Undergraduate Research Experience Award Program Report

The Impact of Nonsteroidal Anti-inflammatory Drugs on Endothelial Cell and Platelet Production of Microvesicles During Strenuous Exercise in Humans

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Introduction

Microvesicles (MVs) are anucleate extracellular vesicles derived from plasma membrane as a result of cytoskeletal reorganization and in response to stimuli[4,6] such as exercise or disease[6,8]. Interestingly, the concentration of circulating MVs is altered following strenuous exercise[7-9] and the relatively large diameter of MVs enables lipids, proteins, mRNA, and miRNA transport from where they are produced to distant recipient cells[3,6,8]. This suggests MVs play a role in physiology by facilitating cell-to-cell communication that leads to structural and functional changes[4-8]. MVs are taken up by the cells lining the walls of blood vessels, allowing MVs to modulate vessel functions including proliferation and migration which result in angiogenesis[3-6,8,9]. This capability, along with regulation of thrombosis and inflammation, demonstrate a key role of MVs in vascular adaptation[3-5]. The functions MVs alter in target cells depends on the cell type from which they are derived, endothelial, platelet, smooth muscle, leukocyte, or erythrocyte, and the conditions under which they are formed[3,5-8]. A wide variety of MVs, and substantial differences in concentrations, exist naturally in circulation with platelet derived microvesicles (PMVs) being most abundant[3-6,8]. Similarly, the dynamics of MV response to exercise is also cell specific, with platelets producing the most, particularly following strenuous exercise[6-9]. This response mirrors the increases in vascular stress induced by exercise, as sympathetic nervous system activation and increases in circulating metabolites like adenosine-diphosphate have been suggested agonists of PMV formation[5,7,8]. This pro-angiogenic response in addition to the absence of an increase in endothelial MVs associated with vascular damage, suggests MVs play an integral role in vascular adaption to the stress responses of exercise[6-8]. Therefore, any drug affecting platelet function may also affect MV formation and influence a person's ability to adapt to exercise.

Non-steroidal anti-inflammatory drugs (NSAIDs), such as ibuprofen or aspirin, inhibit platelet function[1,2] by interacting with cyclooxygenase (COX), an enzyme responsible for production of thromboxanes and prostaglandins, which are critical for platelet-endothelium interaction and hemostasis[1,2]. This inhibition actively supresses COX in both endothelial cells and platelets, however, the lack of synthetic capability in platelets[8] results in greater functional impact[2]. Further, *in vitro* investigations have shown some COX inhibitors specifically inhibit MV formation following stimuli similar to that of exercise[3]. The aim of the proposed research is to determine whether taking NSAIDs blunts MV concentrations that are normally elevated following strenuous exercise. We will focus on changes in PMV concentrations, however, as NSAIDs effect both platelets and endothelial cells, endothelial MVs will also be monitored. The proposed research is an important step in connecting MV involvement in vascular adaptation to exercise with the effects of NSAIDs. Additionally, the present study may provide novel insight into the potential determinantal effects of NSAID use on an individual's ability to adapt to exercise training. This is particularly important in the rehabilitation setting where pain relievers like Ibuprofen are used routinely. The ubiquitous accessibility of NSAIDs as over-the-counter medications, demonstrates further importance of investigation into potential negative effects.

Project Aims

This research aimed to address the hypothesis that using non-steroidal anti-inflammatory drugs prior to strenuous exercise blunts circulating concentrations of endothelial and platelet

derived microvesicles during and following the exercise period. Specifically, MV production throughout and after exercise was to be compared in young and healthy individuals prior to and following the use of NSAIDs.

Exercise trials were to be completed by participants 18-35 years of age in good health and include three conditions, on one occasion, a control trial, during which the participant would receive a placebo (sugar pill), on another occasion, an experimental trial, involving a standard 600 mg dosage of ibuprofen two hours prior to the exercise period, and on a third occasion, an experimental trial, involving a 325 mg dosage of aspirin one hour before exercise. Before these experimental and control trials, an exercise tolerance test was to be performed by each participant to establish the intensities of all three experimental and control trials that involve 45 minutes of intense cycling. Analysis was to be performed on venous blood samples taken at various time points following exercise (Dr. Rakobowchuk or affiliated nurse).

