Tissue Plasminogen Activator and Plasminogen Activator Inhibitor-1 Gene Expression in Muscle after Maximal Acute Aerobic Exercise

RICHARD L. CARPENTER¹, JEFFREY T. LEMMER¹, RYAN M. FRANCIS¹, JEREMY L. KNOUS¹, MARK A. SARZYNSKI¹, CHRISTOPHER J. WOMACK¹.

¹Department of Kinesiology, Michigan State University, East Lansing, MI USA.

ABSTRACT

Carpenter RL, Lemmer JT, Francis RM, Knous JL, Sarzynski MA, Womack CJ. Tissue Plasminogen Activator And Plasminogen Activator Inhibitor-1 Gene Expression in Muscle After Maximal Acute Aerobic Exercise. JEPonline 2010;13(6):35-44. Animal studies suggest plasminogen activation in skeletal muscle is necessary for muscle repair. However, plasminogen activators have been studied little in humans. Therefore, the purposes of this study were to: 1) assess changes in skeletal muscle gene expression of fibrinolytic and coagulation factors 2) assess plasma activity of tPA and PAI-1 in response to an acute bout of maximal aerobic exercise, 3) determine if there is any relationship between muscle gene expression of tPA and PAI-1 with plasma activity levels. Six healthy, college-aged males volunteered blood and muscle samples prior to and immediately following a maximal treadmill exercise test. Muscle tissue was homogenized and purified RNA underwent RT-PCR using gene-specific primers for tPA and PAI-1 as well as biotynylation for microarray. Blood was analyzed via biofunctional immunosorbent assays for tPA and PAI-1 activity. A significant increase in tPA mRNA was seen with exercise (p=0.038) while PAI-1 mRNA showed no changes with the exercise. Muscle tPA activity showed no changes with the exercise bout. Plasma tPA showed a significant increase in activity (p<0.0001) while plasma PAI-1 activity had no change with the exercise bout. In conclusion, tPA synthesis increases in muscle following acute, high-intensity exercise and muscle production does not significantly contribute to plasma tPA increases seen with acute, high-intensity exercise.

Key Words: Microarray, Muscle Regeneration, Zymography, Coagulation, Fibrinolysis
INTRODUCTION

Although exercise is an important part of cardiovascular disease prevention, it transiently increases blood coagulation potential (28) and risk for cardiovascular events (21). Fibrinolysis is the dissolution of fibrin networks associated with blood clots and counteracts coagulation. The fibrinolytic response to exercise is crucial for maintaining low risk for cardiovascular events such as myocardial infarction and stroke, which are typically caused by occlusive clots (8,17). Fibrinolysis is initiated by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA; primary activator in mice), which converts plasminogen to plasmin, the active protease that degrades fibrin networks. tPA is inhibited by plasminogen activator inhibitor-1 (PAI-1) providing fine regulation of fibrinolysis. Several studies have observed increases in tPA and decreases in PAI-1 activities in plasma with maximal exercise (3,14,24,26,28,29), suggesting fibrinolytic activity is increased with acute exercise possibly providing protection from occlusive thrombi.

It has been shown in animal models that plasminogen activation occurs locally within skeletal muscle following injury and this activation plays a role in muscle regeneration (23). More specifically, plasminogen, tPA/uPA, and PAI-1 initially increase expression in skeletal muscle following muscle injury in animals (22). Furthermore, inactivation of the plasminogen system in knockout mouse models results in a severe muscle regeneration defect following chemically-induced muscle injury (15,22). Additionally, PAI-1-deficient mice demonstrate accelerated recovery of muscle force, protein levels, and morphology following injury (11). These results suggest local plasminogen activation in skeletal muscle is important for recovery from muscle-damaging exercise or injury. A recent study observed tPA and PAI-1 expression in skeletal muscle in men with metabolic syndrome after aerobic training (9). Coagulation factors also may play a role as certain coagulation factors (Factor Xa and Va) can accelerate tPA-induced plasminogen activation (19). However, responses of fibrinolytic and coagulation factors in human skeletal muscle following muscle-damaging exercise has not been assessed. Understanding the mechanisms of muscle repair and regeneration following muscle injury, such as from intense exercise, could prove to be useful clinically for athletes or patients that are using exercise for rehabilitation.

Expression of tPA has been found in the uterus, brain, and heart but plasma levels have traditionally thought to be primarily from vascular endothelial cell production (10,25). As mentioned above, tPA and PAI-1 expression has been observed in skeletal muscle of humans (9) and animals (15,22). Increases in plasminogen activation in animal muscle following muscle injury combined with the finding of local expression of plasminogen activators in skeletal muscle may suggest muscle contributes to the large increase in tPA seen with exercise (3,14,24,26,28,29). This suggestion is attractive as skeletal muscle provides a highly abundant tissue for release of tPA. However, the contribution of muscle tPA and PAI-1 to plasma levels has not been studied.

