

SKELETAL MUSCLE AND SKIN REPAIR IN DIABETES MELLITUS

THE INHIBITION OF PAI-1 FOR RESTORATION OF SKELETAL MUSCLE AND SKIN REPAIR IN
DIABETES MELLITUS

By

Irena Alexandra Rebalka, HBSc

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AUTHOR: Irena Alexandra Rebalka, HBSc (McMaster University)

SUPERVISOR: Dr. Thomas J. Hawke

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LAY ABSTRACT

Type 1 diabetes is a lifelong condition that affects many of the normal functions of the body. Type 1 diabetic patients have an impaired ability to heal wounds on their skin and repair muscle after damage or exercise, greatly affecting their quality of life. One factor other than blood sugar that is found in abnormally high levels in diabetic patients is PAI-1, which inhibits proper movement of cells and healing of wounds. In this thesis, we investigated the effects of PAI-1 inhibition on tissue repair in diabetic rodents. While a specific therapeutic designed only to inhibit PAI-1 helped skin wounds close, a commercially available drug that has been shown to inhibit PAI-1 as a secondary effect made healing of diabetic skin and muscle worse. The findings in this body of work suggest that specialized PAI-1 inhibitors should be further investigated as a therapeutic to help restore diabetic tissue repair.

ABSTRACT

Type 1 diabetic (T1D) individuals are burdened with many systemic complications, including impairments in skeletal muscle and cutaneous repair following injury. Plasminogen activator inhibitor 1 (PAI-1), the primary inhibitor of the fibrinolytic system, plays a critical role in the regulation and progression of tissue repair via controlling both extracellular matrix breakdown and cell migration. Previous work has demonstrated that elevations in PAI-1 inhibit adequate tissue regeneration, and has shown that PAI-1 is overexpressed in T1D patients regardless of insulin therapy. As such, the purpose of this thesis is to investigate the effects of known PAI-1 inhibitors on tissue repair of skin and skeletal muscle in T1D. To undertake these experiments, a rodent model of T1D was used, and animals were subject to cutaneous and skeletal muscle injuries. PAI-039, a small-molecule PAI-1 inhibitor, and Fluvastatin, a readily available statin that, among other functions, is known to inhibit PAI-1, were administered, and their effects on tissue regeneration were examined. PAI-039 was effective in restoring cutaneous wound closure in T1D. Fluvastatin, on the other hand, not only proved deleterious to both cutaneous and skeletal muscle regeneration, but also caused a pathological change in lipid deposition in the diabetic rodents. Overall, PAI-1 inhibition via a therapeutic agent that has a multitude of other pleiotropic effects is not recommended for improving T1D tissue repair. PAI-1 inhibition via a specific small-molecule inhibitor does, however, prove beneficial to cutaneous regeneration. Taken together, this data lays the foundation for

future studies investigating small-molecule PAI-1 inhibitors as a therapeutic for the restoration of tissue repair in T1D.

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PREFACE

This thesis is a “sandwich” style thesis. Chapter 1 provides a review of the concepts and ideas relevant to this thesis in the form of a general introduction. Chapters 2 and 3 have been published as peer-reviewed research papers, and Chapter 4 has been submitted for publication in a peer-reviewed journal. A preface that describes the significance of each work is found at the beginning of each chapter, along with the individual contributions of all authors included in the work.

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LIST OF ABBREVIATIONS

| | |
|----------|---|
| ACC | American College of Cardiology |
| AHA | American Heart Association |
| ASCVD | Atherosclerotic cardiovascular disease |
| cDNA | Complementary DNA |
| CVD | Cardiovascular disease |
| DM | Diabetes mellitus |
| ECM | Extracellular matrix |
| eMHC | Embryonic myosin heavy chain |
| GGPP | Geranylgeranyl pyrophosphate |
| GPS | Gastrocnemius, plantaris, soleus |
| H&E | Hematoxylin-eosin |
| HMG CoA | 3-hydroxy-3-methylglutaryl-coenzyme A |
| IMCL | Intramyocellular lipid |
| LDL | Low-density lipoprotein |
| LDL-C | Cholesterol-bound low-density lipoprotein |
| MMP | Matrix metalloproteinase |
| MT | Masson's trichrome |
| ORO | Oil Red O |
| PAI-1 | Plasminogen activator inhibitor-1 |
| PCR | Polymerase chain reaction |
| STZ | Streptozotocin |
| T1D/T1DM | Type 1 Diabetes Mellitus |
| TA | Tibialis anterior |
| TIMP | Tissue inhibitor of metalloproteinase |
| tPA | Tissue plasminogen activator |
| uPA | Urokinase plasminogen activator |
| VEGF | Vascular endothelial growth factor |
| WT | Wild-type |

CHAPTER 1

GENERAL INTRODUCTION AND OBJECTIVES

LITERATURE REVIEW

Diabetes

Type 1 Diabetes (T1D), formerly referred to as juvenile diabetes, is a chronic disease state that originates due to the immune destruction of pancreatic beta cells; the cells responsible for insulin production in the body. This predisposition for a dysfunctional immune response in the pancreas and selective auto-destruction of beta cells are said to be influenced by both genetic and environmental factors (Atkinson, Eisenbarth, and Michels 2014). Despite active research into both the mechanisms that cause T1D and the factors that influence these mechanisms, T1D has no cure, and the inevitable destruction of pancreatic beta cells results in a lack of insulin production and an impairment in glucose handling in the body. As such, there is an immediate need for exogenous insulin replacement and therapy, which must continue for the entire life of the affected individual.

Although T1D accounts for only approximately 10% of the total cases of diabetes worldwide (Daneman 2006), the incidence of T1D continues to rise approximately 3-5% each year (Gillespie et al. 2004; Onkamo et al. 1999). This is a great concern as the peak age of onset and diagnosis of T1D is between 10 and 14 years of age (Dabelea et al. 2007), leaving T1D patients to live with the burden of this disease for the majority of their lifetime.

Despite insulin therapy, diabetic individuals suffer events of dysglycemia; blood glucose levels that deviate from the healthy range (Iscoe et al. 2006). Elevations in circulating glucose causes damage to a multitude of cell types including endothelial cells, neurons, skeletal muscle fibers, mesangial cells, keratinocytes, and fibroblasts, to name a few. What's more, the effects of hyperglycemia appear to be a significant contributor to determining the severity of future diabetic complications; alterations in organ and system functions as a result of cumulative cellular glucose damage (The Diabetes Control and Complications Trial Research Group 1993). Evidently, exogenous insulin alone is not a cure for T1D, and systemic disturbances in growth and organ function persist even during insulin therapy.

Two tissue systems that suffer from complications of prominent consequence are the skin and the skeletal muscle. For the duration of this thesis, we will focus on these two tissue systems and investigate their non-pathological processes of repair as well as deviations from healthy repair that are observed with T1D.

Non-Pathological Cutaneous Wound Repair

Cutaneous wound healing is a multi-factorial cascade of cellular events that occur to resurface, replenish and restore the skin after injury. One would be remiss if the steps involved in wound repair were identified as distinct occurrences in space and time, however categorization into four separate phases allows for a better understanding of

the function and importance of each step in the repair process. As such, normal wound repair can be divided into four overlapping phases: hemostasis, inflammation, proliferation/migration and maturation. Table 1 highlights and summarizes the timing of each phase as well as the main events that occur during each step in normal, non-pathological wound repair.

Table 1. Phases of non-pathological cutaneous wound repair.

| Phase of repair | Timing after injury | Main events |
|-------------------------------------|---------------------|---|
| Hemostasis | Minutes- Hours | <ul style="list-style-type: none"> • Fibrin clot formation • Release of chemokines that attract inflammatory factors |
| Inflammation | Hours-Days | <ul style="list-style-type: none"> • Inflammatory cell infiltration • Foreign particle and bacteria clearance • Release of factors that initiate granulation tissue formation and angiogenesis |
| Proliferation/ Migration | Days-Weeks | <ul style="list-style-type: none"> • The “healing” phase • Epidermal cell migration • Formation of granulation tissue • Angiogenesis |
| Maturation | Weeks-Months | <ul style="list-style-type: none"> • Collagen remodeling |

Hemostasis and Inflammation

Soon after injury, platelets aggregate and activate in response to their contact with underlying collagen, now exposed due to injury. Within this clot, aggregating platelets are lodged within a network of fibrin as well as other proteins and glycoproteins (Falanga

2005). This fibrin clot that now populates the wound site acts as a temporary protective barrier, and is the first defense against exsanguination. The activated platelets lodged within the clot have another critical function in the repair process, and release a multitude of cytokines and chemokines, facilitating the initiation and transition into the inflammatory phase of wound repair (Singer and Clark 1999). Furthermore, the fibrin clot acts as a provisional matrix to support the infiltration of inflammatory factors and cells necessary for wound repair (Kurkinen et al. 1980).

Neutrophils are the first circulating inflammatory cells to enter the wound site, and clear the area of bacteria and foreign matter upon arrival. Monocytes infiltrate the wound site and become activated macrophages. Macrophages have a host of responsibilities during the inflammatory phase including phagocytosing neutrophils, releasing cytokines that stimulate fibroblast and smooth muscle cell proliferation, attracting endothelial cells to promote angiogenesis, and releasing macrophage-derived growth factors that instigate and propagate granulation tissue formation (Brown 1995; Hunt et al. 1984; Martin 1997; Polverini et al. 1977). In fact, wound healing in macrophage-deficient animals is delayed, and defective; causing wounds to remain in the inflammatory stage for an extended period of time (Leibovich and Ross 1975). Macrophages are a critical piece in both the events of the inflammatory phase as well as the transition from the inflammatory phase to the next stage of wound repair: proliferation and migration.

Proliferation/ Migration

Upon the achievement of hemostasis as well as the onset of the inflammatory cascade, wounded tissue shifts toward a phenotype of tissue replacement and repair. The formation of granulation tissue, new connective tissue within the wound gap, begins in the end-stages of the inflammatory phase. Granulation tissue formation is a predominant event during the proliferatory and migratory phase of wound repair, and this new infiltrate acts as a replacement matrix network for the fibrin clot. Granulation tissue is composed of residual inflammatory cells, fibroblasts and new vasculature woven into a matrix of collagen, fibronectin and proteoglycans; the extracellular matrix (ECM). While the ECM is mandatory to support cell in-growth, it also has the potential to hinder the infiltration of inflammatory cells, fibroblasts, vascularity and ultimate cutaneous remodeling in the absence of ECM turnover (Lu et al. 2011; Peters et al. 1997; Wysocki, Staiano-Coico, and Grinnell 1993). As such, a fine balance of ECM synthesis and degradation must ensue.

Cellular infiltration into a fibrin clot or a tightly woven matrix requires an active proteolytic system to clear a path for cellular migration. Functionality of the fibrinolytic system, whose primary role is the generation of the active plasmin enzyme from its inactive precursor plasminogen, is critical in this process (Figure 1). The generation of plasmin leads to fibrin degradation as well as the activation of matrix metalloproteinases (MMPs) which are responsible for ECM degradation (Lu et al. 2011). Plasminogen

activator inhibitor-1 (PAI-1; also called SERPINE1) is a known inhibitor of the ECM remodeling enzymes urokinase plasminogen activator and tissue plasminogen activator (uPA and tPA respectively), which are critical for plasmin activation (Falanga 2005; Li et al. 2003). By binding to uPA and tPA, PAI-1 is the major physiological inhibitor of plasmin generation, the subsequent degradation of fibrin and overall regulation of fibrinolytic balance (Dellas and Loskutoff 2005; Loskutoff et al. 1993).

Within the proliferatory/ migratory phase of wound repair, many diverse processes occur in conjunction with ECM synthesis, including re-epithelialization and angiogenesis. Re-epithelialization of the wound begins hours after injury. In order to re-establish the cutaneous barrier, epidermal cells move inward, penetrating the fibrin clot, and moving above viable dermal tissue. Cellular motility of this kind requires cell adhesion, a process that involves integrins; the principal receptors that bind infiltrating cells to the ECM (Holly, Larson, and Parise 2000). The expression of integrin receptors allows epidermal cells to interact with a variety of intergrin-bearing ECM proteins including vitronectin (Clark 1990; Holly et al. 2000). PAI-1 competes for vitronectin affinity and acts to oppose cell adhesion and migration (Kjøller et al. 1997; Stefansson and Lawrence 1996). An excess of PAI-1 would hinder the binding between vitronectin and cell integrins, impairing cell migration and motility of epidermal cells and other infiltrating, reparative cells. ECM and collagen degradation, which is also required for successful inward migration of epidermal cells, depends on the activation of plasmin, whose activation is also impaired

by PAI-1 (Falanga 2005; Li et al. 2003). Upon the initiation of inward epidermal migration, epidermal cell proliferation is triggered, and these cells aggregate at the wound edge. Newly proliferated cells climb inward from these stores, and the leading epidermal edges meet in the center in a zipper-like fashion (Clark et al. 1982). Once these edges meet and cells stop migrating, cellular adhesion ensues, and the newly formed epidermis returns to near-normal thickness by five to seven days post-injury (Odland and Ross 1968).

The establishment of new vascularity within the wound site is also critical to the repair process. After initial damage, the wound center lacks vascular supply and vascular endothelial-cell growth factor (VEGF) is released in response to tissue hypoxia (Ladoux and Frelin 1993; Shweiki et al. 1992). Macrophages also release angiogenic factors that stimulate plasminogen activator release and ECM breakdown (Koh and DiPietro 2011). Vasculature on the periphery of the wounds perfuses tissue at the wound margins, endothelial cells migrate inward, and neovasculature invades the fibrin clot to form a new microvascular network. This inward migration of endothelial cells is also highly dependent on vitronectin-mediated cell adhesion and migration, and requires ECM degradation to bore a pathway for cellular entry into the wound site.

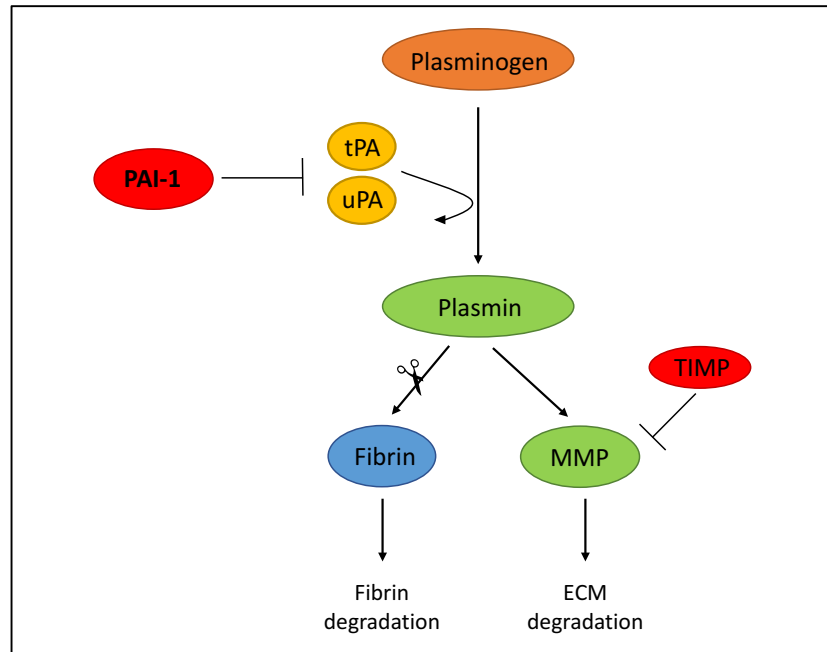


Figure 1. A composite of the fibrinolytic system. PAI-1 (plasminogen activator inhibitor-1) is a known inhibitor of the ECM (extracellular matrix) remodeling enzymes uPA and tPA (urokinase plasminogen activator and tissue plasminogen activator), which are critical for the conversion of plasminogen to the active enzyme plasmin. Plasmin activates MMPs (matrix metalloproteinases), responsible for ECM degradation. TIMPs (tissue inhibitors of metalloproteinase) inhibit MMP function. Plasmin also cleaves fibrin, causing degradation of fibrin clots.

Maturation

Healthy wounds enter the maturation phase approximately three weeks after injury, and remain in this phase for weeks or months to follow depending on the severity, size and depth of the wound. An integral part of the maturation phase of wound repair is the

turnover and remodeling of collagen. Collagenases and MMP's within the wound site work to remove excess collagen while tissue inhibitors of metalloproteinases (TIMPs) limit their enzyme activity and create a balance in collagen flux (see Figure 1). During the remodeling process, fibronectin, a key provisional scaffold in the granulation tissue, gradually disappears as is replaced by collagen (Lenselink 2015). Edema decreases with the progression of wound repair, and collagen fibers move together. This facilitates collagen cross-linking; decreasing the profile of the wound while increasing wound tensile strength (Bailey et al. 1975).

Non-Pathological Skeletal Muscle Regeneration

The health of our skeletal muscle is vital to our physical and metabolic capacities. Not only is skeletal muscle critical to mobility, but it is the largest organ for glucose disposal and storage (Baron et al. 1988; Katz et al. 1983; Kraegen et al. 1985). Skeletal muscle is subject to continuous mechanical stress during contraction, which occurs continuously thanks to the physical function of this tissue type. As a result, muscle damage, turnover and repair, necessary to restore skeletal muscle function, occurs on a regular basis. Similar to cutaneous repair, the repair of skeletal muscle is a complex, methodical operation that includes three phases: the inflammatory response, satellite cell proliferation/migration and a maturation processes (Yin, Price, and Rudnicki 2013).

Skeletal muscle repair begins with the degeneration of damaged muscle in the form of necrosis of damaged myofibers. Myofiber necrosis initiates the inflammatory response and increases cell permeability, allowing the influx of necessary reparative factors (Orimo et al. 1991). The disruption of myofiber integrity is easily observed without biopsy, as the release of muscle proteins such as creatine kinase are apparent in bloodwork (Pearce, Pennington, and Walton 1964; Rebalka and Hawke 2014). Just as in cutaneous repair, neutrophils are the first inflammatory cell type to infiltrate the damaged area, appearing as early as 45 minutes after damage (Fielding et al. 1993). Following neutrophil infiltration, macrophages populate the wound site and secrete a sequence of pro-inflammatory followed by anti-inflammatory cytokines. Pro-inflammatory cytokines are responsible for the phagocytosis of refuse and are present in the days following injury (Cantini and Carraro 1995). Anti-inflammatory cytokines are responsible for the proliferation and differentiation of satellite cells; the myogenic population that not only facilitates repair but also formation of new muscle fibers (Cantini and Carraro 1995; Nathan 1987). Satellite cell proliferation follows, and these progeny both fuse to injured myofibers to initiate repair and fuse to one another to generate new myofibers (Hawke and Garry 2001). Similar to cutaneous repair, there is a vital importance of plasminogen activation and subsequent ECM breakdown for satellite cell activation, migration and subsequent adequate skeletal muscle repair (Bryer et al. 2008; Sisson et al. 2009). In fact, rodents deficient in PAI-1, the primary inhibitor of the fibrinolytic process, exhibit improved skeletal muscle regeneration (Koh et al. 2005). While the comprehensive

details of satellite cell activity will not be explored in depth in the context of this thesis, this is an important part of the regenerative process of skeletal muscle, an extended review is provided in (Hawke and Garry 2001).

Upon cross-sectional investigation, newly formed skeletal muscle fibers undergoing repair are easily distinguished from their uninjured counterparts. New muscle fibers are smaller in diameter due to the continuation of regeneration and growth, and are centrally nucleated due to satellite cell activation and inward migration from their dormant peripheral location. Upon the completion of maturation, these phenotypic hallmarks of repair are no longer evident, nuclei move to the periphery of the cell, fiber size and contractile properties are restored, and newly regenerated fibers appear no different than their neighboring cells that did not undergo repair.

