LOVASTATIN DECREASES TGF-β1 CONCENTRATION OF GLOMERULAR ENDOTHELIAL CELLS CULTURED IN HIGH GLUCOSE

By

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The members of the Committee appointed to examine the thesis of Erinn Claire Lanxon-Cookson find it satisfactory and recommend that it be accepted.

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Abstract

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Background: Diabetic nephropathy (DN) is the leading cause of morbidity, mortality and end stage renal disease in patients with diabetes mellitus.\textsuperscript{1,2} Glomerular fibrosis is a key pathological process in DN and is enhanced in diabetes. Endothelial cells cultured under high glucose conditions can enhance the production of transforming growth factor beta (TGF-β1). HMG-CoA reductase inhibitors (statins) have been shown to decrease markers of fibrosis in cell culture models of diabetes.\textsuperscript{1-3} Lovastatin (LOV) decreases TGF-β production in mesangial cells and various types of endothelial cells cultured in high glucose conditions.\textsuperscript{1-8} However, to date, there are no studies reporting the effects of LOV on the production of TGF-β by rat glomerular endothelial cells (RGEC) cultured under high glucose conditions. The purpose of this study was to investigate the effects of LOV on the production of TGF-β in a diabetic cell culture model using RGEC. We hypothesize that LOV will inhibit the increases in total TGF-β concentration that typically occur in high glucose models. Methods: RGEC were grown to confluence in normal glucose (5.5 mM) media supplemented with 10% FBS. Once confluent, RGEC were exposed to high glucose (25 mM) media alone or with 10 μM LOV, and incubated for 48 h. Media samples were taken at 12, 24 and 48 h and frozen (-80°C) with protease...
inhibitor cocktail and subsequently assayed for total TGF-\(\beta\)1. **Results:** Total TGF-\(\beta\)1 protein produced by RGEC exposed to 10 \(\mu\)M LOV was significantly lower than groups exposed to high glucose alone at 12 h (\(p=0.017\)) and 24 h (\(p=0.020\)). We conclude that 10 \(\mu\)M LOV was effective at decreasing total TGF-\(\beta\)1 concentration in RGEC exposed to high glucose media.
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I. Introduction and Background

Diabetic nephropathy (DN) is a leading cause of morbidity, mortality and end stage renal disease (ESRD) in patients with diabetes mellitus. The total Medicare burden for patients with ESRD in 2005 was approximately 19 billion dollars, lending support to the argument that therapeutic interventions to ameliorate DN are necessary and are being widely studied. Characterized by glomerulosclerosis and renal hypertrophy, DN is a consequence of longstanding diabetes. Diabetic nephropathy is diagnosed by progressive thickening of the glomerular basement membrane and expansion of the mesangial matrix, which correlates with decreased glomerular filtration and dysfunction. Glomerulosclerosis, a pathologic progressive thickening of the glomerular basement membrane and expansion of the mesangial matrix, is a condition described by the excessive deposition of the fibrotic proteins fibronectin and collagen IV, and activation of the profibrotic cytokine transforming growth factor beta (TGF-β1) within the glomerulus.

Transforming growth factor-beta1 is a profibrotic and prosclerotic cytokine known to be naturally expressed in renal mesangial and endothelial cells and its expression enhanced in response to high plasma glucose in DN. Fibronectin, a key extracellular matrix (ECM) protein considered to be a marker of fibrosis, is upregulated by increased TGF-β1 protein expression in various models of diabetes. Alternate pathways to the TGF-β signaling pathway produce TGF-β1, fibronectin, and collagen IV, another protein of the ECM that contributes to fibrosis, have been elucidated; however, this discussion will focus on TGF-β1 protein expression in response to high glucose because of its pivotal role in stimulating fibrosis involved in DN.
In addition to stimulating fibrosis and sclerosis, TGF-β1 downregulates genes encoding for enzymes that degrade ECM proteins known as matrix metalloproteases.\textsuperscript{19, 20} Normally, fibrotic proteins are enzymatically metabolized by matrix metalloproteases within the ECM, thereby inhibiting protein accumulation in the ECM of the glomerulus.\textsuperscript{2} Metabolism of ECM proteins by these proteases is markedly decreased in cells exposed to high glucose due to the accumulation of TGF-β1.\textsuperscript{19, 21-23} The simultaneous actions of TGF-β1 to enhance profibrotic protein buildup and reduce proteolytic activity within the ECM make TGF-β1 a crucial element in glomerulosclerosis and DN.\textsuperscript{8} In vivo studies have shown TGF-β1 involvement in the renal tubular, glomerular, and mesangial cell hypertrophy that often accompanies diabetes.\textsuperscript{24, 25} Furthermore, studies have shown that administration of anti-TGF-β1 antibodies in vitro and in vivo prevent the development of DN.\textsuperscript{21, 26} Therefore, increased glomerular TGF-β1 expression in experimentally induced diabetic animals, as well as diabetic patients, suggests TGF-β1 as a potent mediator of diabetic renal disease and is considered an intervention point in the progression of DN.\textsuperscript{27}

In experimental models using endothelial and mesangial cells, most studies show that high glucose concentrations stimulate the expression of TGF-β1,\textsuperscript{2, 28} fibronectin,\textsuperscript{29, 30} and collagen IV.\textsuperscript{24, 31} However, to date there are two studies showing that a high glucose concentration did not stimulate TGF-β1 expression by human umbilical vein endothelial cells at 12 and 24 hours.\textsuperscript{29, 32} In vitro studies have demonstrated that glucose in high concentrations (25.5 mM) stimulates the expression of the TGF-β isoforms, which upregulate genes encoding for the ECM proteins fibronectin and collagen IV, both overexpressed in DN.\textsuperscript{2, 12, 13}
HMG-CoA reductase inhibitors (statins), commonly known for their cholesterol-lowering effects, have recently been shown to decrease markers of fibrosis in cell culture models of diabetes.\textsuperscript{10-13} A detailed look at the effects of statins on ECM protein accumulation in fibrosis and the fibrinolytic system will be detailed in chapter II. Lovastatin (LOV), a commonly prescribed statin, has been studied for its effect on TGF-\(\beta\)\textsubscript{1} expression in various cell culture models of diabetes;\textsuperscript{1-8, 33-36} however, its effect on TGF-\(\beta\)\textsubscript{1} expressed by glomerular endothelial cells (RGEC) has yet to be established. Statins inhibit the mevalonate pathway, an important metabolic pathway primarily responsible for synthesizing sterol and non-sterol isoprenoids, as well as GTPase intracellular signaling molecules used in TGF-\(\beta\)\textsubscript{1} transcription.\textsuperscript{35, 37-39} Statins inhibit high glucose-induced synthesis of TGF-\(\beta\)\textsubscript{1}, which block the downstream expression of collagen IV and fibronectin through unavailability of GTPase intracellular signaling molecules.\textsuperscript{12,10,11} Furthermore, statins interfere with AP-1 transcriptional binding sites within the TGF-\(\beta\) promoter region, which are necessary for TGF-\(\beta\)\textsubscript{1} transcription.\textsuperscript{40} The inhibitory actions of statins on TGF-\(\beta\)\textsubscript{1} synthesis work through a decreased availability of GTPase molecules, thereby interfering with the gene expression of these fibrotic proteins. A detailed look at the molecular actions of glucose on the TGF-\(\beta\)\textsubscript{1} promoter, as well as the effects of LOV at this site, will be discussed in chapter II.

The purpose of this study was to determine if LOV would decrease the expression of TGF-\(\beta\)\textsubscript{1} in a cell culture model of diabetes using RGEC. Based on findings in types of human umbilical vein and glomerular endothelial cells, as well as mesangial cells exposed to media supplemented with high glucose, it is hypothesized that total TGF-\(\beta\)\textsubscript{1} will increase in response to high glucose media. Therefore, the hypothesis of this study is
that LOV will decrease total TGF-β1 protein concentration in an in vitro model of diabetes using RGEC.
II. Literature Review

Introduction

Diabetic nephropathy (DN) is the leading cause of end stage renal disease worldwide and is a co-morbid factor associated with diabetes. Characterized by angiopathy of the glomeruli, expansion of the mesangial matrix, and basement membrane thickening, DN is a result of excessive accumulation of fibrotic proteins in the glomerular basement membrane and within the extracellular matrix (ECM). Glomerulosclerosis is defined as an aberrant production and accumulation of ECM proteins within the mesangium and basement membrane of the kidney. The glomerular ECM provides essential functions, including structural support, cellular adhesion, and a barrier to the movement of fluid and macromolecules into interstitial spaces. Endothelial cells line the vasculature of the glomerulus and are a crucial component in the pathogenesis of DN. Cell culture models of diabetes using endothelial cells have been used to determine the deleterious cellular and molecular effects of high glucose involved in the initiation and progression of DN. In vitro studies have demonstrated that high glucose concentrations enhance the TGF-β1 promoter region, thereby increasing its expression in mesangial cells and various types of endothelial cells. Although multiple cytokines are involved in the pathogenesis of DN, TGF-β1 is thought to play an integral role in progression of fibrosis and glomerulosclerosis by enhancing the accumulation of ECM proteins. A proponent of fibrosis, TGF-β1 synthesis directly increases expression of fibronectin and collagen IV by activating the gene sequence responsible for these proteins. Moreover, it has been established in vivo that when
anti-TGF-β1 antibody is administered, DN can be reversed by directly decreasing TGF-β1, fibronectin and collagen IV accumulation within the glomerulus.\textsuperscript{21, 26}

