side to determine the ratio of IR to control (I/C) for each day. An I/C ratio of 1, meaning that the IR and control sides are equal flux, is when the hindlimb blood flow is said to return to basal levels. The average differences were also recorded, showing the percent change between the limbs for each day of imaging, essentially quantifying the differences between hindlimbs. First an ANOVA was run to

determine if there were any significant differences between any of the days. Then, if there were differences, they were identified using paired, two-tailed t-tests.

The Western blots were quantified using ImageJ gel analysis. Western blots were normalized using GAPDH, β-actin, or total protein staining. ANOVA tests were run on each normalized protein. Then if ANOVA indicated a significant difference ($p<0.05$), a paired, two-tailed t-test was run to determine which groups differed significantly ($p<0.05$).

2.6 Experiment 1: Preconditioning protection in males and females

This experiment was performed in order to examine whether the IP would elicit any protection against IR injury and whether the protection would be different in male and female mice. The first group of animals had six male mice with weights ranging from 27g - 35g. Three mice were subjected to an IP treatment; the other three received no IP. Then, all six mice were subjected to the same IR as in Section 2.1.

The procedure for males was then repeated in six female mice with weights ranging from 16g -26g. There were a few changes implemented in the experimental procedure. Instead of using the cocktail of Ketamine, Xylazine, and Acepromazine, they were anesthetized using gaseous isoflurane on a heating pad. Instead of doing IR for 1.5 hours, it was done for 2 hours because other lab data showed 2 hours induced better IR injury. Another change was that they were imaged on days 0, 1, 3, 7, 9, and 14. Tissue samples were still taken on day 14 after IR to keep Western blots the same. The same tissues were taken and prepared for testing in the same way as described in Section 2.3. However, none of these samples were run for Western blot. They were placed in the ultra-cold freezer and may be used later. The main idea we were observing in female mice was their IR pattern and seeing if it differed from male IR. Some previous literature has shown that males and females recover from IR in different ways. One

study showed that males had a larger protective effect than females did (Paradis-Deschênes et al., 2017). While a different study showed that males and females had a similar IP protection (Sárközy et al., 2021).

As used later in this paper, the following terminology will be used. IP control (IP-C) refers to the control limb without IR of the mouse that received the IP procedure. IP ischemia (IP-I) refers to the limb with IR of the mouse that received the IP procedure. IR control (IR-C) refers to the control limb of the mouse that received only the IR procedure without IP. And IR ischemia (IR-I) refers to the IR limb of the mouse that received only the IR procedure without IP.

2.7 Experiment 2: Effect of Carvedilol Treatment on Preconditioning Protection

The second experiment was done to investigate if the β-blocker medication Carvedilol may influence the results of IP protection. Since Carvedilol is commonly prescribed to patients with heart problems, we hypothesized that this could be one of the variables altering the IP results of protection from preclinical to clinical trials. Since this was a pilot study, we were more interested in a broader medication. Since Carvedilol is a commonly prescribed non-specific β-blocker, we wanted to identify if there was any difference in IP protection when blocking both β_1 and β_2 receptors. Then if there was, we could further investigate if which β receptor caused the influence on IP protection.

To begin the second experiment, we used six male mice ranging from weights 28g - 32g. One day before the preconditioning treatment, mice were subjected to intraperitoneal injection with Carvedilol at 2 mg/kg daily (Dantas et al., 2013). The Carvedilol injection solution was 0.2mg/ml diluted with saline. This is a dose that acts as a nonspecific β-blocker. The injection volume was 0.1ml/10 g body weight. The control group was injected with saline at the same volume. The treatments started one day before the IP/IR procedures and continued for seven

days after the IP/IR procedures. We decided to do these injections to give the mice time to adjust to Carvedilol before the ischemia was performed. This would not translate exactly to the clinical setting, as most patients would likely have been taking Carvedilol for a longer period of time. However, with the time restraints of this project, we figured it would be the best to expose the mice to Carvedilol treatment before the ischemia. After one day of the treatments, all six mice were subjected to the same IP procedure described in Section 2.7. Next, the mice were subjected to a two-hour IR procedure using new ORBs to ensure the ischemia was complete.

Laser doppler imaging was done during IR and after days 1, 3, and 6. Tissue samples were then taken on day 7 following ischemia. Day 7 was chosen to collect tissues because new data in our lab showed that this was the most critical time point of changes from pro-inflammatory to anti-inflammatory response. As in the previous experiment, soleus, EDL, and TA were taken and prepared in the same way. The soleus and EDL were prepared for Western Blotting and the TA was prepared for tissue staining via cryosectioning.

Western blots were run using the same antibodies listed in Section 2.3 with a few exceptions. GAPDH, β-actin, eNOS, and phosphor-p65 were not used during this experiment. In this study, the total protein staining in the gel was used as the loading control. Data were normalized using total protein staining as loading controls. eNOS was not run because the data were inconclusive for Experiment 1. Additionally, phosphor-p65 was not run because we had trouble getting the antibody to stain the Western blot membrane. Immunofluorescent staining was done on the TA muscle looking at CD11b, CD68, and CD206 from tissue cryosections.

As a note for future terminology, the control group C-C or C-I, respectively, refers to the control or IR hindlimb in the mouse that received the saline treatment. The Carvedilol treatment

group BB-C, or BB-I respectively, refers to the control limb or IR limb in the mouse that received the Carvedilol treatment.