Tissue Repair in T1D

Wound repair following both major and minor cutaneous injury is conducted via complex yet gracefully orchestrated interactions between reparative cells and the wound site environment. While most wounds in healthy non-diabetic persons progress through the phases of wound repair in a linear fashion to heal rapidly and efficiently, this is not often the case for diabetic wounds. Patients with both T1D and Type 2 Diabetes have an impaired ability to heal after injury (Brem and Tomic-Canic 2007; Fahey et al. 1991; Falanga 2005; Greenhalgh 2003; Martin, Komada, and Sane 2003; Stadelmann, Digenis,

and Tobin 1998). Minor cutaneous wounds in diabetic patients are often resistant to healing and perpetuate as chronic open wounds, resulting in a predisposition to infection. These chronic, non-healing wounds are especially common in the lower distal extremities, namely diabetic foot ulcerations. Delayed healing, resulting in an increased risk of infection, leads to several complications, and ultimately, the need for amputation.

Further, skeletal muscle health in the T1D population is far from ideal. Diabetic individuals exhibit smaller muscle fibers as well as decreases in muscle mass and work capacity, resulting from impairments in muscle growth, and altered metabolic capacity (Gordon et al. 2010; Krause et al. 2009; Krause, Riddell, and Hawke 2011; A. Vignaud et al. 2007).

Indeed, human studies have demonstrated alterations in muscle structure, function and contractile proteins with T1DM (Almeida, Riddell, and Cafarelli 2008; Andersen, Schmitz, and Nielsen 2005; Jakobsen and Reske-Nielsen 1986; Reske-Nielsen, Harmsen, and Vorre 1977). What's more, mimicking cutaneous complications, a reduction in the capacity for skeletal muscle regeneration is observed in T1D (Gulati and Swamy 1991; A Vignaud et al. 2007). From a clinical perspective, exercise and an increase in physical activity is considered a vital therapeutic in the blood glucose management and maintenance of a healthy body in T1D individuals (Colberg et al. 2016). Due to the increased susceptibility for contraction-induced muscle damage in the T1D population (Copray et al. 2000; D'Souza et al. 2016), as well as the impairments in skeletal muscle turnover and repair in this population (Gulati and Swamy 1991; Krause et al. 2013; A Vignaud et al. 2007), the

search for a therapeutic that improves muscle health and repair for both daily use and therapeutic exercise is of supreme relevance and importance.

Problems with diabetic healing of both the skin and skeletal muscle are concentrated in the inflammatory and proliferative/migratory phases of repair, and include the prolonged presence of macrophages and neutrophils, impaired angiogenesis, an impaired ability for fibrinolysis and extracellular matrix remodeling, altered growth factor expression, and a decrease in cellular proliferation and migration (Babaei et al. 2013; D'Souza et al. 2016; Diegelmann and Evans 2004; Epstein, Singer, and Clark 1999; Falanga 2005; Gulati and Swamy 1991; Krause et al. 2013; Krause, Riddell, et al. 2011; A Vignaud et al. 2007).

PAI-1 and Diabetic Tissue Repair

When one thinks about T1D, dysregulations in insulin and blood glucose are the first pathological factors that come to mind. Interestingly, there are many other factors that are dysregulated in diabetes, one of which being PAI-1, the previously introduced inhibitor and master-regulator of the fibrinolytic system (Figure 1) (Rabieian et al. 2017). In fact, type 1 and type 2 diabetic patients suffer from elevated levels of PAI-1 regardless of their level of glycemic control (Oishi 2009). Cell culture studies verify increases in PAI-1 mRNA and protein expression in a high glucose environment (Ni et al. 2013), and rodent studies verify elevations in PAI-1 in diabetes-induced mice (Krause, Moradi, et al. 2011; Rebalka et al. 2015).

Dysregulated PAI-1 expression is a causative factor in atypical wound healing, as it regulates matrix breakdown/turnover as well as cell adhesion and migration (Rabieian et al. 2017; Zhou et al. 2003). In fact, dysregulated cutaneous levels of PAI-1 are commonly noted in many repair anomalies, including persistent scarring and non-healing chronic wounds (Ghosh and Vaughan 2012; Providence and Higgins 2004). Elevations in PAI-1 are also implicated in the impairment of skeletal muscle regeneration (Koh et al. 2005; Krause, Moradi, et al. 2011; Suelves et al. 2005). The elevated PAI-1 represses MMP-9 activity and ECM breakdown, resulting in an inability of macrophages, neutrophils and muscle satellite cells to infiltrate the wound site. This attenuation of necrosis and degeneration ultimately delays the transition to muscle regeneration. Intuitively, previous work has shown that a PAI-1 knockout mouse model displays accelerated cutaneous wound healing, and that mice deficient in PAI-1 have elevated MMP-9 activity, increasing fibrinolytic potential and ECM turnover (Chan et al. 2001; Ebrahimian et al. 2012).

Therapeutic Targets for the reduction of PAI-1

PAI-039

PAI-039, also called Tiplaxtinin, is an orally bioavailable PAI-1 antagonist (Elokda et al. 2004). Mechanistically, PAI-039 binds to active PAI-1 in the systemic circulation, blocking the vitronectin binding site (Gorlatova et al. 2007). The blockade of this binding site is twofold in its functionality. As the association of vitronectin to PAI-1 stabilizes and prolongs the lifespan of PAI-1, PAI-039 acts to destabilize active PAI-1 (Zhou et al. 2003).

Additionally, PAI-1 competes for and binds to the ECM protein vitronectin, acting to oppose cell adhesion and migration (Kjøller et al. 1997; Stefansson and Lawrence 1996). Tiplaxtinin's blockade of the PAI-1: vitronectin binding site allows the binding between vitronectin and cell integrins to occur, promoting cell migration and motility. In short, although transcription and translation of PAI-1 are not manipulated by this low molecular weight antagonist, PAI-039 works to both increase cell attachment and migration as well as negate the anti-proteolytic activity of PAI-1.

Previous studies have reported PAI-039 as an anti-thrombotic agent; preventing thrombosis and mitigating the presence of existing thrombi (Hennan et al. 2008). PAI-039 has also been used to reduce PAI-1 levels in T1D rodents, reducing fibrosis and restoring MMP-9 activity (Krause, Moradi, et al. 2011). This resulted in the normalization of ECM remodeling, and restoration of the regenerative capacity of the muscle, even in the absence of insulin.

The fibrinolytic properties restored by the attenuation of PAI-1 have also been deemed a therapeutic target for the reduction of atherosclerotic cardiovascular disease (ASCVD) risk in diabetic patients (Kearney et al. 2017). PAI-039 has more than proved its efficacy in the inhibition of PAI-1, and should be considered as a potential therapeutic for promoting ECM turnover and restoring physiologically normal levels of PAI-1 in the diabetic population.

Statins

Statins are a class of drugs that act to effectively lower blood cholesterol levels by limiting the endogenous production of cholesterol through the inhibition of HMG Co-A reductase (Schachter 2005). As a result of depletions in systemic cholesterol, hepatic expression of low density lipoprotein (LDL) receptor is upregulated, and hepatic cellular uptake of LDL bound cholesterol (LDL-C) is increased, further reducing circulating quantities of LDL-C (Hobbs, Brown, and Goldstein 1992; Schachter 2005). Because high plasma concentrations of LDL-C have been extensively characterized as a primary factor in the development of atherosclerosis, this class of lipid-lowering agents have been proven as key players in the primary and secondary prevention of ASCVD events (Peto et al. 2002; Sacks et al. 1996).

In excess of impairments in cutaneous repair and skeletal muscle impairments, another complication that burdens individuals with diabetes is cardiovascular disease. Indeed, the risk of death from ASCVD is 1.7 times greater in diabetic individuals than their age-matched non-diabetic counterparts (Centers for Disease Control and Prevention (CDC) 2014). In 2011, 47% of diabetic patients were reported to have cardiovascular disease, and this statistic has not faltered since 1997 (Centers for Disease Control and Prevention (CDC) 2014). Due to the aforementioned efficacy of statins in preventing ASCVD coupled with the increased risk of cardiovascular disease in the diabetic population, guidelines released by the by the American College of Cardiology and the American Heart

Association in 2013 recommend that all diabetic individuals above the age of 40 be prescribed statins regardless of the presence of other ASCVD risk factors (Stone et al. 2013). As such, in 2014, 62.8% of adults diagnosed with diabetes were prescribed statins, and this number only continues to rise (Gu et al. 2014).

In addition to their lipid-lowering effects, statins have been shown to have a plethora of pleiotropic functions including anti-inflammatory effects (Diomedea et al. 2001), antibacterial properties (Jerwood and Cohen 2007), and improvement in blood vessel formation (Chen et al. n.d.; Kureishi et al. 2000). Based on what we know about persistent inflammation and vascular deficits in the diabetic population, statins appear as an appealing therapeutic in this population. What's more, it has been suggested that statins have beneficial effects on the wound healing process (Asai et al. 2012; Bitto et al. 2008; Suzuki-Banhese et al. 2015).

In addition to their abundance of systemic effects, statins have been known to inhibit PAI-1 expression in culture (Bourcier and Libby 2000; Mussoni et al. 2000; Ni et al. 2013; Sato et al. 2008) and humans (meta-analysis in (Sahebkar et al. 2016)), as well as enhance tPA expression; effects that favors fibrinolysis (Essig, Nguyen, et al. 1998; Essig, Vrtovsnik, et al. 1998). Statins produce this effect by both reducing PAI-1 gene transcription and reducing activity of the PAI-1 promoter (Bourcier and Libby 2000; Hua et al. 1998).

Statins are known to inhibit post-translational modification of small GTPases such as RhoA, thereby inhibiting the Rho/ROCK pathway (Figure 2). This pathway is critical for the activation of smad3 and subsequent transcription of PAI-1 (Fenton II et al. 2002; Greenwood, Steinman, and Zamvil 2006; Hua et al. 1998). Briefly, the action of statins on RhoA can modulate TGF- β signaling via inhibition of the Rho/ROCK pathway, necessary for the phosphorylation and subsequent activation of smad3 (Burke et al. 2009; Watts and Spiteri 2004). Phospho-smad3 binds to smad4, and the resultant complex translocates into the nucleus, activating transcription of PAI-1 (Leask and Abraham 2004). This inhibitory effect can be reversed by mevalonate (a cholesterol biosynthesis pathway intermediate) as well as geranylgeranylpyrophosphate (a prenylation protein created downstream of mevalonate) supplementation, confirming the requirement of prenylated intermediates in the pathway leading to PAI-1 production (Bourcier and Libby 2000; Ni et al. 2013). Intuitively, downregulation of phospho-smad3 production via the action of statins on the TGF- β -smad pathway ultimately attenuates this transcriptional response.

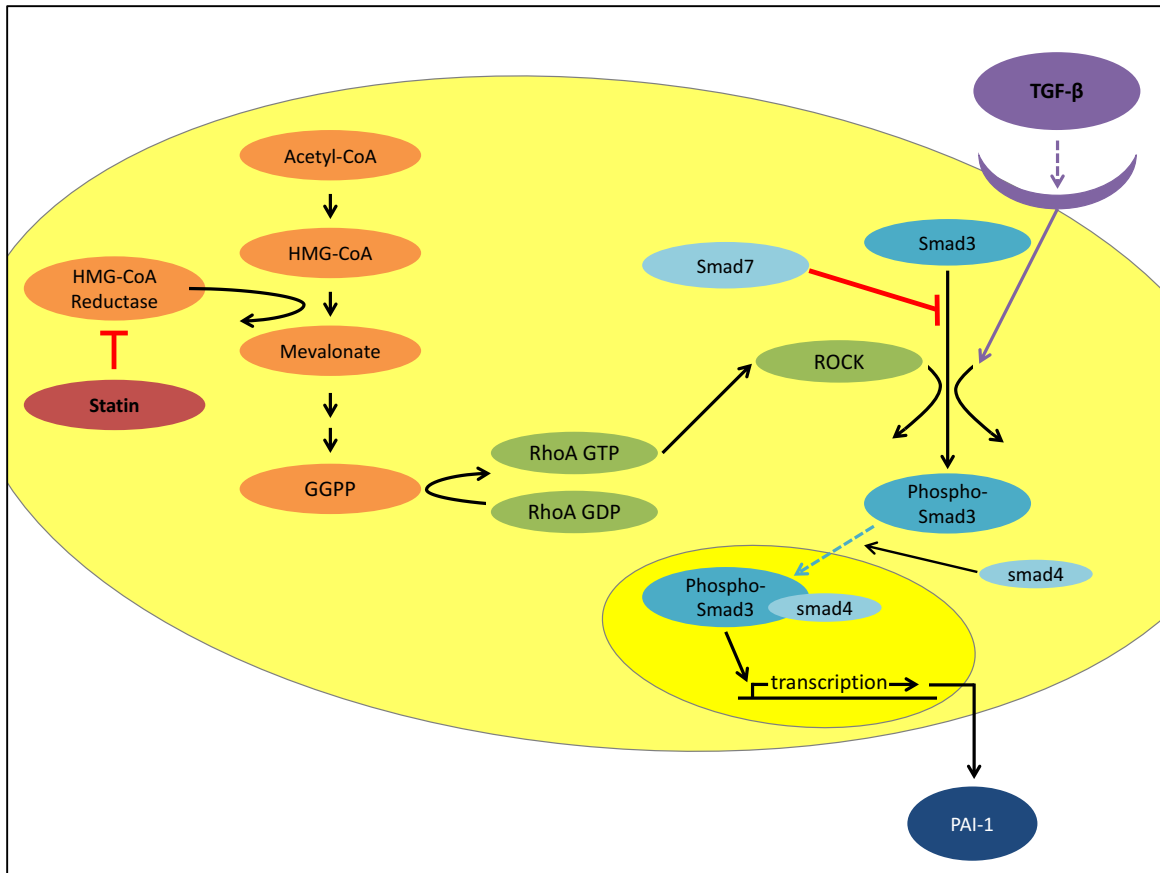


Figure 2. Pathway of statin action on PAI-1 transcription. TGF- β binds to its receptor, allowing for the phosphorylation of smad3 (now phospho-smad3). Phospho-smad3 binds to smad4, and the resultant complex translocates into the nucleus, activating PAI-1 gene transcription. Statins inhibit HMG-CoA reductase, whose inhibition reduces the downstream production of Mevalonate and GGPP. The lack of GGPP hinders the modification of RhoA (Greenwood et al. 2006). The action of statins on RhoA modulates the TGF- β -smad pathway via inhibition of the Rho/ROCK pathway, necessary for the phosphorylation and subsequent activation of smad3 (Burke et al. 2009; Watts and Spiteri 2004).

MAIN OBJECTIVES

Type 1 diabetic individuals are faced with a multitude of systemic complications, including impairments in skeletal muscle health and repair as well as impairments in cutaneous repair following injury (Brem and Tomic-Canic 2007; Falanga 2005; Gulati and Swamy 1991; A. Vignaud et al. 2007). Plasminogen activator inhibitor 1 (PAI-1), the key regulator and inhibitor of the fibrinolytic system, plays a critical role in the regulation and progression of tissue repair via controlling both ECM breakdown and cell adhesion and migration, with elevations in PAI-1 inhibiting adequate regeneration (Dellas and Loskutoff 2005; Li et al. 2003; Stefansson and Lawrence 1996). Interestingly, PAI-1 is overexpressed in type 1 diabetic patients regardless of their level of glycemic control (Oishi 2009). As such, the purpose of this thesis is to investigate the effects of known PAI-1 inhibitors on tissue repair of skin and skeletal muscle in a type 1 diabetic environment.

SPECIFIC AIMS

One: Investigate the effects of PAI-1 inhibition via a small-molecule PAI-1 inhibitor on cutaneous repair in a rodent model of T1D.

Two: Investigate the role of statins, a readily available class of lipid-lowering therapeutics known to inhibit PAI-1, on repair of skin and skeletal muscle in a rodent model of T1D.

Three: Investigate the effects of statin therapy on resting T1D skeletal muscle health.

MAJOR HYPOTHESIS

Repair of both skin and skeletal muscle following injury will be impaired in T1D rodents. Elevations in PAI-1 levels are contributing to the impairments in cutaneous wound and skeletal muscle healing in T1D. Furthermore, the administration of therapeutics that inhibit PAI-1 levels will improve and expedite repair in both tissue types.

MINOR HYPOTHESES

One: Pharmacologically decreasing PAI-1 levels via small-molecule inhibitor PAI-039 will improve wound healing in T1D rodents.

Two: Pharmacologically decreasing PAI-1 levels via a readily-available and prescribed lipid-lowering therapeutic will improve wound healing in T1D rodents.

Three: The combination of T1D and statin administration will be detrimental to muscle health, and exacerbate the myopathy resultant from each factor alone.

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CHAPTER 2

INHIBITION OF PAI-1 VIA PAI-039 IMPROVES DERMAL WOUND CLOSURE IN DIABETES MELLITUS

Authors: Irena A. Rebalka, Matthew J. Raleigh, Donna M. D'Souza,
Samantha K. Coleman, Alexandra N Rebalka, Thomas J Hawke

Corresponding Author: Thomas J. Hawke, Dept. of Pathology and Molecular
Medicine, McMaster Univ., 4N65, Health Sciences Centre,
1200 Main St. W., Hamilton, Ontario L8N 3Z5, Canada (email:
hawke@mcmaster.ca)

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PREFACE

Significance to thesis

PAI-1 is a known inhibitor of tissue remodeling and is constitutively elevated in type 1 diabetic individuals. Inhibition of PAI-1 has been shown to improve cutaneous repair in non-diabetic rodents, and inhibition of PAI-1 via PAI-039 has previously been shown to restore skeletal muscle regeneration in type 1 diabetic rodents. Given this information, PAI-039 was administered to type 1 diabetic rodents following cutaneous injury to determine if PAI-1 was a primary contributor to the impairments in type 1 diabetic wound repair.

Author contributions

Irena A. Rebalka: Designed the study, interpreted the results, performed animal care, performed sample collection, data collection and data analysis, wrote the initial manuscript.

Matthew J. Raleigh: Performed sample collection, data collection, data analysis, interpreted results and edited the manuscript.

Donna M. D'Souza: Performed data collection and analysis and edited the manuscript.

Samantha K. Coleman: Performed sample collection and edited the manuscript.

Alexandra N Rebalka: Performed data collection and data analysis and edited the manuscript.

Thomas J Hawke: Designed the study, interpreted the results, performed animal care and edited the manuscript.

ABSTRACT

Diabetes Mellitus impairs the ability to heal cutaneous wounds, leading to hospitalization, amputations and death. Diabetic patients suffer from elevated levels of Plasminogen Activator Inhibitor-1 (PAI-1), regardless of their glycemic control. It has been demonstrated that PAI-1 deficient mice exhibit improved cutaneous wound healing, and that PAI-1 inhibition improves skeletal muscle repair in T1DM mice, leading us to hypothesize that pharmacologically-mediated reductions in PAI-1 using PAI-039 would normalize cutaneous wound healing in streptozotocin-induced diabetic mice (STZ-diabetic). To simulate the human condition of variations in wound care, wounds were aggravated or minimally handled post-injury. Following cutaneous injury, PAI-039 was orally administered twice daily for 10 days. Compared to non-diabetics, STZ-diabetic wounds healed more slowly. Wound site aggravation exacerbated this deficit. PAI-1 inhibition had no effect on dermal collagen levels or wound bed size, but did elevate dermal macrophage content in STZ-diabetic wounds. PAI-039 treatment failed to improve angiogenesis in STZ-diabetic wounds, and blunted angiogenesis in non-diabetic wounds. Importantly, PAI-039 treatment significantly improved epidermal cellular migration and wound re-epithelialization compared to vehicle-treated STZ-diabetics. These findings support the use of PAI-039 as a novel therapeutic to improve diabetic wound closure, and demonstrates the primary mechanism of its action to be related to epidermal closure.