In tissue culture studies, cellular hypertrophy and matrix production are stimulated by glucose in the culture media,\textsuperscript{16, 28, 50, 61-64} effects that are prevented by anti-TGF-β treatment.\textsuperscript{21, 25, 26} Therefore, due to the apparent status of TGF-β1 as a crucial mediator in the pathogenesis of DN, therapeutic interventions are aimed at alleviating the overproduction of this protein.\textsuperscript{65}

Endothelial dysfunction is an underlying contributor to the progression of fibrotic protein expression and renal dysfunction in DN.\textsuperscript{14, 25, 41, 66} However, the majority of research conducted in vitro has been focused on mesangial cell production of TGF-β1 in DN. Although mesangial cells are responsible for the majority of TGF-β1 production in fibrosis, glomerulosclerosis, and DN, the role of endothelial cells has recently gained attention in the pathogenesis of the disease, due to endothelial dysfunction being present in patient with diabetes.\textsuperscript{25}

In recent years, the pleiotropic effects of statins, including a wide variety of beneficial vascular effects such as inhibiting vascular inflammation,\textsuperscript{36} retarding fibrotic protein accumulation in glomeruli,\textsuperscript{4, 33, 35} ameliorating endothelial dysfunction,\textsuperscript{67} and enhancing fibrinolysis of the fibrinolytic system,\textsuperscript{1} have been recognized. In particular, lovastatin has proven to be efficacious in decreasing the production and expression of TGF-β1 in mesangial cell culture models of diabetes, as well as increasing fibrinolysis in the glomerulus of rats in models of diabetes.
Therefore, the aim of this research study was to elucidate whether TGF-β1 expression increased in RGEC cultured in high glucose media, and if this increase in production could be suppressed by treatment with lovastatin.

The following chapter will outline in a stepwise manner the physiological processes known to cause DN, from initial insult to progressive glomerulosclerosis. A brief overview of the historical significance of statins and their molecular actions in various models of diabetes, as well as their relevance in the proposed model of this study, will follow.

**Historical Overview of Statins**

In 1971, Akira Endo began research on HMG-CoA reductase inhibitors (statins) and hypothesized that inhibiting hepatic production of HMG-CoA would be more effective in reducing serum cholesterol levels than decreasing cholesterol absorption from the diet. Endo postulated that microorganisms would produce secondary metabolites which inhibit HMG-CoA reductase, an integral enzyme within the mevalonate pathway, as a defense mechanism against other microbes. The mevalonate pathway is an important metabolic pathway which plays a key role in multiple cellular processes by synthesizing sterol isoprenoids, dolichol, heme-A, isopentenyl tRNA and ubiquinone, which serve as the basis for the biosynthesis of molecules used in processes as diverse as protein prenylation, cell membrane maintenance, hormones, protein anchoring, and N-glycosylation. The intracellular signaling proteins inhibited by statins are small GTPase proteins, which are classified as the Ras, Rho and Raf proteins, derived downstream of mevalonate-pyrophosphate. These small GTPase proteins function as critical relays
in the transduction of a variety of intracellular signals by cycling between the inactive GDP-bound and an active GTP-bound state.\textsuperscript{37} Isoprenylation of small GTPase proteins are necessary for the translocation of the inactive GTPase protein from the cytosol to the cell membrane where they are activated.\textsuperscript{5} These active GTPase proteins are involved in the translocation of the Smad complex located in TGF-\(\beta\) receptor signaling pathways, the complex primarily responsible for the transcription of genes encoding for fibrotic proteins.\textsuperscript{1} Translocation of the receptor-bound Smad (R-Smad) complex to the nucleus activates transcription of genes encoding for TGF-\(\beta1\), fibronectin, and collagen IV.\textsuperscript{5,22,70}

In early 1978, Akira Endo and Akira Yamamoto showed that mevastatin significantly reduced plasma cholesterol in patients with serious hypercholesterolemia.\textsuperscript{68} This was the first clinical trial to provide hard evidence that statins could reduce elevated levels of serum cholesterol, which was later found to be a significant risk factor for many vascular diseases such as atherosclerosis. In 1982, lovastatin (LOV) was prescribed to patients with serious hypercholesterolemia who were unresponsive to other pharmaceuticals; LOV drastically lowered plasma cholesterol with very few side effects noted.\textsuperscript{68} Subsequently, statins were investigated in various in vitro and in vivo models, and were found to have various efficacious effects in many diseases, such as cardiovascular and renal disease, and more recently diabetes.\textsuperscript{71-73} Epidemiological research also suggests statin efficacy in curbing inflammation,\textsuperscript{52,73,74} maintaining and protecting renal function,\textsuperscript{52,58,72} and reversing endothelial dysfunction in patients with diabetes.\textsuperscript{42,69,75} Statins have also been shown to reduce sclerotic plaque formation throughout the vasculature by interfering with the production and binding of low-density lipoproteins to their cell surface receptors.\textsuperscript{74} Results of these studies led researchers to
explore mechanisms underlying the noted effects of statins on processes involved in cardiovascular and renal atherosclerotic disease.

In the following years, multiple studies investigated the effects of LOV on various cell types and animal models, showing variable results associated with dose dependency.1-3, 6-8, 34, 36 The studies which pertain to cell culture models of diabetes will be discussed in detail at the end of this chapter. A brief discussion of endothelial dysfunction and its importance to diabetes-associated disease will follow in the subsequent section.

**Endothelial Dysfunction in Diabetes**

Regardless of whether the underlying disease is glomerulonephritis, tubulointerstitial disease, or DN, the histology of chronic renal disease is uniformly characterized by a progressive accumulation of ECM proteins that obliterates renal function and leads to organ failure.41 Endothelial injury, dysfunction and damage are common starting points for macroangiopathy and microangiopathy, conditions seen in diabetes.76 Recent studies suggest endothelial dysfunction as the main initiator of fibrosis, inflammation, and glomerulosclerosis,77 and possibly the underlying factor leading to insulin resistance and diabetes.58, 66 Various epidemiological studies have demonstrated that endothelial dysfunction occurs concurrently with diabetes, vascular disease, and elevated risk of heart attack or stroke, therefore implying the importance of endothelial cell function in vascular disease.44, 66, 77

The endothelium produces and responds to multiple mediators that regulate inflammatory processes, such as the recruitment and activation of inflammatory cells.77
Hartage et al\textsuperscript{77} has defined endothelial dysfunction as being characterized by various interactions of numerous pro-inflammatory processes, reduced vasodilatation, and increased vascular permeability. The endothelium is capable of carrying out many physiologic processes, which include the regulation of leukocyte adhesion, extravasation, and subendothelial accumulation of these immune cells; the platelet adhesion resulting in thrombotic processes; and the regulation of vasodilatation processes for maintenance of appropriate blood flow.\textsuperscript{66,77} In diabetic states, the endothelium is chronically exposed to elevated concentrations of reactive products and abundant humoral agents, thus enhancing the production of various fibrotic ECM proteins, leading to a dysfunctional endothelium.\textsuperscript{77,78}

A dysfunctional endothelium results when injury occurs. Injury to the endothelium can happen through various mechanistic pathways, such as hyperglycemia, reactive glucose products, reactive oxygen species, inflammation, fibrotic protein accumulation, macrophage adhesion and diapedesis, all integral processes in the development of DN and glomerulosclerosis.\textsuperscript{70,79} Statins are able to retard endothelial dysfunction via multiplicative mechanisms,\textsuperscript{69} including: lowering circulating low density lipoproteins,\textsuperscript{74} enhancing the generation of nitric oxide,\textsuperscript{61} and retarding the adhesion and signaling of inflammatory molecules.\textsuperscript{72} Nitric oxide exerts important vasodilatory, antiplatelet, antioxidant, anti-adhesive, and anti-proliferative effects on the endothelium and is thus an important vasoprotective agent.\textsuperscript{44,52,58,66,72,77,80} Endothelial cells promote vasodilatation through the production and secretion of nitric oxide through endothelial nitric oxide synthase expression and conversion of L-arginine. Endothelial cell damage results in an inability to produce nitric oxide, a defective dilatory response, and a
upregulation of the profibrotic cytokine TGF-β, which further damages the vasculature by signaling other inflammatory and destructive molecules to sites of injury.\textsuperscript{44} Nitric oxide is also a potent inhibitor of fibrotic protein synthesis and deposition on the endothelium.\textsuperscript{58} Therefore, an increase in nitric oxide is expected to be beneficial in vascular disease. However, nitric oxide in abundant amounts can act as a free radical and damage vascular cells and tissues, as well as upregulate TGF-β1 through translocation of the Smad complex.\textsuperscript{61} Nevertheless, nitric oxide is mainly considered to be a beneficial agent in maintaining vascular health and its presence is not considered deleterious unless in copious amounts.\textsuperscript{77}

**Glomerulosclerosis in Diabetic Nephropathy**

Glomerulosclerosis is a classic characteristic of DN and an essential process in the initiation and progression of the disease.\textsuperscript{42} Although the mechanisms of glomerular change in diabetes are uncertain, they are thought to be a result of enhanced synthesis of the glomerular basement membrane and mesangial matrix proteins with inappropriate incorporation of glucose into non-cellular components of the glomerular structures.\textsuperscript{81} Over production of fibrotic matrix proteins, which are deposited within the mesangium and vascular basement membrane, facilitate intima-media thickening and underlie mesangial expansion in renal fibrosis and DN.

