Endothelium and Cardiovascular Complications of Diabetes Mellitus: the Role of the Glyoxalase System

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Thesis submitted to the

Faculty of Graduate and Postdoctoral Studies

in partial fulfillment of the requirements

for the Doctorate in Philosophy degree in Cellular and Molecular Medicine

Faculty of Medicine

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ABSTRACT

In patients with diabetes, hyperglycemia leads to functional impairment of endothelial cells (ECs) and microangiopathy. Inflammation and endothelial dysfunction (ED) have been associated with the development of several cardiovascular complications. Concentration of methylglyoxal (MG) - a highly reactive aldehyde is increased in diabetes. In a non-pathological state, MG is detoxified by the enzymes glyoxalase-1 (GLO1) and glyoxalase 2 in presence of glutathione.

This thesis examines the role of MG accumulation in ECs and bone marrow cells (BMCs), with the consequences it has for their function. To this end, a transgenic mouse model was used in which the human enzyme GLO1 is overexpressed in the vasculature By using a GLO1 overexpressing mouse model studies described here examined the contribution of MG-induced inflammation in vivo to cardiovascular complications of diabetes, namely diabetic heart failure and peripheral vascular disease.

This study confirmed that accumulation of MG leads to inflammation and cell death, and further explained how MG affects the role of ECs in development of the heart failure and BMCs in the revascularization. Overexpression of GLO1 in the vasculature diminished MG-induced inflammation, reduced EC death and delayed and limited the loss of cardiac function in streptozotocin (STZ)-induced diabetic mice (Chapter 2). The in vitro part of this study showed that MG and tumor necrosis factor (TNF-□□ have a synergistic effect on cell death (Chapter 3). Overexpressing the GLO1 in BMCs only, restored neovascularization in ischaemic tissue of mice with STZ-induced diabetes (Chapter 4).

Taken together, the results of this thesis suggest that hyperglycemia increased MG leads to endothelial inflammation, EC death and decreased angiogenic potential of BMCs. Furthermore, this MG-induced inflammation and reduced cell function observed, identifies a potential target for therapy of the cardiovascular complications seen in diabetes.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVATIONS

3-DG	3- deoxyglucosone
ADP	adenosine diphosphate
AGEs	advanced glycation end products
AP	argepyrimidine
ARE	antioxidant-response element
ATP	adenosine triphosphate
BCA	bicinchoninic acid assay
Bcl-2	B-Cell lymphoma - 2
Bcl-XL	B-Cell lymphoma - XL
BM	bone marrow
BMCs	bone marrow cells
CACs	circulating angiogenic cells
CEL	Nε-carboxyethyl-lysine
CPR	C-reactive protein
CVD	cardiovascular disease
DC	diabetic complications
DM	Diabetes Mellitus
DNPH	dinitrophenylhydrazine
EBM	endothelial basal medium
EC	endothelial cells
ECM	extracellular matrix
ED	endothelial dysfunction

ELISA	enzyme-linked immunosorbent assay
eNOS	endothelial nitric oxide synthase
EPC	endothelial progenitor cells
E-selectin	endothelial-leukocyte adhesion molecule 1
FGF	fibroblast growth factor
FOV	field of view
FS	fractional shortening
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluoresent protein
GLO	glyoxalase
GLUT	glucose transporter
GSH	glutathione
hECs	human endothelial cells
HG	high Glucose
HIF-1	Hypoxia-Inducible Factor-1
HKR	HEPES-Krebs-Ringer's buffer
HMGB1	high mobility group box I
HNE	hydroxynonenal
HPLC	high-performance liquid chromatography
HSCs	hematopoietic stem cells
ICAM-1	intercellular adhesion molecule 1
IL-1	interleukin-7
IL-6	interleukin-1
ISH	in situ hybridization

KD	knock down
LV	left venticular
LVEF	left ventricular ejection fraction
MAP	mitogen-activated protein
MBO	methyl diaminobenzene-BODIPY
MCP-1	monocyte chemoattractant protein-1
MDA	malondialdehyde
MG	methylglyoxal
MG-H1	hydroimidazolone–isomer 1
MG-H2	hydroimidazolone–isomer 2
MG-H3	hydroimidazolone–isomer 3
MI	myocardial ischemia
MODIC	arginine–lysine-derived crosslink 2-NH3-1H-imidazol-5-ylideneamino hexanoate
MOLD	methylglyoxal-derived lysine dimer
NADPH	nicotinamide adenine dinucleotide phosphate oxidase
NF-κβ	nuclear factor-kappa B
NO	nitric oxide
NRF2	nuclear factor-erythroid 2
OCR	oxygen consumption rate
PAI-1	plasminogen activator inhibitor-1
PI	propidium iodide
PVD	peripheral vascular disease
RAGE	receptor for advanced glycation end products
ROS	reactive oxygen species

SDF-1	stromal cell-derived factor-1
SEM	standard error of the mean
sRAGE	soluble receptor for advanced glycation end products
STZ	streptozotocin
T1DM	type 1 Diabetes Mellitus
T2DM	type 2 Diabetes Mellitus
THP	tetrahydropyrimidine
TNF	tumor necrosis factor
TNFR1	tumor necrosis factor receptor 1
TP	triose-phosphate
TUNEL	terminal uridine nick-end labeling
UCP	uncoupling proteins
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
vWF	Von Willebrand factor
WT	wild type

CHAPTER 1 - INTRODUCTION

1.1 Diabetes mellitus – Causes and Prevalence

Diabetes Mellitus (DM) is a chronic metabolic disorder characterised by an insufficient production of insulin (type 1 - T1DM) or by the lack of response to insulin (type 2 - T2DM). Both types of DM are characterized by an increase in blood glucose levels, which subsequently damages the vasculature leading to diminished organ function (Gray and Jandeleit-Dahm, 2014). Prolonged exposure to elevated plasma glucose levels leads to toxic effects in a variety of cell types resulting in pathological changes in organ and tissue function, known as diabetic complications (DC) (Carlos, 2012).

In 2008/09, almost 2.4 million Canadians (6.8% of the population) were living with DM (Government of Canada, 2011). Worldwide, 171 million people have DM, and of those approximately 90% have T2DM, mainly due to obesity and/or the aging population. Rates of T1DM among children and youth have also been on the rise globally, with Canada found to have one of the highest incidence rates of T1DM for children younger than 14 years of age. The total number of people with DM is predicted to increase to approximately 366 million people by 2025 (American Diabetes, 2014).

1.2 Cardiovascular complications of DM

Diabetic individuals have a four-fold increased risk of developing cardiovascular complications (Fox, 2010). Consistently high blood glucose levels can lead to serious diseases affecting the heart and blood vessels, eyes, kidneys, and nerves. The pathophysiology of the link between diabetes and cardiovascular disease (CVD) is complex and multi-factorial. Four main mechanisms implicated in the pathogenesis of DC are: 1)

increased flux through the polyol pathway, 2) activation of the protein kinase C (PKC) pathway, 3) increased flux through the hexosamine pathway (HSP) and 4) increased formation of advanced glycation end products (AGEs) (Brownlee, 2001a). All these mechanisms seem to be a consequence of hyperglycemia-induced overproduction of superoxide by the mitochondrial electron-transport chain (Brownlee, 2001b). The focus of this thesis is on the increased formation of AGEs, which will be discussed in more detail in section 1.3.2.

1.2.1 Diabetes - risk factor for cardiovascular disease (CVD)

A large body of epidemiological and pathological data document that DM is an independent risk factor for CVD in both men and women (American Diabetes, 2014, Abaci et al., 1999, Mercer et al., 2012, Stirban and Tschoepe, 2008, Taylor, 2013). CVD is listed as the cause of death in approximately 65% of patients with DM, as diabetic patients who developed clinical CVD have a worse prognosis for survival than non-diabetic patients (Grundy et al., 1999).

The direct and indirect effects of hyperglycemia and/or insulin resistance on the human vascular tree are the major sources of morbidity and mortality in both type 1 and type 2 diabetes. Generally, the harmful effects of hyperglycemia are separated into macrovascular complications (coronary artery disease, peripheral arterial disease, and stroke) and microvascular complications (diabetic nephropathy, neuropathy, and retinopathy) (Fowler, 2008) (FIG 1.1).

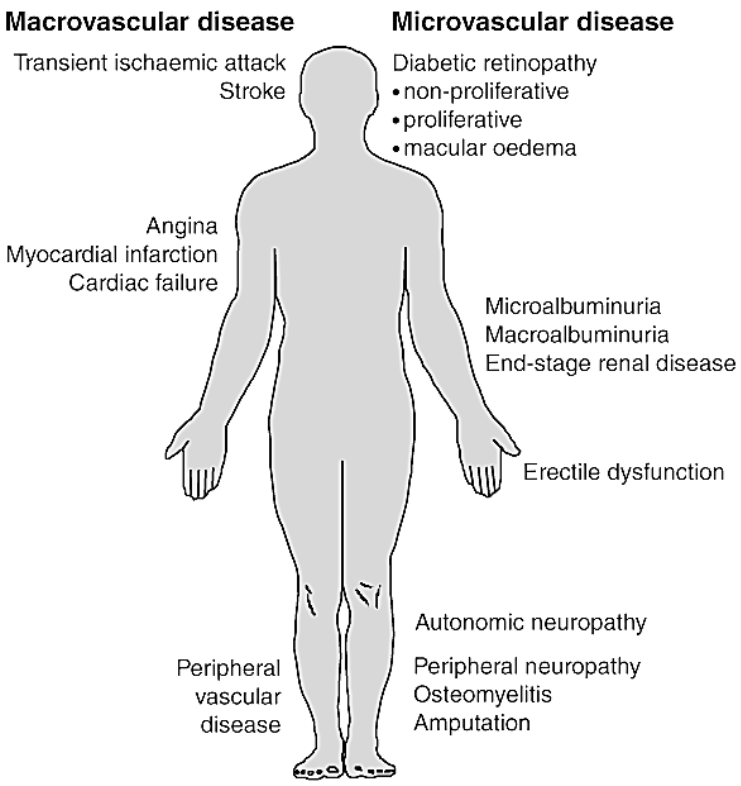

FIGURE 1.1. Complications of diabetes – adopted from "Preventing complications of diabetes" (Bate and Jerums, 2003). Diabetic complications are the major cause of associated morbidity and mortality. They are classified as macrovascular (affecting large arteries) or microvascular (affecting capillaries and small blood vessels). Reproduced with permission from © Copyright 2003 The Medical Journal of Australia.

1.2.2 Macrovascular disease

The central pathological mechanism in macrovascular disease is narrowing of arterial walls throughout the body. Atherosclerosis is thought to result from chronic inflammation and injury to the arterial wall in the peripheral or coronary vascular system (Paneni et al., 2013). In addition to the accumulation of degenerative material in the inner layer of artery walls (atheroma), there is strong evidence of increased platelet adhesion and coagulation in T2DM (Ding and Triggle, 2005). Impaired nitric oxide (NO) generation in the endothelium, increased free radical formation in platelets, as well as altered calcium regulation, may promote platelet aggregation (Ding and Triggle, 2005). Elevated levels of plasminogen activator inhibitor type 1 may also impair fibrinolysis in patients with DM (Pandolfi et al., 2001). In response to endothelial injury and inflammation, oxidized lipids from LDL particles accumulate in the endothelial wall of arteries (Dokken, 2008). Monocytes then infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipids to form foam cells. Once formed, foam cells stimulate macrophages and the attraction of T-lymphocytes. T-lymphocytes, in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation (Fowler, 2008). The net result of all these processes is the formation of an atherosclerotic lesion, which can lead to vessel stenosis and eventual plaque rupture with resulting thrombosis. (Cerletti et al., 2010).

DM is an independent risk factor across all ages for stroke – the incidence of stroke is up to 2- to 4-fold greater in patients with DM (Cade, 2008). DM also impairs recovery from a stroke, with severe neurological deficits and disability, a poorer long-term prognosis, and a higher incidence of stroke recurrence than in patients without DM (Sacco et al., 2006).

Myocardial ischemia (MI) due to coronary atherosclerosis commonly occurs without symptoms in patients with DM. In many cases, a delayed diagnosis of the diabetic condition worsens the prognosis for survival for many diabetic patients that suffered MI. More and more studies have shown that the risk of MI in people with DM is equivalent to the risk in non-diabetic patients with a history of previous MI (Kumar and Cannon, 2009).

Another cause for heart failure that is specific for patients with diabetes is termed diabetic cardiomyopathy (DC). The concept of DC was first introduced by Rubler *et al*, and has later been broadly used by epidemiologists and clinicians (Rubler et al., 1972). DC describes DM-associated changes in the structure and function of the myocardium that are not the direct result of coronary artery disease or hypertension. Several mechanisms have been implicated in the pathogenesis of DC (Boudina and Abel, 2010). Changes in myocardial structure, calcium signaling and metabolism are early defects that have been described mainly in animal models (Boudina and Abel, 2010, Hayat et al., 2004, Poornima et al., 2006). Studies have shown that these abnormalities are independent of ischemic heart disease or systemic hypertension and often occurred asymptomatically (Voulgari et al., 2010, Isabelle Pham, 2015). The possibility of underlying endothelial dysfunction (ED) that contributes to changes in the myocardium has not yet been confirmed in patients, but may precede clinically manifested cardiac dysfunction (Bando and Murohara, 2014, Hayat et al., 2004).

Occlusion of the lower-extremity arteries, which can result in functional impairments and disability, is known as peripheral vascular disease (PVD). PVD is directly related to the duration and severity of DM (American Diabetes, 2003). Hyperglycemia, and specifically the glycation of hemoglobin, has been shown to be a risk factor for PVD that is independent of smoking (Cade, 2008). About 27 million of individuals in Europe and North America

have PVD (Brevetti et al., 2008). Also, these patients are 15 times more likely to have lower extremity amputation than people without DM (Cade, 2008).

Although macrovascular disease that results from diabetes is mainly associated with complications of the large vessels, the role of microvascular abnormalities in altering cardiac structure and large blood vessel function must be considered.

1.2.3 Microvascular disease

The primary cause of microvascular disease in diabetic patients is the exposure of endothelial cells (ECs) to chronic hyperglycemia (Fowler, 2008). Two clinical trials, the UK Prospective DM Study on type 1 diabetes (UKPDS) and the DM Control of Complications Trial (DCCT – with type 2 diabetes patients) have established a clear relationship between microvascular disease and glucose control (Higgins et al., 2007, Nathan and Group, 2014).

Microvascular disease mainly affects the tissues where glucose uptake is independent of insulin activity (vascular endothelium, but also kidney and retina) (Kolluru et al., 2012). In human micro- and macrovascular ECs, insulin does not affect glucose uptake and metabolism (Artwohl et al., 2007), and glucose transport is principally mediated via GLUT1 (Mann et al., 2003). Cells in these tissues are exposed to glucose levels that correlate very closely with blood glucose levels, since they cannot regulate the main cellular glucose transporters (GLUT family). A combination of direct glucose-mediated endothelial damage with oxidative stress, and the production of sorbitol and AGEs cause altered blood flow and changes in endothelial permeability, extravascular protein deposition and coagulation resulting in vascular and organ dysfunction (Vithian and Hurel, 2010).

The microcirculation is regulated by central and local regulatory mechanisms. The central regulation is via autonomic sympathetic and parasympathetic nerves that reach the vascular smooth muscle. Local regulation is carried out by factors produced by the ECs and by local products of metabolism. The endothelium produces both vasodilators and vasoconstrictors (Cines et al., 1998). Normally, the vascular smooth muscle receives continuous regulatory nerve signals and a continual supply of NO from the endothelium regulating vasodilation, as well as a supply of nutrients (Dokken, 2008). These regulatory mechanisms adjust microvascular flow instantaneously to meet the metabolic needs of the tissue. Patients with DM consistently show an impairment of endothelium-dependent vasodilation (Schalkwijk and Stehouwer, 2005).

A very common pathogenic mechanism for microvascular and macrovascular disease is the chemical reaction of by-products of sugars with proteins that produce irreversible adducts and crosslinks – AGEs (Milne and Brownstein, 2013, Goldin et al., 2006, Kalousová et al., 2005, Rabbani and Thornalley, 2012). AGEs can exhibit a wide range of effects on surrounding tissues: direct modification of collagen and endothelium, inhibition of growth, programmed cell death (apoptosis), defective neovascularization, and increased vascular inflammation. In addition, AGEs can increase the expression of the receptor for AGE (RAGE), a cell surface member of the immunoglobulin superfamily that, upon ligand-binding, elicits a cascade of signaling events that further lead to EC dysfunction (Ramasamy et al., 2012, Kalousová et al., 2005, Brownlee, 2001b, Chetyrkin et al., 2011, Yan et al., 2003).

1.2.3.1 Endothelial dysfunction (ED)

Large clinical trials in both T1DM and T2DM have shown that hyperglycemia plays a big part in the pathogenesis of microvascular complications and is a major contributing factor in the development of ED and EC apoptosis (Giles and Sander, 2004, Mercer et al., 2012, Cade, 2008, Institute of Medicine (US) Forum on Drug Discovery, 2010). Understanding and treating ED is a major focus in the prevention of vascular complications associated with all forms of DM.

ECs form a continuous layer on the intimal surface of the entire cardiovascular system. ECs provide an anticoagulant barrier between the vessel wall and the blood. In addition to this, the ECs have metabolic and paracrine functions (Cines et al., 1998). ECs regulate basal vascular tone, vascular reactivity, permeability, the composition of the sub-endothelial matrix, the adhesion and extravasations of leucocytes and the balance between coagulation and fibrinolysis (Favero et al., 2014). ECs produce a wide range of factors that also regulate cellular adhesion, thrombo-resistance, smooth muscle cell proliferation, and vessel wall inflammation. This regulation is accomplished by a complex interplay between the ECs and other cell types: smooth muscle cells and leukocytes (Rao et al., 2007).

ED begins when the endothelium is no longer capable of adequately performing its functions (Endemann and Schiffrin, 2004). One of the first signs is an increased inflammatory state of the endothelium (Steyers and Miller, 2014). This can be evaluated by measuring circulating levels of soluble adhesion molecules (e.g. ED markers) such as: Intercellular Adhesion Molecule 1(ICAM-1), Vascular Cell Adhesion Molecule 1 (VCAM-1), Endothelial-leukocyte Adhesion Molecule 1 (E-selectin), soluble von Willebrand factor, and also plasminogen activator inhibitor-1 (PAI-1) (Hadi and Suwaidi, 2007, Farhangkhoee

et al., 2006, Ruszkowska-Ciastek et al., 2015). An increased number of circulating mature ECs and reduced endothelial progenitor cells (EPCs) is another sign of disturbed endothelial homeostasis (Fadini, 2008).

While acute hyperglycemia and hyperinsulinemia enhance vasodilatation in T1DM without increasing capillary permeability and inducing endothelial dysfunction (Oomen et al., 2002), hyperglycemia-induced AGE formation has a more lasting effect (Ulrich and Cerami, 2001). Within the vessel wall, collagen-linked AGEs can trap plasma proteins, quench NO activity and interact with specific receptors (RAGE) to modify a large number of cellular properties (Milne and Brownstein, 2013, Goldin et al., 2006, Ma et al., 2009, Kalousová et al., 2005). AGEs induce oxidative reactions that can promote the formation of oxidized LDL (Basta et al., 2004). These interactions of AGEs with ECs provide a mechanism to lasting vascular dysfunction.

1.2.3.2 EC inflammation

The identification of chronic inflammation in DM is mainly based on an observed increase in plasma concentrations of C-reactive protein (CRP), fibrinogen, interleukin-6 (IL-6), interleukin-1 (IL-1), and tumor necrosis factor (TNF) (Jain et al., 2011). These inflammatory cytokines increase vascular permeability, change vasoregulatory responses, and increase the adhesion of monocytes, neutrophils, and macrophages, resulting in cell damage. Inflammation also facilitates thrombus formation by inducing pro-coagulant activity, inhibiting anticoagulant pathways and impairing fibrinolysis (van den Oever et al., 2010, van Hinsbergh, 2012).

The TNF-family of cytokines plays an important role in regulation of the immune response, inflammation, and apoptosis. TNF-α is mainly produced by neutrophils and

macrophages, but also by other tissue cells and can induce other cytokines such as IL-6, which in turn regulates the expression of CRP (Grivennikov et al., 2005, Spriggs et al., 1992). CRP increases the expression of endothelial ICAM-1, VCAM-1, E-selectin, and Monocyte Chemoattractant Protein-1 (MCP-1) and intensifies the secretion of endothelin (Endemann and Schiffrin, 2004, van den Oever et al., 2010). It is also possible that TNF up-regulation may contribute to increased apoptosis associated with diabetic complications (van den Oever et al., 2010).

1.2.3.3 Endothelial cell (EC) apoptosis

Apoptosis is a controlled and regulated process in which a cell plays an active role in its own death. There are several mechanisms through which apoptosis can be induced in cells: binding of death-inducing ligands to cell surface receptors and intrinsic signals that are produced following cellular stress (like oxidative stress, deprivation of growth factors, or exposure to radiation or chemicals) (Elmore, 2007). The sensitivity of cells to apoptosis is regulated by the balance of the pro- and anti-apoptotic bcl family of proteins. Bcl-2 and Bcl-XL are anti-apoptotic, while Bad, Bax and Bid are pro-apoptotic proteins (Tzifi et al., 2012). Important for the vasculature, NO has also been demonstrated to inhibit apoptosis in a number of cell types including ECs (Suenobu et al., 1999).

The increased and accelerated rate of apoptosis of ECs seen in diabetes is probably a crucial factor in the development of DC. There are many pathways involved in activating EC apoptosis and all of these pathways can in turn be activated in multiple ways. Specifically in the development of DC, a common mechanism causing EC apoptosis is oxidative stress (Farrugia and Balzan, 2012, Kannan and Jain, 2000).

1.2.3.4 Abnormal angiogenesis in DM

In a healthy tissue, vasculature repair occurs by the spontaneous growth of collateral blood vessels leading to blood vessel renewal. Initially, ischemia-induced neovascularization was thought to result solely from the angiogenic process which involved hypoxia and inflammation-related pathways (Isner and Asahara, 1999). In diabetic patients, ischemia, and often non-traumatic limb amputation, is the consequence of insufficient collateral blood vessel formation around occluded arteries (Silvestre and Levy, 2006).

It has been suggested that the alteration in vascular endothelial growth factor (VEGF) expression and signaling due to hyperglycemia affects blood vessel formation in the tissue (Benjamin, 2001). VEGF plays a crucial role in EC motility, proliferation, and survival. VEGF-A has been shown to promote adult vasculogenesis, in part, via BM-derived EPC mobilization (Wong and Crawford, 2013). A key mechanism that regulates VEGF expression is the hypoxia-inducible factor-1 (HIF-1) pathway. Studies have demonstrated that impaired HIF-1 binding to its co-activator p300 may underlie diabetic impairments in wound healing (Ceradini et al., 2008)

Recent studies demonstrate that postnatal neovascularization does not rely exclusively on sprouting of pre-existing vessels, but also involves BM-derived progenitor cells (Urbich and Dimmeler, 2005, Carmeliet, 2000, Asahara et al., 1999). Signals from ischemic tissues promote the mobilization of both vascular and hematopoietic progenitors into the peripheral circulation from which they get recruited to the ischemic site (Takahashi et al., 1999). Adult BM is known to contain a population of cells that can be divided into lineage positive (Lin^+) and lineage negative (Lin^-) categories with regard to their potential to differentiate into formed elements of the blood. Lin^- hematopoietic stem cells (HSCs) have

been shown to contain a population of endothelial progenitor cells (EPC) capable of forming blood vessels (Li Calzi et al., 2010). Endogenous BM-derived progenitor cells also release pro-angiogenic growth factors that promote blood vessel growth.