INTRODUCTION

Diabetes Mellitus (DM) is a family of metabolic disorders characterized by elevated blood glucose levels and impaired insulin signaling. Currently, it is estimated that over 29 million individuals in the USA, and greater than 347 million individuals worldwide have diabetes (1, 2). By 2030, the worldwide prevalence of diabetes will approach 8 percent of the world's population (3). Individuals with DM are at a significantly elevated risk for a number of comorbidities including nephropathy, neuropathy, peripheral artery disease, stroke and retinopathy. Another major complication associated with DM is non-healing dermal wounds. These wounds are especially common in the lower distal extremities, namely, diabetic foot ulcerations. Foot ulcers are the leading cause of hospital admissions for persons with diabetes, are estimated to occur in 15% of all diabetic patients, and precede 85% of all diabetic lower leg amputations (4, 5). In the United States, the average cost of treating one single infected diabetic foot ulcer is \$17,000, and amputation costs approach \$45,000 per amputation (5, 6). Approximately 72,000 non-traumatic diabetic lower-limb amputations are performed in North America each year (5). Despite significant medical advancements in the treatment of diabetic wounds, these statistics have not faltered significantly in the past 30 years, highlighting the necessity to develop effective strategies to expedite diabetic wound healing in order to avoid amputation (6).

Wound healing in non-diabetics is a multi-factorial process that follows a basic series of overlapping processes: (i) hemostasis (clotting), (ii) inflammation (clean out debris and bacteria), (iii) proliferation (rebuild wound site), and (iv) maturation (regeneration of

damaged tissue and vessels). In DM, it is reported that there are over 100 known physiological factors that contribute to the deficits in wound healing (4). In general, the impairments in diabetic wound healing appear to be concentrated in the inflammatory and proliferatory phases, and include the prolonged presence of neutrophils and macrophages, an impaired angiogenic response, decreased migration and proliferation of fibroblasts and keratinocytes, decreased quantity/quality of granulation tissue, and altered growth factor/cytokine expression (4). Though DM is characterized by altered blood glucose regulation and dysregulation of insulin (and/or insulin signaling), numerous other endocrine factors are also known to be differentially regulated. One hormone of particular interest is Plasminogen Activator Inhibitor-1 (PAI-1), a member of the serine protease inhibitor family. PAI-1 has actions that are largely determined through two distinct, yet interrelated, cascades. First, PAI-1 is involved in fibrinolysis via the inhibition of plasminogen activators, and second, in cell migration, survival and proliferation, through binding to urokinase plasminogen activator (uPA), the uPA receptor, vitronectin, and low-density lipoprotein related protein (LRP). Plasma PAI-1 levels are significantly elevated in diabetes mellitus, obesity and insulin-resistance, and have been implicated in the development of vulnerable atherosclerotic plaques and nephropathy (7, 8, 9).

Based on the work of Chan et al. (10), who demonstrated that PAI-1 deficient mice display accelerated wound healing, we hypothesized that elevations in PAI-1 were contributing to the impairments in dermal wound healing in DM. Furthermore, we hypothesized that pharmacologically decreasing PAI-1 levels means may serve as a useful

therapeutic strategy to improve wound healing in DM. The results of the present investigation demonstrate that PAI-1 inhibition significantly improves epidermal closure in diabetic wounds, a process critical for the formation of a protective barrier atop the wound site, preventing bacterial entry and infection, and allowing the expedition of the healing process. These findings support the use of PAI-1 inhibitors as a novel therapeutic to improve diabetic cutaneous wound repair.

RESEARCH DESIGN AND METHODS

Animal Handling

Male C57BL6/J mice [Jackson Laboratories (Bar Harbor, ME)] were provided enrichment material, chow [D12450K; OpenSource Diets; New Brunswick, NJ] and water ad libitum. Animal housing conditions were maintained at 21°C, 50% humidity and a 12h/12h light/dark cycle. Experimentation was approved by the McMaster University Animal Research Ethics Board, in accordance with Canadian Council for Animal Care guidelines. At 10-12 weeks of age, animals were randomly assigned into Streptozotocin-diabetic (STZ) or control (WT) groups. Cohorts were then subdivided into aggravated and minimally-handled wounds. Aggravated Wounds: STZ animals received three daily injections of STZ anomer [Streptozocin; Sigma-Aldrich, Oakville, ON] dissolved in sterile saline at 50 mg/kg, and one final injection at 200 mg/kg. Minimally-handled Wounds: Streptozotocin [Calbiochem; Gibbstown, NJ] was dissolved in sodium citrate buffer, pH

4.5, and one injection at 150 mg/kg was administered. No significant differences were observed between groups in body mass ($P=0.49$) or blood glucose values as a result of the two distinct STZ protocols (**Supplemental Figure 1**). Six weeks after DM onset (blood glucose $>14\text{mM}$), punch biopsies were conducted. Hair removal took place two days prior to punch biopsy. On the day of biopsy, all mice were anaesthetized via isoflurane. Following surgical preparation (sequential application of 0.5% povidone scrub, 70% ethanol, 1% povidone solution), each animal received two 6 millimeter diameter full-thickness wounds via punch biopsy [Miltex; York, PA] on their dorsal scapular region. Oral analgesic [Tempra; Bristol-Myers Squibb Canada; Montreal, QC] was administered two hours prior to biopsy, and topical analgesic [Emla Cream; AstraZeneca, Mississauga] was applied prior to surgical preparation. Oral analgesic was also provided to all animals every 4-6 hours for the first 48 hours post-biopsy.

PAI-1, PAI-039 Treatment and Wound Care

Changes in PAI-1 were confirmed using mRNA expression levels observed in cardiac tissue (11). Briefly, cDNA was prepared from WT and STZ-diabetic cardiac tissue. Semi-quantitative PCR was performed using sequence-specific primers for PAI-1, ACGTTGTGGAAGTGCCTAC and GCCAGGGTTCCTAAACAT, and $\beta 2$ Microglobulin, ATCCAAATGCTGAAGAACGGG and CATGCTTAACTCTGCAGGCG, and quantified using CareStream Imager software. Consistent with others (11), PAI-1 levels were significantly

elevated in STZ mice (0.55 ± 0.03 WT vs 0.76 ± 0.03 STZ, $P < 0.01$). To determine if elevations in PAI-1 were contributing to impaired cutaneous wound repair, PAI-039 [Axon Medchem; Groningen, Netherlands], an orally-effective PAI-1 inhibitor, was administered. STZ and WT mice were randomly assigned to receive vehicle (V) (0.5% Methylcellulose, 2% Tween-80 in sterile H₂O) or PAI-039 (2 mg/kg PAI-039, in vehicle) treatment at both 11:00 a.m. and 3:00 p.m. daily, beginning on the day of wounding, and terminating on the day of harvest, 10 days post-biopsy. This PAI-039 administration protocol has previously been demonstrated to reduce PAI-1 levels to that of non-diabetic mice (12). Animals received two variations of wound care: aggravation or minimal handling. In order to model improper wound care, wounds were manually grasped and held during treatment administration via oral gavage, twice daily. In order to model the proper care of diabetic wounds and remove wound site aggravation (13), treatment was combined with sugar-free cherry-flavored Tempra [Bristol-Myers Squibb Canada; Montreal, QC], a solution that allowed simple oral administration via dropper without handling of the dorsal scapular wounds.

Tissue Collection

Ten days post-biopsy, animals were euthanized via cervical dislocation, wounds were isolated, bisected, fixed in 4% paraformaldehyde, processed [Leica TP 1020 tissue processor; Leica Biosystems; Wetzlar, Germany], and paraffin embedded. All macroscopic

images were taken with a Canon Powershot SX 200 IX camera [Canon; Tokyo, Japan] at a standardized height and magnification. All microscopic images were obtained with a Nikon 90i Eclipse Microscope [Nikon, Inc.; Melville, NY]. All analysis was completed using NIS Elements Analysis Software [Nikon, Inc.; Melville, NY].

Macroscopic, Histochemical and Immunofluorescent Analysis

Macroscopic Analysis

Healing was assessed by imaging and quantifying eschar size throughout the 10-day healing period. Animals were briefly anaesthetized via isoflurane, and images were taken at a standardized height at 0, 2, 4, 6, 8 and 10 days post-wounding. To obtain percent decrease in eschar size at each time point, eschar area was measured and transformed via **Formula A**. Highest and lowest values were removed.

$$\left(\frac{A_x}{A_0} \times \frac{100}{1}\right) = \% \text{ original eschar size}$$

Formula A: Eschar size. A_x represents eschar area measurement taken at either day 2, 4, 6, 8 or 10. A_0 represents wound area measurement at day 0.

Histology

6 μ m paraffin sections were air dried overnight, deparaffinized, and rehydrated.

Hematoxylin and eosin (H&E) and Masson's Trichrome (MT) staining were performed using standard protocols. H&E stained sections taken from the center of the excised wounds were analyzed. If leading epidermal edges were fully fused, wounds were considered fully closed. Number of fully closed wounds per group were tallied and graphed accordingly. MT stained sections taken from the center of the excised wounds were used to determine epidermal thickness differences between wound edges and unwounded tissue (**Formula B**, below), total presence of collagen in the wound bed dermis (signal threshold settings used as the detection method), and dermal depth of wound bed (6 evenly-spaced depth measurements provided an average wound depth).

$$E_8 - U_4 = \textit{Difference in epidermal thickness}$$

Formula B: Difference in thickness between wound edges and unwounded epidermis. The average of 4 cross-sectional measurements of unwounded epidermis (U_4) was subtracted from the average of 8 epidermal cross-sectional measurements from the wound edges (E_8 ; 4 measurements taken on each side of the wound).

Immunofluorescent Staining

6 μ m sections were air dried overnight, deparaffinized, and rehydrated. Heat-mediated antigen retrieval of sections took place in citrate buffer (pH 6) at 65 $^{\circ}$ C for 30 minutes.

CD206 and NOS2/CD86 Staining: Sections were incubated in 0.2% Triton-x 100 for 30

minutes, 5% Normal Goat Serum for 40 minutes, and CD206 antibody (1:2500) [Abcam; Cambridge, MA], or CD86 and NOS2 antibody (1:50 each) [Santa Cruz Biotechnology; Dallas, TX] for 2 hours at room temperature. To visualize CD206, sections were incubated in goat-anti-rabbit Alexafluor 488 (1:250) [Abcam] for 60 minutes. To visualize NOS2 and CD86, sections were incubated in goat-anti-rabbit Alexafluor 594 or goat-anti-mouse Alexafluor 488, respectively (1:250) [Abcam] for 60 minutes. DAPI (1:10,000) was used to stain and identify nuclei. Analysis included determination of CD206, NOS2 and CD86 positive area via manual quantification. If a macrophage stained via the CD86/NOS2 co-stain produced a positive signal for both antibodies, it was classified as an M1 macrophage, however, if a macrophage only stained positive for CD86, it was classified as an M2b macrophage. CD31, Collagen I and Collagen III Staining: Sections were incubated with CD31 antibody (1:50), Anti-Collagen I antibody (1:200) or Anti-Collagen III antibody (1:500) [Abcam] for 1 hour at 37°C, biotinylated anti-rabbit IgG (1:1000) [Vector; Burlingame, CA] for 40 minutes, and counterstained with hematoxylin. Analysis included determination of CD31, Collagen I or Collagen III positive area using signal threshold settings as the detection method.

Statistics

All statistical analyses were performed using GraphPad Prism 6 [GraphPad Software Inc.; La Jolla, CA]. For all analysis normality tests were conducted. For all analyses apart from analysis of epidermal closure, statistical significance was determined using a 2-way

ANOVA followed by Tukey's multiple comparison post-hoc test. Statistical significance for analysis of epidermal closure was determined using Pearson's chi-square test. Statistical significance was defined as $P \leq 0.05$.

RESULTS

Eschar Size

Figure 1 highlights improvements in eschar size from the day of wounding (day 0) to the day of harvest (day 10). Significant differences in eschar size of aggravated wounds (**Figure 1A, C**) between groups at 10 days post-wounding was noted. Significant differences in percent eschar area between groups at days 2, 4, 6 and 8 were revealed in minimally-handled wounds (**Figure 1B, D**). Wound aggravation delayed reductions in eschar size regardless of treatment (**Figure 1A, C**). The coupling of minimal-handling (**Figure 1B, D**) with PAI-039 treatment proved most effective in reducing eschar size. In order to glean information on the cellular processes in the dermis and epidermis of the wound, further microscopic analysis was conducted.

Dermal Analyses

In order to observe the effects of PAI-039 treatment on the structure and composition of the sub-eschar layers of tissue, histochemical/immunohistochemical analysis was conducted on excised wound tissue 10 days post-wounding. Given the role of PAI-1 in

extracellular matrix remodeling, it was critical to investigate the role of PAI-039 treatment in the remodeling and restoration of the dermis. Collagen content (**Figure 2A, B**) was analyzed, however no significant differences in total collagen content were revealed as a result of PAI-039 treatment. Further collagen analysis (**Supplemental Figure 2**) revealed no significant differences in either collagen I or collagen III content as a result of PAI-039 treatment. Small but statistically significant differences were noted as a result of diabetes in collagen III content in aggravated and minimally-handled wounds. Information regarding depth of the dermal portion of the wound bed (**Figure 2C, D**) was gathered, however no significant differences were revealed as a result of treatment. These results indicate that the administration of PAI-039 did not facilitate repair in the dermis.

Wound Angiogenesis

To assess angiogenesis, immunofluorescent staining for CD31, an endothelial cell marker, was conducted on both aggravated (**Figure 3A**) and minimally-handled (**Figure 3B**) wounds 10 days post-injury. In both aggravated and minimally-handled WT wounds, PAI-039 significantly reduced CD31 content, indicating a negative effect on wound angiogenesis. In both aggravated and minimally-handled wounds, STZ-V displayed lower levels of CD31 than WT-V animals, indicating a negative effect of diabetes on angiogenesis. This reached statistical significance in minimally-handled wounds ($P=0.0038$) but not in aggravated wounds. As expected, given its effect on WT wounds,

PAI-039 treatment was not seen to restore CD31 content in either aggravated or minimally-handled STZ wounds.

Macrophage Infiltration

A co-stain of CD86 and NOS2, used to visualize M1 macrophages, revealed no significant differences in either aggravated or minimally-handled wounds (**Figure 4A,B**). CD206 staining, used to visualize M2a macrophages, revealed greater amounts of CD206 in aggravated diabetic wound beds compared to WT (**Figure 4C**) and were notably greater than in minimally-handled (**Figure 4D**) wounds regardless of diabetic state. CD206 levels were elevated in STZ-PAI-039 aggravated wounds, and although a significant main effect of treatment (PAI-039; $P=0.0283$) was revealed, no significant interactions were observed in minimally-handled wounds (**Figure 4D**). CD86, used to visualize M2b macrophages, revealed no significant differences in aggravated wounds (**Figure 4E**), and although a significant main effect of PAI-039 ($P=0.0049$) was revealed, no significant interactions were observed in minimally-handled wounds (**Figure 4F**). A sum total of all aforementioned macrophage quantification was calculated ($M1+M2a+M2b$), and no significant differences between groups were found (**Figure 4G,H**). In an effort to identify differences between aggravated and minimally-handled groups in terms of total macrophages, we undertook a series of unpaired t-tests. While no differences between WT groups were noted, this analysis revealed wound aggravation to exacerbate the

presence of macrophages, namely, as a significant increase in sum total macrophage content in aggravated wounds in both STZ and STZ with PAI-039 ($P=0.01$ and $P=0.05$ respectively) compared to minimally-handled wounds.

Epidermal Analyses

Wounds were either observed to have an uninterrupted epidermal layer, and were classified as fully closed, or were observed to have an interrupted epidermal layer over the wound bed, and were classified as 'not fully closed'. In WT aggravated wounds, 75% of wounds were fully closed 10 days post-wounding, compared with only 17% of STZ wounds (**Figure 5A**). This dramatic decrease in the amount of fully closed wounds is consistent with the negative effect of DM on wound repair (for review see 14). In animals with aggravated wounds, the administration of PAI-039 to WT mice resulted in all wounds being fully closed 10 days post-wounding. Importantly, a significant 300% increase in the number of fully closed wounds was observed in STZ-treated mice administered PAI-039. This data demonstrates that systemic reduction of PAI-1 levels markedly improved epidermal closure, thus, significantly improving diabetic wound healing in the presence of aggravation. In minimally-handled wounds (**Figure 5B**), 100% of WT-V wounds, and 80% of STZ-V wounds were fully closed. This finding emphasizes the importance of proper wound care in DM, but still illustrates the decreased wound healing ability of the diabetic population. Coupled with minimal handling, the administration of PAI-039 to STZ animals

allowed for closure of 100% of wounds at day 10 post-biopsy, a 20% improvement over STZ-V wounds. It has been demonstrated that DM negatively impacts cellular migration (4, 14, 15, 16, 17). It was hypothesized that the negative impact on cellular migration, particularly epidermal migration, would result in 'thicker' wound edges, and that the increase in epidermal closure with PAI-039 treatment was the result of improved epidermal migration. As can be seen in **Figure 6**, diabetes significantly impaired cell migration resulting in a thicker epidermis at the wound edges in both aggravated and minimally-handled wounds. PAI-039 treatment reversed this defect, resulting in comparable wound edge thicknesses to that of the WT cohort, regardless of level of handling.

DISCUSSION

One of the most prominent diabetic complications is the impaired ability to heal wounds (14, 18, 19, 20, 21). In the current investigation, decreases in epidermal closure and CD31 content, as well as an increase in epidermal edge thickness exemplify the impairments in healing associated with DM. In 2001, Chan and colleagues reported an accelerated rate of cutaneous wound healing in non-diabetic PAI-1 deficient mice (10). As previously mentioned, diabetic patients suffer from elevated levels of PAI-1 regardless of their level of glycemic control (10, 22). Due to the increased expression of PAI-1 in the diabetic population, as well as the aforementioned improvements in WT dermal wound repair

observed as a result of genetically-mediated knockout of PAI-1 (10, 22), investigations into the pharmacological reduction of PAI-1 levels for the restoration of diabetic wound healing were executed. To the best of our knowledge, this is the first study to analyze the effects of PAI-1 inhibition on wound repair in the diabetic state. Epidermal migration prompts wound re-epithelialization, and is consequently responsible for initiating the process of wound repair (23). Therefore, epidermal closure is the most important step in wound healing, as all other cellular proliferative and migratory events in the healing cascade follow epidermal closure (24). After wound closure, newly formed epidermis around the wound edges becomes hyperplastic due to the accumulation of cells that will migrate inward to close the wound gap (23). The thickness of this hyperplastic epidermal edge is expected to return to “nearly normal” by five to seven days post-injury in a non-diabetic environment (25). Consistent with the literature (14, 15, 16, 17), impairments in cellular proliferation and migration associated with diabetic wound healing, namely, attenuation of epidermal closure and increased epidermal thickness, have been shown in the current study. Importantly, the administration of PAI-039 to diabetic mice accelerated epidermal cellular migration, facilitating the restoration of epidermal thickness and total wound closure to a level consistent with WT wounds.

Given the prominent role of PAI-1 in regulating the fibrinolytic pathway and in the remodeling of the extracellular matrix, a significant impact of PAI-1 inhibition on fibrosis in the regenerating wounds was expected. This expectation was consistent with the previously reported attenuation of skeletal muscle collagen accumulation in diabetic mice

post-injury following PAI-039 treatment (12). What we found, however, was that PAI-039 treatment had no effect on dermal collagen content post-wounding, suggesting that the mechanism of PAI-039 action does not include mediating changes in the wound dermis.