Glomerulosclerosis is a stepwise disease process resulting in part from a concomitant increase in blood glucose and amino acids.\textsuperscript{82} In addition to high blood glucose, diabetic patients also often have an increased concentration of amino acids in the blood due to diabetic ketosis or increased protein consumption to alter serum glucose
concentrations. Increased concentration of amino acids leads to the creation of the reactive protein metabolites called advanced glycated end products. These reactive metabolites can activate the signaling of various profibrotic cascades, leading to aberrant synthesis of glomerular ECM proteins by glomerular endothelial and mesangial cells. Although the insult from protein glycation and accumulation is important in DN, the current discussion will focus on the deleterious actions of glucose on glomerular endothelial and mesangial cells.

The vascular response to injury model demonstrates that glomerulosclerosis and atherosclerosis are disease processes initiated by injurious products [e.g. low density lipoproteins, glucose, reactive oxygen species, advanced glycation end products, and immune cells]. Research has demonstrated these products to be in abundance in the blood of diabetic patients, leading to injury of the endothelial cell layer and resulting in glomerulosclerosis. Hyperglycemic conditions inflict damage to the renal structures by increasing the production of fibrotic proteins, thereby decreasing the glomerular filtration rate and increasing the glomerular intracapillary pressure. Increased pressure leads to enlargement of intracapillary pores, thus negatively altering the size-selective barrier function of the kidney, allowing for infiltration of blood components into the subendothelial layer. Increased renal pressure activates the renin-angiotensin-aldosterone system to vasodilate the renal arterial system. Chronic activation of this system, i.e. chronic vasodilatation, increases the production of TGF-β1, fibronectin, and collagen IV to compensate for increased pore size within the glomerular tuft. Fibrotic proteins are deposited within the ECM and on the glomerular basement membrane, thus impeding the normal filtration function of the kidney.
Hyperglycemic damage to the glomerular endothelium initially enables filtration of damaging agents into the subendothelial layer, leading to sclerotic capillaries; however, in end stages of the disease filtration is inhibited by fibrotic protein accumulation.\textsuperscript{88} Infiltration through the endothelium by cytokines and inflammatory molecules promotes endothelial cell death, consequently exposing the subendothelial layer to blood components and reactive metabolites which can cause additional vascular damage.\textsuperscript{25} Exposure of this layer facilitates mesangial smooth muscle cell migration from the media to the intima layer of the vessel.\textsuperscript{77} Mesangial cell proliferation and migration are integral steps in the pathogenesis of renal fibrosis and hypertrophy.\textsuperscript{66}

Leukocyte migration and infiltration into the subendothelial layer of the glomerulus is primarily responsible for the initiation and amplification of glomerular injury, and is mediated by adhesion molecules and chemokines which can be locally synthesized by mesangial cells upon cell injury.\textsuperscript{47, 70, 79} The cytokine TGF-\(\beta\)1 has a chemoattractant ability to signal for recruitment of inflammatory cells such as monocytes, macrophages, platelets, and lymphocytes to the endothelium.\textsuperscript{70} Damaged endothelial cells signal an increase in chemokines to damaged endothelium in part by TGF-\(\beta\)1, thereby facilitating inflammation, platelet accumulation, and adherence of these molecules, leading to sclerotic lesions seen in glomerulosclerosis and DN.\textsuperscript{42, 82, 90} Macrophages, leukocytes, and neutrophils are capable of producing various reactive species, pro-inflammatory cytokines, matrix metalloproteases, and growth factors, which modulate the inflammatory response to injury.\textsuperscript{79, 88, 89} Upon macrophage migration into the subendothelial layer, foam cells are formed through endocytosis of lipids, in particular low density lipoproteins.\textsuperscript{89} These foam cells are capable of signaling pro-inflammatory
and pro-fibrotic cytokines, such as TGF-β1. The cyclical ability of TGF-β1 to create sclerotic lesions and attract inflammatory molecules outlines its importance in diabetic glomerulosclerosis.

The final step in glomerulosclerosis is smooth muscle cell proliferation and deposition of fibrotic proteins within the ECM and on the mesangium, resulting in the formation of an atheromatous plaque with a lipid core. The presence of atheromatous plaque and damaged endothelial cells impede normal functioning of the endothelial cell layer, thus increasing systemic pressure and decreasing the functional status of the glomerulus.

As discussed previously, endothelial dysfunction involves many dysregulated physiologic processes, one of which is the inability to break down fibrotic proteins deposited in the ECM and mesangium. Reduced fibrinolysis and glomerulosclerosis is apparent in patients with DN. The overproduction and accumulation of fibrotic proteins in the glomerulus are expressed, in part, through TGF-β1. A potent contributor to DN, TGF-β1 is an injurious cytokine with cellular and molecular actions that have been studied extensively. The following sections will detail TGF-β activation and its receptor signaling cascade in the pathogenesis of DN.

**TGF-β Activation and Signaling in Endothelial and Mesangial Cells**

Transforming growth factor-beta1 is generally considered to be the major or predominant isoform involved in fibrosis, with the roles of TGF-β2 and -β3 being less clear. The TGF-β1 molecule is a profibrotic cytokine that acts in an autocrine and paracrine manner in DN by binding to specific cell surface receptors throughout the
kidney. The three isoforms are secreted in their latent form and cannot exert any biologic activity until activated. The TGF-β1 precursor cleaved of its peptide signal is called pro-TGF-β and comprises 361 amino acids. An endoprotease, furin convertase, recognizes a motif in pro-TGF-β, and cleaves the molecule between the two arg residues 278/279. The two parts remain non-covalently associated and in dimeric form, constituting small latent TGF-β1 approximately 100 kD in size. The N-terminal remnant of pro-TGF-β1 is sufficient to confer latency and is referred to as the latency associated peptide (LAP). Latent TGF-β was first discovered through the effect of acid pH treatments of cell-conditioned media from normal and transformed fibroblasts and of extracts of human blood platelets. It was also shown that alkaline pH, heating to 100°C, and urea would similarly afford activation, suggesting that breaking hydrogen bonds was involved in such activation. From these results it was proposed that activation of latent TGF-β in vivo might be a crucial regulatory step in controlling the biological activity of this peptide.

In order for TGF-β1 to exert any biologic effect, it first must disassociate from its LAP and bind to its receptor. Disassociation, referred to as activation of TGF-β1, can occur in the presence of various proteins, including other TGF-β1 proteins and thrombospondin 1 (TSP-1). The TGF-β receptor includes type I and type II subunits, which signal through the Smad family of proteins. The TGF-β receptor has dimeric binding properties; binding of TGF-β to its type II receptor leads to the phosphorylation of the type I receptor, which then phosphorylates and activates the Smad2 protein. Upon dimerization, Smad2, in combination with the receptor bound Smad4 (R-Smad), are translocated to the nucleus of the cell. Within the nucleus, the Smad complex recruits
other transcription factors that together activate the expression of target genes which mediate the biological effects of TGF-β.\textsuperscript{50} Congruently mediated through this receptor are the expression of the fibrotic proteins fibronectin and collagen IV, some of the fibrotic proteins found excessively within the ECM and glomerular basement membrane in DN.\textsuperscript{17} Transcription of the TGF-β gene sequence regulates apoptosis, growth and proliferation of many different cell types.\textsuperscript{30,101,102}

In normal functioning cells, TGF-β1 plays an important role in wound healing and tissue repair by stimulating cells to synthesize fibronectin and collagen, as well as decreasing production of metalloproteinase enzymes.\textsuperscript{17,28} These metalloprotease enzymes degrade ECM proteins, such as fibronectin and collagen IV, thereby maintaining the physical integrity of the ECM. As stated throughout this paper, excessive deposition and accumulation of ECM proteins on the endothelium damages endothelial cells leading to endothelial dysfunction. Endothelial cells, as well as mesangial cells, produce fibronectin and collagen products which are naturally degraded by matrix metalloproteases in euglycemic states. In DN, the overexpression of TGF-β1 downregulates matrix metalloprotease function in renal cells inducing ECM protein accumulation and promoting damage to the glomerulus.\textsuperscript{91}