Circulating angiogenic cells (CACs), also referred to as early EPCs, are believed to represent a cell population enriched in monocytes that execute their angiogenic effects via paracrine and signaling mechanisms, critical for the maintenance and repair of ECs (Rehman et al., 2003, Yoder et al., 2007). The number of CACs is decreased in both type 1 and 2 DM patients, which is a partial cause of the pathogenesis of vascular complications (Avogaro et al., 2011). In diabetic patients with vascular complications, there is a marked reduction of CACs compared to those patients without vasculopathy, and the BM-derived CACs are dysfunctional, producing fewer ECs with reduced proliferative and migratory potential due to oxidative stress (Case et al., 2008). Studies performed *in vitro* show that CACs from diabetic populations have reduced capacity to form tubules, and a poorer ability to revascularize damage tissues (Loomans et al., 2009, Case et al., 2008).

Impaired endothelial regeneration and defective neovascularization contribute to the progression of diabetic vascular complications and, as a consequence, the potential role of altered EPC function in diabetes is now an important area of investigation (Georgescu, 2011, Jarajapu and Grant, 2010).

1.2.3.5 Role of microangiopathy in macrovascular disease

Inflammation and apoptosis caused by ED probably play a significant role in the pathophysiology of heart failure (FIG 1.2), atherosclerosis, PVD and stroke. In cardiac diseases, damage to ECs disrupt their regulation of heart function (van den Oever et al.,

2010). Endothelial damage and dysfunction have been observed in myocardial capillaries in hypertension, hyperlipidemia and ischemia/reperfusion linked to DM (Bauters et al., 2003, Frustaci et al., 2000, Vithian and Hurel, 2010). ED can lead to repeated episodes of ischemia and small infarcts that ultimately contribute to the development of heart failure and stroke. The poor prognosis of recovery depends on new vessel formation and remodeling of the pre-existing vasculature. The negative outcome recorded in patients with DM may be partly related to the inflammatory processes and the abnormalities in endothelial function (Billinger et al., 2002, Katon et al., 2004).

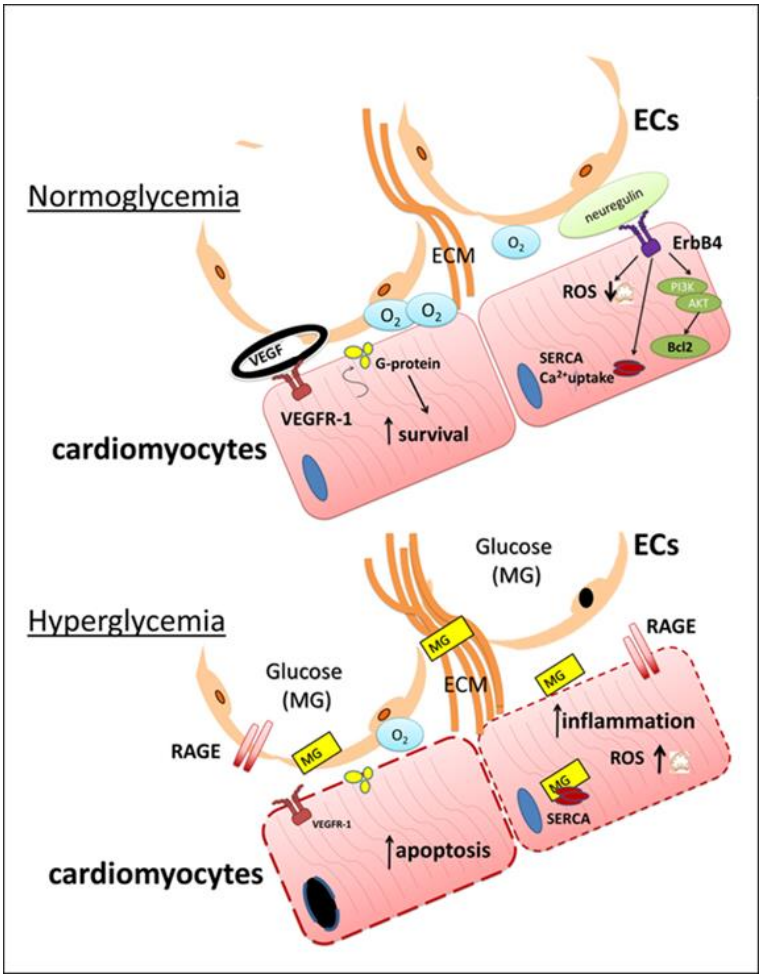

FIGURE 1.2. Model of the development of diabetic heart disease. Cardiac microvascular endothelial cells (ECs) express neuregulin, a pro-survival cytokine that acts on adjacent cardiomyocytes through the Akt pathway, promoting expression of anti-apoptotic factor Bcl2. It also reduces ROS production in mitochondria. VEGF directly protects cardiomyocytes from apoptosis through activation of G-protein. In diabetes, high blood glucose through non-enzymatic glycolysis generates methylglyoxal (MG). MG accumulation causes EC dysfunction, and increases RAGE expression and inflammation. EC death and dysfunction causes ischemia, and reduced neuregulin and VEGF-B levels results in greater cardiomyocyte apoptosis.

1.3 METHYLGLYOXAL (MG), THE GLYOXALASE (GLO) SYSTEM, AND AGES IN DM

The formation and accumulation of AGEs is related to diabetes and is one of the main causes of DC (Milne and Brownstein, 2013, Basta et al., 2004, Goldin et al., 2006, Münch et al., 2012, Kalousová et al., 2005). AGE formation is believed to be mainly a result of various reactive metabolites that derive from glucose, and less so from the glucose itself (Attia, 2010). Perhaps the most potent glycating glucose-derived metabolites is methylglyoxal (MG) (Ramasamy et al., 2006). In normal physiological conditions it is metabolized by the glyoxalase (GLO) system, while in conditions of hyperglycemia MG accumulates and affects the conformation and function of cell proteins and nucleic acids (Rabbani and Thornalley, 2011, Rabbani and Thornalley, 2012, Thornalley, 2008a). In this way MG leads to inflammation, altered cell function and ultimately cell death. MG is primarily detoxified by the GLO system, present in the cytosol of all cells. It comprises two enzymes, glyoxalase I (GLO1) and glyoxalase II (GLO2), and a catalytic amount of glutathione (GSH), and in a healthy physiological setting it reduces MG in the cell to a non-toxic level (Thornalley, 2003).

1.3.1 METHYLGLYOXAL (MG) and GLYOXALASE 1 (GLO1)

1.3.1.1 Methylglyoxal (MG)

MG (2-oxopropanal) has long been thought of simply as a by-product of glycolysis (Allaman et al., 2015). MG is a reactive dicarbonyl compound known to form covalent adducts with proteins and nucleic acids (Oya et al., 1999, Rabbani and Thornalley, 2012). In diabetes, cells contain considerable amounts of MG that accumulate with time. The majority

of the MG not detoxified by GLO system is quickly bound to proteins (Rabbani and Thornalley, 2012).

In aqueous solutions it is present in three forms, the non-hydrated, monohydrate and dihydrate forms, which are in equilibrium (Wren et al., 2015). The presence of physiological concentrations of glutathione (GSH) shifts this equilibrium towards the formation of the hemithioacetal, and MG is then efficiently removed by the GLO system (Thornalley, 2003).

The main sources of MG (FIG 1.3) are triose-phosphate (TP) glycolytic intermediates (Martins et al., 2001). Non-enzymatic fragmentation and elimination of phosphate from glyceraldehyde-3-phosphate (G3P) and dihydroxyacetonephosphate (DHAP) contribute most to the formation of MG. The metabolism of acetone and threonine also add to the formation of MG, but under normal metabolic conditions both are of minor importance (Reichard et al., 1986). MG can also be formed enzymatically by MG synthases, although the significance of these enzymes has not yet been investigated in higher eukaryotes (Rose and Nowick, 2002).

An important regulator of intracellular MG levels is glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Reduced activity of GAPDH leads to the accumulation of TP, leading to a subsequent increase in MG formation (Beisswenger et al., 2003). GAPDH activity is in turn influenced by oxidative stress and by MG modification. This suggests that MG is able to propagate its own formation.

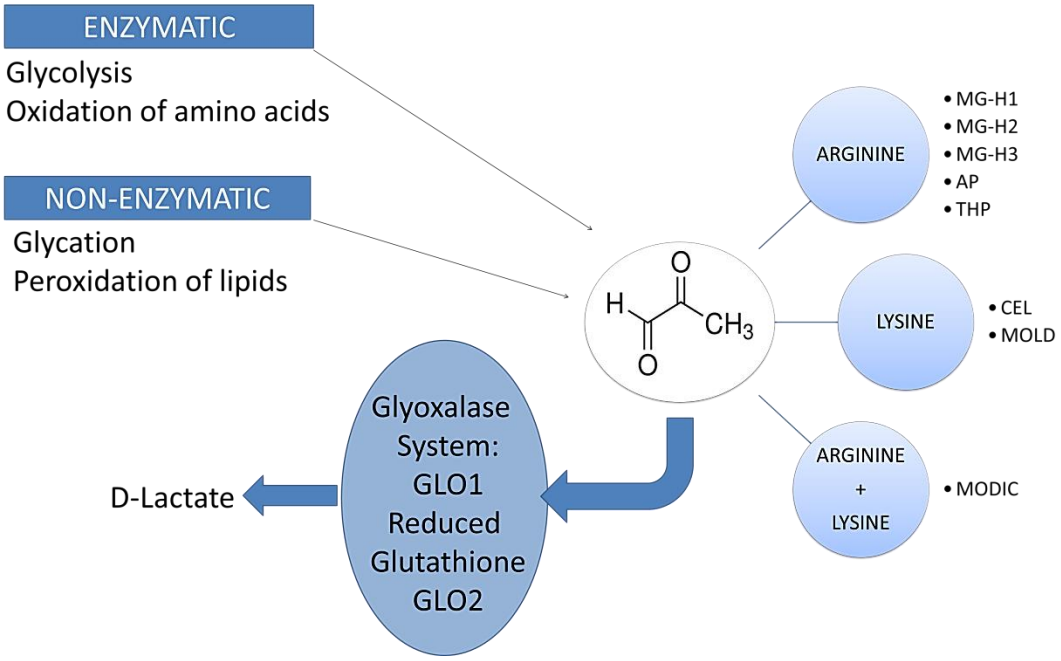

FIGURE 1.3 – Methylglyoxal (MG) origin and metabolism. MG is a highly reactive dicarbonyl molecule derived both enzymatically and non-enzymatically from the metabolism of glucose, proteins and lipids. Under physiological conditions, the glyoxalase system (GLO) degrades MG into D-lactate in the presence of reduced glutathione, keeping plasma and cellular levels of MG low. GLO I catalyzes the conversion of hemithioacetyl (adduct of reduced glutathione and MG) into S-D-lactoylglutathione, which is acted on by GLO II to regenerate GSH. Free MG interacts with arginine and lysine to produce advanced glycation end products (AGEs) – MG-H1 (hydroimidazolone–isomer 1), MG-H2 (hydroimidazolone–isomer 2), MG-H3 (hydroimidazolone–isomer 3), AP (argepyrimidine), THP (tetrahydropyrimidine), CEL (Nε-carboxyethyl-lysine), MOLD (methylglyoxal-derived lysine dimer), MODIC (arginine–lysine-derived crosslink 2-ammonio-1H-imidazol-5-ylideneamino hexanoate).

1.3.1.2 Brief history of glyoxalase

For a little over a hundred years the GLO system has been known to exist in animals. The discovery dates back to 1913, and from then on, research focussed on the possible role it might have in glycolysis. A definite proof that the GLO system was not involved in glycolysis was that the end product of the GLO system was D-lactate and not L-lactate (L-lactate being the end product of glycolysis). The ubiquitous nature of the GLO system, however, suggests its essential and conserved role (Sousa Silva et al., 2013).

Today it is known that the GLO system represents an enzymatic MG detoxification pathway (FIG 1.4). During the first step in this pathway a hemithioacetal is formed non-enzymatically by the reaction of MG with GSH. The rate limiting enzyme GLO1 facilitates the formation of S-D-lactoylglutathione, after which GLO2 catalyses the hydrolysis of S-D-lactoylglutathione to D-lactate, thereby restoring the GSH. Glyoxal is also detoxified by the GLO system, resulting in the formation of S-glycolylglutathione and consequently glycolate as the end product (Thornalley, 2003, Thornalley, 1993).

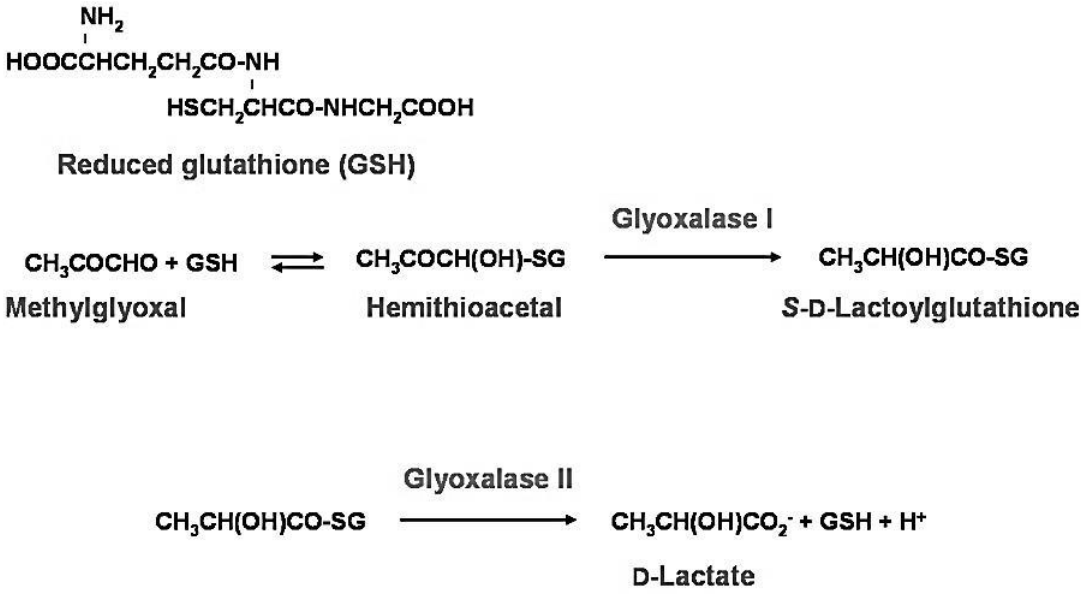

FIGURE 1.4. Glyoxalase system. Under physiological conditions, the glyoxalase system (GLO) degrades MG into D-lactate in the presence of reduced glutathione. A hemithioacetal is formed non-enzymatically by the reaction of MG with glutathione (GSH). The rate limiting enzyme GLO1 facilitates the formation of S-D-lactoylglutathione, after which GLO2 catalyses the hydrolysis of S-D-lactoylglutathione to D-lactate, thereby restoring the GSH.

1.3.1.3 Physiological vs. pathological role of glyoxalase

It is evident that the detoxification of α-oxoaldehydes, and MG in particular, is a very important function for the GLO system. However, the fact that the GLO system includes two enzymes, that are ubiquitous and conserved, and that there are several other mechanisms capable of the detoxification of MG (although minor by comparison), suggest that other functions for the GLO system might exist, yet the exact possible role of GLO system is yet to be discovered. Suggested alternative functions of GLO are the regulation of cell division and the reduction of stress (Reddy and Sopory, 1999, Deswal et al., 1993, Ranganathan et al., 1995).

A possible function in cell division was first discovered in plants. In the dividing cells of the plant, the concentration of MG was low and GLO1 activity was high. In non-dividing cells, the level of MG was higher and GLO1 activity was undetectable (Reddy and Sopory, 1999). In yeast, GLO1 activity correlates with the overall growth status, although it is not involved in the regulation of the cell cycle (Aguilera and Prieto, 2004). In addition, some animal studies describe a correlation between cell proliferation and activity of the GLO system (Thornalley, 1990). Also, after partial hepatectomy, the regenerating liver expresses high GLO1 activity and low GLO2 activity (Principato et al., 1983). These activities return to baseline levels after regeneration. During the maturation of human erythrocytes it was found that GLO1 activity increases, while a decreased activity of GLO1 was seen in mature, old erythrocytes (Thornalley et al., 1989).

High expression levels of GLO1 are considered a sign of advance in growth of certain types of cancer, and various tumours show a significant increase in levels and activity of GLO1 and a high GLO1 to GLO2 activity ratio in comparison to the corresponding non-

tumour and surrounding tissues (Zhang et al., 2014, Hosoda et al., 2015, Baunacke et al., 2014). Therefore, the GLO system is considered to be a 'marker for tumorous cell growth and division', but a mechanistic connection between the GLO system and cell growth is still to be discovered.

The GLO system may also have a pro-survival role. It has been reported that tumors with high levels of GLO1 are more resistant to anti-tumor induced-apoptosis (Sakamoto et al., 2001). Consequently inhibitors for GLO1 are being developed to be used as anti-tumor agents (Geng et al., 2014).

Still, because of its ability to reduce levels of MG in the cell, the GLO system is mainly studied in the settings of diabetes and aging, and lately as a possible therapeutic target for neurodegenerative diseases.

1.3.2 Advanced glycation end products (AGEs)

AGEs are proteins or lipids that become non-enzymatically glycated and oxidized after contact with aldose sugars (Goldin et al., 2006). Early glycation and oxidation processes result in the formation of Schiff bases and Amadori products. Further glycation of proteins and lipids causes molecular rearrangements that lead to the generation of AGEs. AGEs may produce reactive oxygen species (ROS), induce specific cell surface receptor expression, and form cross-links (Ahmed and Thornalley, 2007).

AGEs formed in hyperglycemic environments or during the aging process contribute to the pathophysiology of vascular disease in DM (Ahmed and Thornalley, 2007). AGEs can accumulate in the vessel wall, where they may disturb cell structure and function, and have

been implicated in both the microvascular and macrovascular complications of DM (Cade, 2008, Goldin et al., 2006). AGEs may modify the extracellular matrix (ECM), and the action of hormones, cytokines, and free radicals through engagement of cell surface receptors and changes of the function of intracellular proteins (Yan et al., 2010, Hegab et al., 2012a).

1.3.2.1 Formation of AGE

The non-enzymatic reaction of an amino group with a reducing sugar, resulting in the formation of complex brown pigments and protein-protein cross-links, was first described in the early 1900s by Louis-Camille Maillard, and the reaction was named the Maillard reaction (Robert et al., 2010). Haemoglobin's glycosylated isoform HbA1c is a product of a Maillard reaction, and is increased in subjects with DM (Singh et al., 2014). The glycation is subdivided into three main stages: the early, intermediate and late stages (FIG 1.5). In the early stage, a carbonyl group (aldehyde or ketone) of glucose (or other reduced sugars such as fructose and pentose) reacts with free amino groups of proteins, and also nucleic acids and lipids, to form a Schiff base. In the intermediate stage, rearrangements of these Schiff bases gives rise to a stable ketoamine, known as the Amadori product. Finally, in the late stage these Amadori products undergo further rearrangement, dehydration, and condensation to from stable AGEs (Rabbani and Thornalley, 2008).

Because of their slow formation, it was long believed that AGEs accumulate only on long-lived extracellular proteins, such as skin collagen and lens proteins. But, AGEs can also be formed intracellularly from glucose- or lipid-derived dicarbonyl precursors, and this is believed to be of even greater importance (Giacco and Brownlee, 2010). Glucose-derived glycolytic intermediates - glucose-6-phosphate, G3P, DHAP, and the dicarbonyl compounds

glyoxal, MG and 3- deoxyglucosone (3-DG) - form many more glycated proteins, and do so more rapidly (Forbes et al., 2005). They play an important role in the fast intracellular Maillard reaction. Furthermore, the sorbitol pathway generates reactive intermediates such as fructose, fructose-3-phosphate and 3-DG, and these intermediates may also react with proteins and substantially contribute to fast intracellular AGE formation (Giacco and Brownlee, 2010). Therefore, it is likely that it is the intracellular hyperglycemia that initiates the formation of AGEs and contributes to the increase in circulating AGEs.

Another major route of AGE formation originates from lipids. Lipid peroxidation of polyunsaturated fatty acids leads first to the formation of lipid peroxides, which then decompose into a large variety of reactive carbonyl compounds (Turk, 2010). Some of them are identical to those formed from glucose-derived glycolytic intermediates, such as glyoxal and MG and others are uniquely lipid peroxidation products, such as malondialdehyde (MDA) and hydroxynonenal (HNE) - called advanced lipid end-products (Nowotny et al., 2015).

AGEs have a crucial role in inflammation, activating monocytes or macrophages and inducing the subsequent synthesis and secretion of TNF-α, interleukin-1β, and monocyte colony stimulating factor, or activating transmembrane receptors belonging to the immunoglobulin family - i.e. RAGE (Sparvero et al., 2009).