Therefore, the purposes of this study were: 1) assess changes in skeletal muscle gene expression of fibrinolytic and coagulation factors 2) assess plasma activity of tPA and PAI-1 in response to an acute bout of maximal aerobic exercise, 3) determine if there is any relationship between muscle gene expression of tPA and PAI-1 with plasma activity levels.

METHODS
Subjects
Six healthy college-aged males participated in this study. Subject characteristics are listed in Table 1. Subjects were excluded if there was previous diabetes or cardiovascular disease including bleeding disorders and hypertension. Subjects were not required to be trained and average VO$_2$max can be
seen in Table 1. Health and exercise history was determined by questionnaire. All subjects agreed to participate in the study and signed informed consent. This study was approved by the Institutional Review Board at Michigan State University.

**Procedures**

**Exercise Testing**
The maximal treadmill exercise test began at a speed of 2.5 miles per hour and increased at a rate of 0.5 mph per minute until reaching a maximum speed of 6.0 mph. Once this speed was reached, treadmill elevation increased continuously at a rate of 3% per minute until subjects achieved volitional exhaustion. All subjects had heart rate monitored via Polar Heart Rate monitors (Polar Electro Inc., Lake Success, NY, USA) and oxygen uptake measured continuously using indirect calorimetry (Sensor Medics 2900 Metabolic Cart, Yorba Linda, CA, USA). The highest one-minute oxygen uptake achieved during the test was defined as maximal oxygen uptake ($\text{VO}_2\text{max}$). To ensure a maximal effort, subjects achieved two out of the following three established criteria (4): 1) achievement of 95% of age-predicted maximal heart rate, 2) respiratory exchange ratio greater than or equal to 1.15, and 3) plateau of $\text{O}_2$ consumption ($\dot{\text{O}}_2 = 150 \text{ mL O}_2/\text{min}$) (2).

**Blood and Muscle Tissue Collection**
All blood and muscle samples were obtained following a 12-hour fast. Samples were collected between 7 and 10 a.m. to minimize the effects of diurnal variation in fibrinolysis (1). Prior to baseline blood sampling, subjects assumed a semi-recumbent position for 30 minutes to eliminate postural effects on fibrinolysis (27). Blood samples of 5 ml were collected from the antecubital vein into an acidified citrate solution. Blood was spun to obtain platelet-poor plasma and stored at –80°C until assayed.

The site for muscle biopsies was at the mid-line of the quadriceps in the vastus lateralis. Muscle samples were obtained using a 5 mm Bergstrom biopsy needle and applied suction (5). The muscle sample was immediately placed on an ice-chilled watch glass and dissected of all visible blood, adipose, and connective tissue. A portion of the sample was flash frozen in liquid nitrogen and another stored in RNAlater (Ambion, Austin, TX, USA). Both samples were stored at –80°C until analysis.

Following baseline collection, subjects completed the exercise test with Steri-strips, sterile gauze, and an elastic pressure bandage over the biopsy incision. Immediately following the exercise test, subjects were placed on the examination bed in a supine position where blood was drawn and muscle tissue extracted from the same incision as the baseline biopsy. All blood and muscle samples were collected within 4 minutes of cessation of the exercise test.

**Muscle tPA and PAI-1 Expression Analysis**

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**
A portion of the muscle biopsy tissue was homogenized in Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) using a polytron homogenizer. Total RNA was extracted and purified using an RNeasy column per manufacturer’s instructions (RNeasy kit, Qiagen Inc., Chatsworth, CA, USA). RNA was then subjected to DNase treatment (DNase Free, Ambion, Austin, TX, USA). RNA was quantified spectrophotometrically at 260:280 nm to determine concentration and purity. Total RNA
Figure 1. Muscle tPA and PAI-1 mRNA Expression with Exercise.

Muscle tissue was collected from subjects via percutaneous biopsy just before and immediately following acute maximal aerobic exercise. Tissue was homogenized and total RNA was collected and analyzed for mRNA content via semi-quantitative RT-PCR. A) Quantified tPA mRNA from muscle tissue as seen with a representative gel from one subject. n=6. B) Quantified PAI-1 mRNA from muscle tissue as seen with a representative gel from one subject. n=6. C) tPA mRNA from muscle tissue for each study subject for pre- (white bars) and post-exercise (black bars). D) tPA mRNA from muscle tissue quantification with the exclusion of subject 2. n=5. *Represents p<0.05.