**Glucose Increases the Expression of TGF-β1**

Until recently, the molecular actions of glucose to promote TGF-β1 production were unclear. However, it was apparent that certain endothelial cells and all mesangial cells cultured in high glucose exhibited an increase in TGF-β1 production compared to control. A seminal study by Hoffman et al. demonstrated that mesangial cell gene
expression of TGF-β1 was transcriptionally regulated by ambient glucose concentration. These authors demonstrated that the region of maximal glucose responsiveness was between -835 and -406 of the murine TGF-β1 promoter. Within this region, the murine TGF-β1 promoter contains an E-box that exhibits increased binding of transcription factors from cells cultured in high glucose. From this experiment, the molecular actions of glucose on TGF-β1 transcription were clarified, however the pertinent proteins and transcription factors involved remained unclear. To further the investigation into the glucose binding sequence, Zhu et al. evaluated the role of upstream stimulatory factors (USF) in mesangial cells cultured in varying glucose concentrations. These authors demonstrated for the first time that USF-1 protein was stimulated and produced in the presence of media supplemented with high glucose concentrations in mesangial cells; furthermore, USF1 exhibited binding properties to a glucose-responsive element in the murine TGF-β1 promoter region. Increased expression of USF1 stimulated the promoter activity of TGF-β1. The actions of glucose to increase USF and directly activate the promoter of TGF-β1 gene clearly identified a pathway for glucose-induced gene expression of TGF-β1. Furthermore, recent studies have implicated USF factors in the regulation of human TGF-β1 promoter, and in glucose-induced regulation of TSP-1 in rat mesangial cells.

Investigating the argument for USF action in glucose-induced expression of TGF-β1, Liu et al. considered USF and its action in diabetic kidney injury. These investigators found that mesangial cells exposed to high glucose stimulated the accumulation of nuclear USF2. Furthermore, these authors found that the excess USF2 induced TSP-1 gene expression, inducing the activation of latent TGF-β1 and further
suggesting USF2 has a role in renal mesangial cell complications. Until recently the molecular inducing actions of glucose on TGF-β1 gene sequence and activation were unknown, however, now through the work of Lie et al. and Zhu et al, the molecular activities of glucose on the TGF-β1 promoter region are clear. Furthermore, it seems apparent that mesangial and endothelial cells cultured in high glucose conditions will increase their expression and protein production of TGF-β.

Although the glucose induced expression of TGF-β1 has been well established in mesangial and endothelial cells, Caglieri et al. that glucose did not induce a significant increase in TGF-β1 mRNA or protein production for human umbilical vein endothelial cells. Furthermore, another study using the same cells and same conditions only reported significance in TGF-β1 expression between groups at 48 to 72 hours, not at 12 or 24, implying TGF-β1 expression in response to glucose was time-dependent. However, there is still a wide base of literature which supports the theory of a glucose-stimulatory effect of TGF-β1 mRNA and protein from endothelial cells exposed to high glucose. Therefore, we hypothesize RGEC exposed to high glucose media will increase total TGF-β1 expression.

Recently, statins have been used in cell culture models of high glucose and have shown an inhibitory effect on TGF-β1, fibronectin and collagen IV synthesis, as well as the fibrinolytic system. The following section will detail these models and provide an argument for lovastatin (LOV) use in tissue culture models of high glucose. Moreover, the molecular actions of LOV will be discussed and an argument made for how LOV could theoretically inhibit glucose-induced TGF-β1 production seen in DN.
The Effects of Lovastatin in Experimental Models

In recent years, statins have become a beneficial agent in combating fibrosis, atherosclerosis, inflammation and endothelial dysfunction, stroke, and undoubtedly hypercholesterolemia. Important to this discussion is the action of LOV on fibrosis where it inhibits the deposition of extracellular matrix proteins, and its ability to enhance fibrinolysis.

Lovastatin has been used experimentally on multiple cell types including: coronary endothelial cells, glomerular mesangial cells, epithelial cells, and human umbilical vein endothelial cells. As stated previously, statins function by inhibiting 3-hydroxy-3-methylglutaryl coenzyme A reductase, a rate-limiting enzyme in the cholesterol biosynthesis pathway as well as the small GTPase proteins used in intracellular signaling. Statins effectively prevent the isoprenylation of small GTPase proteins, such as the Ras and Raf proteins, used in various signaling pathways. In the last 20 years, statins have gained attention for their pleiotropic effects, such as interfering with the adhesion of inflammatory molecules to the endothelium, reducing the production of TGF-β1 and fibronectin in mesangial cells, inhibiting fibrotic protein accumulation within the ECM, and enhancing the fibrinolytic system in models of fibrosis. A detailed discussion of these experiments as they pertain to this research design will follow.

The actions of LOV in high glucose models using mesangial cells have been well documented. Mesangial cells exposed to high glucose have consistently shown increased expression of TGF-β1, fibronectin, and collagen IV. In each of the previous studies, treatment with LOV markedly decreased the glucose-induced
increases in TGF-β1 compared to control levels. Kim et al.\textsuperscript{2} investigated the effects of LOV in vivo and in cell culture models of diabetes. First, the investigators induced diabetes in Sprague Dawley rats weighing 200-250 kg with a single injection of streptozotocin and then treated with 4mg/kg of lovastain. The investigators took blood samples taken at 7 and 14 days, as well as 1, 3, 6 and 12 months. Animals were sacrificed at defined time points and TGF-β1 mRNA and protein were measured.\textsuperscript{2} The TGF-β1 mRNA was obtained from the glomeruli at 4 days, 1, 4, and 12 weeks. Northern blot analysis showed a 1.3-fold (4 day) to 1.9 fold (12 week) increase in glomerular TGF-β1 mRNA in the untreated compared to control rats. Lovastatin effectively suppressed glomerular TGF-β1 mRNA expression in diabetic glomeruli to 1-fold of control, approximately 0.3 to 0.9 fold below diabetic controls. These results showed in vivo administration of LOV was effective in inhibiting TGF-β1 protein accumulation in the glomerulus compared to control, as well as suppressing the clinical measures of DN.

Another part of their study investigated rat mesangial cells cultured under control (5.5 mM) and high (25 mM) glucose conditions with 10 μM LOV alone, 100μM mevalonate alone, or both, to determine the cellular effects of LOV. Media supplemented with LOV showed a 1-fold decrease in TGF-β1 mRNA and protein expression, as well as a 1.4 fold decrease in fibronectin expression compared to control. The inhibitory effect of LOV was effectively reversed by supplementation of 100 μM of mevalonate to the media. These results suggest that LOV has a direct cellular effect on TGF-β1 independent of a cholesterol-lowering effect and also suggest LOV may delay the onset and progression of DN, at least in part, through suppression of glomerular mesangial expression of TGF-β1 and fibronectin.\textsuperscript{2}
However, the cellular and molecular mechanisms of LOV on TGF-β1 and fibronectin were unclear. Therefore, these investigators used the same in vivo model, although focused on small GTP binding proteins produced from the mevalonate pathway, which is inhibited by LOV. Kim et al. found that the down regulation of TGF-β1 and fibronectin were linked to signaling of small guanine triphosphate (GTP)-binding proteins and mediated by the limitation of isoprenoids such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP). The isoprenoid FPP is responsible for the post-translational modification and membrane targeting of the Ras protein, while GGPP is responsible for those of the Rho family small GTP binding proteins. To determine if isoprenoid and small GTP-binding proteins were involved in high glucose-induced TGF-β1 and fibronectin expression, FPP and/or GGPP were added to rat mesangial cells cultured under normal (5.5 mM) or high glucose (30 mM) conditions. Rat mesangial cells, were incubated with high glucose media supplemented with LOV (10 μM) and incubated for 48 h. Glucose increased the expression of TGF-β1 and fibronectin mRNA and protein expression in mesangial cells, all of which were effectively inhibited by LOV. These results were in agreement with their previous study. Suppression of fibronectin expression by LOV was completely reversed when GGPP or mevalonate were added to the cultured media; however, when GGPP and FPP were added together, only partial reversal of the suppressive effect of LOV on fibronectin expression occurred, thus suggesting that the intracellular signaling pathway for fibronectin is regulated differently than TGF-β1 expression.