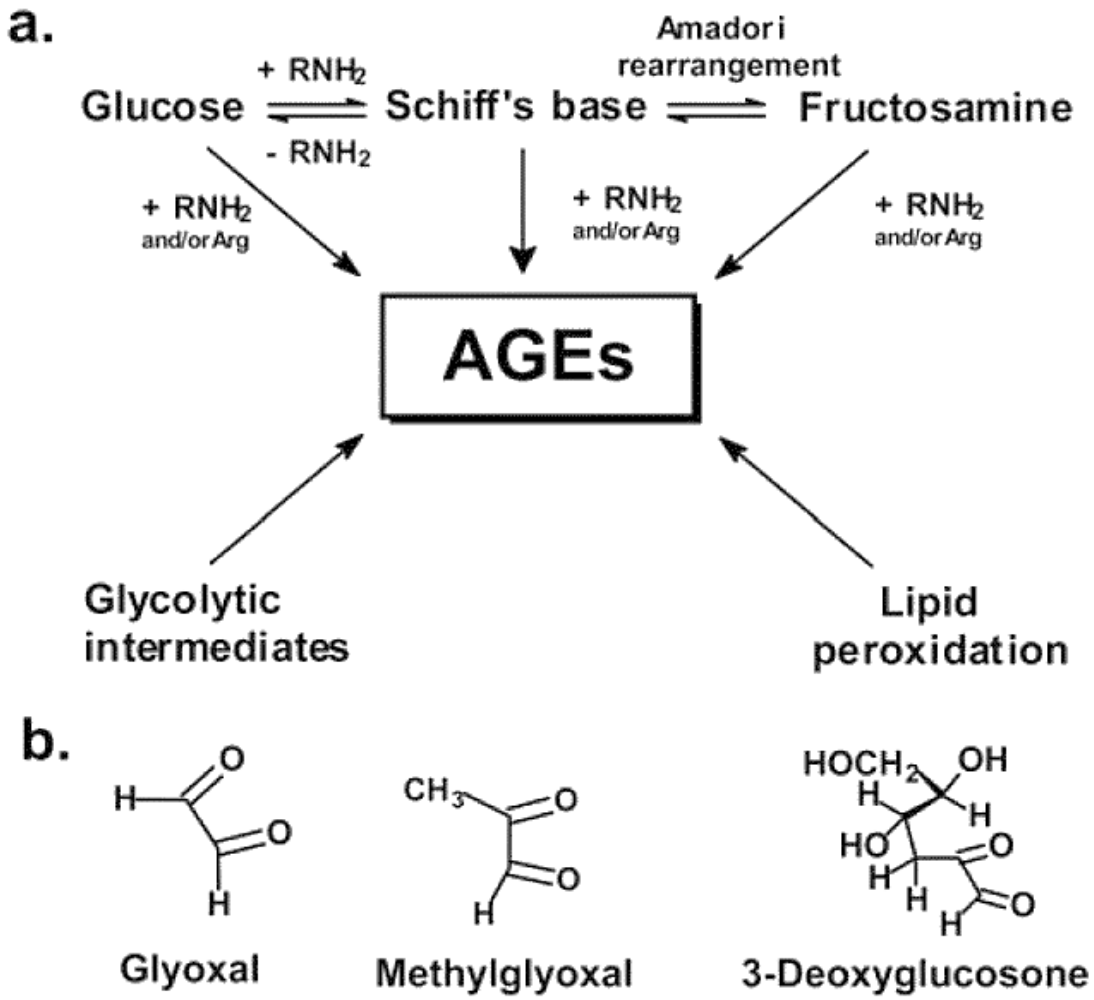

FIGURE 1.5. Major pathways for the formation of AGEs in physiological systems and precursor dicarbonyl metabolites. (a) Formation of early and advanced glycation adducts from glucose and glycolytic intermediates and products of lipid peroxidation. (b) Physiological reactive dicarbonyl-glycating agents (Rabbani and Thornalley, 2008). Reproduced with permission, Rabbani, Naila, and Paul J. Thornalley, "Dicarbonyls Linked to Damage in the Powerhouse: Glycation of Mitochondrial Proteins and Oxidative Stress." Biochemical Society transactions 36.Pt 5 (2008): 1045–1050. PMC. Web. 6 July 2015. © the Biochemical Society.

1.3.2.2 Receptor for AGE - RAGE

RAGE was first isolated from bovine lung extract by affinity purification using AGE-modified albumin (Yan et al., 2010). Its expression has been detected in all tissues, but at very low levels under physiological conditions. Under pathological conditions, RAGE expression is strongly up-regulated (Sparvero et al., 2009, Ramasamy and Schmidt, 2012, Ramasamy et al., 2011, Ott et al., 2014).

A striking feature of RAGE signaling is its multi-ligand character. There is some controversy over whether AGEs are, in fact, physiological ligands for RAGE, or whether they just up-regulate its expression (Xue et al., 2014, Sparvero et al., 2009, Leclerc et al., 2009). The binding of AGE to RAGE has so far been shown only *in vitro*, and with high, non-physiological concentrations of AGEs. Other ligands for RAGE include amyloid-β peptide (Aβ) and β-sheet fibrils, several members of the S100/calgranulin family, amphoterin (also referred to as high mobility group box I (HMGB1)), and Mac-1, a member of the β2-integrin family (Fritz, 2011, Leclerc et al., 2009, Sparvero et al., 2009). The major AGE Nε-(1- carboxymethyl)lysine (CML) has been identified as a RAGE binding modification, resulting in an increased expression of VCAM-1 and ICAM-1, thereby assisting the binding of monocytes to the endothelium of blood vessels (Singh et al., 2014, Boulanger et al., 2002).

The outcome of RAGE signalling is dependent on the cell type, the available ligands, their relative concentrations and the presence of additional splice variants and isoforms. Several of these signalling cascades are important in the regulation of cell proliferation and survival. RAGE stimulates secondary messenger pathways such as protein kinase C (Zhao et al., 2014). After its activation, the key target NF-κB is translocated to the nucleus were it

increases transcription of various proteins including ICAM-1, E-selectin, VEGF, proinflammatory cytokines and also RAGE itself (Goldin et al., 2006, Hegab et al., 2012b) (FIG 1.6).

RAGE activation is an important step in the development of cardiovascular diseases. The prevention of RAGE activation by infusion with the soluble form of RAGE (sRAGE), or by knocking out the RAGE gene, prevents atherosclerosis in apolipoprotein E-null mice, improves diabetic wound healing, and prevents nephropathy, and early retinopathy (Lee et al., 2013a, Schmidt et al., 1999).

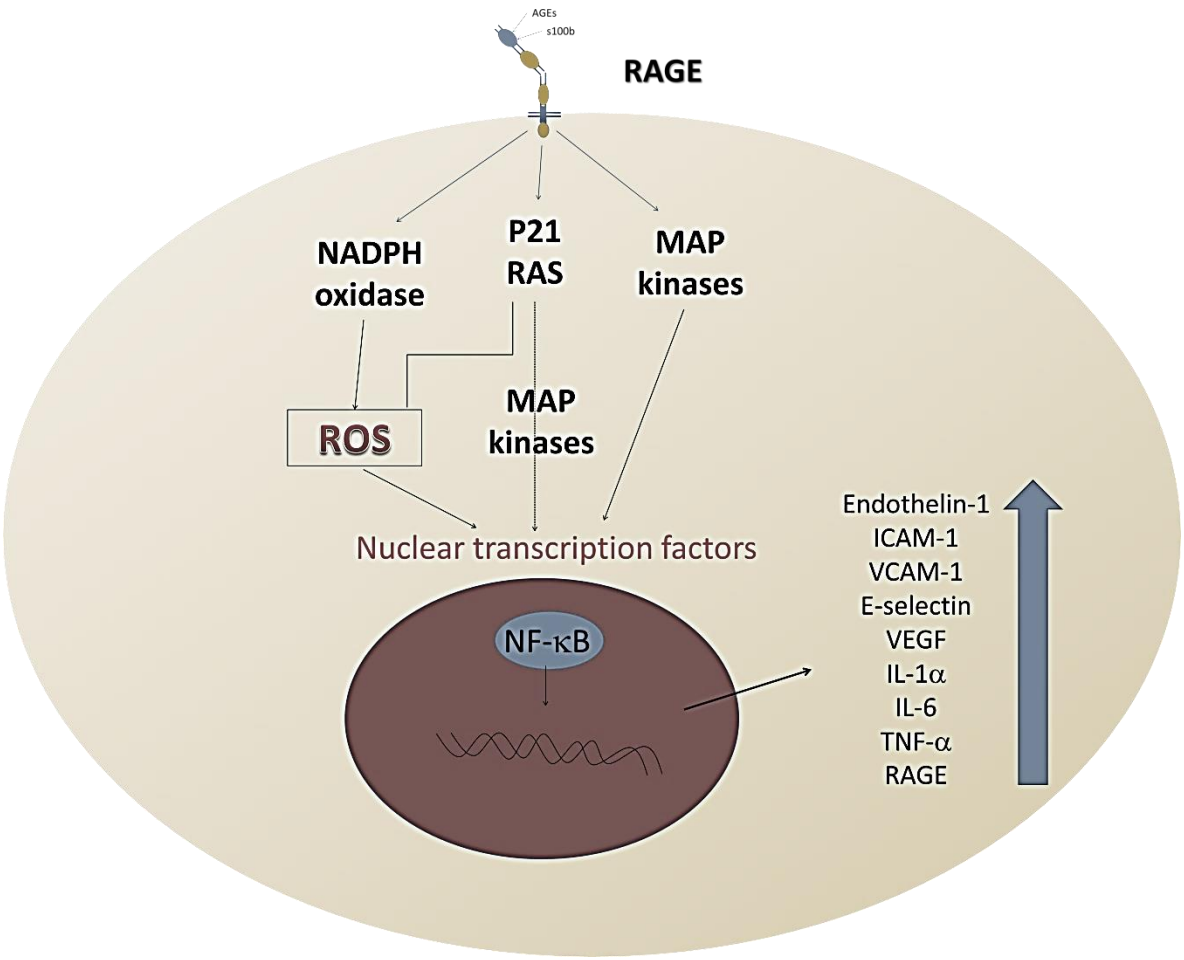

FIGURE 1.6. Intracellular effects of AGEs after AGE/RAGE binding. Representation of AGE/RAGE interaction on the surface of an endothelial cell leading to transduction of a signalling cascade; nicotinamide adenine dinucleotide phosphate oxidase (NADPH) is activated and enhances ROS production and phosphorylates p21, RAS and mitogen-activated protein (MAP) kinase. Moreover, the AGE/RAGE interaction induces signalling through activation of MAPK. A main step in AGE/RAGE signalling is the activation of NF-kB and its translocation to the nucleus, where it enhances transcription of target genes as endothelin-1, ICAM-1, E-selectin and inflammatory tissue factors.

1.3.2.3 DC caused by AGE accumulation

Despite the various mechanisms that cells possess for the detoxification of MG, some diseases still lead to elevated levels of MG and thus MG-derived AGEs. AGEs then affect cell function in two different ways: extracellular matrix and intracellular modifications (Goldin et al., 2006). AGE accumulation and AGE receptor (RAGE) up-regulation have been shown to contribute to the onset of DC (Ma et al., 2009).

The formation of AGEs in the ECM occurs predominantly on proteins with a slow turnover rate. The major matrix proteins collagen, elastin, vitronectin and laminin can all be glycated via the formation of cross-links (Duran-Jimenez et al., 2009). This cross-linking increases the ECM area, which results in increased stiffness of the vasculature. AGE formation can also alter the binding function of the ECM. Increased modification of vascular basement membrane type IV collagen by MG causes EC detachment, anoikis, and inhibition of angiogenesis (Berrou et al., 2009, Kuzuya et al., 1998). The main sites of modification are in RGD and GFOGER integrin-binding sites of collagen (Dobler et al., 2006). In addition, a study in diabetic rats showed that neurite outgrowth was also impaired due to glycation of ECM proteins laminin and fibronectin, thereby linking ECM modification to neuropathy (Duran-Jimenez et al., 2009).

As a result of their generation from dicarbonyls derived from glycolysis, AGEs can also accumulate intracellularly (Giacco and Brownlee, 2010, Yamabe et al., 2013). It is possible that besides modifying the function of intracellular proteins, these AGEs also induce a cascade of altered intracellular signalling pathways. Facchiano *et al* showed that basic fibroblast growth factor (FGF) is one of the main AGE-modified proteins in ECs, which markedly decreases its mitogenic activity (Facchiano et al., 2002). Since then several

proteins have been shown to be glycated, with MG as the major precursor in this process. In both tumour cells and ECs, heat-shock protein 27 has been identified as one of the MG-modified proteins (Oya-Ito et al., 2011). The modification of proteins and antioxidant enzymes in the mitochondrial membrane directly results in increased oxidative stress (Giacco and Brownlee, 2010, Nowotny et al., 2015). Furthermore, MG can influence cell metabolism by the modification of transcription factors. Yao *et al.* demonstrated that in mouse kidney ECs, high glucose led to increased MG modification of the co-repressor mSin3A (Yao et al., 2007). This modification resulted in increased angiopoietin-2 expression and subsequently the expression of ICAM-1 and VCAM-1. Similarly, elevated MG levels modify co-activator p300, which causes impaired HIF-1α binding, resulting in decreased VEGF expression in cases of hypoxia (Ceradini et al., 2008). In addition to the altered gene expression pathways, intracellular AGE accumulation can also directly influence other signalling pathways. Bucala *et al.* showed that the bioavailability of NO in the vascular bed can be significantly decreased by a direct chemical reaction with AGE-modified proteins (Bucala et al., 1991). In addition, Queisser *et al.* demonstrated that hyperglycemia induces the formation of MG, which then covalently modifies the 20S proteasome, thereby decreasing its proteasomal chymotrypsin-like activity, leading to the functional and structural changes of diabetic nephropathy (Queisser et al., 2010).

1.3.3 Oxidative stress caused by MG

Oxidative stress results from an excessive production of ROS, the loss of antioxidant defenses, or both. The two major sources of ROS are mitochondrial or cytosolic (Morgan and Liu, 2011, Lee et al., 2013b). The mitochondrial inner membrane contains five main enzymatic and electron transporting complexes (Baughman and Mootha, 2006) (FIG 1.7). The specific dehydrogenases use the energy of electrons derived from glucose or free fatty acid oxidation to catalyze the conversion of ADP to ATP by ATP synthase. However not every high energy electron is used for ATP synthesis. During passages through the electron chain, energy is gradually released from electrons, but also transferred to O_2, thereby generating ROS (Chen and Zweier, 2014). The principal mitochondrial sites of oxidative stress generation are complex I and III. The mitochondrial proton gradient is partly modulated by uncoupling proteins (UCPs), which are localized in the inner-membrane and function as proton carriers, bypassing ATP synthesis. Up-regulation of UCPs by oxidative stress indicates that this mechanism is important in the defense against mitochondrial ROS production (Aledo, 2014).

The major route by which MG generates ROS is by the direct modification of proteins like mitochondrial and antioxidant enzymes (Giacco and Brownlee, 2010, Sena et al., 2012). Besides being a major precursor in the formation of AGEs, MG is probably also a key factor in the acceleration of oxidative stress. MG can be subjected to auto-oxidation and can thereby generate ROS. Additional free radicals can be generated from MG via various pathways. Studies have shown that during the conversion of aminoacetone and acetol to MG, H_2O_2 can be formed (Yim et al., 1995). In addition, the breakdown of MG also produces free radicals. Interestingly, MG formation is dependent on ROS, thereby creating a vicious cycle (Giacco and Brownlee, 2010, Brownlee, 2001a).

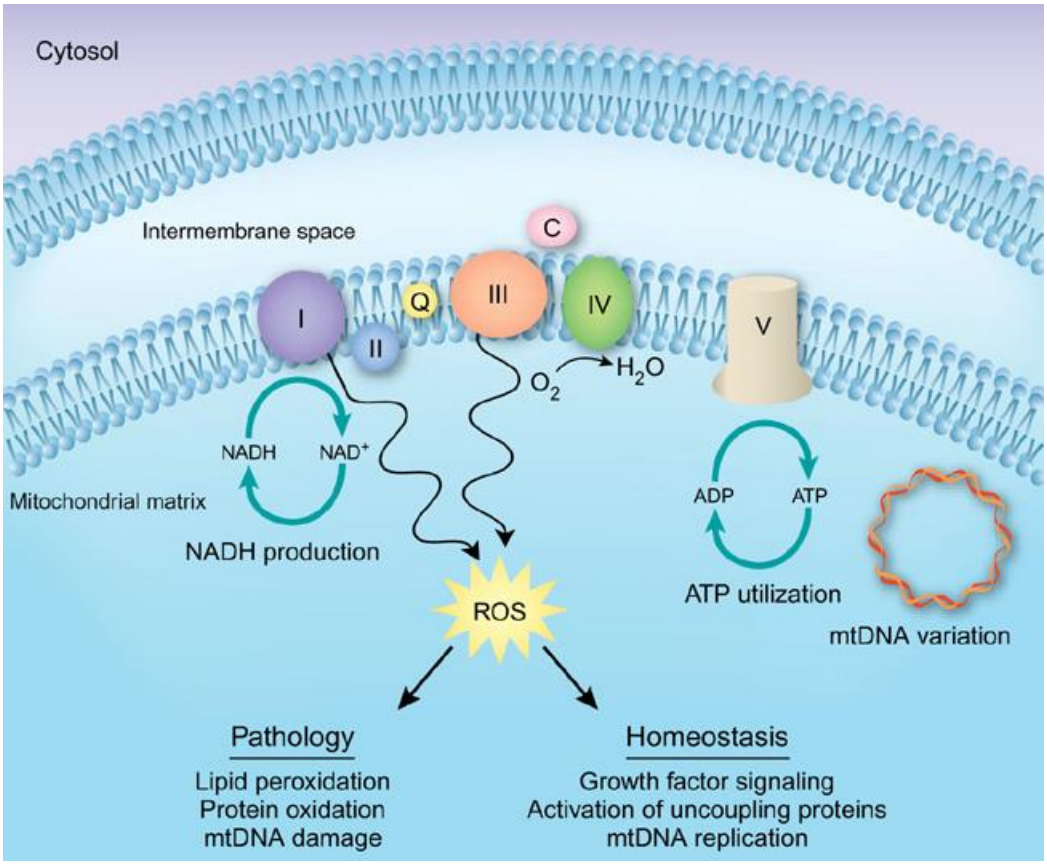

FIGURE 1.7. Generation and consequences of ROS in mitochondria. Mismatches between NADH production and the use of ATP can stress the electron transport chain (complexes I–IV) and modulate the production of ROS. According to Moreno-Loshuertos et al., the mtDNA haplotype can also influence steady-state ROS generation in the cell. Although ROS are traditionally viewed as toxic agents contributing to cellular pathology, emerging evidence suggests that ROS are also critical in cellular homeostasis. (Baughman and Mootha, 2006). Reproduced with permission from Nature Publishing Group.

1.4 STUDIES OF METHYLGLYOXAL AND GLYOXALASE

MG and MG-formed AGEs are potent glycating agents of proteins, nucleotides and basic phospholipids, and these glycation processes contribute to cell and tissue damage in the diabetic environment. By the efficient metabolism of α-oxoaldehydes, GLO1 provides an enzymatic defense. GLO1 activity is also linked with multidrug resistance in cancer chemotherapy. These data encourage further studies into MG and the GLO system as therapeutic targets.

1.4.1 Regulation of GLO1

Although GLO1 is ubiquitously expressed, limited information is available on how this enzyme is regulated in human cells. It is suggested that cells with increased glucose metabolism protect themselves against cellular damage by MG through up-regulation of GLO1 activity (Ayoub et al., 1993). That is true for most tumor cells, such as cells derived from prostate, breast, and colon cancer, where increased expression of GLO1 is combined with enhanced anaerobic breakdown of glucose to lactate (Geng et al., 2014, Sakamoto et al., 2000).

Recently, a functional antioxidant-response element (ARE) was found in the 5'-untranslated region of exon 1 of the mammalian GLO1 gene (Xue et al., 2012). Transcription factor Nrf2 (nuclear factor-erythroid 2) binds to this ARE, increasing basal and inducible expression of GLO1. Also, activators of Nrf2 lead to the induction of GLO1 mRNA transcription, and increased protein levels and activity. It has been shown that the Nrf2

activator, sulforaphane, can prevent nephropathy in a mouse model of type 1 diabetes (Zheng et al., 2011).

1.4.2 Glo1 transgenic animal models

Overexpression studies can improve our understanding of the physiological role of certain enzymes, but they can also increase our knowledge of their roles in disease, helping to identify possible therapeutic targets.

The overexpression of GLO1 prevents the accumulation of MG and AGE in ECs exposed to high glucose concentration *in vitro* (Ahmed and Dobler, 2008). GLO1 reduction of hyperglycemia-induced damage is shown by a recent study by Morocos *et al.* performed in the nematode C. elegans (Morcos et al., 2008a). This study shows that detoxification of MG by GLO1 overexpression prevents hyperglycemia-induced oxidative stress, and thereby improves the lifespan in this nematode model.

Studies done using a GLO1 overexpressing rat have revealed a role for GLO1 in the reduction of hyperglycemia-induced levels of AGEs and oxidative stress in diabetes (Brouwers et al., 2011). A study with a transgenic mouse line that expressed high levels of human GLO1 in the eye lens provided direct evidence that GLO1 activity plays an important role in the regulation of AGE synthesis in the lens (Mailankot et al., 2009). Another transgenic murine model, the GLO1 overexpressing rat, showed hyperglycemia-induced impairment of vasorelaxation in mesenteric arteries and intracellular MG levels (Brouwers et al., 2010). This same model was also used to link mild cardiac alterations, and increased oxidative stress, inflammation, fibrosis and MG-glycation (Brouwers et al., 2011).

In another study, using knockdown of GLO1 in non-diabetic mice, it was shown that, in the absence of hyperglycemia, MG increased to diabetic levels and modified glomerular

proteins and induced oxidative stress, leading to alterations in kidney morphology very similar to those caused by diabetes and resembling early diabetic nephropathy (Giacco et al., 2014). In the same study, the overexpression of GLO1 prevented changes in kidney morphology and microalbuminuria in streptozotocin (STZ) treated mice. These combined observations highlight the importance of MG in diabetic kidney disease. All these studies helped to reveal some of the *in vivo* physiological and pathological functions of MG and the GLO system.

1.4.3 Methods of MG measurement

Since high levels of MG are linked to various diseases, although mainly studied in DM so far, standardized methods to measure MG in body fluids and tissues are more and more necessary. The most widely accepted method for the measurement of MG involves the derivatization of MG with 1,2-diaminobenzene derivatives, followed by quantification of the resulting quinoxaline product using high-performance liquid chromatography (HPLC) (Rabbani and Thornalley, 2014). Recently, a fluorescent sensor called methyl diaminobenzene-BODIPY or "MBo" that can detect MG in living cells has been developed (Wang et al., 2013).

The concentration of MG in the plasma of diabetic patients has been measured to be approximately 30% higher than in healthy subjects (Lapolla et al., 2003). Unfortunately, variations in sample treatment protocols between studies has resulted in significant differences in the amount of MG detected and reported in plasma, cultured cells and liver samples (Rabbani and Thornalley, 2014, Phillips and Thornalley, 1993, Chaplen et al., 1996, Wang et al., 2012, Nemet et al., 2006, Kalapos, 2013). The plasma concentration of MG has

been reported in a range varying from 100nM up to 400uM (Thornalley, 2008b, Kilhovd et al., 2003, Wang et al., 2007). Free MG is present at low levels in most cells. Intracellular levels of MG have been estimated to be from 1 to 5 μM (Chaplen, 1998, Dhar et al., 2008). A possible explanation for the differences between measured free intracellular and extracellular MG levels is that the assay for detecting free intracellular MG may also measure some reversibly bound MG. These inconsistencies in MG concentration data lead to a wide range of MG concentrations being tested in *in vitro* studies of MG.

1.4.3 Potential for development of therapy

The harmful effects of MG accumulation can be reduced by the prevention of AGE formation, the scavenging of MG and/or by enhancing the expression of the detoxifying enzyme GLO1. This reduction may be beneficial in the prevention and treatment of not just T1DM and T2DM vascular complications, renal failure and CVD, but also in supporting healthier ageing. In cancer therapy the goal is the opposite - cell permeable GLO1 inhibitors could find use as anti-tumor and anti-microbial agents for the treatment of resistant tumors and microbial infections (Taniguchi et al., 2012, Sakamoto et al., 2001, Jervis-Bardy et al., 2011). MG scavengers tested so far have shown a degree of toxicity and instability, which tempered the enthusiasm for the development of this kind of therapeutic (Dhar et al., 2010b) Still, metformin – widely used as a support therapy of T2DM – significantly reduced MG-modified proteins in patients, possibly through both an increase in GLO1 activity, and its MG scavenging properties (Beisswenger and Ruggiero-Lopez, 2003). The discovery of GLO1 inducers through activation of Nrf2 (e.g. sulforaphane bardoxolone) also provides a promising direction for more effective and safe treatments (Xue et al., 2012).