Increases in PAI-1 expression, as well as alterations in the inflammatory response (including prolonged inflammation and defective macrophage function) are associated with impaired wound healing in DM (10, 17, 19, 22, 24, 26, 27, 28). In a genetically-induced diabetic mouse model (Akita), elevated PAI-1 levels attenuated collagen turnover and, ultimately, impaired macrophage infiltration into damaged skeletal muscle. This impairment in infiltration was restored with the systemic administration of PAI-039 (29).

Similarly, previous findings indicate an increase in lung exudate macrophages in the presence of PAI-1, while contrarily, the administration of a small molecule PAI-1 inhibitor attenuated renal macrophage migration in non-diabetic animals (30, 31). In the current investigation, PAI-039 treatment consistently decreased macrophage content (M2a and M2b) in minimally-handled wounds. This effect was not consistently observed in aggravated wounds, however differences between aggravated and minimally-handled wounds were also observed in other ways. A lesser overall amount of CD206, a marker of M2a macrophages with a wound-healing phenotype, was observed in minimally-handled wounds. As CD206 is indicative of repair and regeneration, this data, most notably in diabetic wounds, suggests that minimally-handled wounds may have progressed further in the wound healing process, and thus have less CD206 macrophages. Furthermore, when the sum total of all macrophages was compared between diabetic groups,

aggravated wounds displayed significantly more macrophages, regardless of treatment, than their minimally-handled counterparts. Taken together, however, as both aggravated and minimally-handled wounds still displayed great improvements in epidermal closure in the presence of these macrophages, it is likely that the changes in dermal macrophage content did not hinder the ability for PAI-039 to facilitate wound closure.

Peripheral vasculature complications are prevalent in patients with DM and pose as a significant hurdle to wound healing, as the formation of vasculature in the wound bed is critical for sustaining migrating cells and newly formed granulation tissue (32, 33).

Elevations in PAI-1 are seen to increase angiogenesis in cancerous tissue (34, 35). In non-cancerous tissue however, thickening of the basement membrane due to matrix metalloproteinase inhibition and a subsequent decrease in extracellular matrix turnover would be expected to impair the ability for endothelial cells to infiltrate the wound site. Mice overexpressing PAI-1 display significantly increased numbers of venous occlusions resulting in necrotic tails and swollen limbs (36) and it has been known for some time that there is a connection between depressed fibrinolysis (through elevated PAI-1) and venous thrombosis (37). Collectively, it is evident that PAI-1 is involved in the regulation and dysregulation of angiogenesis. In the present study, PAI-039 treatment failed to restore CD31 content and improve angiogenesis, however, epidermal wound closure was not hindered as a result. Although there is no animal model that fully reflects all aspects of diabetes and its complications in humans, STZ is the most widely used rodent diabetogenic chemical agent, and has been so since it was first described in 1963 (38). It

is for this reason that we chose the STZ-induced diabetic model, as it is the most widely studied, and would allow our results to be compared to the hundreds of published research studies investigating STZ-diabetic wound healing. Clearly, future studies investigating other animal models of diabetes (such as the db/db or NONcNZO10 polygenic mice; (39)) with and without PAI-039 would provide fruitful in further validating the efficacy of this inhibitor. What's more, given the lack of concomitant vascular disease in STZ-induced diabetic mice, further experimentation using other models of diabetes that more closely mimic the vascular aspects of diabetes may provide further insight into the impact of PAI-039 administration on angiogenesis.

An important consideration of the present study is the fact that two models of wound care were undertaken. The continuous disruption of the wounds with routine handling would be reflective of the repeated aggravation of foot ulcers in diabetic humans who continue to ambulate and wear improper footwear after suffering a dermal wound. In contrast, the minimal handling model exemplifies offloading, a critical component of diabetic wound care. Resting the wound site and minimizing pressure on the lesion or ulcer is, to date, the most effective intervention to promote diabetic wound healing, and is the first step in treatment of a neuropathic diabetic injury (13, 40). To avoid wound chronicity, activities that may lead to wound site aggravation should be avoided until proper healing has occurred (13). Consistent with these recommendations, wound site aggravation used in this study exacerbated the wound-healing deficit observed in STZ-diabetic mice. This is, to our knowledge, the first histological investigation that combines

pharmacological treatment with variations in wound care, highlighting the benefit to wound closure as a result of a reduction in wound-site aggravation. This study also identifies the effectiveness of PAI-039 treatment in promoting wound closure to non-diabetic levels even in the presence of wound aggravation. In fact, the greatest impact of PAI-1 inhibition on epidermal wound closure was observed in aggravated wounds. These findings are of particular clinical importance, as it is estimated that more than half of diabetic individuals with foot ulcers do not follow the recommended guidelines for wound monitoring and maintenance (41).

Coupled with the absence of apparent changes in the wound dermis, the restoration of epidermal closure and epidermal edge thickness in STZ-diabetic mice as a result of PAI-039 treatment reveals the mechanism of PAI-039 action to be primarily epidermal in origin. PAI-039 treatment facilitates the migration of epidermal cells, accelerating the initiation of epidermal migratory edges and facilitating wound closure, allowing the proceeding steps in the healing cascade to commence in the presence of diabetes.

FIGURES

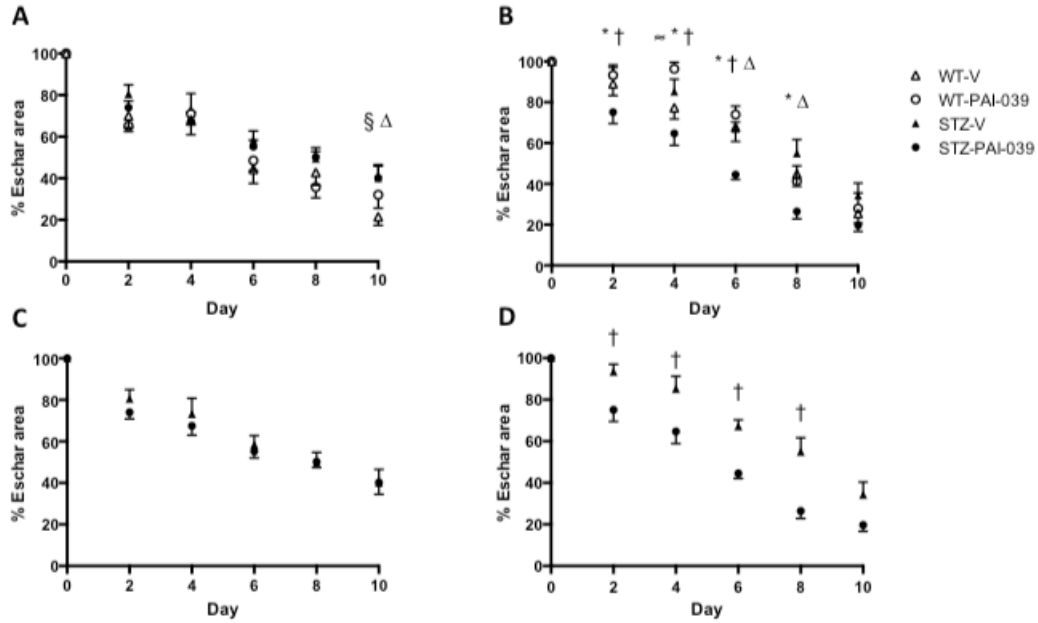


Figure 1. Post-wounding improvement in eschar size. Both aggravated wounds (A, C) and minimally handled wounds (B, D) for all WT and STZ groups (A, B) and only STZ groups (C, D) are displayed above. Wound aggravation (A, C) impedes reduction in eschar size regardless of treatment. The coupling of PAI-039 with minimal handling (B, D) results in maximal eschar size reduction. White triangle= WT-V; white circle= WT-PAI-039; black triangle= STZ-V; black circle= STZ-PAI-039. Significant differences between groups, as determined by Tukey's post hoc comparison (A, B) or Sidak's post hoc comparison (C, D), denoted by symbols: \approx WT-V and WT-PAI-039, \S WT-V and STZ-V, Δ WT-V and STZ-PAI-039, \dagger WT-PAI-039 and STZ-PAI-039, *STZ-V and STZ-PAI-039. All data presented as mean \pm SEM. n=4-6 for each point in A. n=4 for each point in C. n=10 for each point in B and D.

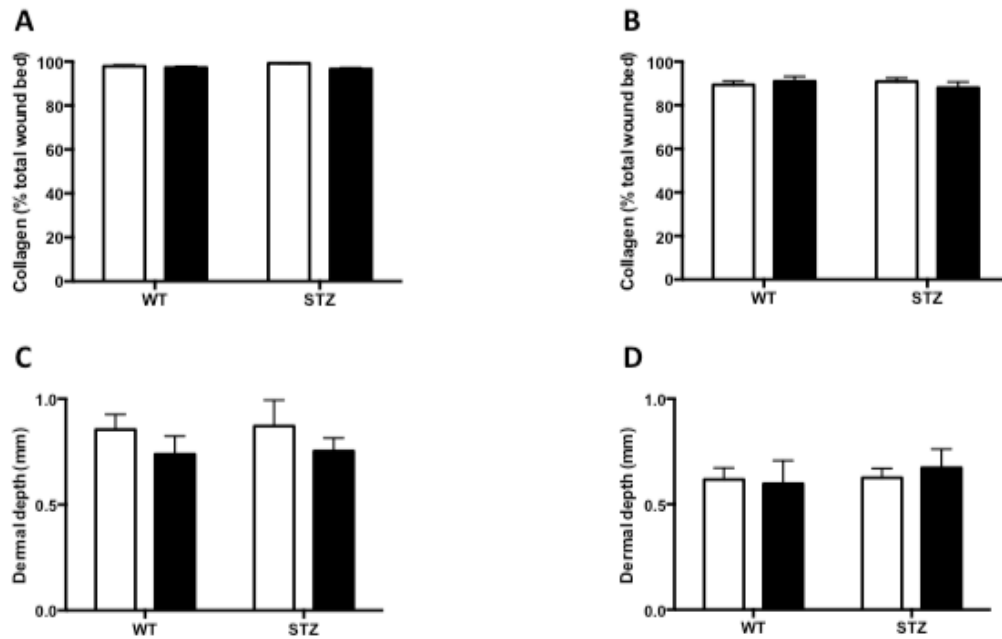


Figure 2. Presence of collagen and dermal depth of wound bed. No significant differences were seen in aggravated wounds (**A, C**) or minimally handled wounds (**B, D**), indicating that PAI-039 treatment had no effect on collagen remodeling or dermal healing. White bars indicate V treatment, black bars indicate PAI-039 treatment. All data presented as mean \pm SEM. n=4-6 for each bar in A. n=6-10 for each bar in B. n= 6-9 for each bar in C. n= 10 for each bar in D.

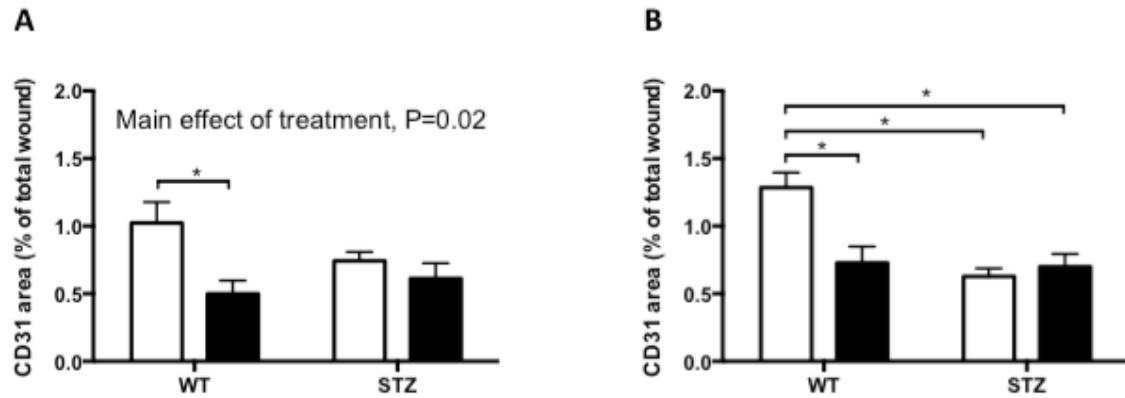


Figure 3. Presence of CD31 in wounds 10 days post-injury. In both aggravated wounds (**A**) and minimally handled wounds (**B**), PAI-039 administration significantly reduced WT CD31 content, indicating a negative effect on angiogenesis. In STZ animals, administration of PAI-039 did not rescue CD31 content in either aggravated (**A**) or minimally-handled (**B**) wounds. In aggravated wounds (**A**), two-way ANOVA revealed a significant effect of treatment ($P= 0.02$). White bars indicate V treatment, black bars indicate PAI-039 treatment. *Significant differences, as determined by Tukey's post hoc comparison. All data presented as mean \pm SEM. $n=4-6$ for each bar in A. $n=6-10$ for each bar in B.

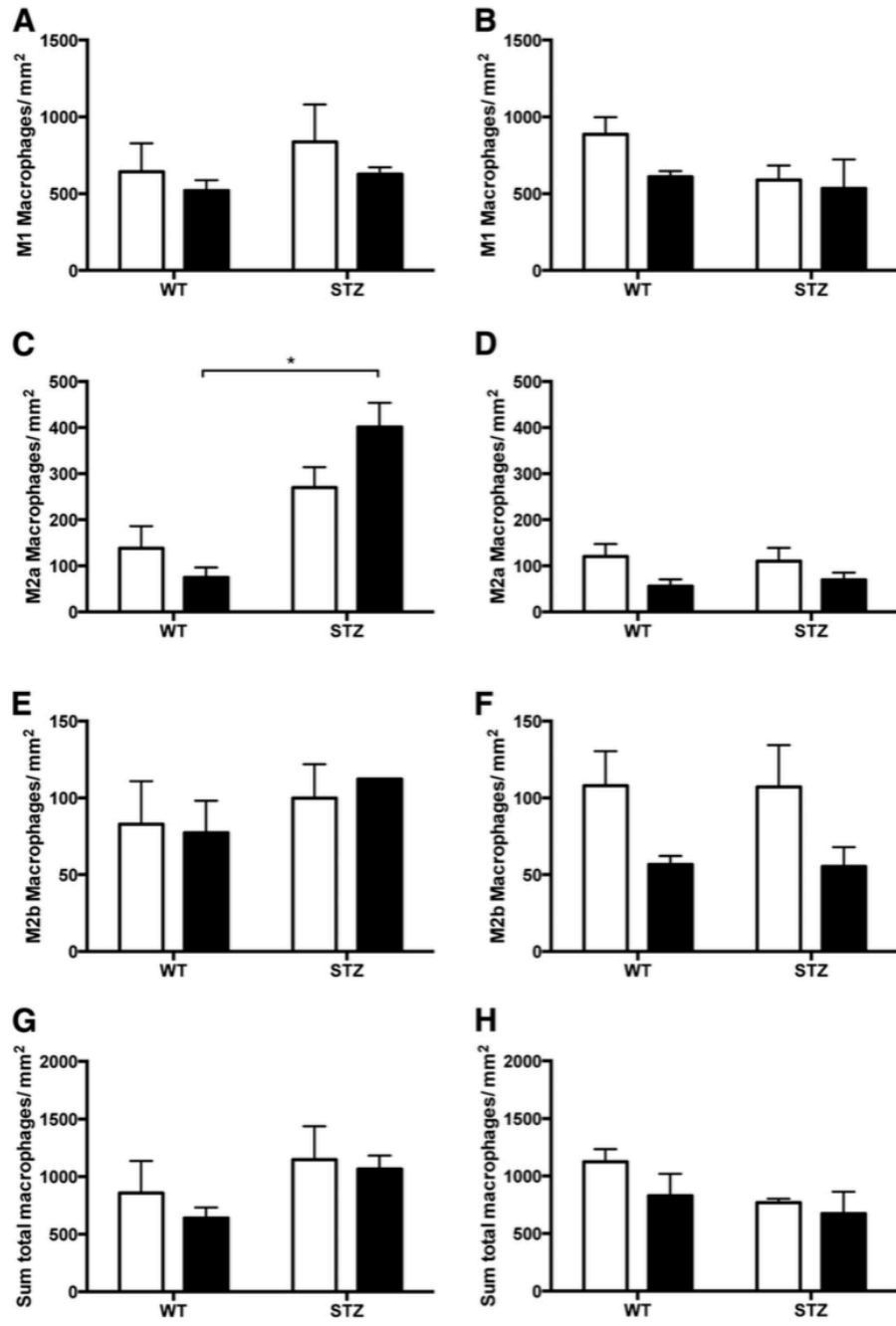


Figure 4. Presence of macrophages in wounds 10 days post-injury. No difference in M1 macrophage content is observed in aggravated (A) or minimally-handled wounds (B).

Aggravated STZ-PAI-039 wounds display elevated levels of M2a macrophages when compared to WT-PAI-039 wounds (**C**), while a significant effect of treatment ($P=0.028$) is observed in minimally-handled wounds (**D**). No difference in M2b macrophage content is observed in aggravated wounds (**E**), while a significant effect of treatment ($P=0.005$) is observed in minimally-handled wounds (**F**). No significant differences in sum total macrophage content were observed in aggravated (**G**) or minimally-handled wounds (**H**). White bars indicate V treatment, black bars indicate PAI-039 treatment. *Significant differences, as determined by Tukey's post hoc comparison. All data presented as mean \pm SEM. $n=3-11$ for each bar in Figure 4.

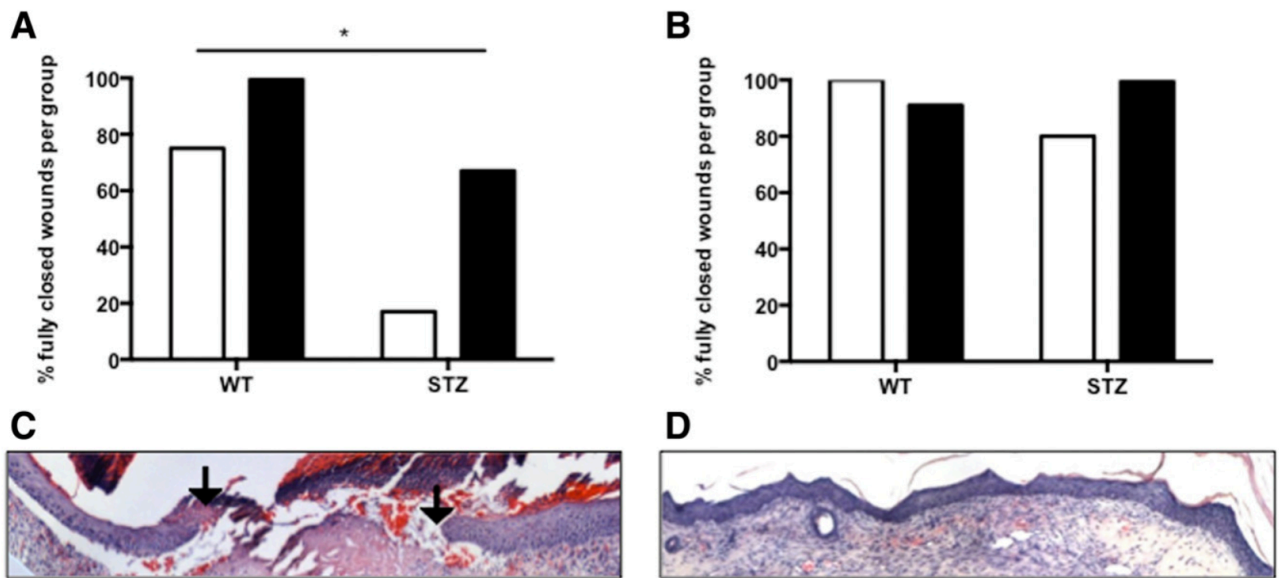


Figure 5. Epidermal closure of wounds 10 days post-injury. (A & B) Wounds were scored as either ‘fully closed’ or ‘not fully closed’. Percentage of wounds per group displaying complete epidermal closure are noted above. In aggravated wounds **(A)**, only 17 % of STZ-V wounds were fully closed, compared to 100% of WT-PAI-039 wounds. In minimally-handled wounds **(B)**, 80% of STZ-V wounds were fully closed, compared to 100% of WT-V and STZ-PAI-039 wounds. **(C)** H&E stain showing leading epidermal edges (arrows) that are not fully closed. **(D)** H&E stain showing fully closed epidermis. White bars indicate V treatment, black bars indicate PAI-039 treatment. *Significant differences, as determined by Pearson’s Chi-Square test. n=6-8 for each bar in A. n=10-12 for each bar in B.