These investigators demonstrated that LOV inhibits TGF-β1 and fibronectin by decreasing the production of intracellular signaling GTP proteins FPP and GGPP created
in the mevalonate pathway.\textsuperscript{5, 37} The production of FPP and GGPP post-translationally modify the intracellular signaling proteins Ras and Rho. The Rho family proteins activate stress-activated protein kinase/c-Jun N-terminal protein kinase (SAPK/JNK) and p38 MAPK.\textsuperscript{5} These kinases activate various transcription factors, such as AP-1. There are three AP-1 binding sites in the promoter region of TGF-β1, which are responsible for the activation and autoinduction of this profibrotic protein.\textsuperscript{31, 43} Therefore, the decrease in membrane-bound small GTP binding proteins reduces expression and activation of various transcription factors, leading to the down regulation of TGF-β1, fibronectin, and collagen IV gene expression.\textsuperscript{1, 5, 37}

**Lovastatin Effects on Fibrosis and the Fibrinolytic System**

One of the beneficial effects of LOV in regards to fibrosis is its ability to increase the enzymatic properties of the fibrinolytic system.\textsuperscript{1} The fibrinolytic system, also referred to the plasminogen activator-plasmin system, naturally functions to break down fibrotic proteins which accumulate within the ECM, for instance after wound healing, a function which is dampened in diabetes.\textsuperscript{69} The fibrinolytic system is important, due to plasmin having a broad spectrum in degrading a number of ECM components, and activating metalloproteases to amplify proteolysis.\textsuperscript{1} Tissue type plasminogen activator (tPA) is a serine protease primarily responsible for the breakdown of the pro-enzyme plasminogen to plasmin, a fibrinolytic enzyme responsible for fibrotic protein degradation.\textsuperscript{1, 36} Therefore, plasmin and tPA have both become intervention points in combating fibrosis. A dysfunctional fibrinolytic system, as seen in diabetes, is therefore incapable of metabolizing matrix proteins and fibrotic proteins, thus facilitating ECM
protein accumulation and creating sclerotic plaques that can occlude the vasculature.\textsuperscript{42} People afflicted with type I and II diabetes have increased risk of atherosclerosis through accumulated plaque formation.\textsuperscript{9, 42} Epidemiological studies of human and animal models have shown that when atherosclerosis is caught early, excessive fibrotic accumulation can be reversed by statin treatment.\textsuperscript{42, 69, 73, 76, 110, 111} Therefore, therapies which increase the proteolytic actions of the fibrinolytic system have been implemented in people afflicted with diabetes.\textsuperscript{71}

Wiesbauer et al.\textsuperscript{8} investigated six different statins (atorvastatin, cerivastatin, fluvastatin, lovastatin, pravastatin, and simvastatin) for their effect on the fibrinolytic system in human umbilical vein endothelial cells, smooth muscle cells, and hepatoma cells in vitro. All statins used except pravastatin significantly decreased plasminogen activator inhibitor type-1 (PAI-1) production in human endothelial and smooth muscle cells, where as no effect was found on the hepatoma cells. Furthermore, only simvastatin and lovastatin increased t-PA production in endothelial cells, an effect which was reversed by the addition of mevalonate to conditions.

Further demonstrating LOV as a fibrinolytic agent, Essig et al\textsuperscript{1} demonstrated that LOV induced an increase in tPA and a decrease in PAI-1. Male Wistar rats received daily injections of LOV at 4 mg/kg body weight, a consistent dose given throughout the literature, for approximately two days before sacrifice. Proximal tubular cells were isolated from control and treatment animals induced to diabetes with streptavadin. Histological examination showed an increase in tPA and urokinase plasminogen activator (uPA) activity in animals treated with LOV, but not in the control. In addition, tubular cells were isolated and cultured from control rats, and incubated with a range of 0.1-10.0
μM doses of LOV for 48 h. Cells cultured with LOV showed a concentration-dependent increase (0.1 to 10 μM) in tissue type plasminogen activator (tPA) and a decrease in PAI-1. Furthermore, LOV effects on tPA activities were completely reversed by addition of 100 μM of mevalonate to the media of LOV-treated cells, clearly showing that LOV was the affecting agent in TGF-β1 inhibition. Therefore, increased production of plasmin via statin action decreases the accumulation of ECM proteins, such as fibronectin and collagen IV, on the ECM and basement membrane. Thus, by increasing t-PA and decreasing PAI-1 at sites of vascular lesions, statins may reduce fibrin formation and thrombus development in vascular lesions seen in diabetic patients. These results suggest that LOV could enhance the fibrinolytic system, thereby decreasing the ECM protein accumulation most often seen in DN.

Taken together, these studies establish LOV as a potent inhibitor of TGF-β1 and fibronectin production in mesangial cells, as well as an accelerator of fibrinolysis by increasing tPA and plasmin in proximal tubule, mesangial and endothelial cells. Abundant production of TGF-β1 is a focal mediator in fibrotic protein accumulation in the renal glomerulus and extracellular matrix. However, high glucose has not consistently been shown to induce TGF-β1 synthesis by multiple types of endothelial cells. Furthermore, LOV has not been applied to a high glucose model using glomerular endothelial cells. Therefore, the current study was designed to assess the effects of LOV on total TGF-β1 concentration by RGEC cultured in high glucose media. Numerous studies outlined here show glucose-induced synthesis of TGF-β1, however the effect of high glucose on glomerular endothelial cells remains unclear and will be assessed in this study.
Conclusion

Diabetic nephropathy (DN) is characterized by glomerulosclerosis with thickening of the glomerular basement membrane, mesangial matrix expansion, tubulointerstitial fibrosis, and renal hypertrophy.\textsuperscript{2,3} Hyperglycemia upregulates the expression of a fibrogenic growth factor, TGF-\(\beta\)\textsubscript{1}, contributing to the development of DN.\textsuperscript{106} Activation of the TGF-\(\beta\) signaling cascade upregulates genes encoding for ECM proteins, such as fibronectin and collagen IV.\textsuperscript{105} Deposition of these proteins within the ECM is mediated in large part through the glucose-induced activation of TGF-\(\beta\)\textsubscript{1} transcription and TSP-1 activation of latent TGF-\(\beta\).\textsuperscript{2,106} With studies showing prevention of DN through use of anti-TGF-\(\beta\)\textsubscript{1} antibody, TGF-\(\beta\)\textsubscript{1} has clearly been established as an important contributor to the pathogenesis of the disease.\textsuperscript{21}

Lovastatin inhibits the rate limiting enzyme mevalonate, a mediator responsible for production of the intracellular signaling proteins FPP and GGPP. These small GTP binding proteins are crucial elements in the TGF-\(\beta\)\textsubscript{1} signaling pathway, leading to TGF-\(\beta\)\textsubscript{1} transcription and protein prenylation.\textsuperscript{5,35,109} Therefore, the ability of LOV to decrease the production of membrane bound small GTP binding proteins, reduces expression and activation of various transcription factors, leading to the down regulation of TGF-\(\beta\)\textsubscript{1}, fibronectin and collagen IV gene expression.\textsuperscript{1,5,37}

In models of diabetes, LOV inhibits the production of fibronectin and TGF-\(\beta\)\textsubscript{1} mRNA and protein expression in mesangial cells,\textsuperscript{2,5,7} and enhanced the fibrinolytic system in human umbilical vein endothelial cells.\textsuperscript{8} However, the effects of LOV on TGF-
β1 expression by glomerular endothelial cells in high glucose conditions has yet to be
established, and is therefore the aim of this research study. The anti-fibrotic effects of
statins have been assessed in multiple endothelial cell culture models, including the heart
and kidney. Because TGF-β1 is a prime mediator in the pathogenesis of fibrosis leading
to DN, inhibiting its deleterious effects through blocking its production is crucial.
Although important, fibronectin and collagen IV, which are expressed by the
translocation of the Smad complex to the nucleus within the TGF-β receptor signaling
cascade, were outside the scope of this study and were not assessed. The specific aim of
this research study was to elucidate if LOV would decrease the expression of TGF-β1
protein in RGEC exposed to media supplemented with high glucose concentrations.
III: Research Design and Methods

All experiments were done using rat glomerular endothelial cells (RGEC) grown to confluence in 100 mm cell culture plates (Cellstar®, Frickenhausen, Germany) and exposed to reduced serum media for 24 h before being exposed to experimental conditions. The purpose of this study was to investigate the effects of high glucose media (25 mM) and lovastatin on total TGF-β concentration within media in an in vitro model of diabetes.

A. Cell Culture

Rat glomerular endothelial cells (RGEC) were previously isolated from glomeruli obtained by sieving the cortex of kidneys excised from 6-month-old Sprague-Dawley rats.112 These cells were a gift from Dr. William Couser from the University of Washington Medical School. RGEC were characterized as endothelial cells using a panel markers specific to endothelial cells; these markers included: vascular endothelial cadherin, FLT, FLK, and endothelial nitric oxide synthase. Cells at passages 14-25 were frozen in liquid nitrogen and used for experiments.

To establish cell cultures, frozen RGEC were quickly thawed in a warm (37°C) water bath for approximately 1 min and immediately washed by slowly pipetting 14 mL of cold Dulbecco’s Modified Eagle Medium (DMEM, Gibco Life Technologies, Rockford, MD) containing 10% FBS (Atlas Biologicals, Ft. Collins, CO), 2 mM sodium pyruvate, 90 μg/mL heparin, 100 IU/mL penicillin, 100 μg/mL streptomycin, 3.5 grams of glucose (25.5 mM), and a total concentration of 4 mM MgSO₄ (complete DMEM). Cells were then centrifuged at 1000 x g, for 5 min in a chilled centrifuge at 4°C. The
media was then aspirated and approximately 1 mL of complete warm DMEM (37°C) was applied to the cell pellet; the solution was aspirated gently and repeatedly to disperse the cell clump. The cell suspension solution was added to 100-mm tissue culture plates (Greiner, ISC Biologicals, Kaysville, UT) that were pre-coated for 30 min with a 5 μg/mL human, plasma-derived fibronectin solution (Roche Biochemicals, Indianapolis, IN) in phosphate buffered saline (PBS). The cells were incubated in a Sanyo Biomedical (Wooddale, IL) incubator at 37°C in 5% CO2 and a humidified atmosphere. Complete DMEM was changed 18-24 h after the initial plating, and every 48 h thereafter until cells were 75-80% confluent on the culture plate, a process that usually took 3-5 days.