Future studies of the glyoxalase pathway may contribute not only to a better understanding of MG and MG-AGE pathways, but may also serve as a foundation for the development of therapies for CVD, ageing and neurodegenerative diseases, and cancer.

1.5 SUMMARY

Diabetic complications, based on the size of blood vessels affected, can be roughly divided in two major categories: microvascular and macrovascular complications. The damage to the small arteries occurs very early and is hard to diagnose. As a consequence, organs vascularized by the affected blood vessels suffer functional loss or failure, leading to disease such as retinopathy, nephropathy and neuropathy, and also possibly to heart failure and PVD.

In patients with DM, ED appears to be a consistent finding, since hyperglycemia and DM lead to an impairment of EC function and microangiopathy. MG likely plays a critical role in the development of these long-term microvascular complications. Elevated MG levels have been shown to promote a series of inflammatory responses that activate ECs leading to vascular damage, ED, cell death and a poor angiogenic repair response.

In summary, this thesis sought to better understand the role of MG in the development of cardiovascular complications in diabetes.

1.6 HYPOTHESIS AND OBJECTIVES

1.6.1 Hypothesis

This thesis tests the hypothesis that the accumulation of MG in ECs and the bone marrow (BM) contributes to the development of EC inflammation and cardiovascular complications in diabetes using a diabetic mouse model.

1.6.2 Global Objective

The global objective is to investigate the role of MG-induced endothelial inflammation in the development of cardiovascular complications, namely defective angiogenesis and diabetic heart failure. To assess this, a human glyoxalase 1 (hGLO1) overexpressing mouse model was used.

1.6.3 Specific objectives

1. To determine if a reduction of MG in the endothelium can prevent the development of endothelial inflammation and heart failure in a STZ-induced diabetic mouse model.
2. To investigate possible synergistic effects of MG and the pro-inflammatory cytokine TNF-α in the apoptotic cell response to inflammation.
3. To assess if a reduction of MG in BM cells can improve neovascularization in the ischemic hindlimb of a STZ-induced mouse model of T1DM.

CHAPTER 2 –MANUSCRIPT #1

Methylglyoxal-Induced Inflammation Contributes to the Development of Diabetic Cardiomyopathy

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Running title: Role of MG-induced endothelial cell inflammation in diabetic cardiomyopathy

Contributions of Authors

This study is a product of collaboration between the University of Ottawa Heart Institute, and the Diabetes Research Center at the Albert Einstein College of Medicine. Immunocytochemistry, in situ hybridization, tissue and cell preparation, part of the GLO activity assays, Western blots, ELISA and the carbonyl stress assay described here were conducted by me. Echocardiography and other animal work were done with valuable help of Brian McNeill, Ferdinando Giacco and Richard Seymour. Part of the GLO activity assay experiments were done by lab technician Michele Geoffrion. Experiments, data analysis and manuscript preparation were done under the supervision and guidance of Drs Erik Suuronen, Ross Milne and Michael Brownlee.

Abstract

Inflammation and endothelial dysfunction (ED) have been associated with the development of diabetic cardiomyopathy, but the mechanisms remain largely unknown. Methylglyoxal (MG) can accumulate and promote inflammation and ED in diabetes. We examined if overexpression of the MG-metabolizing enzyme glyoxalase-1 (GLO1) in the vasculature could reduce MG-induced inflammation and prevent diabetic heart failure. Hyperglycemia increased the circulating levels of inflammatory markers in wild-type (WT), but not GLO1-overexpressing mice. Endothelial cell number was reduced in WT-diabetic hearts compared to non-diabetic controls, whereas GLO1 over-expression preserved capillary density. Endothelial cell function, assessed by neuregulin production and eNOS dimerization, was maintained in the hearts of GLO1-diabetic mice and corresponded to less myocardial cell death compared to the WT-diabetic group. GLO1 over-expression also reduced cardiac inflammation: lower RAGE and TNF-α levels were observed in GLO1-diabetic mice vs. the WT-diabetic group. Over a period of 8 weeks of hyperglycemia, GLO1 over-expression delayed and limited the loss of cardiac function. Taken together, these results suggest that MG in diabetes increases endothelial inflammation, leading to the loss of endothelial cell number and function. This contributes to the development of diabetic cardiomyopathy, and identifies MG-induced inflammation as a target for therapy.

INTRODUCTION

In patients with both type 2 and type 1 diabetes, cardiovascular complications are the main cause of morbidity and mortality. While an increased incidence of atherosclerosis and coronary artery disease (CAD) is a primary reason for this (Collaboration et al., 2010), many patients suffer from clinically significant ventricular dysfunction even in the absence of these conditions. This ventricular dysfunction has been termed diabetic cardiomyopathy, and is defined as myocardial left ventricular (LV) dysfunction independent of atherosclerosis and CAD (Hayat et al., 2004). Diabetic cardiomyopathy (DC) is a major cause of heart failure in people with diabetes. Despite the lack of CAD, a strong association exists between diabetic cardiomyopathy and the presence of microvascular complications (Poornima et al., 2006). For example, endothelial dysfunction (ED), inflammation, abnormal vascular remodeling and an impaired angiogenic response have all been linked to the myocardial apoptosis, fibrosis and hypertrophy seen in DC (Boudina and Abel, 2010, Khazaei et al., 2011), although the mechanisms involved have not been clearly defined.

The activation of endothelial cells (ECs) from a quiescent phenotype to a vasoconstrictive, pro-inflammatory and pro-apoptotic state leads to the inability of the endothelium to regulate vascular homeostasis (Xu and Zou, 2009). In diabetes, ECs are directly exposed to excessive and/or fluctuating blood glucose levels, and hyperglycemia is a known contributing factor to the loss of endothelial function (Hadi and Suwaidi, 2007). This exposure can stimulate the generation of reactive oxygen species (ROS), as well as the production of toxic by-products of glycolysis, primarily methylglyoxal (MG), leading to the formation of advanced glycation end-products (AGEs) (Giacco and Brownlee, 2010). Under normal physiological conditions, MG is metabolized by the glyoxalase system, whereby glyoxalase-1 (GLO1) together with glyoxalase-2 and glutathione reduce MG to D-lactate,

thus preventing MG accumulation (Thornalley, 2003). In diabetes, the production of MG is accelerated, while its detoxification is slow (due to reduced GLO1 activity), leading to MG accumulation (Abordo et al., 1999, Lapolla et al., 2003). Elevated MG levels have been shown to promote inflammatory responses that activate ECs and lead to EC dysfunction and vascular damage (Brouwers et al., 2010, Turkseven et al., 2014). In fact, MG alone can cause endothelial damage similar to that induced by high glucose (Brown et al., 2010, Dhar et al., 2010b, Sena et al., 2012).

The endothelium plays an important role in cardiomyocyte viability and function, and in myocardial homeostasis (Lemmens et al., 2007, Tirziu et al., 2010). EC death can lead to repeated episodes of ischemia and myocardial infarction, the death of cardiomyocytes, and the development of heart failure (Rössig et al., 2000). However, the link between MG-induced inflammation, EC damage and cardiac function in diabetes remain unknown. The aim of the current study was to use mice that overexpress human GLO1 in the vasculature (Geoffrion et al., 2014, Giacco et al., 2014, Vulesevic et al., 2014a) to investigate the connection between increased vascular MG, the ensuing inflammation in ECs, and the development of heart failure in the diabetic heart.

RESEARCH DESIGN AND METHODS

Animal model. As described previously (Geoffrion et al., 2014, Giacco et al., 2014, Vulesevic et al., 2014a), mice that overexpress hGLO1 were used. The cDNA encoding hGlo1 with an amino terminal c-myc epitope tag was cloned into the Not1-digested PEP8 plasmid, so that the hGlo1 insert was under the control of the murine pre-proendothelin promoter. Experiments were performed on heterozygous GLO1 mice and their wild type (WT) littermates. All studies were performed according to protocols approved by the University of Ottawa Animal Care Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Mice were fed the Teklad Global 2019 Extruded Rodent Diet (Harlan) and kept in a 12/12h light/dark cycle.

Induction of diabetes using streptozotocin. Mice received intraperitoneal injection of 50mg/kg of streptozotocin (STZ; Sigma) or vehicle only (citrate buffer control) for 5 consecutive days. Four groups of mice were generated: WT-control, GLO1-control, WT-diabetic and GLO1-diabetic. Fasting blood glucose measurements were taken 7 days after the last STZ injection and again following animal sacrifice. Mice with glucose levels above 15 mmol/l, measured 7 days after the last STZ injection, were considered hyperglycemic (diabetic). Both diabetic groups had increased fasting blood glucose levels at the end of the study (25±4 mmol/l) with no significant difference between them.

Serum analysis. Using ELISA assay kits (RayBiotech), blood serum levels of markers for endothelial inflammation (soluble ICAM, VCAM and E-selectin) were measured at 8 weeks post-STZ following the manufacturer's protocol and are presented as pg/ml of serum.

Heart digestion and cell separation. Whole hearts from sacrificed animals were harvested and digested using digestion buffer as described (Molgat et al., 2014). After 30 min of digestion at 37^{o}C, the cell suspension was filtered and then plated onto tissue culture dishes for 2 h. This period of time allows for the removal of the adherent fibroblast population, while the non-adherent cells were then incubated with Dynabeads® Sheep Anti-Rabbit IgG magnetic beads (Invitrogen) coated with CD31 antibody (Abcam) for 30 min at 4^{o}C. The CD31^{+} cells were then pulled down using a magnet and constituted the endothelial cell fraction. The remainder of the cells from the CD31^{+} cell sort was enriched for cardiomyocytes, with 80% of the cells confirmed to be cardiomyocytes by troponin staining and flow cytometry (data not shown).

Glyoxalase activity. GLO1 activity was determined by measuring the rate of formation of S-D-lactoylglutathione from hemi-thioacetal, as previously described (Vulesevic et al., 2014a). Briefly, the assay mixture containing 7.9mM MG was equilibrated to room temperature and the reaction was initiated by the addition of lysate (10-50mg). GLO1 activity is recorded as the mM concentration of S-D-lactoylglutathione formed/min/mg of lysate protein (concentration determined by the BCA protein assay). GLO1 activity was measured in the whole heart, enriched cardiomyocyte cell population and primary EC cell culture. As an insufficient number of ECs could be obtained from myocardial tissue for performing the GLO1 activity assay, ECs isolated from the aorta were used. GLO1 activity is presented as fold-change compared to the WT mice.

Western blot. Protein expression analysis was determined by Western blots, as described previously (Vulesevic et al., 2014a). The levels of c-myc, tumor necrosis factor (TNF)-α,

receptor for AGE (RAGE) and neuregulin in the heart were assessed (antibodies from Abcam). For the detection of endothelial nitric oxide synthase (eNOS) dimers, polyacrylamide electrophoresis was performed using monomer/dimer-specific antibodies (Abcam) at low temperature (4ºC), as previously described (Yang et al., 2009). All densitometry data were analyzed using ImageJ software and normalized to tubulin expression.

Carbonyl stress measurements. Using an OxiSelect™ Protein Carbonyl ELISA kit, whole heart lysates were probed for total protein carbonyl content, as per the manufacturer's protocol. Briefly, equal concentrations of protein lysates were loaded onto the plate and then transformed chemically to DNP hydrazone and probed with an anti-DNP antibody, followed by an HRP conjugated secondary antibody. The protein carbonyl content of the samples was determined by comparison against a standard curve of the predetermined reduced and oxidized BSA standards, and expressed as nmol of carbonyl protein per mg of total protein.

Histology and immunohistochemistry. Hearts were collected, perfused with saline, and then either flash-frozen in OCT (for immunostaining of ECs (using von Willebrand factor (vWF) and CD31 antibodies; Abcam) and cell death (using a TUNEL kit; Roche) or fixed in 4% formalin and paraffin-embedded (for ISH (see below) and RAGE staining (Abcam)). Visualization was performed as described previously (Vulesevic et al., 2014a), using a Zeiss Axiophot microscope equipped with a Hamamatsu C5985 chilled CCD camera, and Metamorph imaging software 4.01 (Molecular Devices).

In situ hybridization (ISH). ISH was performed as previously described (Wang, 2005) using Digoxigenin (DIG)-labeled antisense RNA riboprobes prepared by in vitro transcription from linearized plasmids containing complete or partial cDNA sequences for hGlo1 and endothelin. Briefly, sections of heart tissue were hybridized overnight at 65^{o}C in a humidified chamber, washed stringently and incubated with an alkaline phosphatase-conjugated anti-DIG antibody. Staining was performed using Nitro blue tetrazolium (Roche) and 5-bromo-4-chloro-3-indolyl phosphate (Roche) and analyzed on an Axioplan microscope and digital images were captured using an AxioVision camera 2.05 (Zeiss).

Echocardiography. At 4 and 8 weeks post-STZ treatment, left ventricular ejection fraction (LVEF), fractional shortening (FS), and other heart function measurements were determined by echocardiography on long-axis views with a Vevo770 system (VisualSonics) in B mode with the use of a 707B series real-time microvisualization scanhead probe.

Statistical analysis. Results are expressed as means ± SEM. Statistical analyses were performed using SigmaStat software. Comparisons between two groups were made by an unpaired two-tailed Student t-test. For multiple group comparisons, a one-way analysis of variance with a post-hoc Student-Newman-Keuls test was performed. Statistical significance was given for $p<0.05$.

RESULTS

Increased GLO1 expression and activity in the hearts of GLO1 mice. It was confirmed that hGlo1 is not overexpressed in the cardiomyocytes of our transgenic mice. By ISH it was demonstrated that only the vasculature in myocardial sections of GLO1 mice stained positive for hGlo1, while hGlo1 staining was not observed in WT mice. The expression pattern of hGlo1 was similar to that of endothelin and vWF, indicating that hGlo1 is restricted to the vasculature (FIG 2.1A). When GLO1 activity was examined, the whole heart of transgenic mice exhibited a 1.8-fold increase in activity compared to the WT hearts (FIG 2.1B). Following heart digestion and cell isolation, no difference in GLO1 activity was detected in cardiomyocytes between GLO1 and WT mice, while aortic ECs extracted from GLO1 mice showed a 5.5-fold increase in GLO1 activity compared to the WT group (FIG 2.1B). The presence of hGLO1 in aorta ECs was also confirmed by Western blot for the c-myc tag, which was not detected in the cardiomyocyte population (FIG 2.1C).

Hyperglycemia-induced endothelial inflammation is reduced by GLO1 overexpression. After 8 weeks of hyperglycemia, WT-diabetic mice had increased circulating levels of the EC inflammation markers E-selectin, VCAM-1 and ICAM-1 compared to WT non-diabetic mice (by 1.5-fold, 1.2-fold and 1.4-fold, respectively; $p \leq 0.04$; Table 1). GLO1 overexpression in the vasculature restored circulating levels of E-selectin, VCAM-1 and ICAM-1 to that of the non-diabetic mice ($0.7 \geq p \geq 0.2$). Compared to the WT-diabetic mice, there was a significant reduction in the level of VCAM-1 ($p<0.001$) and a trend for reduced E-selectin and ICAM-1 (both $p=0.1$) in the serum of GLO1-diabetic mice (TABLE 1).

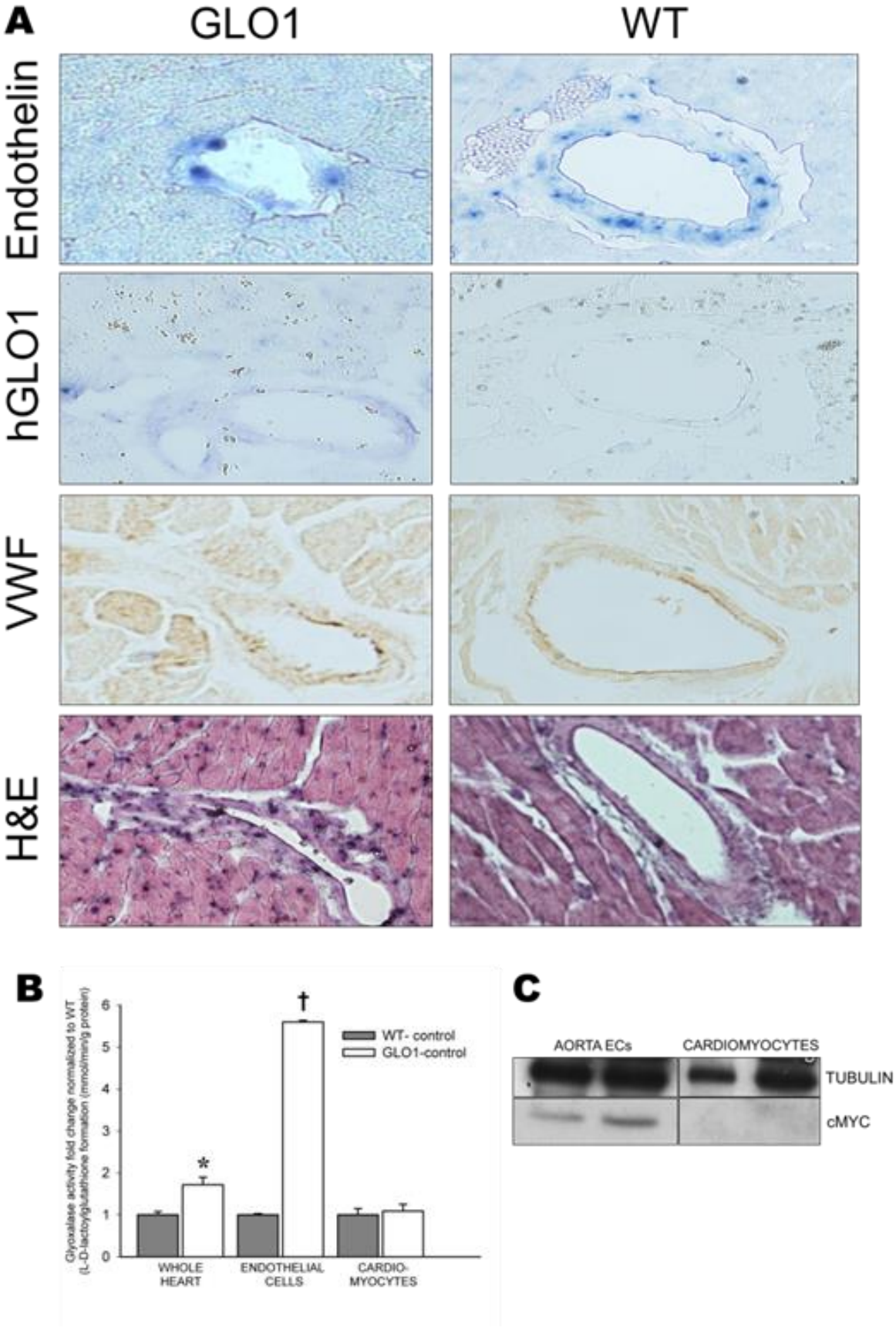

A
GLO1
WT
Endothelin
hGLO1
VWF
H&E
B
Glyoxalase activity fold change normalized to WT
(L-D-lactoylglutathione formation (mmol/min/g protein)
WT- control
GLO1-control
*
†
WHOLE HEART
ENDOTHELIAL CELLS
CARDIO-MYOCYTES
C
AORTA ECs
CARDIOMYOCYTES
TUBULIN
cMYC

FIGURE 2.1. Overexpression of hGlo1 was confirmed in the ECs of GLO1 mouse hearts. A: In situ staining of pro-endothelin and hGlo1 showed mRNA expression of endothelin in both groups (WT and GLO1), while hGlo1 mRNA was present only in GLO1 transgenic mice. Staining of ECs/vasculature was confirmed by vWF and H&E staining. B: GLO1 activity measured in the whole heart, ECs and cardiomyocytes (*p=0.01 vs. whole heart WT-control; $^{\dagger}p$<0.01 vs. endothelial cells WT-control, n=5). C: Representative cMYC Western blots of aortic ECs and cardiomyocytes from GLO1 mice show the presence of the tagged GLO1 protein only in ECs (each lane represents a different mouse, n=2).

	WT	GLO1	WT-D	GLO1-D
E-selectin (pg/ml)	758.9±22.6	956.1±156.0	1149.4±51.5*****	825.7±172.4
VCAM-1 (pg/ml)	39.0±0.7	39.1±0.7	45.0±0.6†	39.8±1.3
ICAM-1 (pg/ml)	14.3±0.3	15.4±1.1	19.9±1.7‡	17.4±0.1

TABLE 2.1. Concentration of soluble endothelial adhesion molecules in blood serum. ELISA measurement of EC inflammation markers in blood serum of non-diabetic WT and GLO1 mice (WT, GLO1) and diabetic WT and GLO1 mice (WT-D, GLO1-D) at 8 weeks post-STZ (*p=0.002 vs. WT; $^{\dagger}p$<0.001 vs. all other groups; $^{\ddagger}p\leq$0.04 vs. WT and GLO1 group, n=3).

Increased GLO1 activity promotes EC survival in the diabetic mouse heart. The number of ECs in the myocardium was assessed in tissue sections by vWF and CD31 staining. At 8 weeks post-STZ treatment, the number of vWF^+ ECs per field-of-view (FOV) was reduced in WT-diabetic hearts (by 40%) compared to the other groups ($p \leq 0.001$, FIG 2.2A). Similar results were obtained with $CD31^+$ staining (data not shown; $p \leq 0.02$). Notably, despite the presence of hyperglycemia, GLO1-diabetic mice had no loss of ECs compared to the non-diabetic animals.

EC function in GLO1-diabetic mice is maintained. Signaling functions of ECs important for the support of a healthy myocardium (eNOS and neuregulin) were maintained in GLO1-diabetic mice. Specifically, the protein levels of eNOS, implicated in the regulation of vascular tone and promotion of cardiomyocyte survival (Hare and Stamler, 2005), were preserved in GLO1-diabetic mice (FIG 2.3A). Current research suggests that only the dimeric form of eNOS is able to generate NO, while the ROS-induced monomeric form of eNOS produces superoxide instead (Yang et al., 2009). While eNOS dimerization was reduced by 50% in WT-diabetic mice ($p=0.04$), GLO1-diabetic mice maintained a level of dimeric eNOS similar to non-diabetic mice ($p=0.63$, FIG 2.3A). In WT-diabetic mice, neuregulin levels were 75% less than in WT non-diabetic ($p \leq 0.05$, FIG 2.3B), whereas its expression was not significantly different between the GLO1-diabetic mice and controls.