Microarray Gene Expression

A portion of the total RNA described above was used for microarray analysis. The pre-exercise samples for all subjects were pooled as one sample such that equal amounts of RNA from each subject were used obtaining 5 µg of RNA for the pre-exercise sample (0.83 µg RNA/subject with 6 total subjects) that was used on one microarray chip. The post-exercise samples were also pooled with equal amounts of RNA from each subject totaling 5 µg of RNA. These pooled RNA samples underwent biotin labeling using the BioArray™ Single-Round RNA and Biotin Labeling System (Enzo, Life Sciences, Farmingdale, NY, USA). The biotinylated RNA for pre- and post-exercise were then quantified to determine concentration and purity. Biotinylated RNA quality was then analyzed using an Agilent 2100 Bioanalyzer and an Agilent RNA 6000 Pico chip (Agilent Technologies, Santa Clara, CA, USA) per manufacturer’s instructions. Human genome expression of biotinylated RNA for pre- and post-exercise pooled samples was then quantified using an Affymetrix (Santa Clara, CA, USA) U133 Plus 2.0 GeneChip kit per manufacturer’s instructions. Fragmentation and analysis were performed by the
Genomics Technology Support Facility at Michigan State University. Coagulation and fibrinolysis proteins were selected from the NetAffx database on the Affymetrix website (http://www.affymetrix.com/analysis/index.affx). Fibrinolysis pathway genes assessed were: tPA, uPA, plasminogen, PAI-1, and fibrinogen. Coagulation pathway genes assessed were: coagulation factors II, III, V, VII, VIII, IX, X, XI, XII, XIII, Von Willebrand factor, protein S, and protein C.

**Muscle tPA Activity Analysis:**
Total protein was extracted from the muscle tissue homogenate using TriReagent™ and was resuspended in 0.1% SDS following the manufacturer’s instructions. Protein concentration was assessed using the DC Protein Assay Kit (Bio-RAD, Hercules, CA, USA). Five micrograms of protein was loaded into a 12% polyacrylamide gel. A tPA standard of 0.1 ng was used as a positive control. Several optimization studies were performed with tPA standards and tissue samples (data not shown). The amount of protein (5 µg) and gel conditions used were optimal to see differences between positive controls. Gels then underwent electrophoresis at 4°C for 5 h at 180 V followed by two 30-minute washes with 2.5% Triton-X to reactivate tPA. Between washes, gels were briefly rinsed with distilled water. Gels were then incubated for 19 h at 37°C with a collagenase soaking buffer (pH=7.5) to allow any active tPA present to catalyze the breakdown of plasminogen copolymerized in the gel. A 0.2% stain stock solution of PhastGel Blue-R (Pharmacia, NY, NY, USA) was prepared according to manufacturer’s protocol. A final 0.025% staining/destaining solution was prepared by mixing 26.5 mL of stain stock with 184 mL destaining solution (1:3:6 glacial acetic acid:methanol:distilled water) (12). Each gel was placed in 200 mL of the final staining/destaining solution and gently rocked for 4 h. KODAK 2000R imaging station and KODAK 1D image software (version 4) was used to analyze band net intensity to quantify plasminogen breakdown for each sample.

**Plasma Analysis:**
Plasma tPA and PAI-1 activities were quantified using bio-functional immunosorbent assays (Biopool International, Ventura, CA, USA) per manufacturer’s instructions. Plasma concentrations of tPA antigen were assessed using enzyme-linked immunosorbent assays (American Diagnostica, Greenwich, CT, USA) per manufacturer’s instructions and were not corrected for plasma volume changes.

**Figure 2. tPA and PAI-1 Protein Activity.** A) Muscle tissue was collected before and immediately following maximal acute aerobic exercise via percutaneous biopsy. Tissue was homogenized and protein was isolated. Equal amounts of protein were used in a zymography assay (see Materials and Methods). Quantified muscle tPA activity is from gel as seen with a representative banding result. B) Blood was collected before and immediately following acute aerobic exercise. tPA activity was measured via ELISA. C) Blood was analyzed for PAI-1 activity via ELISA.
Statistical Analyses
Changes in gene expression from pre- to post-testing by microarray were assessed by GeneChip Operating Software (GCOS) version 3.1 and were performed by the Genomics Technology Support Facility at Michigan State University. Changes in muscle and plasma dependent variables from pre- to post-testing were assessed using paired t-tests. Dependent variables included muscle mRNA (tPA and PAI-1), muscle tPA activity, and plasma tPA and PAI-1 activities. Correlations between muscle variables and plasma variables were assessed using Pearson correlation coefficient. These tests were performed on SPSS version 11. Statistical significance was set at p < 0.05.