Cells were passaged at 75-80% confluence using 0.025 % trypsin/EDTA (Gibco BRL, Rockford, MD) in PBS. After the trypsin/EDTA solution was added to the cells and allowed to incubate at room temperature for 5 min, the cells were scraped using a cell scraper. Approximately 5 mL of warm complete DMEM was applied to the trypsin/EDTA mixture to terminate proteolysis of the endothelial cell attachments. The cells in solution were then placed in a 15 mL centrifuge tube and centrifuged at 1000g, for 5 min. The cell pellet was re-suspended in 3 mL of warm complete DMEM, and 1 mL of cell solution was applied to each of three fibronectin-coated plates. Approximately 8 mL of warm DMEM was added to the plate and the cells were incubated for 24 h. After the initial 24 h incubation after plating, media was replaced. Media was then replaced every 48 h until the cells reached confluency for experimental conditions.
B. Activation of Lovastatin

Inactive lactone forms of lovastatin were converted to active forms following manufacturer’s instructions briefly described here. Approximately 25mg of lovastatin was purchased from Calbiochem (Merk, Darmstadt, Germany). The inactive lovastatin was dissolved in 1 mL of absolute ethanol, and 0.1 M NaOH was added to the mixture. This mixture was heated at 50°C for 2 h. Following incubation, 4.5 mL of an aqueous solution containing 81mM Na₂HPO₄ and 15 mM of NaH₂PO₄ was added and incubated for 30 min in a water bath at 30°C. After incubation, the solution was neutralized with 0.1 M HCl to a pH of 7.2. The final volume was 8.5 mL and a final concentration of 2.941 mg/mL. Appropriate calculations were conducted to yield corresponding concentrations of lovastatin per 9 mL of media. The prepared lovastatin was aliquotted into 1 mL centrifuge vials and stored at -20°C until use.

C. Experimental Conditions and Treatments

At confluence, cells were made quiescent by 24 h serum reduction in DMEM supplemented with high glucose (25 mM) and 2% FBS prior to the initiation of experiments. Because endothelial cells are particularly sensitive to reduced serum, we measured LDH release into medium to determine if cell viability was acceptable. The dose of lovastatin was deemed optimal by use in current literature. Cells were treated with 10 μM of lovastatin in high glucose media only. At 12, 24 and 48 h, 500 μL samples of cell media were taken and frozen in -80°C with 125 μL of EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). These samples were used for assessing cell viability and TGF-β1 production.
Culture plates were then distinguished into one of the three following conditions:

1.) Control incubated in complete DMEM media supplemented with 2% FBS and 5.5 mM glucose;
2.) High glucose incubated in complete DMEM media supplemented with 2% FBS and 25 mM glucose;
3.) High glucose with lovastatin incubated in complete DMEM media supplemented with 2% FBS, 25.5mM glucose and 10μM of lovastatin

Each sample was run in triplicate.

D. Measuring TGF-β Proteins in Supernatant

Secreted TGF-β1 protein was quantified by using a double-antibody sandwich ELISA kit (Duoset, R&D Systems, Minneapolis, MN). The kit included: matched capture antibody, detection antibody for TGF-β, TGF-β protein as a standard, and streptavidin-horseradish peroxidase for detection. Manufacturer’s directions were followed and are detailed below.

Approximately 100 μL of the capture antibody was applied to a sterile 96-well plate (Corning Life Sciences, The Netherlands), covered with an adhesive strip, and incubated at room temperature overnight. After incubation, the plate was washed three times with wash buffer (PBS and .05% tween 20 [Bio-Rad Laboratories, Hercules, CA]). After wash buffer was aspirated, block buffer (PBS, 5% tween 20) was applied for a minimum of 1 h. After 1 h incubation, repeat wash in step 1. Stored samples were thawed in a 37°C water bath for approximately 5 min and centrifuged at 100g for 5 min. Sample Activation: approximately 100 μL of the sample was applied to a clean, sterile 96-well
plate in triplicate wells. Samples were activated by adding 25 μL of 1N HCl for 10 min. The samples were neutralized with 25 μL of 1.2N NaOH and immediately applied to the previously coated plate. The plate was covered with an adhesive strip and incubated for 2 h at room temperature. After the 2 h incubation period, the samples were discarded.

Repeat wash in step 1. Approximately 60 μL of the detection antibody was reconstituted in reagent diluent (1.4% delipidized bovine serum concentrate (R&D Systems) reconstituted with 0.05% Tween [BioRad Laboratories, Hercules, CA]). Approximately 100 μL of the detection antibody was applied to the assay plate with a multi-channel pipette and covered with an adhesive strip. The plate was incubated at room temperature for 2 h. After 2 h, the detection antibody was discarded. Repeat wash in step 1.

Streptavidin-horshradish peroxidase (HRP) was activated within 30 min of use in the assay. Reconstituted HRP consisted of approximately 55 μL of HRP was added to 10.995 mL of reconstituted reagent diluent for a dilution ratio of 1:20. HRP was applied and covered with an adhesive strip to protect the plate from light. The plate was incubated at room temperature for 20 min. Substrate solution was made using a 1:1 ratio of reagent A and reagent B (R&D Systems, Minneapolis, MN). Approximately 100 μL was added to each well, and incubated for 20 min at room temperature. Stop solution consisted of 2N sulfuric acid. Approximately 50 μL of stop solution was applied to each well and read immediately with a spectrophotometer. The plate was read with a spectrophotometer (SpectraMax Pro384 (Software: Softmax Pro v. 3.1.2); Molecular Devices, Sunnyvale, CA) at 450 nM and 560 nM and analyzed using the standard curve.
E. Determination of Cell Injury/Viability

Viability of cells and cellular injury was assessed in each condition/treatment group using the Cytotoxicity Detection KitPLUS (Roche Applied Science, Germany) for lactate dehydrogenase (LDH) in cell supernatant following manufacturer’s directions. One kit included: catalyst, lysing solution, start solution and stop solution. Start solution was prepared by dissolving the catalyst in 1 mL of double deionized water. Approximately 250 μL of the catalyst solution was added to 11.25 mL of start solution to constitute the reaction solution. The reaction solution was used immediately.

Frozen media samples (for conditions see section C) were thawed in 37°C water bath and inverted once to gently mix the contents. To ensure the samples were mixed, the tip of the centrifuge tube was tapped once while the tube was inverted. Samples were then centrifuged at 1000g to remove cell debris. Approximately 50 μL of each sample was applied in triplicate to a 96-well plate. Positive control (PC) sample was obtained from the media of a lysed plate using the manufacturer’s lysing solution supplied with the kit. Approximately 5 mL of lysing solution was applied to RGEC at 100% confluence and media samples were obtained as described above. Samples were frozen in -80°C until use. The low control (LC) sample was obtained from 10% FBS complete DMEM used for feeding the cell culture in this study. Approximately 100 μL of the reaction mixture was applied to each well in the 96-well plate and incubated for 20 to 30 min at room temperature. After 20-30 min incubation, approximately 50 μL of stop solution was applied to each well and read immediately at 490 nM with a reference wavelength of 650 nM using a spectrophotometer (SpectraMax Pro384 (Software: Softmax Pro v. 3.1.2;
Molecular Devices, Sunnyvale, CA). Percent cytotoxicity was determined by using the following equation: 

\[
\frac{(\text{Sample} - \text{LC})}{(\text{PC} - \text{LC})}
\]

F. Statistical Analyses

SPSS Version 16 (Chicago, IL) was used for statistical analyses. Data are expressed as means plus/minus the standard error. A one-way repeated measures Analysis of Variance (ANOVA) test was used to evaluate the outcome measurements in response to experimental conditions. A two way repeated measures ANOVA test was performed to measure differences among groups exposed to different experimental conditions. Statistical significance was set at probabilities less than 5 percent (p<0.05). Samples were run in triplicate for all experiments.
IV: Results, Discussion and Conclusion