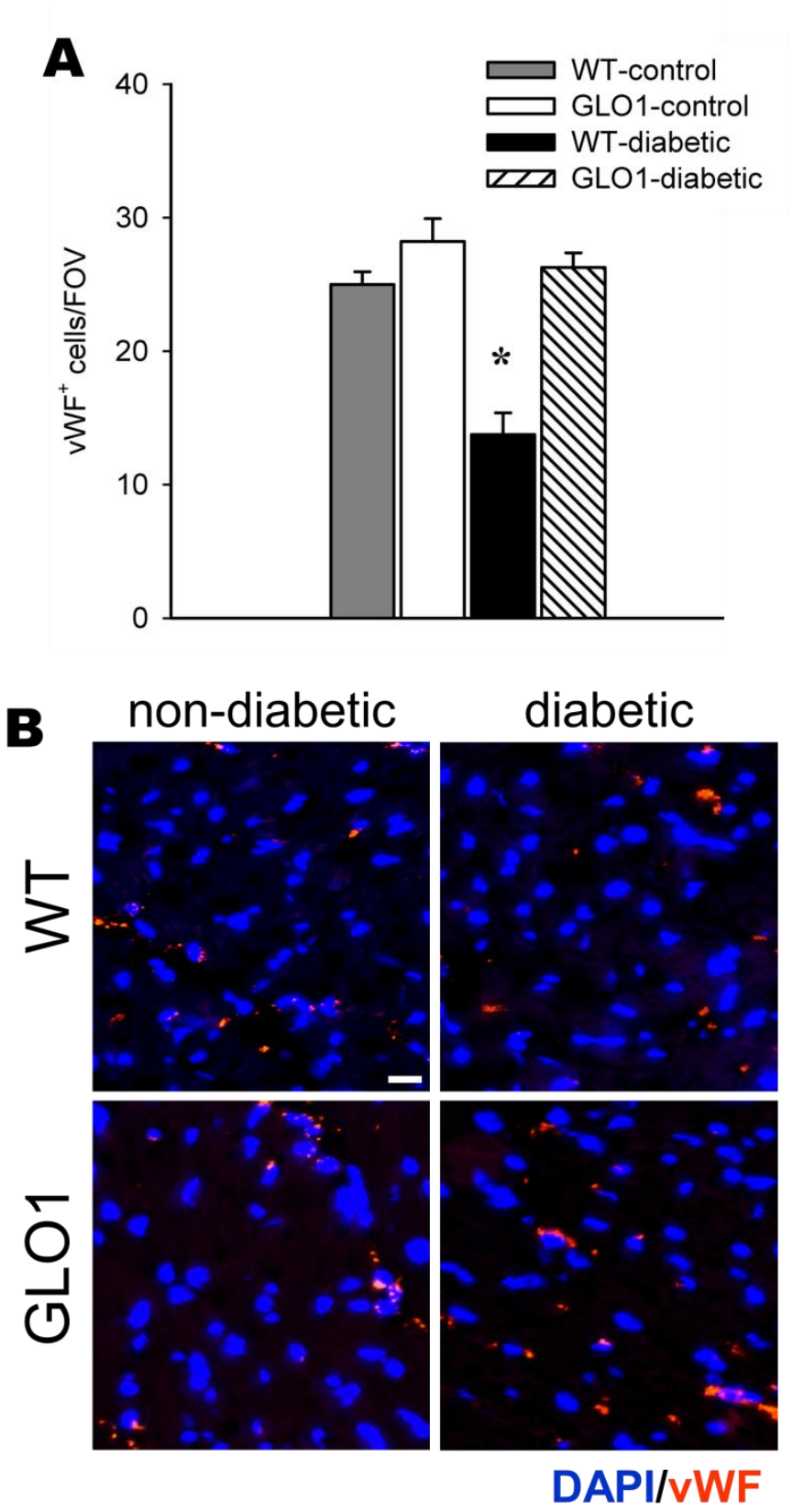

A
WT-control
GLO1-control
WT-diabetic
GLO1-diabetic
vWF+ cells/FOV
40
30
20
10
0
*
B
non-diabetic
diabetic
WT
GLO1
DAPI/vWF

FIGURE 2.2. GLO1 overexpression promotes EC survival in the myocardium of diabetic mice. A: Number of vWF^+ ECs per FOV in myocardial tissue sections at 8 weeks (*$p \leq 0.001$ vs. all other groups, n=3-4 per group). B: Representative images of vWF staining (red) with DAPI-stained cell nuclei (blue). Scale bar=10μm.

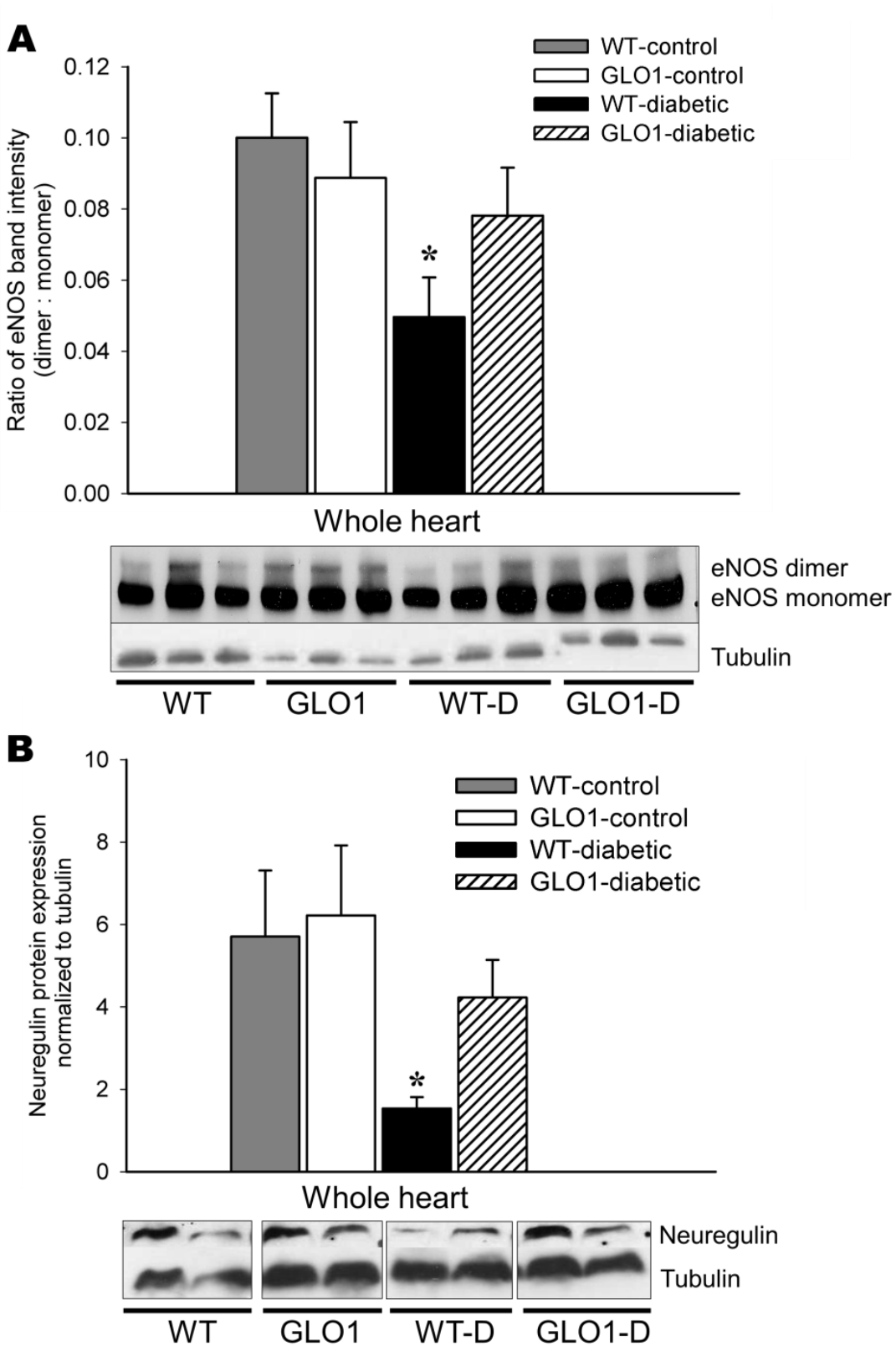

A
WT-control
GLO1-control
WT-diabetic
GLO1-diabetic
Ratio of eNOS band intensity (dimer : monomer)
0.12
0.10
0.08
0.06
0.04
0.02
0.00
*
Whole heart
eNOS dimer
eNOS monomer
Tubulin
WT
GLO1
WT-D
GLO1-D
B
WT-control
GLO1-control
WT-diabetic
GLO1-diabetic
Neuregulin protein expression normalized to tubulin
10
8
6
4
2
0
*
Whole heart
Neuregulin
Tubulin
WT
GLO1
WT-D
GLO1-D

FIGURE 2.3. The production of eNOS and neuregulin is maintained in the hearts of GLO1-diabetic mice. A: Representative Western blot and quantification of the eNOS dimer:monomer ratio in the myocardium at 8 weeks (*$p \leq 0.04$ vs. WT- and GLO1-control, n=3). B: Representative Western blot and quantification of neuregulin protein in the myocardium at 8 weeks (*$p \leq 0.05$ vs. WT- and GLO1-control, n=4).

Carbonyl stress and inflammation are reduced in the heart of diabetic GLO1 mice. To further explore the consequence of oxidative stress and hyperglycemia in the heart, carbonyl stress was evaluated. ROS accumulation (not related only to MG accumulation) leads to protein oxidation and the formation of carbonyl groups on proteins which can be measured by protein carbonyl assays. Carbonyl stress was increased in the whole heart of both WT-diabetic and GLO1-diabetic groups (by 2.5-fold and 1.8-fold respectively; $p \leq 0.008$) compared to the non-diabetic mice (FIG 2.4A). However, there was trend for less carbonyl stress in the GLO1-diabetic vs. WT-diabetic mice (by 20%, $p=0.1$). The same pattern was observed for the levels of the inflammatory cytokine TNF-α. Both diabetic groups had greater TNF-α expression (3.5-fold for WT-diabetic, and 2-fold for GLO1-diabetic) compared to non-diabetic controls ($p \leq 0.02$, FIG 2.4B), and the GLO1-diabetic mice exhibited lower TNF-α vs. the WT-diabetic group ($p=0.04$). The myocardial protein content of RAGE was increased only in the hearts from WT-diabetic mice vs. all other groups (≥1.8-fold, $p \leq 0.02$, FIG 2.4C). Immunohistochemistry staining of tissue sections also showed increased RAGE expression throughout the tissue in the WT-diabetic heart compared to the other groups (FIG 2.4D).

Protecting the vasculature from MG reduces overall cell death in the diabetic heart. Apoptotic cells, determined by TUNEL staining, were more numerous in the hearts of WT-diabetic mice as early as 4 weeks post-STZ injection (up to 8.6-fold greater compared to all other groups, $p \leq 0.02$; FIG 2.5A). By 8 weeks post-STZ, the number of $TUNEL^+$ cells in WT-diabetic mice increased to 10-fold greater than controls ($p=0.006$). For GLO1-diabetic mice, there was no difference in the number of apoptotic cells at 4 weeks compared to the non-diabetic mice. However, at 8 weeks, GLO1-diabetic mice had a 4.1-fold increase in

TUNEL$^+$ cells compared to control mice (p=0.045), but this was still fewer (about 67% less) than in WT-diabetic mice (p=0.008; FIG 2.5A).

GLO1 overexpression in the vasculature delays STZ-induced cardiac dysfunction. There was no difference in cardiac function between WT and transgenic mice at baseline. Four weeks post-STZ, WT-diabetic mice exhibited reduced FS compared to all other groups (p≤0.045; FIG 2.6A). At 8 weeks, decreased LVEF and FS were observed in both WT-diabetic and GLO1-diabetic mice compared to the non-diabetic mice (p≤0.03); however, LVEF and FS were significantly greater in GLO1-diabetic vs. WT-diabetic mice (p≤0.01; FIG 2.6A, B). At 8 weeks, an increase in the heart-to-body mass ratio was found in both diabetic groups (5±0.09mg/g in WT-diabetic and 5±0.21mg/g in GLO1-diabetic mice, compared to 4.4±0.1mg/g in non-diabetic mice; p≤0.02). This was most probably associated with body mass differences between the groups (non-diabetic mice weighed 26±0.4g, while diabetic mice were 20±0.5g, p<0.001). Furthermore, the heart weight was reduced in both diabetic groups (p=0.04). At 4 weeks, WT-diabetic mice showed reduced stroke volume compared to all other groups (p≤0.001; TABLE 2.2). The WT-diabetic group also had reduced cardiac output and lowered diastolic end pressure at 4 weeks compared to the other groups (p≤0.02; TABLE 2.2), showing the first signs of heart failure. While there was no difference in LV wall thickness or systolic end volume between the groups, the cardiac output, stroke volume and diastolic end volume were all decreased at 8 weeks in both diabetic groups compared to control animals (p≤0.03; TABLE 2.2). Still, between the two diabetic groups, cardiac output and stroke volume were significantly better in the GLO1 mice (p≤0.04; TABLE 2.2), confirming the partial preservation of heart function by protection of the vasculature from MG.

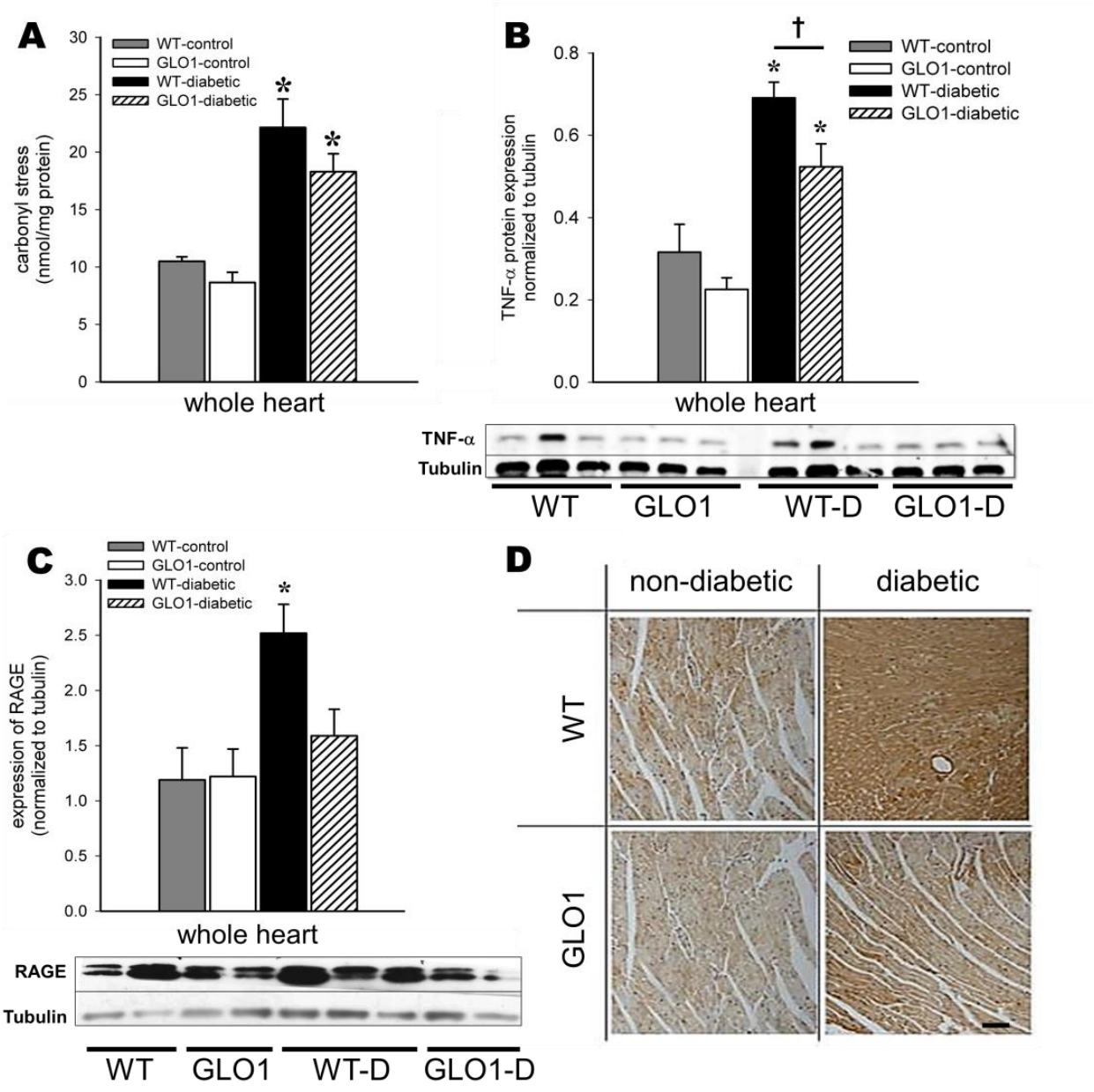
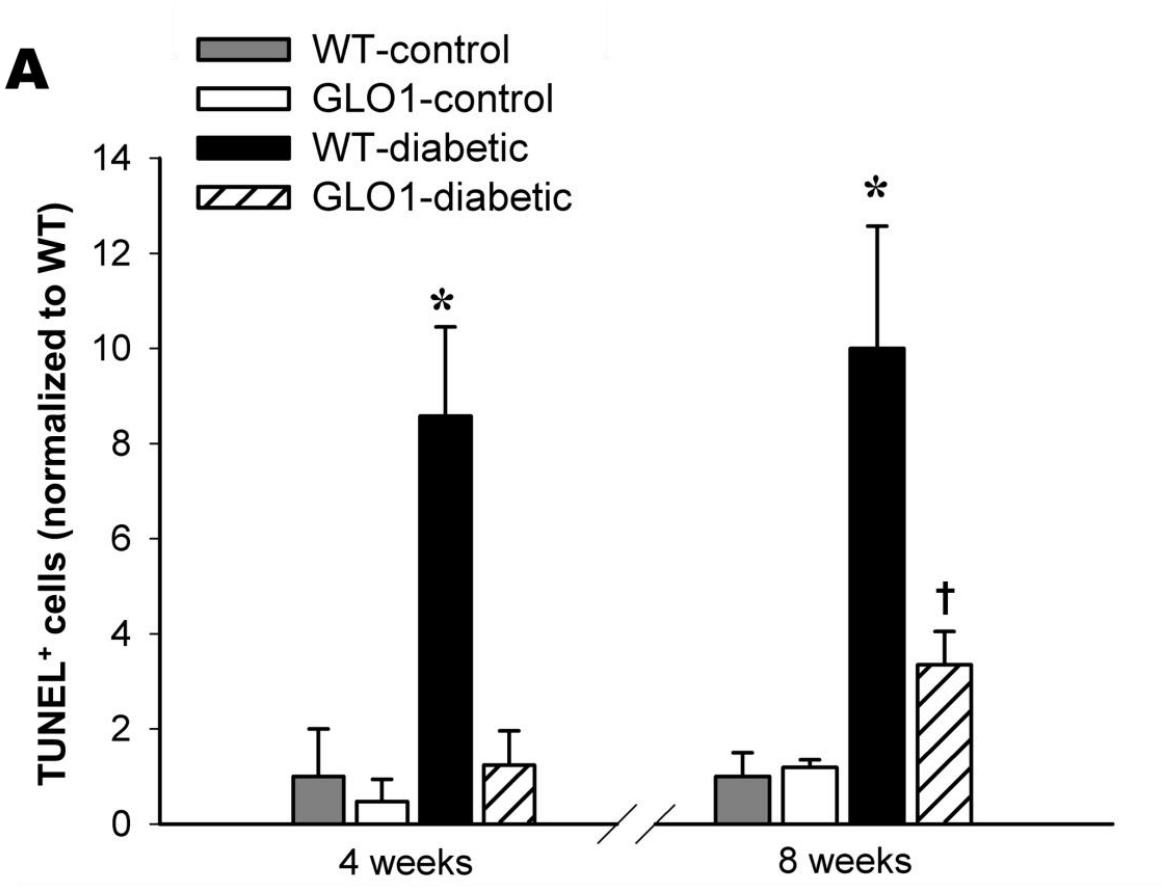

A
WT-control
GLO1-control
WT-diabetic
GLO1-diabetic
carbonyl stress (nmol/mg protein)
whole heart
B
TNF-α protein expression normalized to tubulin
whole heart
TNF-α
Tubulin
WT
GLO1
WT-D
GLO1-D
C
expression of RAGE (normalized to tubulin)
whole heart
RAGE
Tubulin
WT
GLO1
WT-D
GLO1-D
D
non-diabetic
diabetic
WT
GLO1

FIGURE 2.4. GLO1 overexpression reduces carbonyl stress and inflammation in the myocardium of diabetic mice. A: ELISA analysis of protein carbonyl in the myocardium at 8 weeks as a measure of oxidative stress (*$p \leq 0.008$ vs. WT- and GLO1-controls, n=3-4 per group). B: Representative Western blot and quantification of TNF-α in myocardial tissue at 8 weeks (*$p \leq 0.02$ vs. WT- and GLO1-controls; $^{\dagger}p=0.04$, n=3). C: Representative Western blot and quantification of RAGE protein in myocardial tissue at 8 weeks (*$p \leq 0.02$ vs. all other groups, n=5). D: Representative images of RAGE staining in the myocardium at 8 weeks, scale bar=50μm.


A
WT-control
GLO1-control
WT-diabetic
GLO1-diabetic
TUNEL+ cells (normalized to WT)
14
12
10
8
6
4
2
0
*
*
†
4 weeks
8 weeks

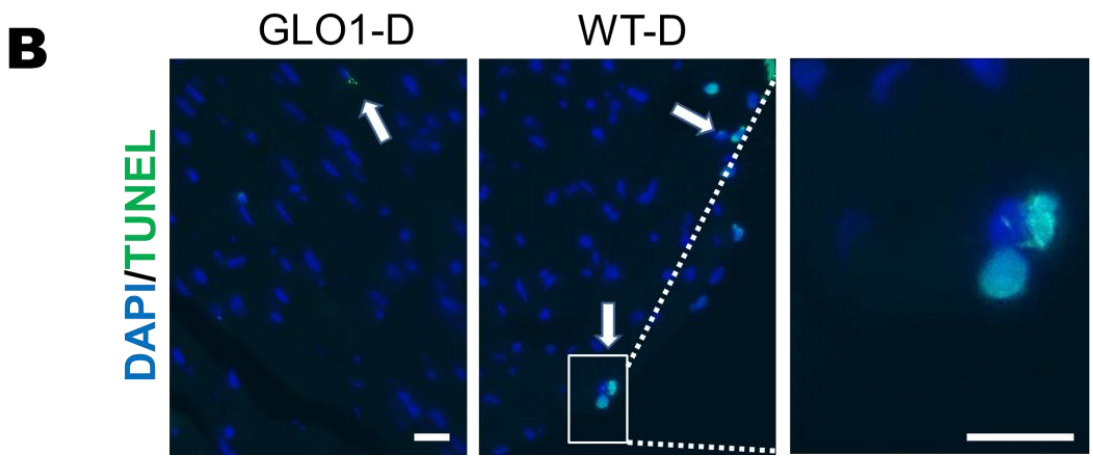

B
GLO1-D
WT-D
DAPI/TUNEL

FIGURE 2.5. Cell death is reduced in the myocardium of GLO1-diabetic mice. A: The percentage of TUNEL^{+} apoptotic cells per FOV normalized to the percent observed in WT mice at 4 and 8 weeks (*$p \leq 0.02$ vs. all other groups at 4 weeks; *$p \leq 0.006$ vs. all other groups at 8 weeks $^{\dagger}p=0.045$ vs. WT and GLO1 controls, n=3-4 per group). B: Representative images of TUNEL staining. Scale bar=10um.