3. Results

3.1 Experiment 1 – Effect of IP

3.1.1 Effect of IP on perfusion in males and females following IR

We first compared the hindlimb perfusion in male groups following IR. The perfusion, measured as a ratio of the ischemic side over the control side (Figure 1A), or as a percent of the change in ischemic side over the control side (Figure 1B), was lower in the ischemic side than the control side at all time points in the mice without IP, suggesting impaired perfusion after IR. In the mice with IP, however, the perfusion in the IR limb was recovered to the basal level at day 7 at a significant increase compared to IR only, suggesting a protective effect of IP against the impaired perfusion by IR to some extent (Figure 1).

Then we compared the hindlimb perfusion in female groups following IR. In both control and preconditioning groups, the perfusion in the ischemic limb was higher than the control side on days 1 and 3 following IR but returned to the control level on day 7. Overall, the perfusion change showed no difference between the control and preconditioning groups, although there was a trend of increased perfusion in the preconditioning group on day 14. These results suggest that the effect of preconditioning on perfusion is insignificant in female mice. (Figure 2).

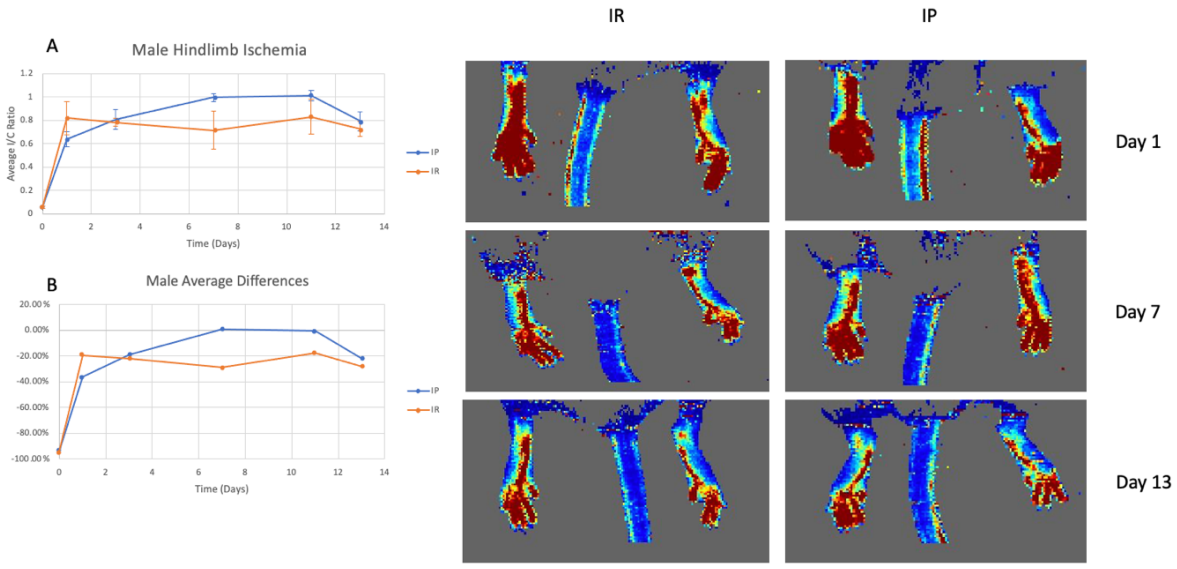

Figure 1: A – Time course of the bloodflow changes as the ratio of IR side over control side in mice with preconditioning (blue) or without preconditioning (orange). Error bars of ± 2 standard errors, $p < 0.05$ on day 7. The other days had no significant differences in recovery. n=3 per group. B – Time course of the bloodflow percent change in the IR side compared to the control side. Laser Doppler perfusion images of hindlimbs – the right limb is the limb that received IR or IP/IR treatment, left is the control limb. Red indicates higher bloodflow through the limb.