Abstract

Background: Diabetic nephropathy (DN) is the leading cause of morbidity, mortality and end stage renal disease in patients with diabetes mellitus.\textsuperscript{1,2} Glomerular fibrosis is a key pathological process in DN and is enhanced in diabetes. Endothelial cells cultured under high glucose conditions can enhance the production of transforming growth factor beta (TGF-\(\beta\))\textsuperscript{1}. HMG-CoA reductase inhibitors (statins) have been shown to decrease markers of fibrosis in cell culture models of diabetes.\textsuperscript{1-3} Lovastatin (LOV) decreases TGF-\(\beta\)\textsubscript{1} production in mesangial cells and various types of endothelial cells cultured in high glucose conditions.\textsuperscript{1-8} However, to date, there are no studies reporting the effects of LOV on the production of TGF-\(\beta\) by rat glomerular endothelial cells (RGEC) cultured under high glucose conditions. The purpose of this study was to investigate the effects of LOV on the production of TGF-\(\beta\) in a diabetic cell culture model using RGEC. We hypothesize that LOV will inhibit the increases in total TGF-\(\beta\) concentration that typically occur in high glucose models. Methods: RGEC were grown to confluence in normal glucose (5.5 mM) media supplemented with 10% FBS. Once confluent, RGEC were exposed to high glucose (25 mM) media alone or with 10 \(\mu\)M LOV, and incubated for 48 h. Media samples were taken at 12, 24 and 48 h and frozen (-80\textdegree C) with protease inhibitor cocktail and subsequently assayed for total TGF-\(\beta\)\textsubscript{1}. Results: Total TGF-\(\beta\)\textsubscript{1} protein produced by RGEC exposed to 10 \(\mu\)M LOV was significantly lower than groups exposed to high glucose alone at 12 h (\(p=0.017\)) and 24 h (\(p=0.020\)). We conclude that 10
μM LOV was effective at decreasing total TGF-β1 concentration in RGEC exposed to high glucose media.

**Background**

Diabetic nephropathy (DN) is a leading cause of morbidity, mortality, and end stage renal disease (ESRD) in patients with diabetes mellitus. The total Medicare burden for patients with ESRD in 2005 was approximately 19 billion dollars lending support to the argument that therapeutic interventions to ameliorate DN are necessary.

Diabetic nephropathy is characterized by progressive thickening of the glomerular basement membrane and expansion of the mesangial matrix, which correlates with decreased glomerular filtration and dysfunction as a result of elevated blood glucose. Glomerulosclerosis, a pathologic progressive thickening of the glomerular basement membrane and mesangial matrix expansion, is a condition characterized by the excessive deposition of the fibrotic proteins fibronectin, collagen IV, and transforming growth factor beta1 (TGF-β1) within the glomerulus and is present in DN. Transforming growth factor beta1 is a profibrotic and prosclerotic cytokine known to be naturally expressed in renal endothelial cells and excessively expressed in response to high glucose in DN.

In high glucose (25 mM) experimental cell culture models using human umbilical vein endothelial cells, glucose stimulates the expression and production of TGF-β1. Activation of TGF-β1 upregulates genes encoding for the ECM proteins fibronectin and collagen IV, both overexpressed in DN. In addition to stimulating fibrosis and sclerosis, TGF-β1 downregulates genes encoding for ECM-degrading enzymes known as matrix metalloproteases. Fibrotic proteins are enzymatically metabolized by MMP
within the ECM, thereby retarding protein accumulation in the ECM within the
glomerulus.\textsuperscript{2} Increased expression of TGF-\(\beta\)1 markedly decreases metabolism of ECM
proteins by matrix metalloproteases in mesangial and endothelial cells cultured under
high glucose concentrations.\textsuperscript{19, 21-23} Therefore, the simultaneous actions of TGF-\(\beta\) to
enhance production of ECM and reduce MMP activity within the ECM make TGF-\(\beta\) a
crucial element in the accumulation of ECM proteins.

HMG-CoA reductase inhibitors (statins), commonly known for their cholesterol
lowering effects, have been shown to decrease fibrosis in cell culture models of
diabetes.\textsuperscript{10-13} Statins inhibit the mevalonate pathway, an important metabolic pathway
primarily responsible for synthesizing sterol and non-sterol isoprenoids, as well as
GTPase intracellular signaling molecules involved in the TGF-\(\beta\) signaling cascade.\textsuperscript{35, 37-39}
Therefore, statins could be beneficial in inhibiting the high glucose-induced synthesis of
TGF-\(\beta\)1, through the inhibition of the GTPase intracellular signaling molecules.\textsuperscript{12,10,11}
Lovastatin (LOV) decreases TGF-\(\beta\)1 expression in a dose-dependent manner in various
cell culture models of diabetes.\textsuperscript{1-8, 33-36} However, its effect on TGF-\(\beta\)1 by rat GEC has yet
to be established.

\textbf{Methods}

\textbf{Endothelial Cell Culture}

Rat glomerular endothelial cells (RGEC) were used for all experiments. RGEC were a
gift from Dr. William Couser from the University of Washington Medical School. Cells
were previously isolated from glomeruli obtained by sieving the cortex of kidneys
excised from 6-month-old Sprague-Dawley rats then frozen in liquid nitrogen until use.
Cell panel of markers indicative of endothelial cells were used to assess phenotypic characteristics; the cell panel included: FLT, FLK, eNOS and VE-Cadherin. Cells at passages 14-25 were used for all experiments. Frozen RGEC were quickly thawed in a warm (37°C) water bath and washed with 14 mL of cold Dulbecco’s Modified Eagle Medium (DMEM, Gibco Life Technologies, Rockford, MD) containing 10% fetal bovine serum (FBS, Atlas Biologicals, Ft. Collins, CO), 2 mM sodium pyruvate, 90 μg/mL heparin, 100 IU/mL penicillin, 100 μg/mL streptomycin, 3.5 grams of glucose was added to yield a concentration of 25.5 mM, and a total concentration of 4 mM MgSO₄ (complete DMEM). Cells were centrifuged (1000x g, 5 min) and the media aspirated. Complete DMEM (8 mL) was added to the cell pellet and the solution aspirated repeatedly to break up the cell clump. The cell suspension solution was added to 96 mm tissue culture plates (Greiner, ISC Biologicals, Kaysville, UT) that were pre-coated for 30 min with a 5 μg/mL human, plasma-derived fibronectin solution (Roche Biochemicals, Indianapolis, IN) in phosphate buffered saline (PBS). Complete DMEM was changed 18-24 h after the initial plating and every 48 h thereafter. Cells were passaged at 75-80% confluence using 0.025 % trypsin/EDTA (Gibco BRL, Rockford, MD) in PBS and centrifuged (1000x g, 5 min), resuspended in complete DMEM, and plated onto the fibronectin-coated plates.

**Experimental Conditions and Treatments**

Once confluent, RGEC were incubated for a total of 48h in one of the three following conditions: 1) Control: complete DMEM containing 5.5 mM glucose; 2) High glucose: complete DMEM containing high glucose (25.5 mM); 3) High glucose with 10 μM LOV
treatment. Supernatant samples from the three above conditions were taken at 12, 24 and 48 h and stored frozen at –80°C until assayed. Each sample was run in triplicate.

**Measurement of TGF-β1**

Supernatant samples were thawed and immediately centrifuged to remove cell debris. Total TGF-β1 protein was activated and quantified in media using double-antibody sandwich ELISA kits (Duoset, R&D Systems, Minneapolis, MN) following manufacturer’s instructions. Each kit included matched capture and detection antibodies for TGF-β1 protein specific to rat TGF-β1, TGF-β1 protein as a standard, and streptavidin-horseradish peroxidase for detection. Samples were run in triplicate wells to increase statistical power. Absorbance was read with a spectrophotometer and compared to a standard curve for total concentration.

**Determination of Cell Viability**

Viability of cells was assessed in each group with the Cytotoxicity Detection KitPLUS (Roche Applied Science, Germany) for lactate dehydrogenase (LDH) following manufacturer’s directions. Assays were done using cell supernatant and cell lysate. Supernatant LDH release was expressed as a percent of total LDH in a completely lysed plate. A spectrophotometer was used to assess absorbance of samples.

**Activation of Lovastatin**

The inactive lactone forms of lovastatin was purchased from Calbiochem® (Darmstadt, Germany) and converted to its active form following manufacturer’s instructions. Briefly,
25 mg of lovastatin was dissolved in 1 mL of absolute ethanol, and 0.1 M NaOH was added to the mixture. This mixture was heated at 50°C for 2 h. Following incubation, 4.5 mL of an aqueous solution containing 81 mM Na2HPO4 and 15 mM of NaH2PO4 was added and incubated for 30 min in a water bath at 30°C. After incubation, the solution was neutralized with 0.1 M HCl to a pH of 7.2. The final volume was approximately 8.5 mL and a final concentration of 2.941 mg/mL. Appropriate calculations were conducted to yield corresponding concentrations of lovastatin per 9 mL of media. The prepared lovastatin was aliquotted into 1 mL centrifuge vials and stored at -20°C until use.

**Statistical Analyses**

SPSS Version 16 (Chicago, IL) was used for statistical analyses. Data are expressed as means plus/minus the standard error. One-way repeated measures Analysis of Variance (ANOVA) was used to evaluate TGF-β1 and LDH in response to experimental conditions. Specific tests between conditions were conducted using a priori contrasts. Statistical significance was set at probabilities less than 5 percent (p<0.05).