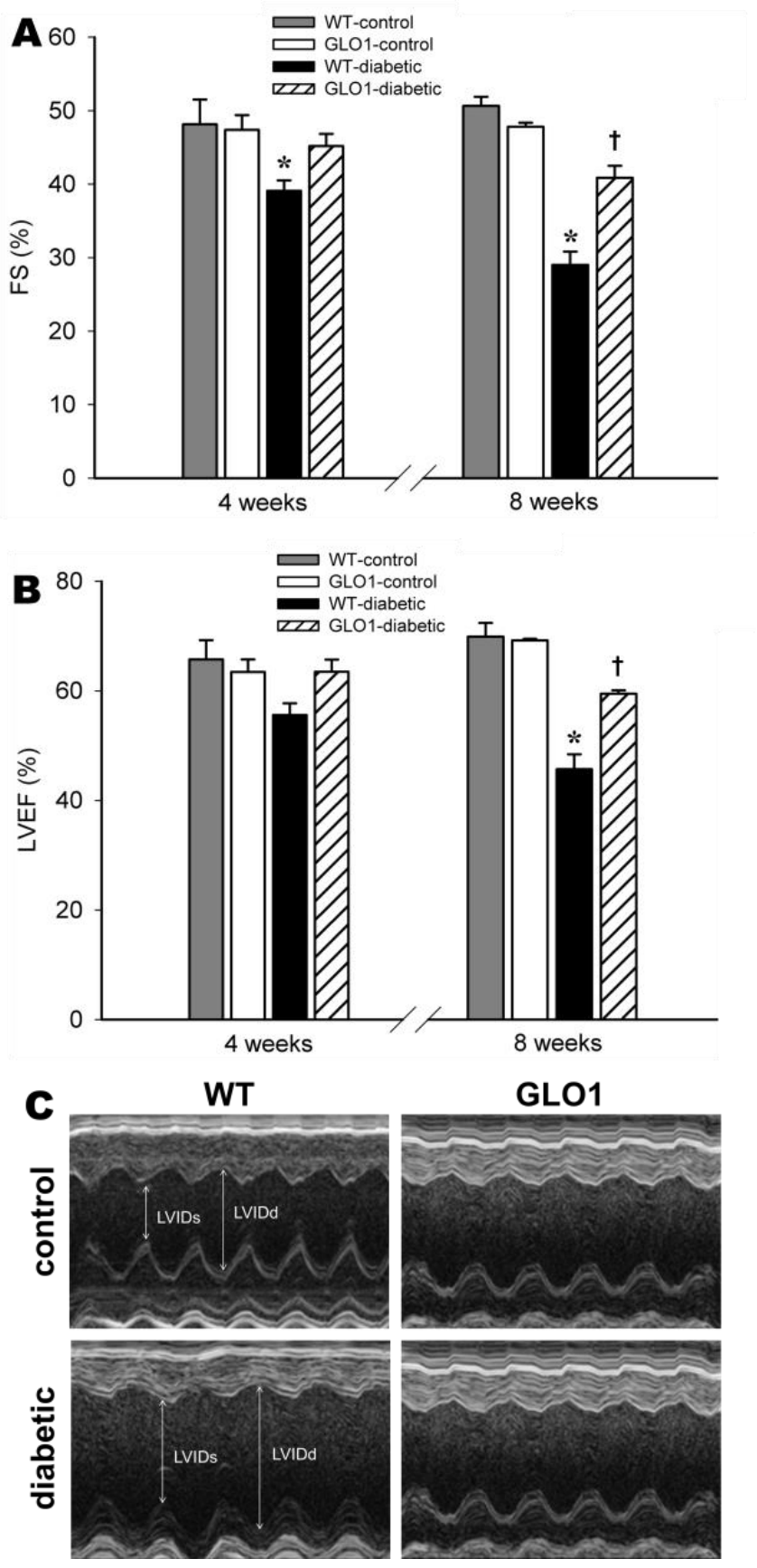

A
WT-control
GLO1-control
WT-diabetic
GLO1-diabetic
FS (%)
60
50
40
30
20
10
0
*
*
†
4 weeks
8 weeks
B
WT-control
GLO1-control
WT-diabetic
GLO1-diabetic
LVEF (%)
80
60
40
20
0
*
†
4 weeks
8 weeks
C
WT
GLO1
control
diabetic
LVIDs
LVIDd
LVIDs
LVIDd

FIGURE 2.6. GLO1 overexpression delays and limits the loss of cardiac function in diabetic mice. A: FS measured by echocardiography at 4 and 8 weeks (*$p \leq 0.045$ vs. all other groups at 4 weeks; *$p < 0.001$ vs. all other groups at 8 weeks; $^{\dagger}p \leq 0.02$ vs. WT and GLO controls at 8 weeks, n=8). B: LVEF measured by echocardiography at 4 and 8 weeks (*$p \leq 0.01$ vs. all other groups at 8 weeks; $^{\dagger}p \leq 0.03$ vs. WT and GLO controls at 8 weeks, n=8). C: Representative echocardiogram recordings for non-diabetic and diabetic mice.

	WT		GLO1		WT-D		GLO1-D	
	4W	**8W**	**4W**	**8W**	**4W**	**8W**	**4W**	**8W**
Wall thickness (mm)	0.87±0.01	0.88±0.01	0.87±0.01	0.87±0.01	0.83±0.01	0.83±0.01	0.85±0.01	0.85±0.01
Cardiac output (ml)	13.6±0.7	12.2±1.4	12.5±0.7	11.2±0.7	9.6±0.2*****	6.8±0.5†	10.9±0.2	8.4±0.19‡§
Stroke volume (ml)	34.6±1.9	33.6±1.8	32.4±0.9	30.4±1.8	21.4±0.9*****	20.5±0.9†	29.6±0.4	24.8±1.2‡§
End volume diastole (ml)	50.1±1.6	49.7±2.3	50.7±3.2	48.9±2.8	37.9±0.8*****	38.4±1.1†	46.0±2.2	39.3±2.4‡
End volume systole (ml)	16.5±0.7	18.1±2.7	15.9±2.1	18.5±1.8	16.5±0.8	16.7±1.7	17.8±0.2	15.8±1.0
Blood glucose levels	n/a	5.4±0.2	n/a	5.8±0.3	19.5±1.92	20.67±1*	14.85±1.7	19.05±1.3*

TABLE 2.2. The effect of diabetes and GLO1 overexpression on cardiac function. Echocardiography results for various cardiac function parameters assessed at 4 and 8 weeks post-STZ (*$p \leq 0.02$ vs. all other groups at 4 weeks; $^{\dagger}p \leq 0.01$ vs. WT- and GLO1-control at 8 weeks; $^{\ddagger}p \leq 0.03$ vs. WT- and GLO1-control at 8 weeks; and $^{\S}p \leq 0.04$ vs. WT-diabetic at 8 weeks, n=8).

DISCUSSION

The aim of this study was to elucidate a link between MG, EC inflammation and the development of diabetic cardiomyopathy. To this end, we used a transgenic mouse model in which the vasculature is protected from MG by GLO1 overexpression. We demonstrated that vascular GLO1 overexpression in hyperglycemic (type I diabetic) mice: 1) prevented the development of inflammation in the endothelium; 2) decreased carbonyl stress; 3) preserved cardiac EC viability and function (eNOS and neuregulin); 4) reduced overall cell death in the myocardium; and 5) delayed and limited the loss of cardiac function. These results highlight the importance of MG-induced endothelial dysfunction in the development of heart failure in diabetes.

To establish that our GLO1 mice provided a good model for this study, we confirmed that both the localization of hGlo1 and the associated increase in GLO1 activity were restricted to the ECs and vasculature of the myocardium. Furthermore, Western blot analysis of whole heart tissue showed no difference in the levels of MG-H1 (the major product of MG-specific glycation) between GLO1 and WT mice (data not shown). Thus, cardiomyocytes in GLO1 mice are expected to still be susceptible to the harmful effects of high glucose and MG accumulation, and changes in heart function after treatment with STZ can be attributed to protection conferred by the ECs and vasculature.

To assess the presence and severity of EC activation and inflammation, we measured the levels of the soluble adhesion molecules VCAM-1, ICAM-1 and E-selectin in blood serum. VCAM-1, ICAM-1 and E-selectin regulate leukocyte adhesion and migration across the vascular endothelium, and their release into the circulation occurs due to EC inflammation and/or cell death (Hwang et al., 1997, Horstman et al., 2004). Notably, GLO1 overexpression protected the ECs in diabetic mice, as increased VCAM-1, ICAM-1 and E-

selectin levels were not present in GLO1-diabetic mice, as they were for the WT-diabetic group. The loss of EC number and function in diabetes is well-described (Kageyama et al., 2011), and occurs in large part due to the accumulation of MG (Baden et al., 2008, Takahashi et al., 2010). In our study, the detrimental effect of hyperglycemia on ECs was evident: there was a reduced number of vWF^+ and $CD31^+$ ECs in the hearts of WT-diabetic mice, as well as decreased neuregulin and eNOS expression. However, the number of vWF^+ and $CD31^+$ ECs was preserved in the GLO1-diabetic mice compared to the non-diabetic groups, suggesting that increased MG caused EC loss in the heart. This is consistent with the observation that the levels of neuregulin and eNOS in cardiac ECs were reduced in WT-diabetic but not in GLO1-diabetic mice compared to the non-diabetic controls.

In addition to its role during heart development, neuregulin produced by ECs closely adjoined to cardiomyocytes in the adult heart helps to regulate cardiomyocyte contractility and survival under stress conditions (Pentassuglia and Sawyer, 2009). It has been shown that neuregulin is reduced in diabetes (Gui et al., 2012), but ours is the first study to show that protecting ECs from MG can prevent this effect. Diabetes also reduces the amount of dimeric eNOS (Yang et al., 2009). The lack of this form of eNOS reduces NO production, which negatively affects the regulation of blood vessel tone and the response of cardiomyocytes to stress, while increasing the deleterious production of superoxide. Changes in NO/redox-based signaling contribute to cardiovascular dysfunction and programmed cell death (Hare and Stamler, 2005). Mechanisms by which MG affects eNOS status have been reported. For example, MG modifications of HIF-1α have been shown to reduce eNOS expression in bone marrow-derived endothelial progenitor cells, which was prevented by GLO1 overexpression (Ceradini et al., 2008). GLO1 can reduce the inhibitory phosphorylation of eNOS caused by MG and neutralize vascular aging (Jo-Watanabe et al., 2014). In our study, the

overexpression of GLO1 preserved the eNOS dimerization process. It should be noted that neuregulin and NO are just 2 components involved in EC-cardiomyocyte signaling, and other factors not evaluated in the present study may contribute to the superior cardiac function observed in GLO1-diabetic mice. However, since GLO1 overexpression maintained EC number and function, altered expression of these other potential contributing factors must also likely be a consequence of modified MG levels.

Myocardial inflammation is one of the main contributors to heart failure (Anker and von Haehling, 2004). In the present study, the increased carbonyl stress, RAGE receptor expression and inflammatory cytokine production (TNF-α) provided evidence of inflammation in the WT-diabetic mice. The overexpression of GLO1 reduced this inflammatory state, but did not completely prevent it. The level of carbonyl stress is an indirect measure of oxidative stress through the detection of carbonylated proteins (Dalle-Donne et al., 2003). Since the cardiomyocytes in GLO1 mice were not directly protected from MG or any oxidative stress, it is reasonable to expect that their level of carbonyl stress would be similar to that of WT-diabetic mice. Cardiomyocytes represent the largest contributor to myocardial mass, yet the hearts of GLO1 mice exhibited less carbonyl stress. Whether this result is exclusively from reduced dicarbonyl stress in the vasculature or also through an undetermined secondary protective effect conferred to cardiomyocytes by the ECs was not elucidated in the present study. Regardless, this highlights the importance of ECs and the vasculature in the overall regulation of oxidative stress in the diabetic heart.

A similar observation was made for TNF-α expression: levels were elevated in the hearts of WT-diabetic mice and reduced by overexpressing GLO1 in the ECs/vasculature. TNF-α is produced mainly in macrophages, and elevated levels of TNF-α in diabetic patients are associated with micro- and macrovascular complications (Dinh et al., 2009). MG has

been shown to stimulate the production and secretion of TNF-α from macrophages (Fan et al., 2003). Notably, macrophages from GLO1 mice have increased GLO1 expression (Geoffrion et al., 2014), potentially making them less susceptible to MG-induced TNF-α production and providing a plausible explanation for the reduced TNF-α level in the hearts of GLO1-diabetic mice. Consequently, since TNF-α induces endothelial cell dysfunction and apoptosis (Kleinbongard et al., 2010), less TNF-α secreted by macrophages may contribute to the improved health of the ECs and vasculature observed in these mice. Although reduced compared to WT-diabetic mice, TNF-α levels in GLO1-diabetic mice were greater than in the non-diabetic groups. This suggests that macrophages are still producing TNF-α, albeit in a reduced amount, and/or that TNF-α is originating from another source such as cardiomyocytes, which has been previously reported (Chen et al., 2010).

Increased RAGE and RAGE ligand expression can further propagate inflammation and result in long-term complications of diabetes (Xue et al., 2014). RAGE is expressed in cardiomyocytes, vascular cells, fibroblasts, and in infiltrating inflammatory cells in diabetes (Ramasamy and Schmidt, 2012). Ligand binding of RAGE initiates a positive feedback loop whereby the receptor-ligand interaction triggers increased RAGE expression (Schmidt et al., 1999). Previous studies have shown that increased GLO1 activity reduces the accumulation of MG-derived AGEs (Brouwers et al., 2013, Geoffrion et al., 2014). Furthermore, the production of endogenous RAGE ligands such as S100 calgranulins and high mobility box 1 (HMGB1) is increased by hyperglycemia, which can be prevented by GLO1 overexpression (Yao and Brownlee, 2010). In our study, the expression of RAGE protein in GLO1-diabetic mice was reduced compared to the WT-diabetic group, despite similar levels of hyperglycemia. This may be associated with lower levels of RAGE ligands (e.g. MG-derived AGEs, HMGB1, S100 calgranulins) in the GLO1 mice, thus reducing the positive-feedback

up-regulation of RAGE expression. Such a mechanism may be contributing to the observed decrease in inflammation in the GLO1-diabetic mice.

Protecting the vascular system from the effects of MG through GLO1 overexpression resulted in reduced overall cell death in the mouse heart. While apoptosis was increased in WT-diabetic mice at 4 and 8 weeks post-STZ injection, GLO1-diabetic mice exhibited an increase in apoptotic $TUNEL^{+}$ cells only at 8 weeks, but were still fewer than in the WT-diabetic group. Cardiomyocyte loss is an important mechanism in the process of myocardial remodeling, cardiac fibrosis, reduced heart function and ultimately heart failure (Kehat and Molkentin, 2010). A previous study showed that ubiquitous overexpression of GLO1 in rats could attenuate cardiac fibrosis that otherwise developed in WT diabetic rats after 16 weeks (Brouwers et al., 2013). We did not detect significant fibrosis in our WT-diabetic mice (1.3-fold, p=0.1, data not shown), possibly because the 8 week time-point may be premature for full fibrosis development. Nevertheless, we were able to observe a reduction in heart function in WT-diabetic mice as early as 4 weeks post-STZ injection, which was prevented in GLO1-diabetic mice.

Consistent with previous studies (Poornima et al., 2006, Brouwers et al., 2013), the data presented herein demonstrate the damaging effect of diabetes on cardiac function in mice. Evaluation of our mice at 4 and 8 weeks post-STZ allowed us to detect differences in the early stages of heart failure development. LVEF and FS were already reduced after 4 weeks of hyperglycemia, which worsened along with other measures of cardiac function (cardiac output, systolic and diastolic end volume, heart weight) by 8 weeks compared to non-diabetic mice. Heart function of the GLO1-diabetic mice was superior to that of the WT-diabetic group, with a delay in the onset of functional loss (observed only at 8 weeks), which was also of lesser magnitude.

In summary, our findings suggest that preventing EC inflammation can improve myocardial function in the setting of hyperglycemia and MG accumulation. Therefore, protecting the vasculature from MG may be a target for therapy to postpone and reduce the development of heart failure in diabetes.

ACKNOWLEDGMENTS

This work was supported by operating grants from the Heart & Stroke Foundation of Canada (to EJS and RWM), the Canadian Institutes of Health Research (to EJS and RWM), and the National Institutes of Health grant DK020541 (to MB and FG). The authors would like to thank Rick Seymour (University of Ottawa Heart Institute) for technical support. BV was supported by a Canadian Graduate Scholarship from the Canadian Institutes of Health Research, and BM by a Research Fellowship from the Heart & Stroke Foundation of Canada.

CHAPTER 3 – MANUSCRIPT #2

Dicarbonyl Stress Increases the Sensitivity of Endothelial Cells to TNF-Mediated Apoptosis

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Running title: Role of MG and TNF in cell apoptosis

Contributions of Authors

This study is collaboration between the University of Ottawa Heart Institute and the Diabetes Research Center in Albert Einstein College of Medicine. Microscopy, cell preparation, live/dead assay and carbonyl stress assay described here were done by me. The dose response study was completed by Kay Maeda, flow cytometry was performed by Ferdinando Giacco. Seahorse experiments were conducted by Xueliang Du. Experiments, data analysis and manuscript preparation was done under the supervision and guidance of Drs. Erik Suuronen and Michael Brownlee.

Abstract

Dicarbonyl stress (DS) is caused by the build-up of α-oxoaldehyde metabolites, such as methylglyoxal (MG). Normally MG is detoxified by the enzyme glyoxalase-1 (GLO1). However, in diabetes MG accumulates and reacts with proteins to form advanced glycation end-products, contributing to the vascular complications in diabetes. The present study aimed to link DS with increased inflammation and death of endothelial cells (ECs), which has been associated with endothelial dysfunction in diabetes. ECs were isolated from the aortas of mice that overexpress GLO1 and their wild type (WT) littermates. In culture, the ECs were exposed to normal or high glucose (HG)±TNF-α. HG+TNF-α treatment decreased cell viability by 30% in WT-ECs, while GLO1-ECs did not show any loss in viability, suggesting that reduced MG protects ECs from TNF-α mediated death. A possible mechanism for increased EC death in the presence of DS and TNF-α was further examined using human ECs (hECs) cultured ± HG, TNF-α and/or MG. At 24h of culture, a live/dead assay revealed that TNF-α in the presence of MG increased EC death by 5-fold compared to ECs exposed to TNF-α alone. Protein carbonylation level measurements showed that both HG and MG significantly increased oxidative stress in hECs, independent of TNF-α. However, increased hEC death was observed only when HG or MG cultured cells were also exposed to TNF-α. hECs exposed to HG or MG for 24h had increased TNF-α induced apoptosis compared to cells treated with TNF-α only (by 2- and 3-fold, respectively), as detected by flow cytometry. Using a Seahorse flux analyzer, it was shown that HG or MG exposure reduced mitochondrial oxygen consumption in hECs by 50%. Our results suggest that DS together with inflammation (TNF-α) leads to the loss of ECs acting through increased oxidative stress and reduced mitochondria respiration. This provides a potential new therapeutic target for preserving endothelial function in diabetes.

INTRODUCTION

Numerous studies have shown that the accumulation of methylglyoxal (MG) has a role in diabetic vascular complications through, among others, endothelial cell (EC) apoptosis (Chan and Wu, 2008, Allaman et al., 2015, De Vriese et al., 2000, van den Oever et al., 2010). Several studies revealed MG-mediated apoptosis in macrophage-derived cell lines (Okado et al., 1996) and neural cells (Suzuki et al., 1998). MG increases cell death and induces liver toxicity, as a result of ROS-mediated mitochondrial dysfunction and oxidative stress (Seo et al., 2014).

MG is readily detoxified, in non-pathological conditions, by the glyoxalase enzyme system that includes two enzymes glyoxalase-1 (GLO1), glyoxalase-2, along with the cofactor glutathione (Thornalley, 2003). The decrease in GLO enzymatic activity promotes accumulation of MG-derived advanced glycation end products (AGEs) and oxidative stress markers (Giacco et al., 2014). On the other hand, over-expression of GLO1 in the C. elegans decreases MG modifications of mitochondrial proteins and mitochondrial ROS production, and prolongs C. elegans lifespan (Morcos et al., 2008b). In the hearts of diabetic GLO1 overexpressing mice cell survival was greater than in wild type (WT) diabetic mice (Vulesevic, McNeill *et al*. unpublished data). Interestingly, despite the difference in cell survival, both groups had increased levels of tumor necrosis factor (TNF-α).

TNF-α is a potent inducer of gene transcription in ECs and a powerful pro-inflammatory cytokine (Kleinbongard et al., 2010, Madge and Pober, 2001). Especially within immune cells, TNF-α is capable of inducing a cascade of downstream cytokines and chemokines (Sedger and McDermott, 2014). TNF-α induced cell signaling leads either to

gene activation, primarily through the NF-kB pathway, or to cell death by either apoptosis or necrosis (Wajant et al., 2003). TNF-α is reported to be increased in the tissues of diabetic patients (Madge and Pober, 2001, Lampropoulou et al., 2014, Hussain et al., 2013). Despite being known as a pro-apoptotic cytokine, TNF-α plays a minor role in apoptosis *in vivo* compared to its considerable function in the regulation of inflammatory processes (Wajant et al., 2003). Pro-apoptotic TNF-α pathway is induced through TNFR1 (tumor necrosis factor receptor 1) - expressed in most tissues, and, among others, involves increase in reactive oxygen species (ROS) production along with the caspase cascade and altered mitochondria function (Wajant et al., 2003, Sidoti-de Fraisse et al., 1998). ROS induce caspase-activation and apoptosis by inducing mitochondrial membrane permeabilization, and it has been proposed that ROS production seems to act as an amplification mechanism in TNF-α signaling (Webster, 2012, Marchi et al., 2012, Baughman and Mootha, 2006). It is important to mention that production of ROS has not only a role in apoptosis but also in necrosis, and is closely related to inflammation *in vivo* (Leist and Jaattela, 2001, Mittal et al., 2014). In diabetes, one of the main effects of hyperglycemia is the production of ROS (Brownlee, 2001a, Giacco and Brownlee, 2010, Pitocco et al., 2013), and MG itself has been shown to increase ROS production in cultured ECs (Dhar et al., 2010a).

We hypothesized that increased MG accumulation may play a role in TNF-induced EC death through increased ROS production. To this end, we used bone marrow (BM) derived ECs from GLO1 overexpressing mice and primary human ECs to examine the synergistic effect of MG and TNF-α in the induction of apoptosis and cell death.

RESEARCH DESIGN AND METHODS

Chemicals. Murine and human TNF peptides were obtained from Abcam. Methylglyoxal solution in H_2O (40 wt. %) and D-glucose were purchased from Sigma.