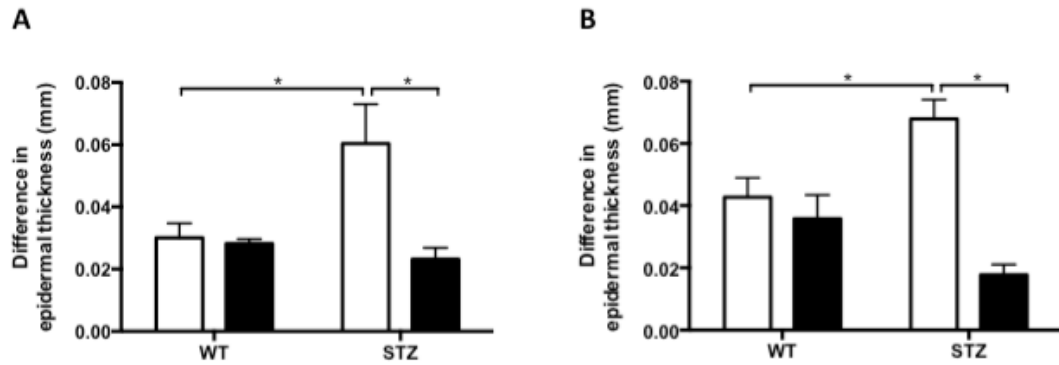
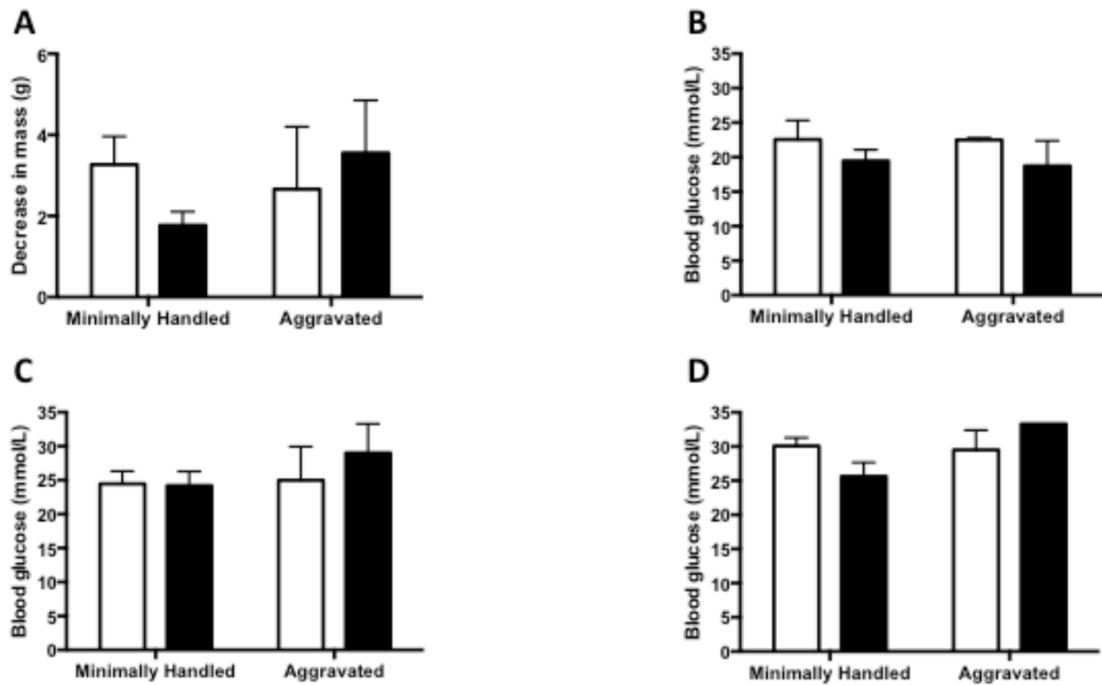
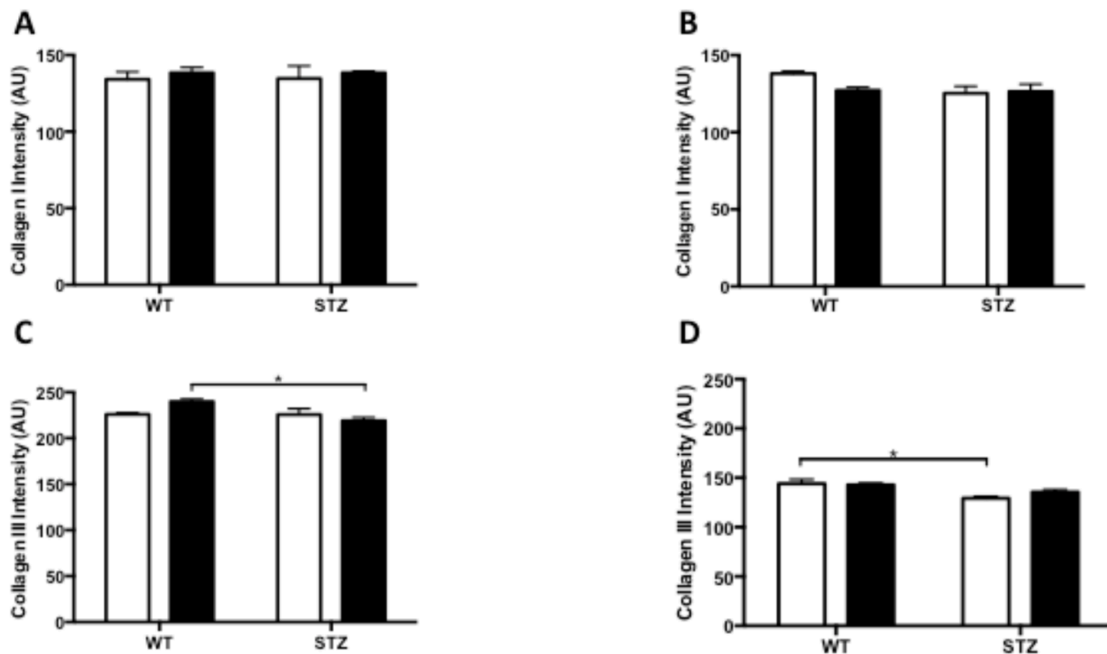


Figure 6. Difference in epidermal thickness between wound edges and unwounded epidermis. Both aggravated wounds (A) and minimally-handled wounds (B) display a significant thickening of wound edge epidermis as a result of diabetes, and a significant restoration of epidermal thickness as a result of PAI-039 treatment in STZ animals. White bars indicate V treatment, black bars indicate PAI-039 treatment. *Significant differences, as determined by Tukey's post hoc comparison. All data presented as mean \pm SEM. n=6-7 for each bar in A. n=10 for each bar in B.



Supplementary Figure 1. Comparison of mass and blood glucose levels of STZ animals, indicating no difference in body condition as a result of two different STZ administration protocols. No significant differences in body mass decreases from time of STZ injections to time of harvest (**A**) were observed as a result of two different STZ administration protocols ($P=0.49$). No significant differences in blood glucose levels at diabetic onset (**B**), pre-injury (**C**), or at time of harvest (**D**), were observed as a result of two different STZ administration protocols ($P=0.89$, $P=0.41$, and $P=0.11$, respectively). White bars indicate STZ-V animals, black bars indicate STZ-PAI-039 animals. All data presented as mean \pm SEM. $N=3-7$ for each bar in A, B, C and D.



Supplementary Figure 2. Presence of collagen I and collagen III in the wound bed, indicating no differences as a result of PAI-039 treatment. No significant differences were seen between V and PAI-039 treated animals in either aggravated (A, C) or minimally handled wounds, (B, D), indicating that PAI-039 treatment had no effect on collagen I content (A, B), or collagen III content (C, D). Upon analysis of collagen III content, small but statistically significant differences were seen as a result of diabetes in aggravated (C) and minimally handled (D) animals. White bars indicate V treatment, black bars indicate PAI-039 treatment. All data presented as mean \pm SEM. n=3-4 for each bar in A, C. n=6-10 for each bar in B, D.

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CHAPTER 3

STATIN THERAPY NEGATIVELY IMPACTS SKELETAL MUSCLE REGENERATION AND CUTANEOUS WOUND REPAIR IN TYPE 1 DIABETIC MICE

Authors: Irena A. Rebalka¹, Andrew Cao¹, Matthew J. Raleigh¹, Brandyn D. Henriksbo², Samantha K. Coleman¹, Jonathan D. Schertzer², Thomas J Hawke¹

1: Department of Pathology and Molecular Medicine,
McMaster University. Hamilton, ON, Canada.

2: Department of Biochemistry and Biomedical Sciences,
McMaster University. Hamilton, ON, Canada.

Corresponding Author: Thomas J. Hawke, Dept. of Pathology and Molecular Medicine, McMaster Univ., 4N65, Health Sciences Centre, 1200 Main St. W., Hamilton, Ontario L8N 3Z5, Canada (email: hawke@mcmaster.ca)

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PREFACE

Significance to thesis

Individuals with type 1 diabetes invariably develop complications including cardiovascular disease, myopathy and delayed wound healing. Type 1 diabetics are generally prescribed cholesterol-lowering 3-hydroxy-methylglutaryl coenzyme A reductase inhibitors (i.e. statins) to reduce their cardiovascular disease risk. Statins not only inhibit cholesterol biosynthesis but exhibit other pleiotropic effects, including reductions in the anti-fibrinolytic factor PAI-1. The purpose of this study is to investigate if statin administration, through its pleiotropic effects on PAI-1, can improve skin and muscle repair in a diabetic rodent model. As most of the diabetic population is already prescribed a statin to attenuate cardiovascular disease risk, investigating the therapeutic effects of statins on PAI-1 inhibition and tissue repair is of great interest.

Author contributions

Irena A. Rebalka: Designed the study, interpreted the results, performed animal care, sample collection, data collection and data analysis, wrote the manuscript.

Andrew Cao: Performed data collection, data analysis, and interpreted results.

Matthew J. Raleigh: Performed sample collection, data collection, data analysis, interpreted results and edited the manuscript.

Brandyn D. Henriksbo: Performed data collection, data analysis, interpreted results.

Samantha K. Coleman: Performed data collection.

Jonathan D. Schertzer: Designed the study and edited the manuscript.

Thomas J Hawke: Designed the study, interpreted the results and edited the manuscript.

ABSTRACT

Those with diabetes invariably develop complications including cardiovascular disease (CVD). To reduce their CVD risk, diabetics are generally prescribed cholesterol-lowering 3-hydroxy-methylglutaryl coenzyme A reductase inhibitors (i.e. statins). Statins inhibit cholesterol biosynthesis, but also reduce the synthesis of a number of mevalonate pathway intermediates, leading to several cholesterol-independent effects. One of the pleiotropic effects of statins is the reduction of the anti-fibrinolytic hormone plasminogen activator inhibitor-1 (PAI-1). We have previously demonstrated that a PAI-1 specific inhibitor alleviated diabetes-induced delays in skin and muscle repair. Here we tested if statin administration, through its pleiotropic effects on PAI-1, could improve skin and muscle repair in a diabetic rodent model. Six weeks after diabetes onset, adult male streptozotocin-induced diabetic (STZ) and WT mice were assigned to receive control chow or a diet enriched with 600 mg/kg Fluvastatin. Tibialis anterior muscles were injured via Cardiotoxin injection to induce skeletal muscle injury. Punch biopsies were administered on the dorsal scapular region to induce injury of skin. Twenty-four days after the onset of statin therapy (ten days post-injury), tissues were harvested and analyzed. PAI-1 levels were attenuated in diabetic regenerating tissue when compared to control-treated tissue, however no differences were observed in non-diabetic tissue as a result of treatment. Muscle and skin repair were significantly attenuated in Fluvastatin-treated STZ-diabetic mice as demonstrated by larger wound areas, less mature granulation tissue, and an increased presence of smaller regenerating muscle fibers. Despite attenuating PAI-1 levels

in regenerating diabetic tissue, Fluvastatin treatment impaired cutaneous healing and skeletal muscle repair in STZ-diabetic mice.

INTRODUCTION

Individuals with Type 1 Diabetes (T1D) are at a significantly elevated risk of developing atherosclerotic cardiovascular disease (CVD), and the risk of death from CVD is 1.7 times greater in diabetic individuals than their age-matched non-diabetic counterparts (Centers for Disease Control and Prevention (CDC), 2014). In 2011, 47% of diabetic patients were reported to have CVD, a statistic that has not changed since 1997 (Centers for Disease Control and Prevention (CDC), 2014). In 2014, this information led to the release of guidelines by the American College of Cardiology and the American Heart Association recommending that all diabetic individuals above the age of 40 be prescribed statins, regardless of the presence of other atherosclerotic CVD risk factors (Stone et al., 2013). As such, in 2014, 63% of adults diagnosed with diabetes were prescribed statins, and this number continues to rise (Gu et al., 2014).

Statins are a class of drugs that act to lower blood cholesterol levels by limiting the endogenous production of cholesterol and promoting clearance of cholesterol-containing lipoproteins through the inhibition of HMG Co-A reductase. This class of lipid-lowering agents has been proven as a key player in the primary and secondary prevention of atherosclerotic CVD events (Peto et al., 2002; Sacks et al., 1996). Evidence has suggested

that statins may have additional beneficial properties independent of lowering blood cholesterol. Indeed, the idea of the pleiotropic effects of statins has created quite a large and engaged discussion (Farmer, 2000; Liao and Laufs, 2005; Zhou and Liao, 2010). One of the pleiotropic effects of statins is the inhibition of plasminogen activator inhibitor-1 (PAI-1, also called SERPINE1) transcription via inhibition of the RhoA/ROCK axis (Bourcier and Libby, 2000; Mussoni et al., 2000; Ni et al., 2013; Sahebkar et al., 2016; Sato et al., 2008). PAI-1 is a serine protease inhibitor that acts to impede fibrinolysis, cell adhesion and migration, as well as extracellular matrix breakdown through the inhibition of tissue plasminogen activator and urokinase plasminogen activator (tPA and uPA, respectively) activity (Dellas and Loskutoff, 2005; Falanga, 2005; Kjølner et al., 1997; Li et al., 2003; Stefansson and Lawrence, 1996).

Numerous studies have reported elevated serum and tissue levels of PAI-1 in diabetic individuals (Krause et al., 2011a; Oishi, 2009; Rebalka et al., 2015), and recent studies from our lab have identified PAI-1 as a key component of delayed skeletal muscle and skin repair in T1D rodent models (Krause et al., 2011a; Rebalka et al., 2015). Furthermore, therapeutic strategies to reduce PAI-1 were effective in improving muscle regeneration and dermal wound closure in these animal models (Krause et al., 2011a; Rebalka et al., 2015). Thus, it was the purpose of this study to determine if statin therapy, through its pleiotropic actions on PAI-1, would restore skin and muscle repair in a T1D rodent model. Our findings indicate that although statin-treated regenerating T1D tissue displayed decreases in PAI-1 levels,

these reductions alone were insufficient to attenuate the impaired muscle and skin regeneration that characterizes those with T1D. In fact, in the current investigation, statin therapy impaired muscle regeneration and cutaneous wound healing in diabetic rodents.

RESEARCH DESIGN AND METHODS

Animal handling: Male C57BL6/J mice [Jackson Laboratories; Bar Harbor, ME] were provided enrichment material, chow and water ad libitum. Animal housing conditions were maintained at 21°C, 50% humidity and a 12h/12h light-dark cycle. Experimentation was approved by the McMaster University Animal Research Ethics Board, in accordance with the Canadian Council for Animal Care guidelines. At 10 to 12 weeks of age, animals were randomly assigned into control (WT) or Streptozotocin diabetic (STZ) groups, and Streptozotocin [one 150 mg/kg injection dissolved in sodium citrate buffer, pH 4.5; Calbiochem; Gibbstown, NJ] was administered to the STZ group. Four weeks after diabetes onset (blood glucose >14mM), STZ and WT mice were randomly assigned to receive diet enriched with 600 mg/kg Fluvastatin or control chow [D12081101, D12450K respectively; OpenSource Diets; New Brunswick, NJ]. Six weeks after the onset of diabetes, and two weeks after the start of diet administration, animals were injured. In order to induce skeletal muscle damage, 10 uM Cardiotoxin [Latoxan; Valence, France] was injected into one tibialis anterior (TA) muscle, as previously reported (Hawke et al., 2007). In order to induce cutaneous damage, each animal received two 6-mm-diameter full-thickness wounds via punch biopsy [Miltex; York, PA] in their dorsal scapular region, as previously

reported (Rebalka et al., 2015). Ten days post-injury (24 days of statin or control diet administration), animals were euthanized via cervical dislocation, and tissues were weighed, collected, and stored appropriately for future analyses.

Fluvastatin quantification: Fluvastatin was quantified in 50 μ L of mouse serum, where Atorvastatin (40 ng/ml) was spiked into collected serum for quantification of loss yield following sample preparation. Acetonitrile was added (3:1) to precipitate proteins out of solution and serum was centrifuged to remove particulates. Samples were nitrogen evaporated and suspended in 70:30 ratio of 10 mM ammonium acetate and 90% Acetonitrile, 10% 10mM ammonium acetate. Samples were loaded into the Agilent 2100 series HPLC [Agilent Technologies; Santa Clara, CA], separated using an Eclipse XDB-C18 column (3.5 μ m, 2.1x100mm) and detected with the Bruker micrOTOF II [Bruker; Billerica, MA]. The protocol mirrored that detailed by Agilent Technologies Application Notes by Srividya Kailasam; Determination of fluvastatin in plasma using the Agilent 6410B Triple Quadrupole LC/MS system coupled with the Agilent 1200 Series Rapid Resolution LC system.

Histochemical and immunofluorescent analysis of tibialis anterior skeletal muscle: 8 μ m frozen TA muscle sections were stained via embryonic myosin heavy chain (eMHC; signal threshold settings used as the detection method [neat; Abcam; Cambridge, MA]) Hematoxylin and eosin (H&E; used to quantify fiber area), F4/80 (used to assess total

macrophage content; number of F4/80 positive macrophages per unit area of muscle [1:100; Abcam]) and Alkaline phosphatase (used to assess vascular content; signal threshold settings used as the detection method) staining using standard protocols.

Macroscopic, histochemical and immunofluorescent analysis of cutaneous tissue: Healing was assessed on a macroscopic level by imaging and quantifying eschar size 0, 2, 4, 6, 8 and 10 days post-wounding as previously described (Rebalka et al., 2015). 6 μ m paraffin embedded sections of skin, taken from the center of the wound, were sectioned and air-dried overnight. Masson's Trichrome staining (to measure wound area and assess granulation tissue), CD31 staining (used to assess vascular content; [1:50; Abcam]), and F4/80 staining (used to assess total macrophage content; number of F4/80 positive macrophages per unit area of muscle [1:100; Abcam]) were conducted using standard protocols.

Histological classification of cutaneous wounds: All Masson's Trichrome images were assigned arbitrary numbers and examined without knowledge of the treatment or group (i.e. blinded analysis). Epidermal and dermal regeneration, as well as granulation tissue formation were evaluated. Unwounded wound margins were used as comparison for scoring (Table 1). This procedure was adapted from previous literature (Altavilla et al., 2001; Galeano et al., 2003).