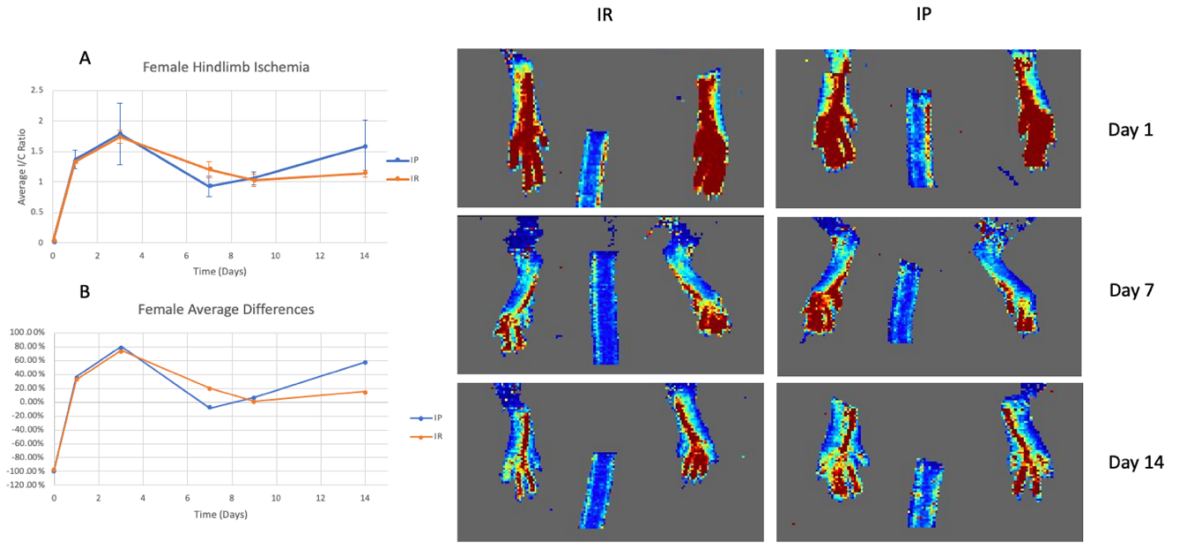

Figure 2: A – Time course of the bloodflow changes as the ratio of IR side over control side in mice with preconditioning (blue) or without preconditioning (orange). Error bars of ± 2 standard errors, no days with $p<0.05$. n=3 per group. B – Time course of the bloodflow percent change in the IR side compared to the control side. Laser Doppler perfusion images of hindlimbs – the right limb is the limb that received IR or IP/IR treatment, left is the control limb. Red indicates higher bloodflow through the limb.

3.1.2 Effects of IP on inflammatory signaling pathways

IR injury causes muscle damage and sterile inflammation. Western blot was performed to measure the major inflammatory signaling pathways to understand whether IP affects the inflammatory responses following IR. Since the IP showed protective effects only in male mice, tissues from the male mice were studied in the following experiments.

NF-κB is the major signaling pathway involved in inflammatory responses via the canonical p65 pathway or non-canonical p100/p52 pathway (Peng et al., 2020). The Western blot (Figure 3) showed that the protein levels of p100 and p52 were significantly increased in the IR-I limb compared to the IR-C limb, suggesting the activation of the p100/p52 pathway by the IR injury. Notably, the protein levels of p100 and p52 were reduced in the IP-I limb compared to the IR-I limb, suggesting that IP attenuated the activation of the p100/p52 pathway. Surprisingly, the phosphorylation of p65, the canonical NF-κB pathway, was not significantly upregulated in the IR-I limb compared to IR-C, suggesting p65 may not play a major role in IR-induced inflammation. IP significantly reduced the phosphorylation of p65 compared to IR-I, suggesting IP nevertheless inhibits p65 activation. Additionally, phosphorylation of JNK, another inflammatory pathway (Hammouda et al. 2020), and inflammatory cytokine TNFα and chemokine MCP-1 also showed a similar trend, i.e., increased in IR-I but reduced in IP-I, consistent with the change of p100/p52, further suggesting the inhibitory effect of IP on IR-induced inflammation. Western blot (Figure 3) also showed that IL-6 was increased in the IP-I limb compared to the IP-C limb.

Monocyte and macrophage infiltration at the site of injury is the hallmark of inflammation (Abbas, 2020). Tissues were stained using the procedures with monocyte and macrophage markers described in Section 2.4. There is evident increased staining with CD11b,

CD68, and CD206 on the IR sides versus the control, indicating IR-induced inflammation. Noticeably, CD68 staining was weaker in IP-I compared to IR-I, suggesting IP reduced pro-inflammatory macrophages. In contrast, the staining with CD206 was stronger in IP-I tissue than in IR-I tissue, suggesting that IP enhanced the anti-inflammatory macrophage (Figure 4). These data point towards the protective effect established by the IP procedure.