Cell culture. Two groups of mice were used for BM isolation – transgenic GLO1 overexpressing mice (GLO1) and their wild type (WT) littermates. All studies were performed under protocols approved by the University of Ottawa Animal Care Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. As described previously, GLO1 mice overexpress hGLO1 under the control of the murine pre-proendothelin promoter (Geoffrion et al., 2014, Vulesevic et al., 2014a). Our previous studies confirmed that the GLO1 transgenic mice have bone marrow (BM) with a 30% increase in GLO1 activity (Vulesevic et al., 2014a). Mouse BM cells were extracted from femurs and tibias as previously described (Whitman et al., 2004). After gradient centrifugation, the fraction containing the mononuclear cells and ECs were seeded on collagen-coated plates in EC medium (endothelial basal medium (EBM), Lonza) for 3 weeks to differentiate and expand the EC population. After 3 weeks, cells were lifted with trypsin, and using CD31 (EC cell surface marker) antibody-coated DYNA beads (Life Technologies), the EC population was pulled down and re-plated for another 10 days on collagen-coated Lab-Tek® Chamber Slides™. The BM-derived mouse ECs were then used for the LIVE/DEAD assay. Primary human aortic endothelial cells (HAECs) (Cambrex) were maintained in EBM-2 medium (Lonza) with all the supplements. Cryopreserved Clonetics™ Human Cardiac Endothelial Cells (HCECs) from Lonza were grown in EGM™ with 2MV BulletKit™ media. Cells were grown and all experiments were performed in standard

incubator conditions at 37ºC and 5% CO_2 under normoxic conditions. For all human cell work, passages 2, 3 or 4 were used.

LIVE/DEAD assay. After 24 h of exposure to 30mM high glucose (HG) or 5μM MG in the media, with the presence or absence of TNF-α, cells were washed with PBS and stained using a LIVE/DEAD kit (Invitrogen) for 30 minutes as per manufacturer protocol. The number of cells was compared to the number of cells in the low glucose without TNF-α condition. Cells were imaged using Zeiss Axiophot microscope equipped with a Hamamatsu C5985 chilled CCD camera, and Metamorph imaging software 4.01 (Molecular Devices). Cells whose nuclei were stained with propidium iodide (PI) (red) were considered dead. The average number of PI-positive cells were calculated from 4 random fields-of-view (FOV) per well.

Apoptosis Assay. The Annexin V/Dead Flow cytometry Cell Kit (Abcam) was used for the apoptosis assay. The kit provides annexin V conjugated to fluorescein (FITC annexin V) and a ready-to-use solution of the red-fluorescent propidium iodide (PI) nucleic acid binding dye. PI cannot penetrate into live and apoptotic cells, but stains dead cells with red fluorescence, binding tightly to the nucleic acids. After lifting the cells with trypsin (Lonza) and staining cell populations with FITC annexin V and PI in the provided binding buffer, apoptotic cells were detected by green fluorescence, dead cells by both red and green fluorescence, and live cells showed little or no fluorescence. Apoptosis and cell death was induced by the addition of 10ng/ml of TNF-α ±HG (30mM) or ±MG (5uM) to cell media 24 h prior to flow cytometry staining. Apoptosis and cell death was recorded by a fluorescence-activated cell

sorter (Becton Dickinson DxP10 FACSCalibur). Ten thousand cells were routinely analyzed. Apoptotic or dead cells are expressed as the percentage of annexin or PI-positive cells in the total cell population.

TNF-α/MG dose response LIVE/DEAD assay. After 24 h of treatment with various concentrations of TNF-α (0, 10 and 50ng/ml) and MG (0, 0.5, 1 and 5uM), HCECs were washed with PBS and stained with the LIVE/DEAD kit (Invitrogen) for 30 minutes as described above. Cells were imaged using Zeiss Axiophot microscope, and the average number of PI negative (viable) cells was calculated from 5 random FOV per well.

Carbonyl stress measurements. Protein carbonyl groups derivatized with 2,4-dinitrophenylhydrazine (DNPH) form a stable dinitrophenyl hydrazone product, which was detected by an enzyme-linked immunosorbent assay (ELISA; Cell Biolabs, USA). HCECs (50,000/well) were lysed after 30 min and after 24 h exposure to 30mM HG, 5uM MG with or without 10ng/ml TNF-α to determine the speed of carbonyl production and its accumulation. Protein carbonyl content was determined by the Oxyselect ELISA kit as per manufacturer protocol. Values were calculated by comparison with provided standards and presented as nmol of carbonyl formed per mg of total protein.

Mitochondria activity measurements. A Seahorse Bioscience XF24 extracellular flux analyzer (North Billerica) was used to measure mitochondrial function in HAECs treated with HG, MG and/or TNF-α as described above. The XF24 allows for the determination of

oxygen and proton concentrations in real time. Data is expressed as the oxygen consumption rate (OCR) in pmol/min.

Statistical analysis. Results are expressed as means ± SEM. Statistical analyses were performed using SigmaStat software. Comparisons between two groups were made by an unpaired two-tailed Student t-test. For multiple group comparisons, a one-way analysis of variance with a post-hoc Student-Newman-Keuls test was performed. Statistical significance was given for $p<0.05$.

RESULTS

GLO1 reduces cell death caused by HG and TNF. The effect of GLO1 overexpression on cell death induced by HG and TNF-α was determined using a LIVE/DEAD assay. As shown in FIG 3.1A, 24 h of HG in combination with 10ng/ml of TNF-α increased the number of dead cells per FOV in BM-derived ECs from WT compared to untreated ECs ($p=0.03$). The percentage of dead cells per FOV under the same conditions in BM-derived ECs from GLO1 mice was unchanged ($p=0.8$, FIG 3.1B). GLO1 overexpression reduces the concentration of free MG that is produced by glycolysis in cells under hyperglycemic conditions (Geoffrion et al., 2014). The lack of increased cell death in GLO1 overexpressing BM-derived ECs suggests that it was increased MG in the cells that may have an apoptotic effect in combination with TNF-α in WT ECs.

HG or MG together with TNF-α increase the death of HAECs. The effect of 10ng/ml of TNF-α in the presence or absence of 30mM HG or 5μM MG on cell apoptosis and necrosis

was evaluated by flow cytometry. The percentage of apoptotic cells after 24h of treatment, labeled by Annexin, was increased only in the cell group treated with both MG and TNF-α (p=0.05, FIG 3.2A) compared to the cell groups not treated with TNF-α. The percentage of dead cells labeled by PI was significantly higher in the HG+TNF-α and MG+TNF-a groups ($p \leq 0.04$, FIG 3.2B) compared to all other groups. These results support the hypothesis that the presence of HG and MG augments TNF-α induced apoptosis.

MG increases the sensitivity of ECs to TNF-α pro-apoptotic signals. When HCECs were exposed to different concentrations of MG and TNF-α, the presence of MG alone did not increase cell death. The addition of 10 ng/ml of TNF-α induced significant cell death only in combination with 5μM MG (p=0.003, FIG 3.3). A concentration of 50 ng/ml of TNF-α also stimulated significant cell death only in the presence of MG. Notably; a concentration of only 0.5 or 1 μM of MG was needed to increase cell death by 2-fold ($p \leq 0.03$, FIG 3.3). The addition of 5μM MG to 50ng/ml of TNF-α reduced the percentage of viable cells by a third, significantly more than any other condition ($p \leq 0.02$, FIG 3.3).

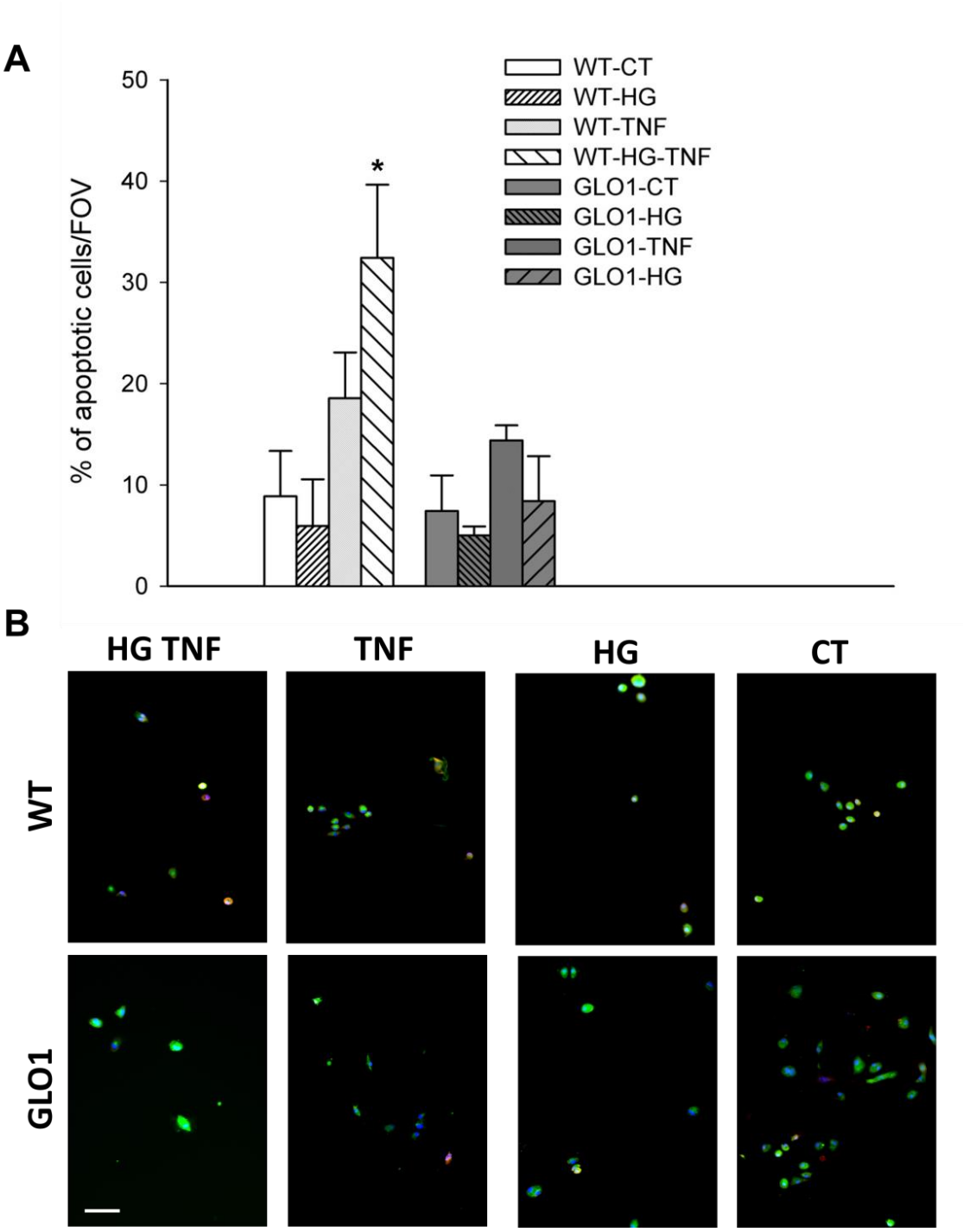

A
50
40
30
20
10
0
% of apoptotic cells/FOV
*
WT-CT
WT-HG
WT-TNF
WT-HG-TNF
GLO1-CT
GLO1-HG
GLO1-TNF
GLO1-HG
B
HG TNF
TNF
HG
CT
WT
GLO1

FIGURE 3.1. GLO1 overexpression reduces HG and TNF-α induced death in ECs. BM-derived ECs were exposed for 24h to 10ng/ml of TNF-α with or without HG (30 mM) and the percentage of PI positive cells per FOV was quantified. A) Apoptotic ECs from WT mice and GLO1 over-expressing mice, *p=0.03, n=4; B) Representative images of LIVE/DEAD staining, blue = DAPI, green = live cell (labeled by calcein AM), red = dead cell (labeled by propidium iodide), scale bar = 20 μm.

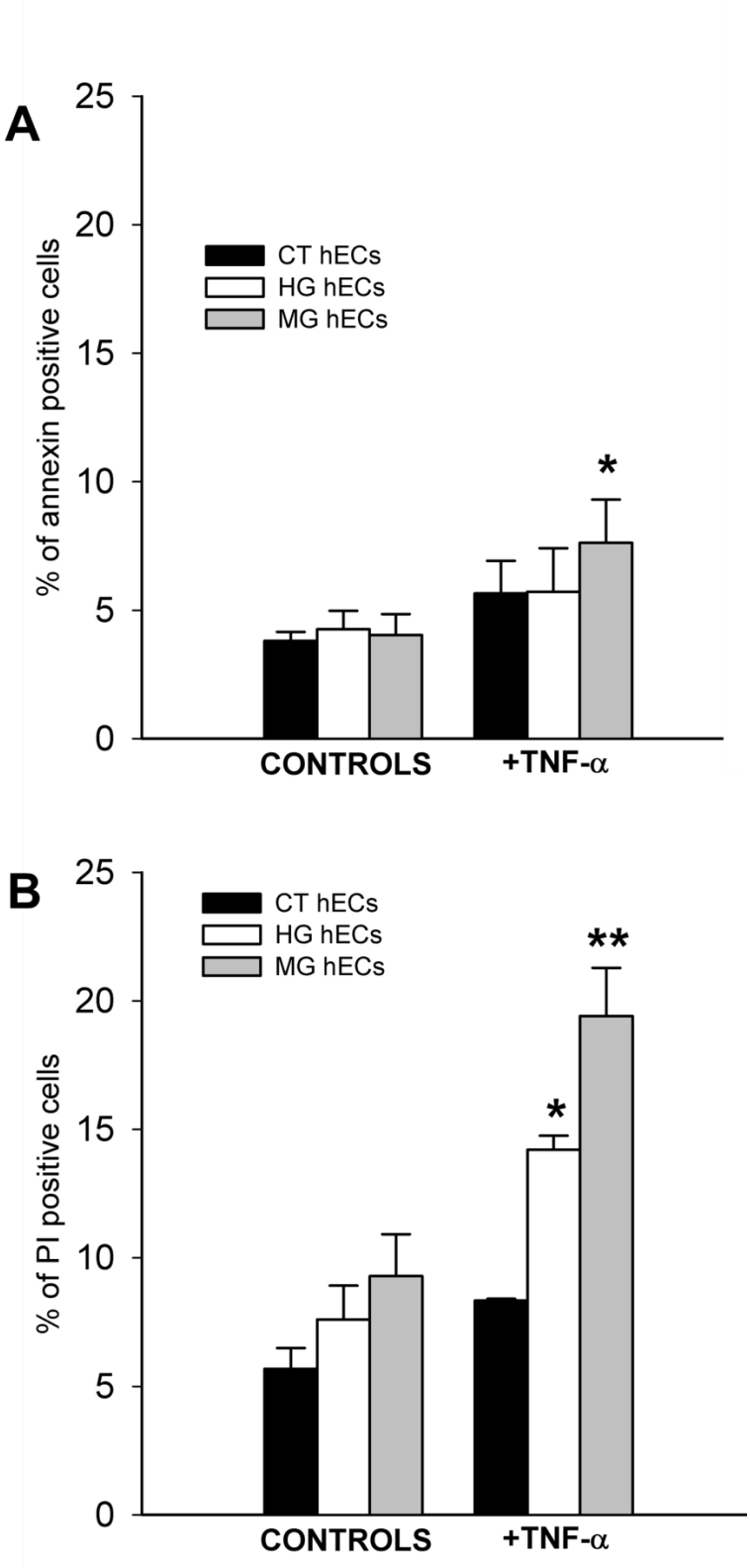

A
% of annexin positive cells
25
20
15
10
5
0
CT hECs
HG hECs
MG hECs
*
CONTROLS
+TNF-α
B
% of PI positive cells
25
20
15
10
5
0
CT hECs
HG hECs
MG hECs
**
*
CONTROLS
+TNF-α

FIGURE 3.2. High glucose and methylglyxal in combination with TNF-α increased the death of HAECs. The effect of 10ng/ml of TNF-α in the presence or absence of high glucose (HG=30mM) or methylglyoxal (MG= 5uM) on cell apoptosis or necrosis after 24h was assessed by flow cytometry. A) The percentage of apoptotic cells labeled by Annexin (*p=0.05 compared to control, n=4); B) The percentage of dead cells labeled by propidium iodide (*p=0.04 compared to controls, **$p\leq$0.03 compared to all other groups, n=4).

HG and MG treatment increases oxidative stress. To elucidate a possible mechanism by which MG and HG affect the cell and render it more sensitive to TNF-α apoptotic signals, oxidative stress was evaluated. The formation of ROS was measured by protein carbonyl ELISA. Protein carbonyls levels (nmol/mg) in the MG+TNF-α treated HCEC group after 30 minutes of exposure were elevated compared to untreated control cells (FIG 3.4A, p=0.03). After 24 h, protein carbonyls were increased in both HG and MG groups, regardless of the presence of TNF-α ($p \leq 0.04$, FIG 3.4B).

Dysfunction of the mitochondria may be a possible origin of oxidative stress. Using the XF24 Extracellular Flux Analyzer (Seahorse Bioscience) to measure mitochondrial respiration in real-time, the function of HAEC treated with HG and MG was compared to untreated control cells. The OCR of control cells was approximately 0.23±0.03 pmol/min/100 cells, and the addition of HG or MG reduced this by 50% (FIG 3.5, p=0.003). During respirometry, wells were sequentially injected with oligomycin to block ATP synthase and assess respiration required for ATP turnover; this oxygen value was also reduced by ~70% in ECs treated with HG or MG (data not shown).

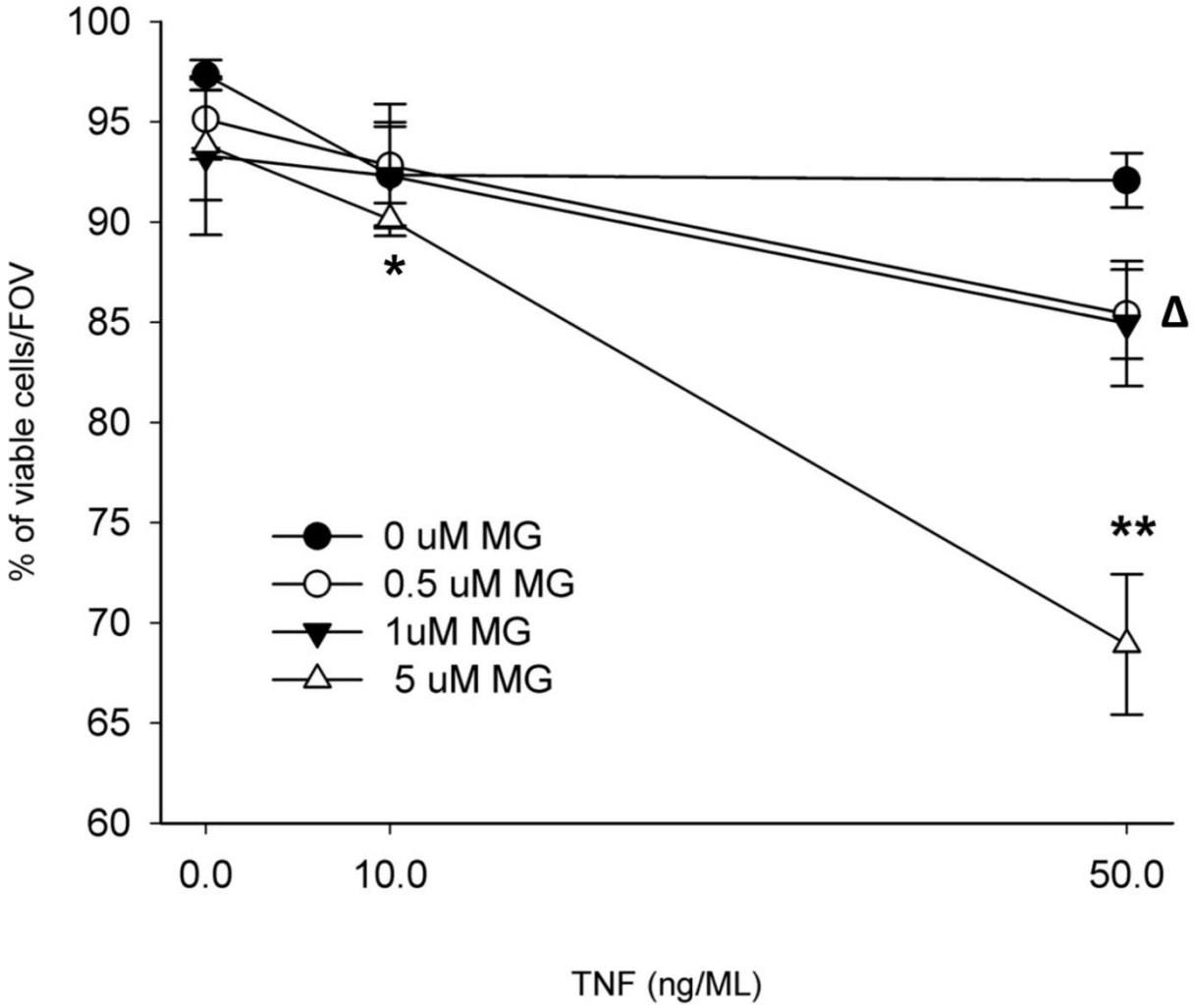
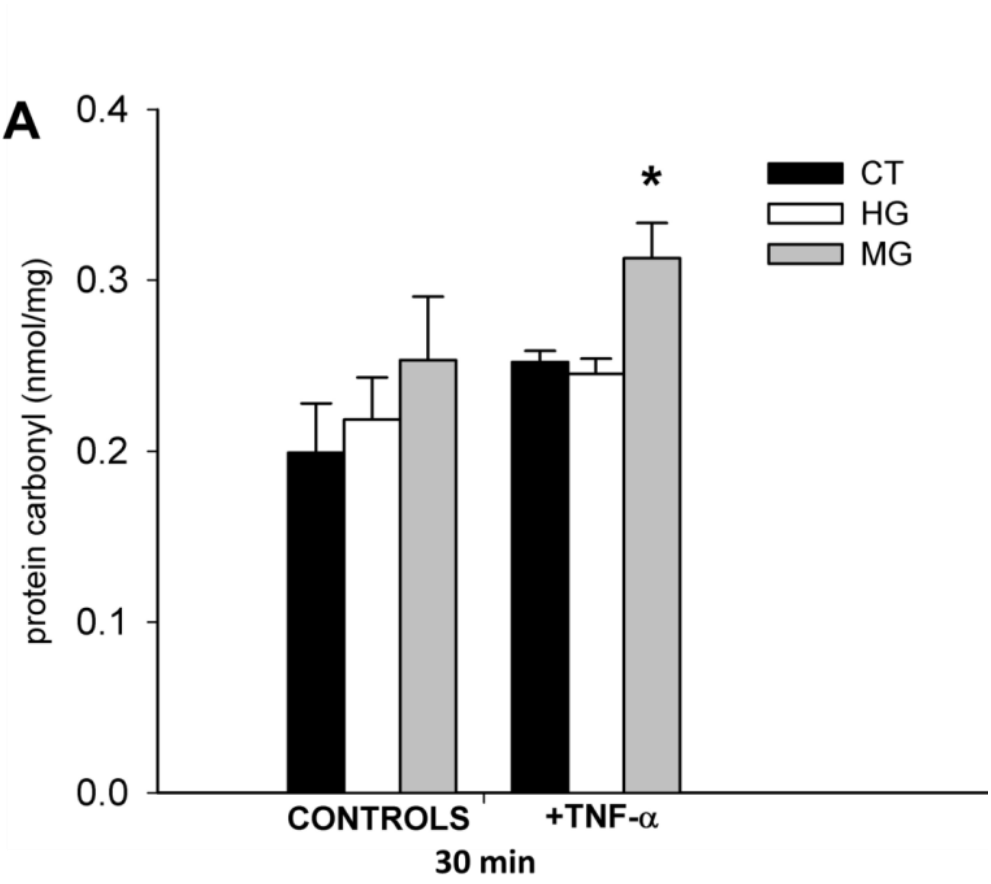

100
95
90
85
80
75
70
65
60
% of viable cells/FOV
0.0
10.0
50.0
TNF (ng/ML)
0 uM MG
0.5 uM MG
1uM MG
5 uM MG
*
**
Δ

FIGURE 3.3. The addition of MG increases the death of ECs exposed to TNF-α. HCECs were exposed to different concentrations of MG (0, 0.5, 1 and 5 uM) and different concentrations of TNF-α (0, 10 and 50 ng/ml) and the percentage of dead cells was assessed using a LIVE/DEAD assay. *p=0.003 compared to the control group (0 µM MG and 0ng/ml TNF-α), $^{\Delta}p \leq 0.03$ compared to controls, **p=0.02 compared to all the other groups, n=3.