Plasma sample analysis includes quantification of MVs, blood lactate and glucose, hormones, and other metabolites. MV concentration was to be determined using the Cytoflex flow cytometer with antibody treatment for derivation-specific quantification. In combination with flow cytometry, MVs may also be quantified through nanoparticle tracking using a custom-built microscope. Portable analyzers, specifically lactate plus, were to be used for measurement of lactate and glucose concentration. Finally, enzyme-linked immunosorbent assays for catecholamines were to be used to quantify hormones present in the samples.

Project Progress

Upon receipt of the Undergraduate Research Experience Award, application for ethical approval of the project was submitted through the Thompson Rivers University ROMEO online portal. While waiting for ethical approval, participant recruitment began along with preliminary method development for sample analysis. Challenges arose during the recruiting process as many of the target age were unavailable during summer months. This in conjunction with the delayed approval of project ethics resulted in an inability to move past the recruitment phase into exercise trials. As a result, the remainder of the project timeline consisted of method development in preparation for sample analysis at a later point. Specifically, the methods developed include sample preparation, total antioxidant capacity (TAC) determination, and quantification of epinephrine/norepinephrine, thiobarbituric acid reactive substances (TBARS), and total nitric oxide (NO) concentrations. These assays all measure different circulating factors thought to modulate the dynamics of MV concentrations in circulation. To develop these assays, samples obtained from another study involving exercise and MVs were used.

Over the course of the summer, I analyzed plasma samples as described above (TAC, catecholamines and NO). The NO assay was a unique aspect of my development since this assay needed to be developed from scratch using published literature methodologies. The first aspect that I worked on was determining the best method to use when preparing plasma samples. Through consulting the published preparation methods and the requirements of each analysis technique, I determined that the anti-coagulant used during blood collection could influence the accuracy of some quantification methods, with the NO assay being the most sensitive in the present study (Griess Assay for total nitric oxide determination)[10]. I determined that a serial centrifugation approach would be needed to first isolate plasma from the venous blood sample, obtain platelet-

free plasma, and isolate MVs. For all the above-mentioned analytical methods, samples would then need to be stored as platelet-free plasma at -80^{o}C and thawed only immediately prior to analysis.

The TAC of plasma as well as concentrations of epinephrine/ norepinephrine and TBARS within samples was determined using commercially available kits. To further evaluate the sample preparation method chosen, plasma samples collected in a similar manner during a previous study were analyzed using the following kits: Epinephrine/ Norepinephrine ELISA Kit (Abnova KA1877), Parameter TBARS Assay (R&D Systems KGE013), and Antioxidant Assay Kit (Cayman Chemical 709001). Both the assays for determination of epinephrine/norepinephrine and TBARS concentrations worked well with the sample preparation used (results have been summarized and are being incorporated into a manuscript). The antioxidant assay kit did not give reproducible results, and upon further investigation it was found that EDTA can interfere with the assay[11]. This is problematic as EDTA is a common anti-coagulant used in blood collection, and sensitivities in the Griess Assay deter use of the other common anti-coagulant, heparin. However, repeated freeze/thaw of plasma samples can interfere with the antioxidant assay and therefore must also be considered.