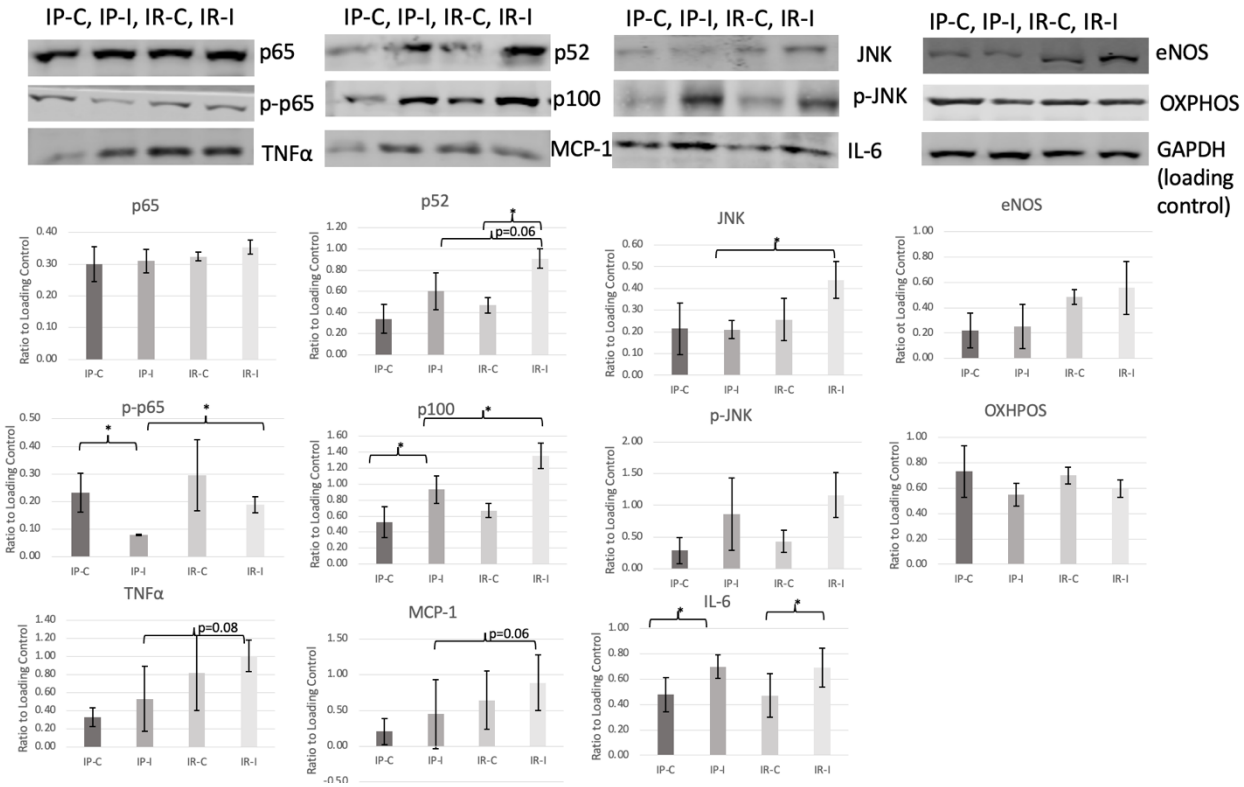

Figure 3: Images and quantifications of Western blots for IP and IR groups. IP-C represents the control limb from the preconditioning group, IP-I represents the IR side from the preconditioning group, IR-C represents the control side from the control group, and IR-I is the IR side from the control group. Error bars of +/- 2 standard errors are included. * Indicates $p<0.05$. n=3 per group. Note: p-p65 and p-JNK are phosphor-p65 and phosphor-JNK, respectively.

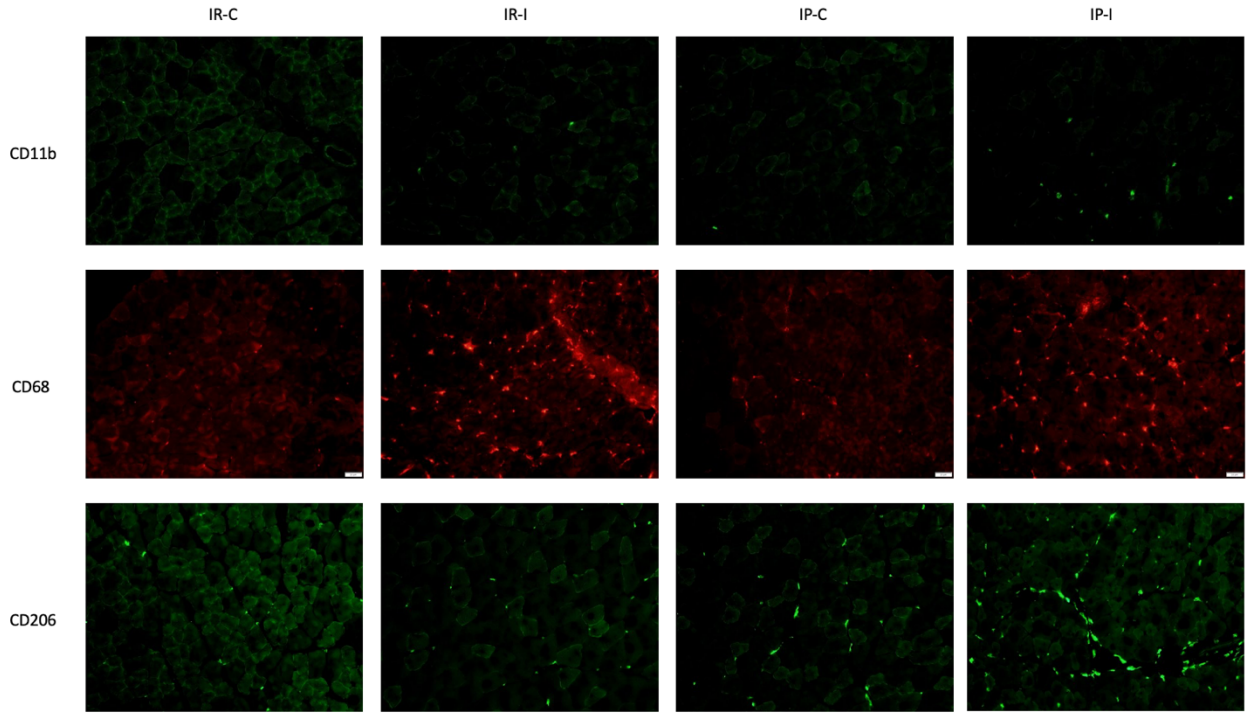

Figure 4: Immunofluorescent staining images comparing IP and IR groups for CD11b, CD68, and CD206.

3.2 Experiment 2 – Carvedilol Treatment

The procedure for Carvedilol treatment described in Section 2.7 was followed for administering Carvedilol to the three male mice and saline as a control to the other three male mice. Then, we compared the hindlimb perfusion in groups using laser doppler imaging, which was done on days 1, 3, and 6 following IP and IR. In the mice without Carvedilol treatment, the perfusion in the IR limb was significantly higher than the control limb on day 1 (Figure 5). In comparison, in the mice with the Carvedilol treatment, the perfusion was lower in the ischemic side than on the control side at all time points. This result suggests that the Carvedilol may be interfering with some of the initial protective effects established by the IP procedure.