RESULTS
Muscle Tissue Analysis

RT-PCR Gene Expression
Mean values for all subjects for tPA and PAI-1 mRNA by RT-PCR are shown in Figure 1A and 1B. There were no significant changes in tPA or PAI-1 expression from pre- to post-exercise. Figure 1C illustrates gene expression for each individual subject illustrating the erroneously high resting value and a differential response for tPA for subject 2 compared with other subjects. As such, when the analysis was performed excluding subject #2, a significant increase (p<0.05) in muscle tPA expression from pre- to post-exercise was observed (Figure 1D). Excluding subject #2 did not change results for PAI-1 mRNA.

Microarray Gene Expression
While changes in gene expression were observed, there were no changes in any fibrinolytic factors that were statistically significant (Table 2) likely indicating expression changes were not large enough to be measured by microarray analysis. Factor V was the only coagulation factor seen to have a statistically significant increase in gene expression in muscle tissue (Table 3).

Muscle tPA Activity
Muscle tPA activity, assessed by plasminogen gel zymography, did not significantly change with exercise (Figure 2A). Exclusion of subject 2 did not alter results for muscle tPA activity.

Plasma Analysis:
Plasma tPA activity showed a significant increase (Figure 2B) with exercise while plasma PAI-1 activity showed no statistical change (Figure 2C). There were no significant associations between plasma variables and any muscle tissue variables (p>0.05).

DISCUSSION
Plasminogen activation clearly plays a role in recovery and regeneration of muscle following injury. Inhibition of uPA results in reduced fusion of myoblasts and differentiation (6,18) and reduced proliferation (6,7). Additionally, chemical muscle damage increases gene expression of uPA and myogenic genes leading to muscle repair and regeneration in mice (15). Further, uPA-deficient mice showed higher fibrin deposition and impaired muscle repair following muscle damage (15). Eliminating fibrin deposits via
the snake venom ancrod following muscle damage resulted in increased muscle repair and regeneration (22). A later study showed PAI-1 knockout mice have an advanced ability for muscle repair and regeneration (11), indicating these plasminogen activators are critical in the face of muscle damage. Studies regarding plasminogen activation in human cells have only used uPA (6,7), however, results from our lab suggest that tPA is the predominant plasminogen activator in humans (13). Regardless, these plasminogen activators appear to be critical for muscle repair following injury.

The present study may further the hypothesis that plasminogen activation plays a role in muscle repair. An increase in tPA has been shown in humans following a chronic exercise program in men with metabolic disease (9). However, the current study is the first to show an increase in tPA mRNA in human skeletal muscle following acute, high-intensity exercise (Figure 1D). Mice also show similar increases, although with uPA, but mice also show an increase in PAI-1 mRNA (22). The present study found an increase in tPA but not in PAI-1 suggesting a possible increase in fibrinolytic potential. Despite these mRNA changes, we did not detect any significant increase in tPA activity from whole muscle samples (Figure 2A). An increase in Factor V gene expression was seen following exercise even though the activated form of Factor V is a known accelerant of tPA-induced plasminogen activation (19). However, an increase in tPA activity may require full protein synthesis as we only detected increases in mRNA. It is possible the current exercise did not induce enough muscle damage to increase tPA activity. However, a recent study with a similar graded treadmill exercise test showed significant increases immediately following the exercise test in plasma levels of creatine kinase, lactate dehydrogenase, and aspartate aminotransferase, all of which are markers of muscle structural damage (20). Despite these inferences, results may be more pronounced using protocols that induce higher levels of muscle damage such as downhill treadmill running.

Sample collection immediately following exercise completion may contribute to the present findings. No significant changes were detected via microarray for fibrinolytic genes despite increases seen for tPA via PCR. Samples were collected from subjects immediately following exercise, which likely limited our detection of changes as up-regulation of genes is a time-dependent process. This limitation accentuates the detected increase in tPA gene expression via RT-PCR and further increases would likely be seen if muscles were sampled longer after the completion of the exercise bout. This time point was chosen as plasma changes in tPA occur during this time interval allowing us to compare muscle production and plasma changes. There were significant increases in plasma