Development and Assessment of NO in Plasma Samples

The total nitric oxide concentration in plasma samples is generally determined indirectly from nitrite and nitrate concentrations using a commercially available kit. Initially, Dr. Rakobowchuk had envisaged using a commercially available kit, but upon reading the details of the assay it was decided that developing the assay from raw reagents would be the more financially viable route in the long-term. As such, I developed a protocol for quantifying nitrates and nitrites in plasma without using an expensive commercial assay kit. I found that current research generally used a diazotization assay based upon the Griess reaction to complete this quantitative analysis[10]. Specifically, the Griess reaction involves the interaction of nitrite with sulfanilamide and N-(1-napthyl)-ethylenediamine to produce a diazonium product[10]. I also determined that prior to this reaction a nitrate reduction step is required to enable spectrophotometric measurement of nitrite concentration and estimation of *in vivo* nitric oxide formation[12]. Use of the Griess reaction on plasma samples, as in the present study, is limited in sensitivity due to the presence of proteins[13]. As a result, I incorporated a deproteination step prior to the reduction of nitrate, to eliminate inflated measurements due to high background absorbance[13]. There are ten commonly used methods of protein precipitation, each of which can influence the Griess assay differently[12]. In accordance with the findings of Ghasemi *et al.*, I decided to use zinc sulphate to deproteinate plasma samples, as it has a similar influence on the assay as the ideal deproteination method, ultrafiltration, without the exorbitant costs (2007). Currently, intra- and inter-assay precision tests as well as recovery assessments have been performed in replicate and results have been consistent with assay validations previously published. Experimental trials have also been performed on plasma samples collected in a previous study to demonstrate appropriateness for the type of sample preparation employed in the present study.

Developing an Assay to Assess Movement of Insulin and the Influence of MVs

Over the summer, I also developed an assay to measure the movement of insulin across endothelial cells. This assay will be used in the present study as a functional bioassay of how MVs could influence glucose regulation in people. We hypothesize that exercise-induced alterations to circulating MV concentrations may contribute to the efficacy of exercise as an insulin resistance countermeasure. If an influence was observed than the hypothesized decrease in concentrations of circulating endothelial cell and platelet derived MVs as a result of NSAID use prior to exercise, must also be considered when suggesting exercise as a corrective. To directly assess whether MVs impact the transcytosis of insulin, a transcytosis assay was performed using human umbilical vein endothelial cells (Ea.hy926 cell line) and transwells containing a polyester membrane with a pore size of 0.4 μm. The confluent monolayer of endothelial cells was representative of the endothelium lining microvasculature of muscle tissue and was exposed to either MV-containing or platelet-poor plasma from pre- or post-high intensity exercise blood draws completed in a previous study[14]. Exposure occurred for one hour and 45 minutes in accordance with the duration of major MV concentration changes following exercise documented in previous studies[8]. Following this, incubation with dextran and insulin conjugated to fluorescent markers allowed quantification of the two molecules movement across the monolayer over a four-hour period. FitC-insulin fluorescence was compared to determine both quantity and rate of insulin transcytosis, whereas observation of TritC-dextran was used as a normalization method for cell monolayer integrity. Previous analysis of the plasma samples using flow-cytometry demonstrated that high intensity exercise increased concentrations of platelet (Pre-cycling: $2.94 \pm 1.16 \times 10^5$/mL [mean ± SEM]; Post-cycling: $6.03 \pm 2.41 \times 10^5$/mL; $p = 0.01$) and not endothelial cell microvesicles (Pre-cycling: $2.62 \pm 1.8 \times 10^5$/mL; Post-cycling: $1.79 \pm 1.51 \times 10^5$/mL; $p = 0.16$)[14]. However, in the transcytosis assays performed thus far, the presence of microvesicles did not alter endothelial insulin transcytosis (total insulin transcytosed: 125.0 ± 11.5 ng and 116.3 ± 8.4 ng for MV-containing and platelet-poor plasma, respectively), nor did alterations in MV concentration as a result of high-intensity exercise (121.7 ± 8.5 ng and 125.0 ± 11.5 ng for pre- and post-cycling, respectively).

Overall, the method development completed thus far will allow the project to continue in a timely manner once participant recruitment can be completed. The plans to disseminate research findings described in the funding application will be carried out upon project completion at a later date. Presently, research findings from the inquiry into the role of MVs in insulin transcytosis by endothelial cells have been submitted in the form of an abstract and will be presented at the Canadian Society for Exercise Physiology Conference in November of 2019.

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