Western blots of IP groups compared to BB groups revealed only one significant difference. This was an increase of IL-6 in the IP-I limb compared to the IP-C limb (Figure 6).

All other measured proteins showed no differences between Carvedilol treated group and saline control group.

All the immunofluorescent staining of the monocyte and macrophage markers showed similar results from the first experiment, with increased staining from the IR hindlimb to the control hindlimb, indicating IR-induced inflammatory responses. There was no marked difference in CD11b and CD206 staining between IP-I and BB-I tissues. However, there was noticeably more staining for CD68 on the BB-I tissue compared to IP-I tissue, suggesting that Carvedilol treatment may enhance the pro-inflammatory macrophage polarization in the ischemic hindlimb (Figure 7).

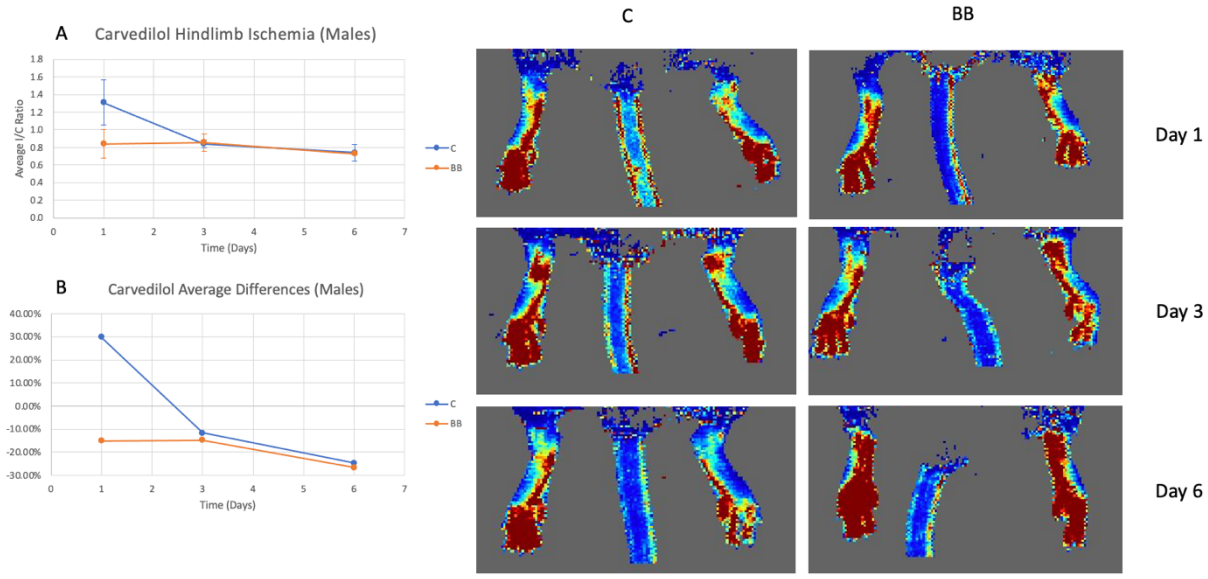

Figure 5: A - Time course of the bloodflow changes as the ratio of IR side over control side in mice with preconditioning (blue) or preconditioning with Carvedilol treatment (orange). Error bars of ± 2 standard errors. Day 1 had $p<0.05$. n=3 per group. B – Time course of the bloodflow percent change in the IR side compared to the control side. Laser Doppler perfusion images of hindlimbs – the right limb is the limb that received IP/IR treatment, left is the control limb. Red indicates higher bloodflow through the limb.