Western blotting: TA muscle was homogenized, analyzed for protein concentration,

separated on acrylamide gels via electrophoresis and transferred to polyvinylidene fluoride membranes as previously described (Hawke et al., 2007). Western blotting was undertaken using an anti-PAI-1 [Abcam] primary antibody, appropriate horseradish peroxidase-conjugated secondary antibody, and visualized with the addition of chemiluminescent reagent [Thermo Scientific; Waltham, MA]. Images were acquired [Montreal Biotech Inc; Dorval, QC], and bands were quantified using Photoshop [Adobe; Mountain View, CA] with equal loading confirmed by Vinculin staining [Abcam].

Statistics: All statistical analyses were performed using Prism 7 software [GraphPad; La Jolla, CA]. For all analyses, apart from western blotting and eschar analysis, statistical significance was determined using an unpaired t-test. Statistical significance for the analysis of western blot bands as well as eschar size were determined using a two-way ANOVA followed by Tukey's multiple comparison test. Statistical significance was defined as $P < 0.05$. N for each experiment is noted in all figure legends.

RESULTS

Fluvastatin Content

Serum Fluvastatin analysis revealed that mice fed a control diet had no Fluvastatin in their serum ($0 \pm 0 \mu\text{M}$ serum Fluvastatin). A significant increase in serum Fluvastatin content was observed in Fluvastatin-treated groups when compared to control-diet-treated groups (Control diet $0 \pm 0 \mu\text{M}$ serum Fluvastatin vs Fluvastatin diet $4.463 \pm 0.795 \mu\text{M}$ serum Fluvastatin, $P=0.004$). No difference in serum Fluvastatin content was observed between

WT-Fluvastatin and STZ-Fluvastatin treated animals (WT-Fluvastatin serum 4.268 ± 1.239 μM Fluvastatin vs STZ-Fluvastatin serum 4.723 ± 1.139 μM Fluvastatin, $P=0.402$).

PAI-1 Content

Consistent with the literature, a significant effect of diabetes on muscle PAI-1 expression was observed in uninjured tissue, namely, an increase in PAI-1 protein expression (**Figure 1A**). Injured muscle tissue was also probed to investigate whether injury would instigate tissue and extracellular matrix turnover, mobilizing PAI-1 and, in turn, enhancing Fluvastatin's action. In this injured/regenerating tissue, Fluvastatin-treated tissue contained less PAI-1 than control treated tissue, but only in the presence of diabetes (**Figure 1C**). Representative blots for these investigations are shown in **Figure 1B** and **D**.

Cutaneous Regeneration

Figure 2A highlights improvements in eschar size from the day of wounding (day 0) to the day of harvest (day 10). Significant improvements in eschar size were observed in STZ-Fluvastatin treated wounds when compared to STZ-control wounds at day 4 post-injury. Ten days post-wounding, STZ-Fluvastatin treated eschar was 28% larger than WT-Fluvastatin treated eschar, suggesting delayed repair in STZ-Fluvastatin treated tissue. To further investigate the effects of Fluvastatin on the subeschar layers of tissue, microscopic histochemical analysis was conducted on excised wound tissue 10 days post-wounding. Wound area analysis of the dermis revealed smaller wounds in WT tissue with Fluvastatin

treatment (**Figure 2B**). Contrastingly, Fluvastatin treatment resulted in larger wound sizes in STZ mice 10-days post-wounding (**Figure 2C**). Histological scoring of granulation tissue (as per **Table 1**) mirrored results of the wound area analysis, showing improvements in tissue repair in WT wounds with Fluvastatin treatment (**Figure 2D**), and an impediment to repair with Fluvastatin treatment in STZ tissue (**Figure 2E**). Representative wound bed images with Masson's Trichrome staining are shown in **Figure 2F-I**.

Muscle Regeneration

When compared to muscle from control-treated rodents, the cross-sectional area of regenerating fibers was significantly reduced following Fluvastatin treatment in both WT (**Figure 3A**) and STZ (**Figure 3B**) muscle, indicating a delay in the regenerative capacity. To confirm the suspected delay in skeletal muscle repair, eMHC immunofluorescent analysis was conducted. eMHC is a myosin isoform that is present during the early stages of skeletal muscle regeneration. A greater presence of eMHC was observed in regenerating Fluvastatin-treated STZ muscle (**Figure 3H**). This effect was not seen in WT muscle, with trace amounts of eMHC present in both treatment groups (**Figure 3G**). This protracted expression of eMHC, which should reach peak expression at 2-3 days post-injury (Schiaffino et al., 2015), supports the conclusion that Fluvastatin treatment delays STZ-diabetic skeletal muscle repair.

Cutaneous Vascularity

To assess vascular density within the wound site, immunofluorescent staining for CD31 (PECAM-1), an endothelial cell marker, was conducted (**Figure 4**). Because statin treatment has been reported to systemically affect angiogenesis (Kureishi et al., 2000; Muck et al., 2004; Urbich et al., 2002; Weis et al., 2002), wound-site vascularity was expressed as a percentage of uninjured vascular density. This measure allows us to glean the effects of Fluvastatin on wound-site vascularity without being skewed by potential confounding effects of pre-injury vascular changes. Vascularity of the wound site was unchanged with Fluvastatin treatment in WT tissue (both at approximately 60% of their unwounded vascular density (**Figure 4A**)). STZ-diabetic wounds, however, displayed 16% less vascularity with Fluvastatin treatment (**Figure 4B**).

Muscle Vascularity

To assess vascular density in regenerating areas of skeletal muscle, alkaline phosphatase staining was conducted (**Figure 4**). Capillary to fiber ratio was investigated, as a higher capillary to fiber ratio allows a greater magnitude of perfusion to each muscle fiber. This comprehensive perfusion can be critical to optimal muscle repair. As in cutaneous repair, vascular density of regenerating muscle was expressed relative to uninjured vascular density to define the effects of Fluvastatin without being skewed by any potential confounding effects of pre-injury vascular changes. Interestingly, capillary to fiber ratio was increased with Fluvastatin treatment in WT tissue (**Figure 4C**), however, regenerating

diabetic muscle displayed no improvements in vascularity with Fluvastatin treatment (Figure 4D).

DISCUSSION

The majority of individuals diagnosed with diabetes are, or will invariably be, prescribed statins due to their known benefits to reduce CVD risk (Gu et al., 2014). While the cardiovascular benefits of statins in the T1D population are well known (Cholesterol Treatment Trialists' (CTT) Collaborators, 2008; Peto et al., 2002), this is the first study, to our knowledge, that investigates the effects of statins on changes in PAI-1 for the restoration of tissue repair in T1D. Due to the substantial number of diabetic individuals currently prescribed statins, defining the pleiotropic effects of statins in this population is of utmost importance. These investigations will not only lead to an understanding and reduction of drug side effects, but may also lead to prescription of new or alternate classes of compounds specifically targeting one pleiotropic stream of statin action.

Consistent with literature (Oishi, 2009), an increase in PAI-1 was seen in STZ-diabetic rodents with only seven weeks of diabetes in the current investigation. Although Fluvastatin did not modify resting muscle PAI-1 levels in WT or STZ-diabetic animals, we hypothesized that changes may be observed in regenerating tissue due to the mobilization and activation of the fibrinolytic system associated with perturbation of the tissue matrix. Interestingly, following injury, statin-treatment attenuated the presence of PAI-1, but only

in the presence of diabetes. As no differences in circulating Fluvastatin levels were observed between WT-Fluvastatin and STZ-Fluvastatin treated animals (4.268 ± 1.239 and 4.723 ± 1.139 μM Fluvastatin, respectively, $P=0.402$), a difference in treatment was not responsible for this effect.

Despite the attenuation in PAI-1 levels in STZ-diabetic tissue post-injury, impaired regeneration persisted in both the skin and muscle. This was an unexpected finding, as it was hypothesized that mitigating the presence of PAI-1 would increase matrix porosity; facilitating the infiltration of reparative cells into regenerating skin and muscle as previously reported (Krause et al., 2011a; Rebalka et al., 2015). Although it is evident that the primary mechanism of statin action in the current investigation is not via PAI-1 attenuation, the muscle and skin examined in the current investigation appears to display similar reparative profiles and changes with Fluvastatin administration. This seemingly parallel phenotype in both tissue types alludes to an alternative yet possibly equivalent mechanism of action.

To complement our findings of delayed diabetic tissue repair with statin treatment, macrophage quantification was completed. Despite the known anti-inflammatory effects of statins (Adami et al., 2012; Jain and Ridker, 2005), when compared to WT animals receiving statin treatment, STZ-diabetic animals treated with Fluvastatin displayed an overabundance of macrophages in both regenerating skin (STZ Fluvastatin 1.87 ± 0.28 %

total wound area vs WT Fluvastatin 0.64 ± 0.15 , $P=0.003$) and skeletal muscle (STZ Fluvastatin $251.40 \pm 53.18/\text{mm}^2$ vs WT Fluvastatin 136.20 ± 17.77 , $P=0.03$). Whereas a deficiency in macrophage content does not allow for adequate tissue repair, the overabundance of macrophages in the wound site is also detrimental to the healing process, chronically leaving wounds in the early phases of tissue repair (Goren et al., 2007). This increased presence of inflammatory cells further supports our aforementioned results of exacerbated impairments in diabetic repair with Fluvastatin treatment. As alterations in the inflammatory phase of wound repair, including prolonged inflammation, are already present in the diabetic environment (Fahey et al., 1991; Wetzler et al., 2000), further delay of inflammatory clearance with statin administration is less than ideal.

It has been observed that statin therapy improves cutaneous healing; having anti-inflammatory effects, accelerating tissue repair, and reducing lesion area in non-diabetic subjects (Adami et al., 2012; Suzuki-Banhese et al., 2015). Indeed, in the current investigation, improved cutaneous repair was observed in non-diabetic rodents. The opposing effect, namely larger wound area and decreased granulation tissue quality, were observed in the presence of diabetes. Due to the transient and dynamic presence of the phases of tissue repair, future extensions of this investigation should focus on exploring the effects of Fluvastatin therapy at various timepoints in the repair process to glean a more comprehensive understanding of the effect of Fluvastatin at each phase in tissue regeneration. Previous reports indicate that all beneficial effects of Atorvastatin, another

lipophilic statin, on cutaneous repair are no longer visible after day 7 post-injury (Suzuki-Banhese et al., 2015). Indeed, in the current investigation, eschar analysis of Fluvastatin-treated STZ rodents revealed a 20% reduction in eschar size at two days post-injury, and a significant 30% reduction in eschar size four days post-injury when compared to STZ control-treated rodents (**Figure 2a**). This result foreshadows a positive effect of statins on the underlying skin at these early timepoints.

Although the effect of statins on the repair of skeletal muscle have yet to be studied extensively, Trapani and colleagues have reported a decrease in myocyte fusion, as well as smaller, thinner myofibers resembling congenital myotonic dystrophy-affected myotubes with statin administration in vitro (Trapani et al., 2012). Furthermore, myoblasts treated with statin maintained higher eMHC levels and lower levels of adult myosin isoforms than their vehicle-treated counterparts (Trapani et al., 2012). This representation of delayed myotube maturation and muscle fiber formation is mirrored in the current investigation by the presence of smaller myofibers and a persistence of eMHC in diabetic muscle receiving statin therapy. Interestingly, Trapani and colleagues were able to reverse the deleterious effects of statins in vitro via mevalonate therapy; restoring the activity of the HMG CoA reductase pathway (Trapani et al., 2012). Similar to cutaneous repair, skeletal muscle repair is impaired in T1D (Gulati and Swamy, 1991; Krause et al., 2011b, 2013; Vignaud et al., 2007). Given information gleaned from previous work as well as this investigation, it is

hypothesized that Fluvastatin is inhibiting myoblast fusion in vitro via a mechanism independent of extracellular matrix remodeling.

We know that statins are effective in reducing the occurrence of macrovascular CVD events in diabetic individuals (Cholesterol Treatment Trialists' (CTT) Collaborators, 2008; Peto et al., 2002), but previous reports have implicated statins in impairing microvascular function in patients with T1D (Tehrani et al., 2013). Indeed, a decrease in vascular presence following cutaneous injury was observed in the current investigation. Contrastingly, statins have been shown to upregulate the presence vascular stimulants in the early phases of tissue repair in type 2 diabetic patients (Bitto et al., 2008; Farsaei et al., 2012). Due to the knowledge that statin therapy modifies angiogenesis (Kureishi et al., 2000; Muck et al., 2004; Urbich et al., 2002; Weis et al., 2002), wound vascularity was normalized to pre-injury vascular presence in the current investigation. No other studies, to our knowledge, look at these absolute wound vascular values, and it would be interesting to draw these comparisons from previously published papers. Given the lack of both cardiovascular and microvascular disease in STZ-diabetic rodents, further experimentation using other preclinical models of T1D that more closely mimic the vascular aspects of diabetes may provide further insight into the impact of statin therapy on microcirculation and its effects on tissue repair. This would allow us to more accurately extrapolate our findings to the clinical diabetic population.

Overall, although Fluvastatin was shown to attenuate PAI-1 levels in regenerating diabetic tissue, impaired cutaneous and skeletal muscle regeneration persisted. In contrast to our hypothesis, the use of statin therapy as a means to inhibit PAI-1 and alleviate impaired cutaneous and muscle regeneration is likely ineffective in those with T1D. Many diabetic individuals, already exhibiting impaired cutaneous and skeletal muscle regeneration, are prescribed statins. As such, the detrimental effects of Fluvastatin on tissue repair observed in the current investigation warrant further investigation.

TABLES AND FIGURES

Table One

Histological scores of wounds

| Scores | Epidermal and dermal regeneration | Granulation tissue thickness |
|--------|--|-------------------------------|
| 1± | Little epidermal and dermal organization. Very thick epidermis. Substantial dermal edema. | Thin granular layer. |
| 2± | Moderate epidermal and dermal organization. Moderate epidermal thickness. Mild dermal edema. | Moderate granular layer. |
| 3± | Complete remodeling of epidermis and dermis. Thin, organized epidermis. Little dermal edema. | Thick granular layer. |
| 4± | Complete remodeling of epidermis and dermis. Epidermis appears as uninjured. | Very thick granulation layer. |

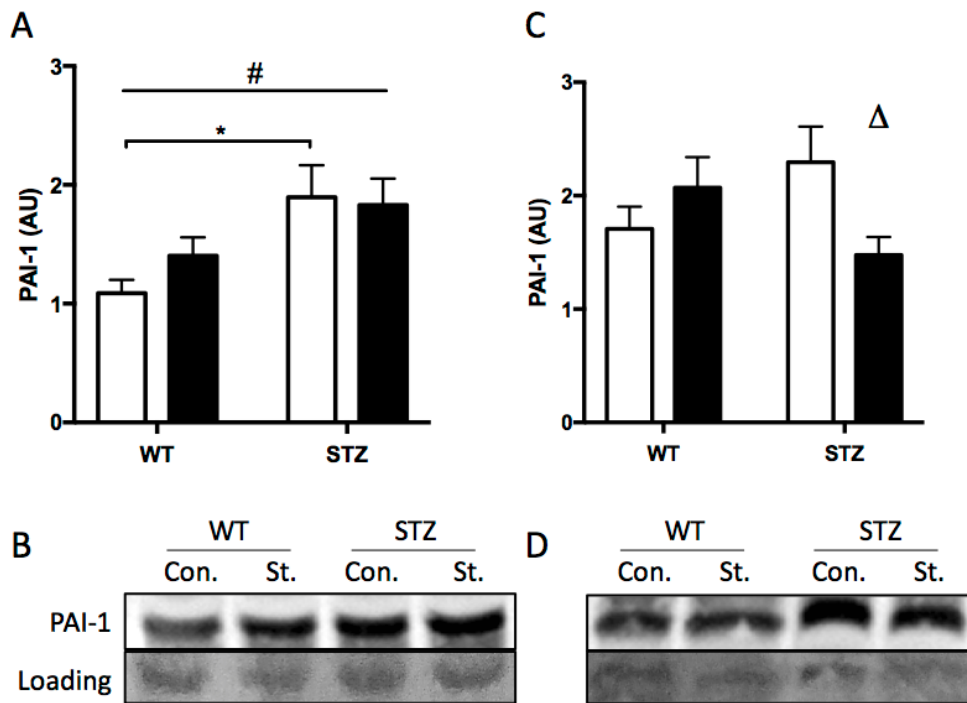


Figure 1. Tissue PAI-1 levels are attenuated by Fluvastatin post-injury, but only in the presence of STZ-diabetes. Two-way ANOVA reveals a significant main effect of diabetes (# = $P < 0.05$) on PAI-1 levels in uninjured tissue (A). A representative blot is shown in B. Injured tissue was probed to investigate whether injury and the instigation of tissue turnover was necessary to observe the statins inhibitory PAI-1 effects. Indeed, regenerating tissue shows attenuation of PAI-1 levels with Fluvastatin treatment, but only in the presence of diabetes (C; $\Delta = P < 0.05$). A representative blot is shown in D. White bars indicate control treatment. Black bars indicate Fluvastatin treatment. *Significant differences ($P < 0.05$), as determined by Tukey's post-hoc test following two-way ANOVA. All data presented as mean \pm SEM. $n = 4-6$ for each bar.

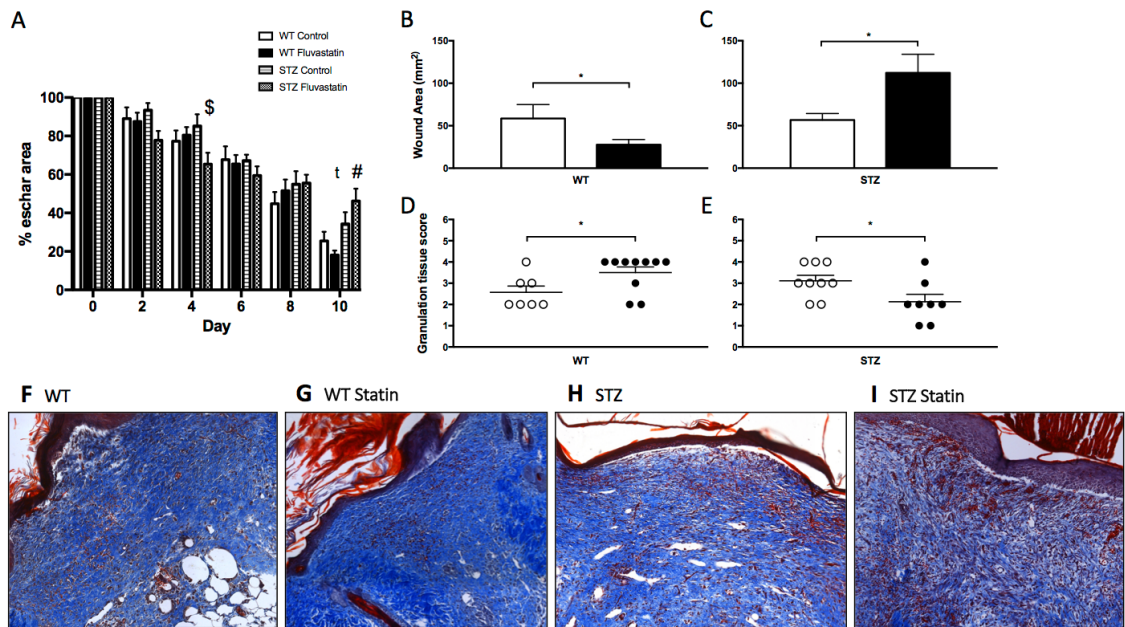


Figure 2. WT cutaneous regeneration is improved by statin treatment, while STZ-diabetic regeneration is impaired by statin treatment. (A) Post-wounding improvements in eschar size for all groups are displayed. \$ indicates significant difference ($P < 0.05$) between STZ Control and STZ Fluvastatin. # indicates significant difference ($P < 0.05$) between WT Control and STZ Fluvastatin. t indicates trending differences ($P = 0.08$) between STZ Control and STZ Fluvastatin. Fluvastatin administration results in a decrease in wound area in WT wounds (**B**), whereas the opposite effect is seen in STZ diabetic wounds (**C**). Similarly, histological assessment of wound healing in WT (**D**) and diabetic (**E**) wounds 10 days after wounding (according to the histological scoring of Table 1) reveal the same effects; an improvement in WT wound repair and a deleterious effect on STZ wound repair with Fluvastatin therapy. (**F-I**) Representative images of wound specimens at ten

days post-wounding are depicted and labeled according to group. White bars/circles indicate control treatment. Black bars/circles indicate Fluvastatin treatment. *Significant differences ($P \leq 0.05$), as determined by two-way ANOVA (A) or unpaired t-test (B-E). All data presented as mean \pm SEM. n=10 for each bar in A, n=10-12 for each bar in B-C, n=7-10 for each bar in D-E.