### Table 3. Gene expression by microarray for coagulation.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Change</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Coag. Factor II</td>
<td>2.14</td>
<td>0.621</td>
</tr>
<tr>
<td>Coag. Factor III</td>
<td>0.354</td>
<td>0.500</td>
</tr>
<tr>
<td>Coag. Factor V (1)</td>
<td>0.574</td>
<td>0.805</td>
</tr>
<tr>
<td>Coag. Factor V (2)</td>
<td>6.96</td>
<td>0.213</td>
</tr>
<tr>
<td>Coag. Factor V (3)</td>
<td>2.14*</td>
<td>0.047</td>
</tr>
<tr>
<td>Coag. Factor VII (1)</td>
<td>1.52</td>
<td>0.169</td>
</tr>
<tr>
<td>Coag. Factor VII (2)</td>
<td>0.660</td>
<td>0.500</td>
</tr>
<tr>
<td>Coag. Factor VIII</td>
<td>0.933</td>
<td>0.500</td>
</tr>
<tr>
<td>Coag. Factor IX</td>
<td>1.41</td>
<td>0.500</td>
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<tr>
<td>Coag. Factor X</td>
<td>1.62</td>
<td>0.704</td>
</tr>
<tr>
<td>Coag. Factor XI (1)</td>
<td>1.00</td>
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</tr>
<tr>
<td>Coag. Factor XI (2)</td>
<td>0.871</td>
<td>0.655</td>
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<tr>
<td>Coag. Factor XI (3)</td>
<td>3.48</td>
<td>0.500</td>
</tr>
<tr>
<td>Coag. Factor XI (4)</td>
<td>1.15</td>
<td>0.494</td>
</tr>
<tr>
<td>Coag. Factor XII (1)</td>
<td>0.250</td>
<td>0.532</td>
</tr>
<tr>
<td>Coag. Factor XII (2)</td>
<td>0.933</td>
<td>0.500</td>
</tr>
<tr>
<td>Coag. Factor XIII</td>
<td>0.812</td>
<td>0.500</td>
</tr>
<tr>
<td>Coag. Factor XIII B</td>
<td>0.812</td>
<td>0.805</td>
</tr>
<tr>
<td>Von Will Factor (1)</td>
<td>0.758</td>
<td>0.999</td>
</tr>
<tr>
<td>Von Will Factor (2)</td>
<td>0.871</td>
<td>0.500</td>
</tr>
<tr>
<td>Protein S</td>
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<td>0.500</td>
</tr>
<tr>
<td>Protein C</td>
<td>0.707</td>
<td>0.787</td>
</tr>
</tbody>
</table>

Values are means for all subjects. Numbers following each gene corresponds to the different probes used on the microarray chip.
tPA immediately following exercise (Figure 2B). However, there were no significant associations found between muscle tPA or PAI-1 mRNA and plasma changes suggesting muscle production of these factors does not significantly contribute to plasma changes seen immediately following exercise.

The present results have interesting implications but have limitations. Having only six subjects decreases statistical power and is compounded by an outlier in a major outcome variable. This outlier was decidedly excluded because his response (Figure 1C) was completely opposite to all other subjects, opposite to well-known plasma tPA changes, and opposite to tPA mRNA responses from animal studies. The differential response led us to conclude this subject was not classified correctly from inclusion/exclusion criteria and therefore should be excluded in the results and conclusions. Post-hoc power calculations showed 32% power to detect differences in tPA mRNA levels at a significance of 0.05. Removing the subject decreased our power by 4%. Removing this subject resulted in a statistically significant increase in tPA mRNA. In addition to this outlier, samples were taken via biopsy of whole muscle, indicating possible contamination of analyzed muscle with protein from microvessels and connective tissue. However, we found no change in muscle protein activity of tPA, which was expected to be at the highest risk for contamination. Furthermore, we have observed tPA expression in single muscle fibers in humans, suggesting these acute changes may be due to inherent changes in the fiber, rather than adjacent tissue (13). The design of the current study also required the post-exercise muscle biopsy to be collected at the same incision as the baseline biopsy in order to collect tissue within the time required. A second collection in the same biopsy site may induce gene expression changes primarily due to the tissue injury. However, previous data indicate that up to three collections from the same site did not induce significant changes in gene expression as detected by real-time PCR (16), suggesting the current data is valid.

CONCLUSIONS

Human skeletal muscle tissue showed an increase in expression of tPA following acute maximal aerobic exercise similar to animal studies suggesting humans also increase plasminogen activation following acute, high intensity exercise. Plasma tPA levels increased dramatically following exercise, however, there was no association between muscle mRNA levels and plasma tPA activity suggesting muscle does not contribute to plasma tPA activity seen with acute, high intensity exercise. Future studies should include more subjects and use an exercise bout to induce higher muscle damage such as downhill running.

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Address for correspondence: Womack CJ, Ph.D., MSC 2303, James Madison University, Harrisonburg, VA 22801. Phone (540)568-6515; FAX: (540)568-3338; Email: womackcx@jmu.edu.
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