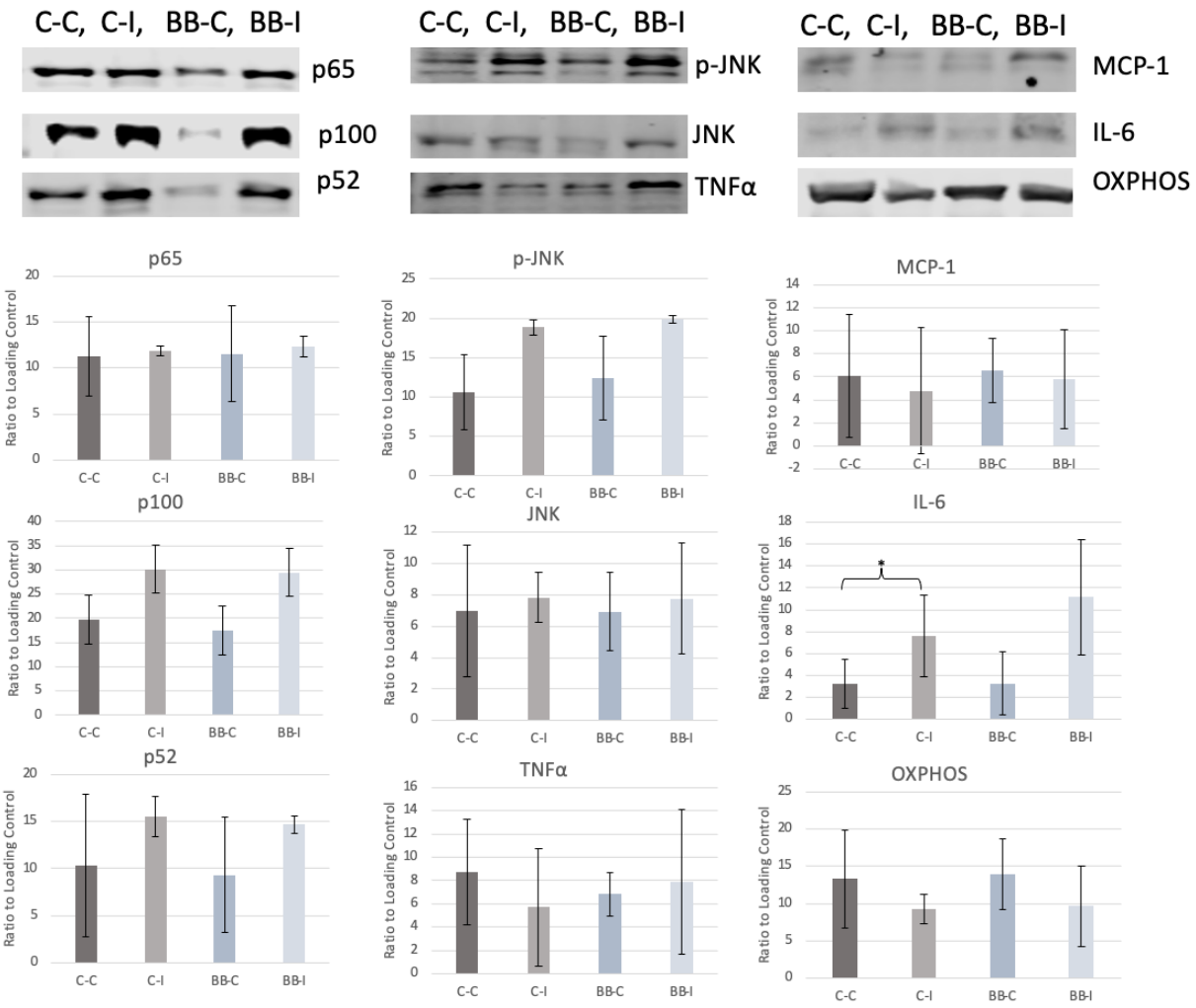

Figure 6: Images and quantifications of Western blots for C and BB groups. C-C represents the control limb from the preconditioning group that received saline, IP-I represents the IR side from the preconditioning group that received saline, BB-C represents the control side from the preconditioning group that received Carvedilol, and BB-I is the IR side from the preconditioning group that received Carvedilol. Error bars of +/- 2 standard errors are included. * Indicates $p<0.05$. n=3 per group. Note: p-p65 and p-JNK are phosphor-p65 and phosphor-JNK, respectively.

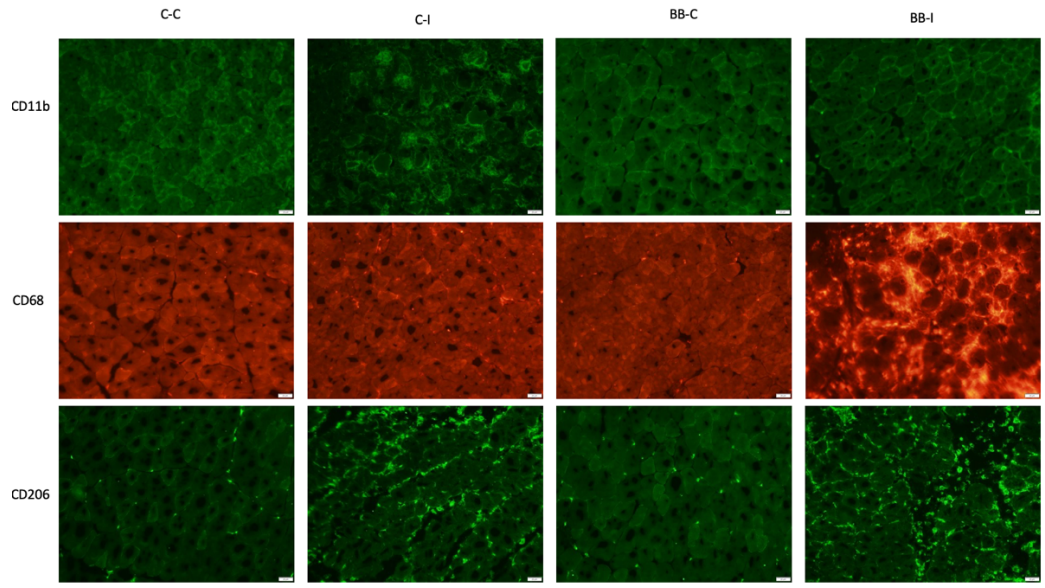

Figure 7: Immunofluorescent staining images comparing C and BB groups for CD11b, CD68, and CD206.

To better understand the effect of Carvedilol treatment on IP protection, we combined the data of the previous groups without IP. Since the C-C and C-I groups received only a saline injection, they were grouped with the IP-C and IP-I groups, respectively. Figure 8 shows the selected four proteins for this – p52, p100, phosphor-JNK, and JNK. As seen in all four graphs of Figure 8, the IR-I was the highest for these four proteins. Both the BB-I and IP-I showed reductions in these four inflammatory proteins. Particularly, as mentioned previously, IP significantly reduced p100 and p52 in the ischemic limb, whereas the treatment with Carvedilol did not change the effects of IP on these two proteins. These results suggest that Carvedilol treatment does not interfere with the tested signaling pathways from the IP procedure.