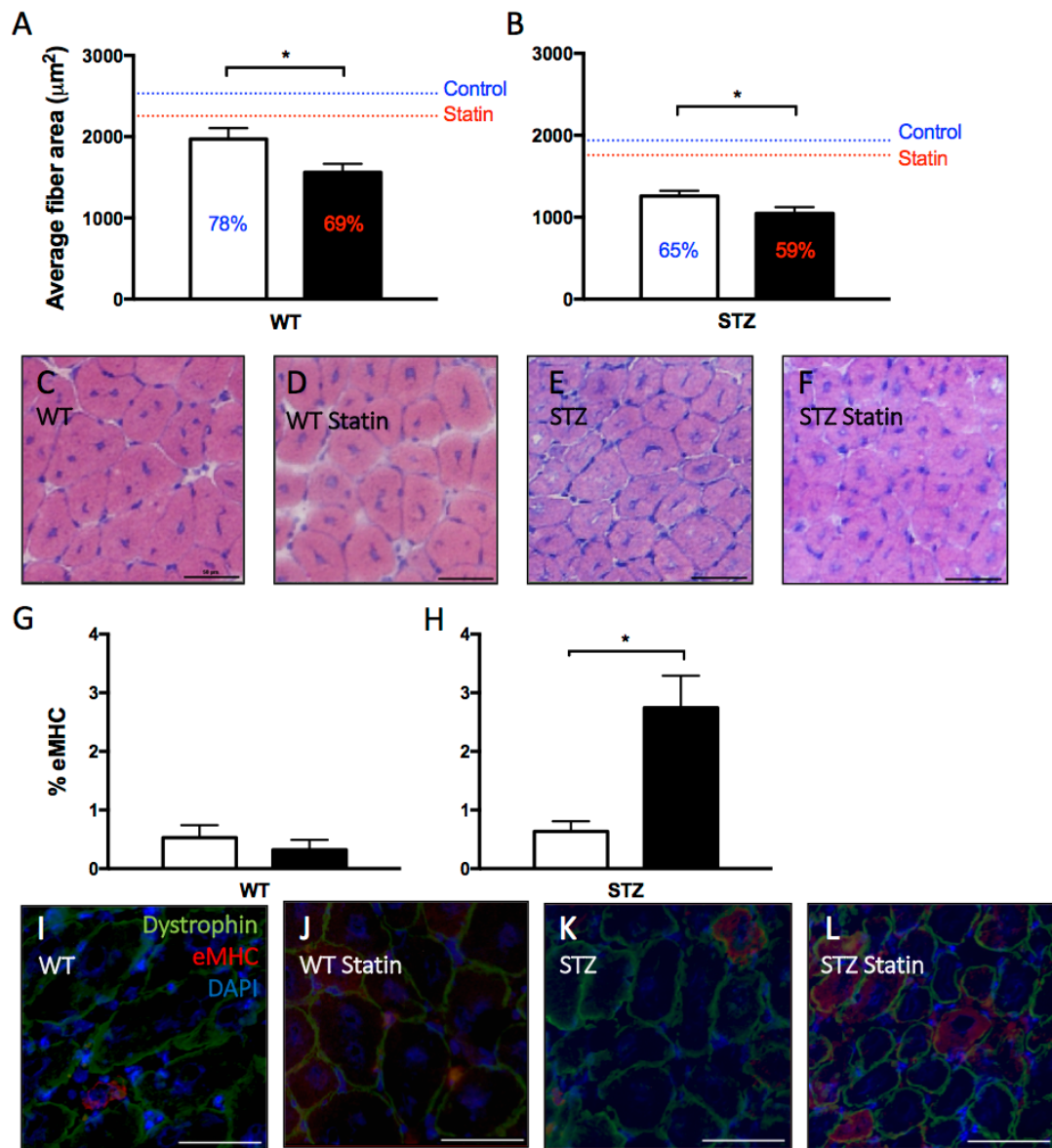


Figure 3. Statin therapy delays STZ-diabetic skeletal muscle regeneration. In both WT (A) and STZ (B) skeletal muscle, smaller average myofiber area, signifying delayed regeneration, is observed in Fluvastatin treated muscle when compared to the respective control. Percent of uninjured muscle fiber size is displayed on each bar, and mean uninjured fiber sizes are indicated by a line for reference. Representative images

depicting fiber size of regenerating myofibers are shown in **C-F**, and are labeled by group. Embryonic myosin heavy chain (eMHC), a myosin isoform present early on in myofiber development, is elevated in Fluvastatin-treated STZ muscle (**H**), but not Fluvastatin-treated WT muscle (**G**) ($P=0.26$) when compared to its control-treated counterpart. (**I-L**) Representative eMHC images, indicating an abundance of eMHC (red staining) in regenerating diabetic statin myofibers, are labeled according to group. White bars indicate control treatment. Black bars indicate Fluvastatin treatment. *Significant differences ($P<0.05$), as determined by unpaired t-test. All data presented as mean \pm SEM. $n=4-6$ for each bar.

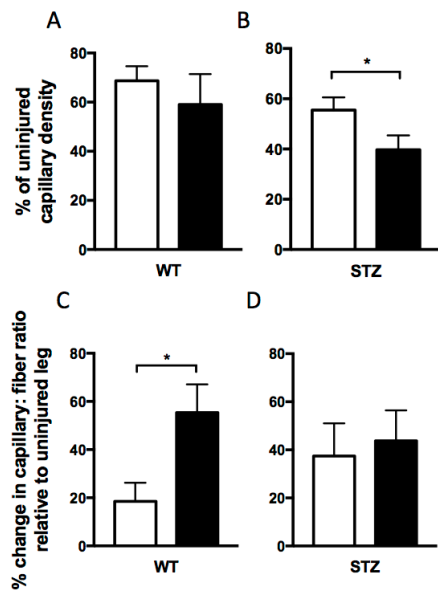


Figure 4. Fluvastatin is deleterious to STZ diabetic cutaneous vascular regeneration and does not improve diabetic skeletal muscle revascularization. Expression of wound-site vascular content as a percentage of uninjured vascular content uncovers an approximate 60% regeneration of vascularity in both control and Fluvastatin treated WT groups **(A)**. In STZ-diabetic wounds, a 16% decrease in the restoration of vascularity is observed with Fluvastatin treatment when compared to control treatment **(B)**. Expression of changes in capillary: fiber ratio relative to uninjured muscle uncovers an exacerbated increase in vascular perfusion with Fluvastatin administration in WT groups **(C)**. When normalized to the uninjured ratio, vascularity is not improved in regenerating STZ diabetic muscle **(D)**. White bars indicate control treatment. Black bars indicate Fluvastatin treatment.

*Significant differences ($P < 0.05$), as determined by unpaired t-test. All data presented as mean \pm SEM. $n=6-10$ for each bar in A-B, $n=5-6$ for each bar in C-D.

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CHAPTER 4

STATIN THERAPY ALTERS LIPID STORAGE IN DIABETIC SKELETAL MUSCLE

Authors: Irena A. Rebalka¹, Matthew J. Raleigh¹, Laelie A. Snook², Alexandra N Rebalka¹, Rebecca E.K. MacPherson², David C Wright², Jonathan D. Schertzer³, Thomas J Hawke¹

1: Department of Pathology and Molecular Medicine, McMaster University. Hamilton, ON, Canada.

2: Human Health and Nutritional Sciences, University of Guelph. Guelph, ON, Canada.

3: Department of Biochemistry and Biomedical Sciences, McMaster University. Hamilton, ON, Canada.

Corresponding Author: Thomas J. Hawke, Dept. of Pathology and Molecular Medicine, McMaster Univ., 4N65, Health Sciences Centre, 1200 Main St. W., Hamilton, Ontario L8N 3Z5, Canada (email: hawke@mcmaster.ca)

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PREFACE

Significance to thesis

The primary goal of this study was to observe the effects of statin administration on skeletal muscle health in type 1 diabetic rodents. Given that statins have a variety of pleiotropic effects beyond their capacity to modulate PAI-1 levels, they are a widely-prescribed pharmaceutical for T1D patients, with 62.8% of diabetic adults prescribed statins in the United States in 2014. The most common complaint surrounding the use of statins is muscle pain, complemented by skeletal muscle damage upon clinical investigation. Due to the vast prescription of statins as well as the susceptibility for T1D skeletal muscle to damage even in the absence of statin therapy, we treated T1D mice with statins to observe their effects on resting skeletal muscle health and metabolic capacity.

Author contributions

Irena A. Rebalka: Designed the study, interpreted the results, performed animal care, performed sample collection, data collection and data analysis, wrote the manuscript.

Matthew J. Raleigh: Performed sample collection, data collection, data analysis, interpreted results and edited the manuscript.

Laelie A. Snook: Performed data collection and data analysis and edited the manuscript.

Alexandra N Rebalka: Performed data collection and data analysis.

Rebecca E.K. MacPherson: Performed data collection and data analysis and edited the manuscript.

David C Wright: Designed the study and edited the manuscript.

Jonathan D. Schertzer: Designed the study and edited the manuscript.

Thomas J Hawke: Designed the study, interpreted the results and edited the manuscript.

ABSTRACT

While statins significantly reduce cholesterol levels and thereby reduce the risk of cardiovascular disease, the development of myopathy with statin use is a significant clinical side-effect. Recent guidelines recommend increasing inclusion criteria for statin treatment in diabetic individuals; however, the impact of statins on skeletal muscle health in those with diabetes (who already suffer from impairments in muscle health) is ill-defined. Here we investigate the effects of Fluvastatin treatment on muscle health in wild-type and streptozotocin (STZ)-induced diabetic mice. Wild-type and STZ-diabetic mice received diet enriched with 600 mg/kg Fluvastatin or control chow for 24 days. Muscle morphology, intra and extracellular lipid levels, and lipid transporter content was investigated. Our findings indicate that short-term Fluvastatin administration induced a myopathy that was not exacerbated by the presence of STZ-induced diabetes. Fluvastatin significantly increased ectopic lipid deposition within the muscle of STZ-diabetic animals, findings that were not seen with diabetes or statin treatment alone. Consistent with this observation, only Fluvastatin-treated diabetic mice downregulated protein expression of lipid transporters FAT/CD36 and FABPpm in their skeletal muscle. No differences in FAT/CD36 or FABPpm mRNA content were observed. Altered lipid compartmentalization resultant of a downregulation in lipid transporter content in STZ-induced diabetic skeletal muscle was apparent in the current investigation. Given the association between ectopic lipid deposition in skeletal muscle and the development of insulin-resistance, our findings

highlight the necessity for more thorough investigations into the impact of statins in humans with diabetes.

INTRODUCTION

Statins inhibit 3-hydroxy-3-methylglutaryl-CoA reductase, thereby inhibiting cholesterol biosynthesis and LDL formation with great efficacy. As reductions in circulating LDL cholesterol levels greatly reduce atherosclerotic cardiovascular disease (ASCVD) risk, statins are among the most widely prescribed pharmaceuticals in the world (Walley et al. 2005; Rotermann et al. 2014; Gu et al. 2013). While considerable evidence supports the benefits of these cholesterol-lowering drugs in reducing ASCVD risk, statin therapies have also been associated with serious side-effects, such as myopathy. Recent reports estimate up to 25% of statin users experience some form of myopathic/myalgia symptoms, ranging from muscle soreness to severe rhabdomyolysis (Nichols and Koro 2007; Stone NJ et al. 2013; Furberg and Pitt 2001). Despite these concerns, the American College of Cardiology (ACC) and American Heart Association (AHA) 2013 guidelines recommend that all diabetic individuals over the age of 40 be prescribed statins regardless of their ASCVD risk (Stone et al. 2013).

Muscle from those with Type 1 diabetes mellitus (T1DM) exhibits myopathic features; a complication termed diabetic myopathy (Krause et al. 2011). Decreases in muscle mass (Andersen et al. 2004) and reduced myofiber size (Jakobsen and Reske-Nielsen, 1986), translating to muscle weakness and reduced physical capacity (Andersen 1998;

Poortmans et al. 1986) have all been shown in individuals with T1DM. While new statin therapy guidelines were implemented to reduce ASCVD risk in diabetic individuals, it has not yet been elucidated if the combination of T1DM and statin administration exacerbates the myopathy resultant from each factor alone. To that end, we fed wild-type (WT) and streptozotocin-treated mice (T1DM mouse model) a control or Fluvastatin-enriched diet for a period of 24 days and harvested their skeletal muscle to assess metabolic and morphologic characteristics.

RESEARCH DESIGN AND METHODS

Animal handling and tissue collection

Experimentation was approved by the McMaster University Animal Research Ethics Board, in accordance with the Canadian Council for Animal Care guidelines. At 10-12 weeks of age, male C57BL6/J mice [Jackson Laboratories, Bar Harbor, ME, USA] were randomly assigned into WT or streptozotocin-diabetic (STZ; single 150 mg/kg intraperitoneal STZ injection [Calbiochem, San Diego, CA, USA]) groups. Six weeks after diabetes onset (blood glucose >14mM), animals were further subdivided and assigned to receive either diet enriched with 600 mg/kg Fluvastatin or control chow [D12081101, D12450K respectively; OpenSource Diets, New Brunswick, NJ, USA]. Diet was administered *ad libitum* for 24 days, after which all animals were euthanized and tissues were embedded and/or snap frozen for later analyses.

Histochemical and immunofluorescent analysis

Frozen TA (tibialis anterior) muscle sections were stained via hematoxylin-eosin (H&E) to quantify centrally-located nuclei, necrotic fibers and myofiber areas. Laminin and dystrophin (both 1:250; Abcam, Cambridge, UK) fluorescent co-stain was used to determine the number of split myofibers. Oil Red O (ORO) staining was used to quantify intramyocellular lipid density. Analysis of perilipin (1:200; Cell Signaling, Danvers, MA, USA) was used for determination of ectopic lipid droplet number and size per unit area. All imaging and analysis was undertaken on a Nikon 90i microscope using Nikon NIS-Elements ND2 software [Melville, NY, USA].

Western blotting

GPS (gastrocnemius, plantaris, soleus) muscle was homogenized in NP40 Lysis Buffer supplemented with phenylmethylsulfonylfluoride and Protease Inhibitor Cocktail. Western blotting was undertaken as previously described (Krause et al. 2009) using anti-FAT/CD36 [Santa Cruz, Dallas, TX, USA] and anti-FABPpm [generous gift from J. Calles-Escandon, Wake Forest University, NC, USA] primary antibodies and appropriate horseradish peroxidase-conjugated secondary antibodies. Bands were quantified via densitometry [Alpha Innotech Fluorchem HD2, ThermoFisher Scientific, Waltham, MA, USA] with equal loading confirmed by PonceauS staining [Sigma-Aldrich, St. Louis, MO, USA].

Real-time PCR

Total RNA was extracted from GPS using Trizol reagent and reversed transcribed into cDNA. Changes in mRNA expression were determined using real time qPCR and Taqman gene expression assays for mouse CD36 (Mm_00432403_m1), FABPpm (Mm00494703_m1), and GAPDH (4352932E) [Applied Biosystems, Foster City, CA, USA] as previously described (Castellani et al. 2014).

Statistics

All statistical analyses were performed using Prism 6 [GraphPah Software, La Jolla, CA, USA]. Statistical significance was determined via unpaired t-test, and defined as $p \leq 0.05$.

RESULTS

Analysis of myopathy

Centrally-nucleated, necrotic and split myofibers were evaluated and summated as 'myopathic fibers' to characterize myopathy. Fluvastatin administration increased total myopathic fibers in TA muscle from both WT (**Figure 1A**) and STZ-treated (**Figure 1B**) mice. Although myopathy was observed in both WT and STZ muscle as a result of Fluvastatin administration, no difference in the severity of myopathy was noted between WT and STZ muscle (**Figure 1C**). When compared to control treated muscle, fiber cross-sectional area was significantly reduced following Fluvastatin treatment in both WT (**Figure 1I**) and STZ (**Figure 1J**) muscle, supporting a greater presence of atrophied,

myopathic fibers. Once again, no differences in myofiber area were noted between WT and STZ muscle as a result of Fluvastatin treatment (**Figure 1K**).

Extracellular and intramyocellular lipid analysis

Histological examination by H&E staining revealed transparent globules adjacent to muscle fibers that appeared to be ectopic lipid deposits, most notably in Fluvastatin treated groups. To verify this hypothesis, perilipin (member of a family of proteins that associate with the surface of lipid droplets/adipocytes) analysis was conducted.

Quantification demonstrated a significant elevation in ectopic lipid content and size within STZ-diabetic muscle following Fluvastatin administration, an effect that was not observed in other groups (**Figure 2A-D**).

With respect to intramyocellular lipid droplets (IMCLs), no differences in content were evident in WT muscle with treatment (**Figure 2E**). In contrast, Fluvastatin-treated STZ-diabetic mice exhibited significantly reduced IMCL content relative to their diabetic counterparts (**Figure 2F-H**).

Lipid transporter analysis

When compared to control treated muscle, no differences in FAT/CD36 content were apparent in WT muscle as a result of Fluvastatin administration (**Figure 2I**), while STZ-diabetic muscles displayed decreases in FAT/CD36 content with Fluvastatin treatment (**Figure 2J**). FABPpm analysis revealed elevations in protein content in WT muscle as a

result of Fluvastatin administration (**Figure 2K**), and, mirroring FAT/CD36 analysis, decreases in FABPpm content in Fluvastatin-treated STZ muscle (**Figure 2L**).

In order to ascertain if Fluvastatin treatment was affecting the transcriptional regulation of lipid transporters, mRNA analysis was conducted. No differences in FAT/CD36 or FABPpm mRNA content were observed between groups (**Figure 2M-Q**).

DISCUSSION

Recent ACC/AHA guidelines recommend the widespread use of statin therapy as a primary means to reduce ASCVD risk in all persons over 40 years old with diabetes mellitus (Stone et al. 2013). While statin therapy lowers ASCVD risk, these HMG-CoA reductase inhibitors are also associated with a risk of myotoxicity (Nichols and Koro 2007), and their impact on skeletal muscle health in those with T1DM has not yet been fully examined. Many rodent studies investigating the impact of statins on myopathy have utilized long-term administration or statins with a stronger myotoxic profile (Pecoraro et al. 2014). The present study, however, utilized short-term exposure of Fluvastatin; a statin generally agreed to have a low clinical risk of myotoxicity (Bruckert et al. 2005). Despite a low myotoxic profile, Fluvastatin has been reported to activate the NLRP3 inflammasome and induce adipose tissue insulin resistance in obese animals at levels comparable to, or greater than Lovastatin, Atorvastatin and Simvastatin (Henriksbo et al. 2014).