**Results**

The total TGF-β1 concentration for RGEC exposed to high glucose media supplemented with 10 μM LOV (n=6) was significantly lower (p=0.04) than cells exposed to high glucose alone (n=5). The total TGF-β1 expression at 12h (p=0.017) and 24h (p=0.020) was significantly lower in cells exposed to lovastatin compared to those in high glucose alone. Statistical significance was lost between these groups at 48h (p=0.063). These results are graphically depicted in Appendix B. Total TGF-β1 concentration was not
significantly different (p=0.65) between cells exposed to normal and high glucose conditions.

Lactate dehydrogenase release from cells exposed to control, high glucose and lovastatin conditions were not significantly different among groups. However, a significant time-dependent increase in LDH release (p=0.00) was seen for each group. Appendix C shows cytotoxicity as a percentage of the whole at 12, 24 and 48 h for all conditions.

**Discussion**

The major finding of this study is that lovastatin is a powerful reducer of total TGF-β1 concentration of glomerular endothelial cells cultured in high glucose. Total TGF-β1 is a profibrotic cytokine excessively expressed in diabetes-associated disease. Increased TGF-β1 concentrations are present in kidneys of patients with renal hypertrophy.\(^1\)\(^2\),\(^4\)\(^3\) Furthermore, decreasing circulating TGF-β1 in patients via administration of anti-TGF-β1 antibodies inhibits the progression of renal fibrotic and cardiovascular sclerotic disease, suggesting its role as a potent mediator of fibrotic-associated diseases.\(^1\)\(^1\)\(^3\)

Statins, most commonly known for their cholesterol lowering effects, are also effective in ameliorating the progression of atherosclerotic and inflammatory diseases.\(^6\)\(^7\),\(^7\)\(^4\),\(^1\)\(^1\)\(^1\)\(^3\) Transcription of TGF-β1 is dependant upon GTPase proteins produced from mevalonate, a rate limiting enzyme blocked by statins.\(^2\),\(^3\),\(^5\) Our study indicates for the first time that glomerular endothelial cells cultured in high glucose media with lovastatin have a decreased total TGF-β1 concentration at 12 and 24 hours compared to high glucose alone.
Surprisingly, a significant difference in total TGF-β1 concentrations was not found between high glucose-treated cells and control cells. The majority of previous studies in endothelial cells, including glomerular endothelial cells, indicate glucose as a potent stimulator of TGF-β1,\textsuperscript{51,53-58,64} although conflicting results have been reported for human umbilical vein endothelial cells (HUVEC).\textsuperscript{29,32} Two studies exposed HUVEC to elevated glucose concentrations and did not find an increase in TGF-β1 mRNA or protein concentrations at 12 or 24 hours compared to control cells.\textsuperscript{29,32} However, in one of these studies Chen et al.\textsuperscript{32} reported a significant increase in TGF-β1 mRNA expression at 72 hours compared to samples taken at 48 hours. Thus, implying that HUVEC needed longer than 48 hours of exposure to high glucose concentrations to produce a measurable increase in TGF-β1 protein concentrations in media. Furthermore, glomerular endothelial cells also appear to be time dependent on glucose exposure. Danne et al.\textsuperscript{51} found that the response to high glucose by glomerular endothelial cells, as measured by collagen IV production, occurs over an extended period of time with maximal response at 78 hours. In the current study, glomerular endothelial cells were exposed to high glucose media for a total of 48 hours, inadvertently limiting glucose exposure time and therefore minimizing the maximal potential of these cells to produce TGF-β1. Therefore, it is possible that glucose exposure time for this study was too short to significantly increase total TGF-β1 concentration.

Lactate dehydrogenase (LDH) release was not significantly different among all groups, but time had a significant inducing effect on LDH release among all groups. However, others have reported LDH release increases in endothelial cells cultured in high glucose models, independent of a time effect. Conceivably, increased LDH release from
cells cultured in high glucose in these studies is in response to excessive TGF-β1, since endothelial cells exposed to abundant amounts of TGF-β1 are induced to go through apoptosis, thereby increasing the release of LDH.\textsuperscript{32, 57} Therefore, the insignificant difference seen in total TGF-β1 concentration between high glucose-treated cells and control cells found in our study, is a potential reason no change in LDH release occurred.

Measuring total TGF-β1 has been highly correlated with active TGF-β1 in cell media.\textsuperscript{83} Thus, the concentration of total TGF-β1 directly reflects the biologically active molecule responsible for the pathogenesis involved with TGF-β1 related renal diseases. This study showed a reduction in total TGF-β1 concentration between cells treated with lovastatin and cells treated with high glucose alone, therefore indicating lovastatin is powerful in decreasing total TGF-β1 concentration in high glucose media.

Statistical power could have been an alternate possibility for not finding a glucose-induced stimulatory effect on total TGF-β1 concentration in our study. The number of samples taken in this study were n=5 for the control group and high glucose group, and n= 6 for the lovastatin-treated group. The number of samples taken for each group was low, and statistical power is generally increased by increasing the number of samples. Therefore, low statistical power is a possible factor for the lack of significant increase in TGF-β1 concentration above control in glomerular endothelial cells treated with high glucose.

Cells of passage numbers 14-24 were used in this study. A panel of cellular markers indicative of endothelial cells was used to establish these cells as endothelial cells. Endothelial cells grown on fibronectin substrate, as done in this study, maintain
their phenotypical characteristics when serially passaged.\textsuperscript{114} Therefore, we believe that phenotypic changes in the cells studied do not explain the lack of significant increase in total TGF-\(\beta\)-1 concentration or LDH release when comparing the high glucose-treated and control cells.

We have shown that glomerular endothelial cells exposed to high glucose concentrations and treated with lovastatin have a decreased total TGF-\(\beta\)-1 concentration compared to control. Although these results indicate that high glucose concentrations are not an inducer of TGF-\(\beta\)-1 production by RGEC, lovastatin elicited a significant decrease of total TGF-\(\beta\)-1 concentrations. These results are significant because a decrease in TGF-\(\beta\)-1 would be expected to decrease the expression of fibronectin and collagen IV, two extracellular fibrotic proteins excessively expressed through the activation of the TGF-\(\beta\) receptor pathway in diabetes.

Since their inception and approval by the Food and Drug Administration, statins have proven themselves to be beneficial agents in combating hypercholesterolemia, stroke, cardiovascular disease, atherosclerosis, and inflammatory diseases with minimal side effects.\textsuperscript{68, 71, 92, 111} Our study suggests that lovastatin may affect TGF-\(\beta\)-1 in high glucose related diseases. Although TGF-\(\beta\)-1 was not induced in glomerular endothelial cells cultured in high glucose, the decrease in total TGF-\(\beta\)-1 by lovastatin was clear. The advantages of this drug being previously approved by the FDA would allow immediate use in epidemiological studies with patients suffering from kidney disease associated with TGF-\(\beta\)-1. The results of this study are important because inhibiting the production of TGF-\(\beta\)-1 would decrease fibrotic extracellular matrix protein production and accumulation within the glomerulus, thereby decreasing the progression of kidney
diseases associated with TGF-β1. Therefore, lovastatin could be a beneficial therapeutic agent in a vastly growing population with diabetes-associated diseases as well as diseases with TGF-β1 as the focal mediator.
Appendix A

Representative Diagram of Experimental Design

Rat glomerular endothelial cells (RGEC) were exposed to one of three experimental conditions:
1. Complete DMEM supplemented with normal glucose (5.5 mM).
2. Complete DMEM supplemented with high glucose (25 mM).
3. Complete DMEM supplemented with high glucose (25 mM) and lovastatin (10 μM).

Cell supernatant samples were taken at 12, 24 and 48 h and frozen at -80°C.

Samples were assayed to quantify TGF-β1 and LDH expression.
Appendix B

Total TGF-β1 Concentration Produced by Glomerular Endothelial Cells Exposed to Control and Experimental Conditions

![Bar chart showing total TGF-β1 concentration among groups at 12, 24, and 48 hours.]

- **Control**
- **High Glucose**
- **High Glucose+Lovastatin**

Figure 1: Mean (± SE) of total transforming growth factor-beta (TGF-β1) concentration in media from rat glomerular endothelial cells (RGEC) exposed to control (5.5 mM glucose; n=5), high glucose (25 mM; n=5), or high glucose supplemented with 10 μM lovastatin (n=6). Compared to high glucose alone, RGEC exposed to HG+LOV showed a significant decrease in total TGF-β1 concentration at 12 h (p=0.017*) and 24 h (p=0.020*), but was not achieved at 48 h (p=0.61).
Appendix C

Lactate Dehydrogenase Release by Glomerular Endothelial Cells

![Graph showing lactate dehydrogenase release](image)

Figure 2: Mean (± SE) of lactate dehydrogenase (LDH) release by rat glomerular endothelial cells (RGEC) exposed to control (5.5 mM; n=5), high glucose (25 mM; n=5), or high glucose media supplemented with 10 μM lovastatin (n=6). No significant difference (p=.61) was found among groups at all time points. However, a significant time effect was seen at 24 and 48 h for all groups (p=0.04).
V. References