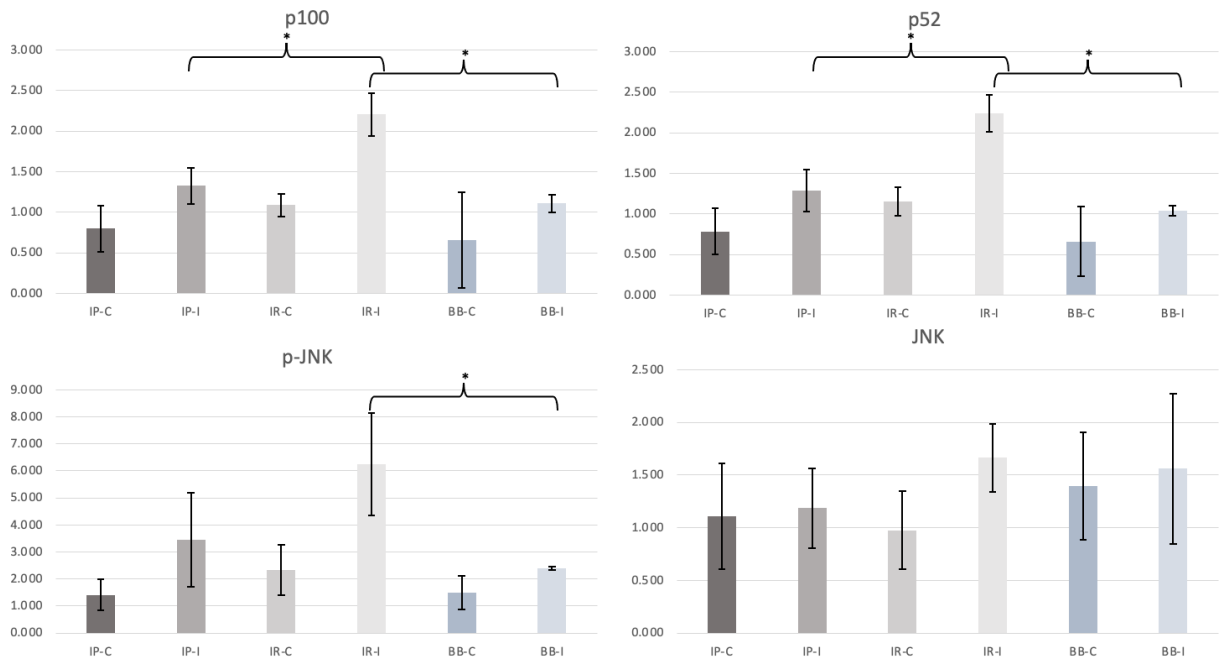

Figure 8: Standardized data for select proteins, error bars for ± 2 standard errors are shown. * indicates $p<0.05$. Note: p-JNK is phosphor-JNK.

4. Discussion

4.1 Experiment 1 – Effect of IP

4.1.1 Males vs. females IR recovery

As seen in Figures 1 and 2, it is clear that the limb reperfusion blood flow following IR is different in male and female mice. When male mice were recovering from the IR procedure, the Moor Laser Doppler Imaging showed that the ischemic limb had much less blood flow than the contralateral hindlimb. This resulted in a fraction below one for ischemic reperfusion divided by the control hindlimb seen in Figure 1. This is to be expected, as IR injury damages the blood vessels in the area, so that the damage would reduce the blood flow to the area. In mice that received the IP procedure, the blood flow returned to the basal level (ratio of IR side vs. control is 1) at day 7, indicating that IP attenuates IR-induced blood vessel damage and improves reperfusion. This was a significant increase in perfusion compared to the IR group (Figure 1).

Though, at day 14, the blood flow in the preconditioning animals showed a trend of reduction. Future studies should investigate the long-term effect of IP on blood flow recovery following IR.

Contrary to the male mice, the blood flow reperfusion after IR in the female mice showed a different pattern, characterized as greater blood flow on the IR side than the control side on day 1, then returning to around basal level. This is an interesting phenomenon but should be more thoroughly tested. More importantly, IP did not show any effects on blood flow after IR in females. This interesting observation needs to be repeated with more animals. If confirmed that sex is a factor that affects IP protection, it should be carefully considered in clinical trials. Because this was a pilot study, we did not take into account the hormonal cycling of female mice. This could have had some influence on the results we had and will need to be accounted for in the future.

Even though these results were obtained using different experimental procedures, they still do seem to match one of the studies regarding sexual dimorphisms. This study showed that males had a stronger response to IP than female mice did (Paradis-Deschênes et al., 2017).

4.1.2 Effects of IP on inflammatory signaling pathways

NF-κB and JNK are major signaling pathways that mediate inflammatory responses. p52 and p100 are part of the non-canonical NF-κB pathway. Figure 4 shows that IP-I and IR-I increase the expression of both p52 and p100, indicating that ischemia is upregulating this inflammatory pathway. However, compared to the IR-I, in the IP-I limb, p52 and p100 proteins were downregulated (significantly in the case of p100 and almost significantly in p52 ($p=0.06$)). This result suggests that IP protection against IR injury is partially established by inhibiting the NF-κB p100/p52 pathway. Phosphor-JNK follows this same trend as p52 and p100, with IP-I

being lowered compared to IR-I, suggesting that inhibition of the JNK pathway is also involved in the IP protective effect.