Here we report that short-term Fluvastatin administration resulted in a myopathic phenotype, the characteristics of which were not exacerbated with STZ-induced diabetes. Statin-treated diabetic mice did however demonstrate a significant increase in the presence of skeletal muscle ectopic lipid droplets, an observation that was not present in statin-treated, non-diabetic mice. In support of this observation, statin-treated diabetic mice displayed significant decreases in lipid transporter content within their skeletal muscle. These findings raise the possibility that statin administration in T1DM humans could contribute to the impairments in skeletal muscle health, including insulin resistance (Borén et al. 2013).

T1DM negatively impacts skeletal muscle health, a complication referred to as diabetic myopathy (Krause et al. 2011). Here we chose an early time point of T1DM progression, where certain myopathic characteristics (centrally-located nuclei, split and necrotic fibers) would be minimal, thus allowing us to assess if statin treatment exacerbated the progression of diabetic myopathy. In contrast to our hypothesis, statin-treated mice did not display a worsened myopathy in the presence of diabetes. Future studies using a more established diabetic myopathy, or a statin with a stronger myotoxicity profile (e.g. Simvastatin (Bruckert et al. 2005)), would address this question more fully.

While we hypothesized that statin administration would worsen the quality of diabetic muscle, the profound effects of statin treatment on ectopic lipid droplet formation within

diabetic muscle was an unexpected and, to our knowledge, novel finding. Muscles of T1DM mice have elevated IMCL content with a concomitant increase in lipid transporters (present study; (Krause et al. 2009)). Interestingly, when diabetic mice are on statin therapy, a significant rise in lipid content outside of the myofibers was noted without an increase in IMCLs, indicating potential complications with myofiber lipid transport. Supportive of this altered lipid compartmentalization, interrogation of lipid transporters demonstrated that FAT/CD36 and FABPpm protein content was significantly downregulated in statin-treated diabetic skeletal muscle. FAT/CD36 and FABPpm are integral membrane proteins that, following the appropriate stimulus, are mobilized from their intracellular pools to be integrated into the plasma membrane (Holloway et al. 2008). As mRNA content of these proteins was not different between groups, these findings suggest the observed changes in lipid transporter content to be post-translational in nature. Specifically, the observed lipidopathy is hypothesized to be a result of attenuated transporter palmitoylation, impairing transporter mobilization and ultimate metabolic functionality. Indeed, previous work has implicated statins in the reduction of palmitoylation (Austen and Sidera 2004) as well as in the impairment of protein trafficking and localization of FAT/CD36 (Thorne et al. 2010).

Elevated lipid transporter content is important in the muscles of poorly controlled Type 1 diabetic individuals to provide an adequate fuel supply in the absence of insulin (and resultant lack of glucose uptake). The present data would suggest that the increased lipid

availability observed in diabetes, coupled with the statin-mediated decreases in lipid transporter availability would hinder lipid uptake and result in an accumulation of ectopic lipids within the diabetic muscle. Given the association between skeletal muscle ectopic lipids and the development of insulin resistance, the adverse effects of statin administration in the presence of overt diabetes could have profound effects on glycemic control in those with insulin-dependent diabetes mellitus.

Future studies should focus on identifying whether statin treatment in humans with diabetes decreases lipid transporter availability and, in turn, increases the presence of ectopic lipids within skeletal muscle. Furthermore, to complement the findings of this investigation, future studies should investigate the exacerbation of lipid deposition in other tissues (heart, liver) as lipid accumulation in these tissues has been linked to dysfunction and insulin resistance (Marchesini et al. 1999).

FIGURES

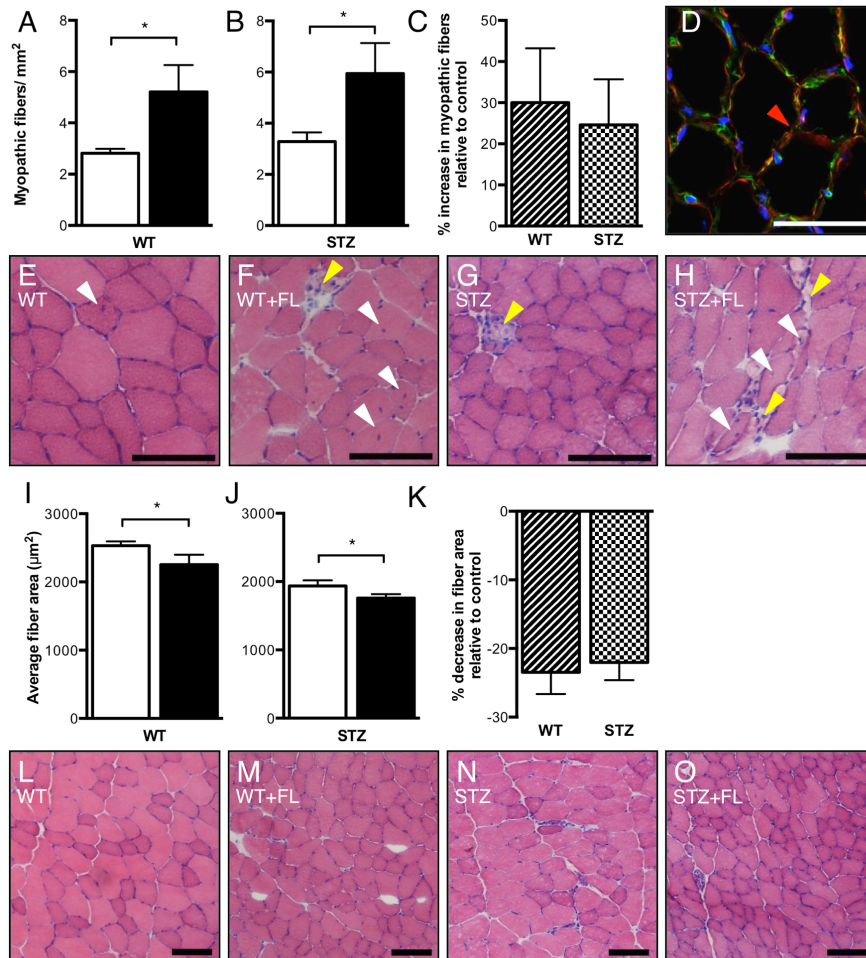


Figure 1: Short-term Fluvastatin administration causes hallmark phenotypes of myopathy.

No differences in severity of myopathy, however, are noted between WT and STZ-

diabetic skeletal muscle. When compared to their control treated counterparts,

Fluvastatin administration results in an increased amount of total myopathic fibers in

both WT (**A**) and STZ (**B**) muscle. No differences in severity of myopathy as a result of

Fluvastatin administration, however, are noted between WT and STZ skeletal muscle ($p=0.38$) **(C)**. A red arrowhead, indicating an example of a split fiber, is shown in **(D)**. Representative myopathic images for each group are shown in **E-H**. White arrowheads indicate centrally nucleated fibers, while yellow arrowheads indicate necrotic fibers. A decrease in average fiber area as a result of Fluvastatin administration is noted in both WT **(I)** and STZ **(J)** muscle, and, once again, no differences in the degree of this decrease are noted between WT and STZ muscle ($p=0.37$) **(K)**. Images depicting representative fiber size for each group are shown in **L-O**. For A, B, I, J: White bars indicate control treatment, black bars indicate Fluvastatin treatment. For C, K: Striped bars indicate percent change in Fluvastatin-treated WT muscle relative to control-treated WT muscle, and checked bars indicate percent change in Fluvastatin-treated STZ muscle relative to control-treated STZ muscle. Scale bar in D= 50 μm . Scale bar in E-H and L-O= 100 μm . *Significant differences ($p\leq 0.05$). All data presented as mean \pm SEM. $n=5-6$ for each bar in A-C. $n=4-6$ for each bar in I-K.

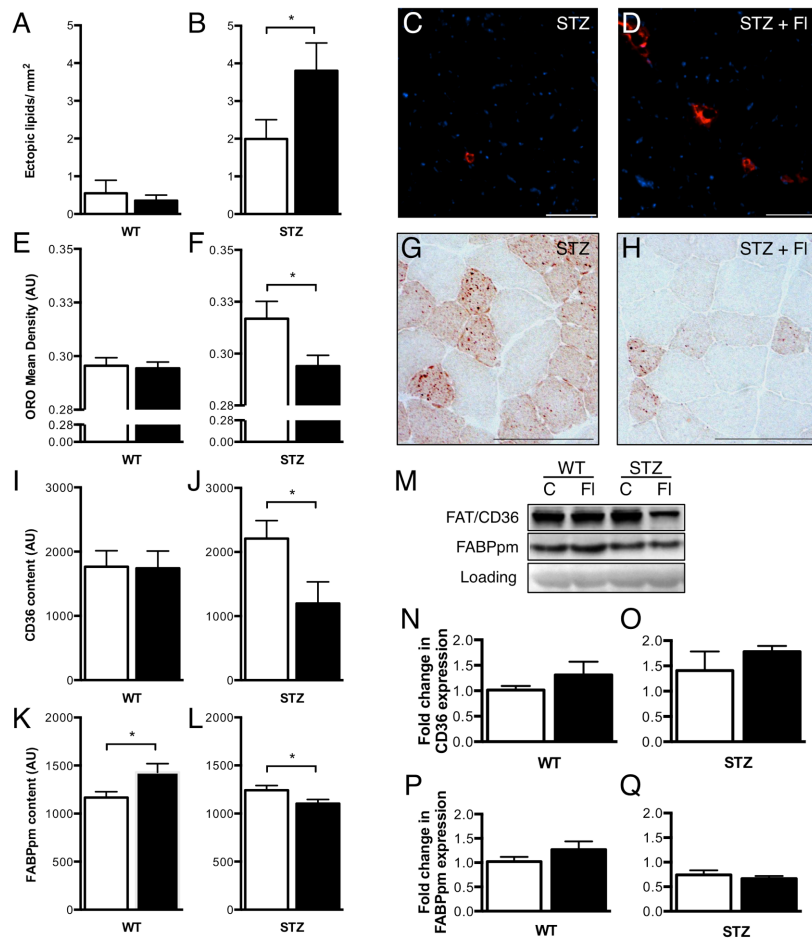


Figure 2: Short-term Fluvastatin administration affects lipid and fatty acid transporter content in STZ-diabetic skeletal muscle. Whereas Fluvastatin administration causes no modifications in ectopic lipid content of WT muscle ($p=0.31$) (A), STZ muscle displays significant increases in ectopic lipid content (and ectopic lipid size ($p<0.05$)) as a result of Fluvastatin administration (B). Representative perilipin-stained (ectopic lipid) images of STZ control and Fluvastatin-treated STZ (STZ+FI) muscle are depicted in (C) and (D), respectively. Oil Red O (ORO) analysis, once again, reveals no differences in WT muscle IMCL content as a result of Fluvastatin administration ($p=0.41$) (E). STZ muscle, however,

(F) displays significant decreases in IMCL density in the presence of Fluvastatin.

Representative IMCL images of STZ control and Fluvastatin-treated STZ muscle are depicted in **G** and **H**, respectively. No differences in FAT/CD36 content were apparent in WT muscle as a result of Fluvastatin administration ($p=0.47$) (**I**), while STZ muscle displayed decreases in FAT/CD36 content with treatment (**J**). FABPpm, contrastingly, was elevated in WT muscle as a result of Fluvastatin administration (**K**), and, once more, STZ muscle displayed decreases in FABPpm content with Fluvastatin treatment (**L**). A representative immunoblot, including loading control (PonceauS), is shown in **M**. No differences in FAT/CD36 or FABPpm mRNA expression were observed with treatment (**N-Q**; graphs depicted as fold-change relative to respective control). White bars indicate control treatment, black bars indicate Fluvastatin treatment. *Significant differences ($p<0.05$). All data presented as mean \pm SEM. $n=5-6$ for each bar in A-Q. Scale bar =100 μ m in C, D, G, H.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSIONS

The average cost of treating one non-healing diabetic foot ulcer during the first 3 years of care is more than \$52,000, and over \$547 million is spent treating diabetic foot ulcerations annually in Canada alone (Hopkins et al. 2015). Despite increasing medical advances, the prevalence of chronic non-healing diabetic ulcerations continues to rise 7.4% per year (Hopkins et al. 2015). It is estimated that 85% of all amputations are the result of a non-healing foot ulcer (Brem and Tomic-Canic 2007), and approximately 72,000 non-traumatic diabetic lower-limb amputations are performed in North America each year (Centers for Disease Control and Prevention (CDC) 2014). The Canadian Diabetes Association reports that 30% of diabetic Canadians will die within one year of amputation, and 51% of diabetic patients with a first amputation will have a second limb amputated within five years. Moreover, approximately 69% of diabetic limb amputees will not survive past five years after amputation (Inderbitzi, Buettiker, and Enzler 2003).

What's more, the inevitable onset and negative effects of diabetic complications make diabetes the 6th leading cause of death in Canada and the 7th leading cause of death in the United States (Heron 2007; Statistics Canada 2013). Due to the immense ability of skeletal muscle to uptake glucose, physical activity is considered a vital therapeutic in muscle maintenance for blood glucose management and the subsequent attenuation of T1D complications (Colberg et al. 2016; Robertson et al. 2009). Interestingly, recent research has reported negative correlations between physical activity and levels of the anti-fibrinolytic hormone, PAI-1, levels; with a sedentary lifestyle being associated with a

higher level of PAI-1 (Lira et al. 2010). Furthermore, physical activity has also been associated with increasing tPA activity and decreasing PAI-1 levels (Cooper et al. 2004; Esmat et al. 2010). T1D patients, individuals who possess elevated levels of PAI-1 in their disease state, would therefore benefit from including physical activity into their lifestyle to naturally combat the elevations of this fibrinolytic inhibitor. Physical activity, however, leads to increased skeletal muscle damage and a concomitant need for repair. With aberrant skeletal muscle regeneration reported in those with diabetes, the necessity for repeated skeletal muscle repair following regular exercise poses a problem for this population (Gulati and Swamy 1991; Krause et al. 2013; Vignaud et al. 2007).

The body of work comprising this dissertation has focused on examining a novel therapeutic strategy for the restoration of cutaneous and skeletal muscle repair following injury; two diabetic complications that are an evident burden to the T1D population. Previous work from our lab has celebrated PAI-039 and its efficacy for PAI-1 inhibition in restoring skeletal muscle repair in a preclinical rodent model of T1D (Krause, Moradi, et al. 2011). In Chapter 2 of the current compilation, we confirmed the dysregulation of PAI-1 as a burden to the regenerative process of skin; a problem that was restored, in part, by PAI-039 treatment and subsequent fibrinolytic balance.

While the use of a specific PAI-1 inhibitor for the restoration of skin and muscle repair seems a promising therapeutic to improve tissue repair in the clinical populace, no small-

molecule PAI-1 inhibitors are as of yet approved for human therapy or being evaluated in the clinical setting (Fortenberry 2013). In this light, due to their availability, acceptable safety profile, and ability to inhibit PAI-1 production, the effects of statins on PAI-1 inhibition and tissue repair were explored (Fenton II et al. 2002; Liao and Laufs 2005; Mussoni et al. 2000; Ni et al. 2013; Sahebkar et al. 2016; Zhou and Liao 2010).

As a result of their efficacy in the reduction of ASCVD, 27% of the Canadian population and 47% of the American population are currently prescribed statins, making this class of lipid-lowering agents among the most widely prescribed pharmaceuticals in North America (Gu et al. 2014; Rotermann et al. 2014). What's more, 63% of diabetics are prescribed statins. So not only is this therapeutic approved for clinical consumption, it is in the homes of millions of people across the continent (Gu et al. 2014). Promising results in the attenuation of PAI-1 and restoration of tissue repair with statin treatment would require no further prescription for many North Americans.

As observed in Chapter 3 above, Fluvastatin did indeed attenuate PAI-1 levels in regenerating diabetic tissue. The subsequent worsening of diabetic cutaneous and skeletal muscle repair was therefore an unexpected finding. By way of their inhibition of GGPP production, statins are known to inhibit post-translational modification of small GTPases such as RhoA. This inhibits the Rho/ROCK pathway and subsequent transcription of PAI-1 (Chapter One, Figure 2) (Fenton II et al. 2002; Greenwood, Steinman, and Zamvil

2006; Hua et al. 1998; Watts et al. 2005). Unfortunately, PAI-1 is not the only factor whose production is inhibited by dampening this pathway, resulting in off-target isoprenoid effects.

While conducting analysis of data for Chapter 3, histological abnormalities in the uninjured skeletal muscle were observed with Fluvastatin administration, leading to the exploration of the effects of Fluvastatin on resting diabetic skeletal muscle.

The most common complaint surrounding the use of statins is myalgia (Bruckert et al. 2005; Cohen et al. 2012; Nichols and Koro 2007). Congruent with clinical findings, controlled trials reveal the myotoxicity of statins, demonstrating myocyte apoptosis and morphological abnormalities in myotubes following statin exposure in vitro. Similarly, statin exposure in vivo results in necrotic rodent muscle fibers, and biopsies from patients symptomatic for statin myopathy demonstrate significant necrosis and inflammation (Huynh et al. 2002; Westwood et al. 2005). Because of the tendencies for diabetic skeletal muscle to display its own myopathic features (Krause, Riddell, and Hawke 2011), we were surprised to note that the myopathy was not exacerbated by the already myopathic tendencies of the diabetic environment. In this preclinical model of T1D, however, a staggering increase in the amount of extramyocellular lipid deposition was observed. Fluvastatin has been historically recognized as a 'mild' statin when observing its causation of muscular symptoms in the clinical population (Bruckert et al. 2005). If short-

term Fluvastatin administration is causing such bold changes in lipid storage in a young rodent model absent of cardiovascular complications and a healthy lipid profile, it is alarming to fathom what changes in lipid handling would occur with long-term administration of a statin, with a more myotoxic profile, in a model of diabetes that more closely resembles the clinical population that would be taking statins on a daily basis (Bruckert et al. 2005). Indeed, the exploration of the effects of a more myotoxic statin such as Atorvastatin or Simvastatin is an intriguing thought, as these are more widely prescribed than Fluvastatin in a clinical setting (Chalasanani et al. 2004; Mason et al. 2005).

The lessons learned thus far are two-fold. First, PAI-1 inhibition via specific small-molecule inhibitors are effective in facilitating diabetic wound closure. Second, PAI-1 inhibition using a compound that has a multitude of pleiotropic effects is not necessarily beneficial to diabetic tissue repair. As such, future research focusing on the advancement of small-molecule PAI-1 inhibitors in the clinical setting are of particular interest. Due to the effects of PAI-1 on not only extracellular matrix degradation but also on fibrin degradation, deficiencies in PAI-1 have pro-thrombotic effects; resisting clotting/thrombus formation and causing unnecessary bleeding (Carmeliet et al. 1993; Fay et al. 1997; Tjärnlund-Wolf et al. 2012). As such, optimizing the dosage of PAI-1 inhibitor therapeutics would be of the utmost importance.

Conclusions

Despite insulin therapy, diabetic complications persist in T1D individuals. In fact, in the present day, the majority of deaths associated with diabetes can be attributed to the presence of diabetic complications (Secrest et al. 2010). While the development of a cure for diabetes is the inevitable goal for diabetes researchers, due to the 3-5% increase in T1D incidence year-over-year, current research should focus on restoring metabolic homeostasis and mitigating the presence of diabetic complications (Gillespie et al. 2004; Onkamo et al. 1999). This would not only increase the healthy lifespan of diabetic individuals, but reduce an incredible burden from the public healthcare system.

The current body of work demonstrates that PAI-1, a factor other than insulin, is dysregulated in T1D. While we were hopeful that all therapeutic avenues for PAI-1 inhibition would attenuate complications in diabetic tissue repair, the results herein identify only a specific small-molecule inhibitor of PAI-1 as effective. Future investigations should focus on extending this knowledge beyond a preclinical model in order to alleviate complications associated with aberrant tissue repair in T1D.

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