The changes in other tested proteins were more variable and inconsistent. For example, phosphor-p65, part of the canonical NF-κB pathway, was not increased in IR-I and IP-I. This result would need further verification. If confirmed, it would suggest that IR injury may selectively activate the p100/p52 pathway over the p65 pathway.

Another unexpected result was that of MCP-1 and TNFα, a major inflammatory chemokine and cytokine. With strong activation of the p100/p52 pathway, it was expected that both MCP-1 and TNFα would be increased in IR tissues. However, this was not the case, as seen in Figure 3. While both of these proteins' levels are decreased in IP-I compared to IR-I, showing some protective effect, the levels of these proteins were higher in the IP-C group than in the IR-C group. This result needs to be further verified. A future study should also test whether the peak levels of these proteins occur at early time points of IR.

IR causes sterile inflammation in IR tissues (Hausenloy and Yellon, 2016). The inflammation is a dynamic process in which macrophages play a critical role. In the early stages of injury, macrophages possess a pro-inflammatory function to promote inflammation. Then macrophages gradually gain anti-inflammatory activity to limit and eventually resolve inflammation. As seen in Figure 4, immunofluorescent staining revealed that the staining for CD68, the marker for pro-inflammatory macrophages, was stronger in IR-I limb than in the IP-I limb. In contrast, the staining for CD206, the marker for anti-inflammatory macrophages, was stronger in IP-I limb than in the IR-I limb. These results indicate that IP reduces inflammatory macrophages and promotes anti-inflammatory macrophages. This is a valuable, novel piece of information to advance our understanding of the mechanism underlying the IP protective effects.

Overall, there are many trends in the IP/IR comparisons that need to be further investigated to determine the underlying mechanism of IP protection. The data gathered in these experiments regarding the NF-κB pathway did seem to fit another study's data (Harari and Liao, 2010). Harari and Liao found that the non-canonical NF-κB was upregulated in ischemic rodents (2010).

4.2 Carvedilol treatment

The goal of the study was to test whether Carvedilol treatment had any impact on IP protective effects against IR injury. Perfusion data in Figure 5 shows that on day 1, the blood flow in the IP-I limb was greater than the BB-I limb, suggesting Carvedilol treatment may impose a negative effect on IP protection. However, this result needs further verification. Furthermore, in future studies regarding any phrenological agent, it will be essential to develop a method for determining if the drug is being absorbed and working. Carvedilol will lower heart rate and blood pressure, so that vital sign could be taken to ensure its efficacy. It is important to note that when comparing Figure 5 to Figure 1, there are some differences in the pattern of perfusion following IR. This may be because a different method of anesthesia was used for ischemia procedures. The previous ischemia was performed under Ketamine/Xylazine/Acepromazine, whereas isoflurane was used in this experiment. Additionally, the time of ischemia was lengthened by 30 minutes from Experiment 1 to Experiment 2. These may have contributed to the differences noted in Figures 1 and 5. These changes in procedures reflect the pilot study nature of this project.

Figure 7 shows the immunofluorescent staining for IP and BB groups. From these images, there is no change in staining for CD11b between IP-I and BB-I groups, indicating that Carvedilol does not influence monocyte level. However, CD68 staining was markedly increased

in BB-I compared to PC-I. This is a piece of evidence that suggests that Carvedilol may attenuate the IP protective effect against the pro-inflammatory macrophages in IR injury. This result is interesting but needs further confirmation. On the other hand, the staining for CD206, an anti-inflammatory macrophage marker, was comparable between the treated and untreated tissues, suggesting that Carvedilol is not negatively interfering with the IP protective effect on the anti-inflammatory macrophages.

As revealed by Experiment 1, IP significantly attenuated the activation of p100/p52 and JNK pathways. Therefore, we wanted to compare whether Carvedilol treatment affects those pathways. Figure 8 shows Western blots for p100, p52, JNK, and pJNK in the mice with preconditioning only (IP) or preconditioning and Carvedilol treatment (BB). Overall, the two groups have no difference in any of those proteins. Since Experiments 1 and 2 were conducted separately, we combined some data from the two experiments to better compare, as shown in Figure 8. Carvedilol treatment may not alter the effects of IP on p100/p52 and JNK pathways.

Overall, the results do not clearly indicate Carvedilol has significant negative effects on IP protection. It should be noted that the IP and ischemia between Experiments 1 and 2 were performed under different anesthesia, and the ischemia length was different. Additionally, the sample size (3 mice per group) is relatively small. In the future, these studies can be done more appropriately using the same procedures across experiments and a larger number of mice.

Conclusion

Overall, the results of this study show that IP has a protective effect against perfusion impairment in IR injury in male but not in female mice. As for the protection mechanism, the data show that IP reduced pro-inflammatory macrophages, promoted anti-inflammatory macrophages, and attenuated NF-κB non-canonical p100/p52 pathway activation. Finally, the

results of this study demonstrate that while Carvedilol treatment enhanced pro-inflammatory macrophages, it had no effect on IP-induced attenuation of the pro-inflammatory signaling pathways. Whether β-blockers can affect IP protection needs to be further investigated.

Through this Honors Thesis project, I have learned different ways to quantify and analyze different proteins and the importance of experimental design as well as critical thinking skills relating to the medical field. These skills are important in my future career as a physician because they will give me insight into how diseases are diagnosed and treated. With all of the research being done in the medical field, it is crucial for me to know how research is done and written in order to effectively be a life-long learner.

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