A
0.4
0.3
0.2
0.1
0.0
protein carbonyl (nmol/mg)
CT
HG
MG
*
CONTROLS
+TNF-α
30 min

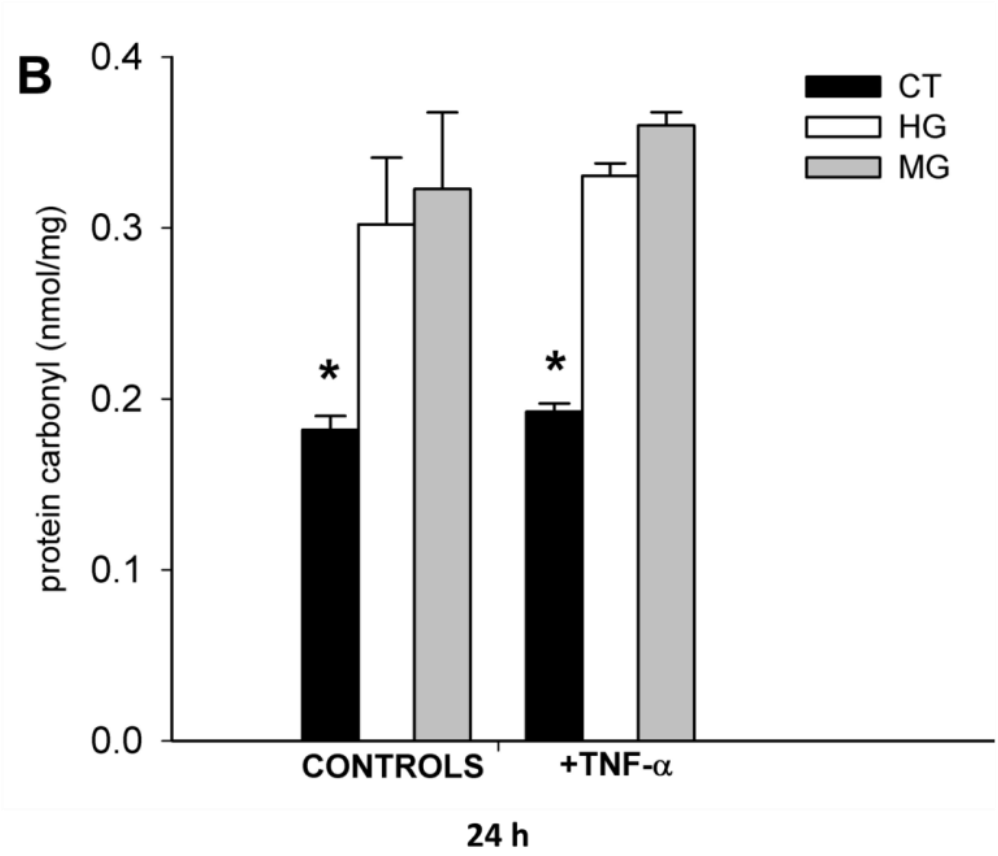

B
0.4
0.3
0.2
0.1
0.0
protein carbonyl (nmol/mg)
CT
HG
MG
*
*
CONTROLS
+TNF-α
24 h

FIGURE 3.4. HG and MG increase oxidative stress in ECs. Protein oxidation induced by reactive oxygen species (ROS) or indirectly by reaction with secondary by products of oxidative stress was measured by ELISA. Protein carbonyl levels were assessed in HCACs after 30 min and after 24h exposure to 30mM HG, 5µM MG with or without 10ng/ml TNF-α. A) Protein carbonyls (nmol/mg) after 30 min of exposure (*p=0.03 compared to the no TNF-α control, n=3). B) Protein carbonyls (nmol/mg) after 24h of treatment (*$p \leq 0.04$ compared to HG or MG treatment with or without TNF, n=3).

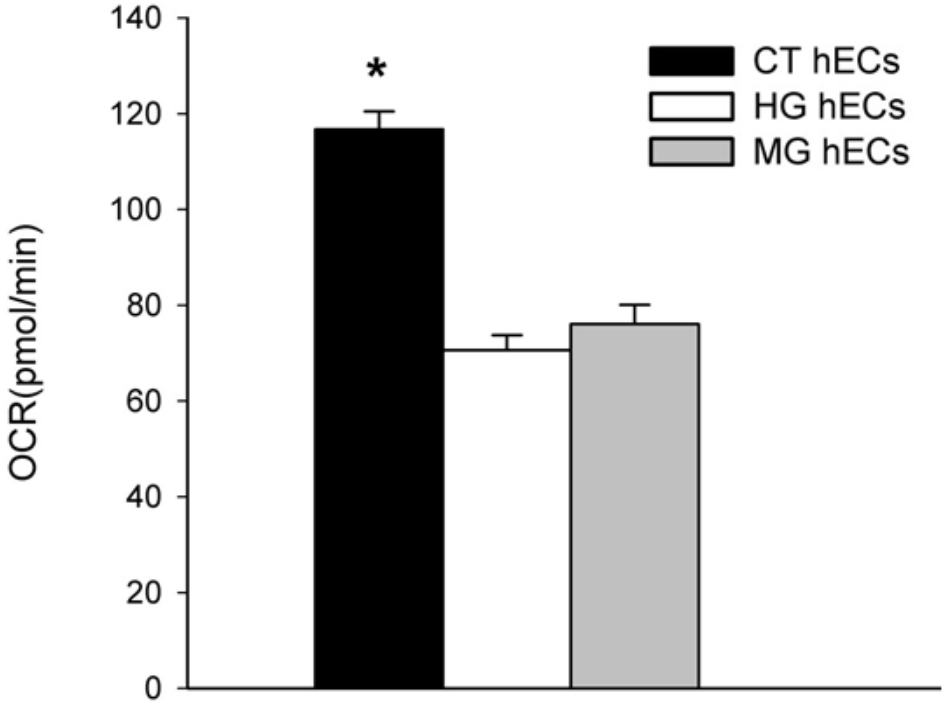


FIGURE 3.5. Methylglyoxal and high glucose reduce oxygen consumption in cell mitochondria. Exposure of HCACs to HG (30mmol) or MG (5uM) for 24h altered the bioenergetic profile (OCR = oxygen consumption rate normalized to cell number, *p=0.003, n=4).

DISCUSSION

This study showed that increased exposure to MG exacerbates TNF-α induced cell death in ECs. Moreover, the response is dose-dependent and characterized by an increased oxidative stress and mitochondrial dysfunction.

The accumulation of MG is mainly recognized in diabetes, but it also occurs in some neurodegenerative disorders, and in normal aging cells (Ramasamy et al., 2006, Rabbani and Thornalley, 2012, Allaman et al., 2015, Kuhla et al., 2005, Tajes et al., 2014). In order to separate the effects of MG alone from other ROS increasing metabolites of glucose, in this study we have exposed cells to either HG or MG alone. There are different reports on the actual MG concentration in diabetic patients, ranging from 300nM up to 8μM. We have chosen to use the dose that does not induce cell death by itself, but that is close enough to the amount of MG found in cells treated with HG (Chan and Wu, 2008, Hsieh and Chan, 2009).

MG is highly reactive and easily modifies proteins and DNA, leading to changes in cell function. It stimulates superoxide production from mitochondria in human ECs (Miyazawa et al., 2010). Metabolism of MG depletes the cells' glutathione content, as it is used by the glyoxalase system, thereby decreasing the elimination of intracellular ROS (Choi et al., 2008). Notably, glutathione is a co-factor in the glyoxalase reaction and glutathione binding is reversible, so this is not the major route of MG ROS induction. Other ways are by direct effect of MG on ROS scavenger enzymes, such as superoxide dismutases, glutathione peroxidases and glutathione transferases (Kang, 2003, Park et al., 2003). MG-induced AGEs can impair mitochondrial function resulting in more production of ROS and further damage (Desai et al., 2010). In our study we confirmed that increased MG (or HG)

levels also increase levels of carbonyl stress, as an indirect measure of oxidative stress in the cell.

By overexpression of GLO1 in BM-derived ECs, we also showed that the reduction of MG only can prevent the pro-apoptotic effect of HG. By reducing MG through increased GLO1 activity we also reduced cell death. The synergistic function of TNF-α and MG or HG in the ECs was confirmed with flow cytometry. After 24 h of MG treatment, a significant amount of early apoptotic annexinV-FITC^{+}/PI^{-} and late apoptotic AnnexinV^{+}/PI^{+} cells was detected. The number of dead (PI positive) cells was increased in both HG and MG groups, indicating that the apoptotic process started early on and that 24 h later most of the affected cells were already dead. It also confirms the possibility that increased ROS production leads directly to necrosis of the cell (Leist and Jaattela, 2001).

Cell death was induced by MG synergistically with TNF-α in a dose-dependent manner. We showed that the death-inducing effect of increasing concentrations of TNF-α on ECs can be amplified by the addition of MG. Five μM of MG lowered significantly the amount of TNF-α needed to induce cell death. These results help explain data from our previous work: hearts from diabetic mice had increased TNF-α levels and death of ECs, whereas mice that overexpress GLO1 in their vasculature had increased TNF-α levels but without a similar loss of ECs (unpublished data).

A study on the apoptotic effect of HG and MG together on human mononuclear cells showed that concentrations of 5 μM MG and 15 mM glucose significantly reduced mitochondrial membrane potential and cell viability (Hsieh and Chan, 2009). In the same study, ECs treated with 5 μM MG and 30 mM glucose appeared to undergo necrosis, which was not observed for cells treated only with 5μM MG. Our data also showed no significant

cell death in the cells treated with only 5 µM MG. This suggests that increased MG levels alone may not be enough to induce cell death, especially not within 24 h of exposure. Another study showed that 5 µM MG did not have an effect on hippocampal neuron viability, but 50 µM did; and this effect was shown to be through the induction of ROS-mediated cell death (Di Loreto et al., 2004). Several studies report that MG-mediated apoptosis occurs via the innate pathway, typically triggered by oxidative stress and growth factor withdrawal (Amicarelli et al., 2003, Okouchi et al., 2005). Our study suggests that MG increased carbonyl stress is not enough to induce apoptosis. Only together with increased levels of TNF-α MG caused significant loss of viability, suggesting that it may serve as an apoptotic signal for the EC undergoing inflammation.

Our results show that HG or MG increases protein carbonylation, a biomarker of oxidative stress. Higher concentrations of MG or HG increased the level of carbonyl stress already within the first 30 minutes of exposure in ECs. This suggests that the susceptibility of ECs to TNF-α related cell death may be exacerbated by MG- or HG-induced oxidative stress.

The mitochondrial content in ECs is modest compared with other cell types with a higher energy requirement (Tang et al., 2014). MG strongly inhibits respiration in ECs. Several studies showed that MG specifically inhibited mitochondrial respiration of malignant and cardiac cells by inhibiting electron flow through complex 1 (Halder et al., 1993, Biswas et al., 1997, Ray et al., 1997). Using the bioenergetics analyser, we showed that HG and MG inhibit mitochondrial respiration in ECs, suggesting that increased carbonyl stress may be a source of the oxidative stress.

Another factor that may be contributing to the effects observed in this study is that TNF-α can also lead to elevated levels of MG and the subsequent formation of specific MG-

derived AGEs (Van Herreweghe et al., 2002). Also, MG-induced oxidative stress has been shown to increase the expression of RAGE (Giacco and Brownlee, 2010, Goldin et al., 2006). RAGE is a pattern recognition receptor that in conditions such as diabetes activates immune cells and the vascular endothelium (Fritz, 2011), and stimulates TNF-α production (Luan et al., 2010). This implies that both MG and TNF-α augment each other's presence in the cell and that they collectively increase the chance of cell death.

In conclusion, this study identifies a potential underlying mechanism contributing to the vascular pathology associated with diabetes. Specifically, our results suggest that the accumulation of MG in a setting of increased inflammation and TNF-α release synergize to increase EC death and tissue damage. This provides a potential new therapeutic target for preserving endothelial function and vascular health in diabetes.

ACKNOWLEDGMENTS

This work was supported by operating grants from the Heart & Stroke Foundation of Canada (to EJS and RWM), the Canadian Institutes of Health Research (to EJS and RWM), and the National Institutes of Health (to MB and FG). The authors would like to thank Brian McNeill (University of Ottawa Heart Institute) for technical support. BV was supported by a Canadian Graduate Scholarship from the Canadian Institutes of Health Research.

CHAPTER 4 – MANUSCRIPT #3

Glyoxalase-1 overexpression in bone marrow cells reverses defective neovascularization in STZ-induced diabetic mice

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Running title: Role of MG-induced ED in diabetic cardiomyopathy

Published in Cardiovascular Research, 2014 Feb 1;101(2):306-16.

Contributions of Authors

The animal work, immunocytochemistry, tissue and cell preparation, part of the GLO activity assays, Western blots, cytokine arrays and carbonyl stress assay described here were performed by me. The QPCR, migration assay and part of the experimental design were contributed by Brian McNeill, and Michele Geoffion maintained the mouse colony, helped with bone marrow transplant and performed some of the GLO activity assays and Westerns. Joanne McBane did LIVE/DEAD assay and eNOS Western staining. Marina Lochhead performed GFP Western analysis and helped with bone marrow cell isolation. The analysis of immunocytochemistry and cytokine array results was done with the help of Drew Kuraitis. Experiments and data analysis and manuscript preparation was done under the supervision and guidance of Drs. Erik Suuronen and Ross Milne. Research design and support was provided by Drs. Erik Suuronen, Ross Milne and Greg Korbutt. A special thank you to Dr Barbara Vanderhyden, who helped design and make the transgenic hGLO1 overexpressing mouse model.

Abstract

Methylglyoxal (MG) accumulates in diabetes and impairs neovascularization. This study assessed whether overexpressing the MG-metabolizing enzyme glyoxalase-1 (GLO1) in only bone marrow cells (BMCs) could restore neovascularization in ischaemic tissue of streptozotocin-induced diabetic mice.

After 24 h of hyperglycaemic and hypoxic culture, BMCs from GLO1 overexpressing and wild-type (WT) diabetic mice were compared for migratory potential, viability, and mRNA expression of anti-apoptotic genes (Bcl-2 and Bcl-XL). In vivo, BMCs from enhanced green fluorescent protein (eGFP) mice that overexpress GLO1 were used to reconstitute the BM of diabetic mice (GLO1-diabetics). Diabetic and non-diabetic recipients of WT GFP^+ BM served as controls (WT-diabetics and non-diabetics, respectively). Following hindlimb ischaemia, the mobilization of BMCs was measured by flow cytometry. In hindlimbs, the presence of BM-derived angiogenic (GFP^+CXCR4^+) and endothelial (GFP^+vWF^+) cells and also arteriole density were determined by immunohistochemistry. Hindlimb perfusion was measured using laser Doppler. GLO1-BMCs had superior migratory potential, increased viability, and greater Bcl-2 and Bcl-XL expression, compared with WT BMCs. In vivo, the mobilization of pro-angiogenic BMCs ($CXCR4^+$, $c\text{-}kit^+$, and Flk^+) was enhanced post-ischaemia in GLO1-diabetics compared to WT-diabetics. A greater number of GFP^+CXCR4^+ and GFP^+vWF^+ BMCs incorporated into the hindlimb tissue of GLO1-diabetics and non-diabetics than in WT-diabetics. Arteriole and capillary density and perfusion were also greater in GLO1-diabetics and non-diabetics.

This study demonstrates that protection from MG uniquely in BM is sufficient to restore BMC function and neovascularization of ischaemic tissue in diabetes and identifies GLO1 as a potential therapeutic target.

INTRODUCTION

Following ischaemia, neovascularization occurs to restore the supply of oxygen and nutrients. Blood vessel regeneration involves signalling from ischaemic tissue and mobilization of both local pro-angiogenic cells and bone marrow-derived circulating angiogenic cells (BM-CACs). BM-CACs are a heterogeneous population consisting mostly of myeloid haematopoietic cells, with a small fraction of true endothelial precursors (Fadini et al., 2012). Although phenotypic and functional characterization of these cells have been controversial, subsets of these cells can contribute to vascular repair through direct participation in the formation of new blood vessels, but primarily through the secretion of pro-angiogenic cytokines and the recruitment and regulation of local angiogenic cells (Fadini et al., 2012).

Decreased vascularity and defective ischaemia-induced neovascularization are major contributors to cardiovascular complications in diabetes. Hyperglycaemia impairs neovascularization through inactivation of the hypoxia-inducible factor-1a (HIF-1a) that regulates angiogenic factors, including vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) (Thangarajah et al., 2010, Ceradini et al., 2008). Furthermore, hyperglycaemia attenuates the tissue's signaling capacity to recruit BM-CACs and, through the generation of toxic metabolic glucose by-products, creates a hostile environment for recruited cells (Fadini et al., 2006). Additionally, bone marrow cells (BMCs) themselves are functionally deficient in diabetes, characterized by a poor ability to mobilize in response to hypoxia and to promote blood vessel growth (Capla et al., 2007, Loomans et al., 2009).

Hyperglycaemia and the related formation of advanced glycation endproducts (AGEs) have been associated with the pathogenesis of diabetic vasculopathy (Schmidt and Stern, 2000). AGEs are formed by non-enzymatic protein glycation, i.e. the addition of

sugars and toxic aldehydes, such as methylglyoxal (MG) and glyoxal. Through interaction with the receptor for AGE, AGEs increase inflammation and oxidative stress by the formation of reactive oxygen species (ROS) (Yao and Brownlee, 2010, Bierhaus and Nawroth, 2009). MG is the major glycating agent in diabetes; and MG-derived hydroimidazolones are the predominant AGEs in tissues of diabetic patients (Ahmed, 2003, McLellan et al., 1994). Detoxification of MG is done primarily by the glyoxalase system: glyoxalase-1 and -2 (GLO1 and GLO2); in the presence of reduced glutathione, MG is converted into D-lactate via the intermediate S-D-lactoylglutathione. Overexpression of GLO1 reduces AGE formation and ROS (Ceradini et al., 2008, Ahmed, 2003, Ahmed and Dobler, 2008). Decreasing intracellular ROS by increasing GLO1 expression can rescue angiogenic cells from hyperglycaemia-induced functional defects in vitro, through reversal of the MG-mediated HIF-1a modification.3 Transgenic rats overexpressing human Glo1 (hGlo1) showed reduced retinal, neuroglial, and vascular pathology following treatment with streptozotocin (STZ) and were resistant to renal ischaemia–reperfusion injury (Berner et al., 2012, Kumagai et al., 2009).

Since BM-CACs are one of the major contributors to vascular repair, the objective of this study was to look specifically at the role of dicarbonyl stress (increased oxidation of glucose and inadequate detoxification) caused by MG in the BM. We examined whether over-expression of hGlo1 in the BM could help restore lost BM mobilization and BM-CAC function and reverse the defective ischaemia-induced neovascularization in an STZ-induced mouse model of Type 1 diabetes.

RESEARCH DESIGN AND METHODS

Transgenic hGlo1 mice. All animal procedures were performed with the approval of the University of Ottawa Animal Care Committee and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The cDNA encoding hGlo1 with an amino terminal c-myc epitope tag was cloned into the Not1-digested PEP8 plasmid, so that the hGlo1 insert was under the control of the murine pre-proendothelin promoter. The PEP8 plasmid was kindly provided by DrWilliam Sessa (Boyer Center for Molecular Medicine, Yale University) with the permission of Dr Mitsuhiro Yokoyama (Kobe University School of Medicine). The 13 kb SpeIlinearized PEP8hGlo1 was micro-injected into the pronuclei of fertilized C57BL/6 mouse eggs that were then transferred to the oviducts of pseudopregnant foster mothers. Transgenic founders were identified by PCR screening. All experiments used mice hemizygous for the hGlo1 transgene from a single line (hGlo1 mice) or their non-transgenic littermates.

Diabetes induction. C57BL/6 mice received an intraperitoneal injection of STZ (50 mg/kg) in 0.05 M sodium citrate or an equal volume of citrate buffer (control nondiabetic mice) for 5 consecutive days. Blood glucose measurements of fasting animals were taken 8–10 days after the first STZ injection and following animal sacrifice. Mice with glucose levels > 15 mmol were considered hyperglycaemic/diabetic; those below this level were excluded. Mice were fed the Teklad Global 2019 Extruded Rodent Diet (Harlan, Mississauga, Canada).

Bone marrow extraction for in vitro studies. Three groups of mice were used for the in vitro study: STZ-treated hGlo1 transgenic mice (GLO1-diabetic) and their wild-type (WT)

littermates divided into STZ-treated (WT-diabetic) and citrate buffer-treated (WT) groups. Mice were anaesthetized under 3% isoflurane, and euthanasia was performed by an overdose of sodium pentobarbital. Bone marrow was harvested from femurs and tibias using a 27-gauge needle and phosphate buffered saline. The BMCs were collected 4 weeks after confirmation of hyperglycaemia, separated by density gradient centrifugation (Histopaque 1119; Sigma, Oakville, Canada), and counted (VicellTM Analyzer; Beckman Coulter, Mississauga, Canada). As a measure of oxidative stress, BMC lysates were prepared for analysis using the OxiSelect protein carbonyl ELISA kit, following the manufacturer's protocol (Cell Biolabs, San Diego, USA). Cells were cultured on fibronectin-coated plates in endothelial basal medium (EBM; Clonetics, Mississauga, Canada) with gentamycin, lacking growth factors and serum, but containing either 30 mmol glucose for BMCs from GLO1-diabetic and WT-diabetic mice or 5 mmol glucose for BMCs from non-diabetic WT mice.

In vitro viability assay. BMCs (n = 3 per group) were cultured for 24 h (as described above) under hypoxic conditions (1%O_2 and5%CO_2), and stained using the LIVE/DEAD Viability Assay Kit (Invitrogen, Burlington, Canada). Cells were imaged using an Olympus BX60 microscope with the Spot Basic software (four images/ sample, ×10 magnification). Viability was calculated per field-of-view (FOV) as: viable cells/total cells.

Chemotaxis migration assay. Migration was assessed using a horizontal fibronectin-coated m-Slide Chemotaxis 2D assay (Ibidi, Verona, USA). Briefly, 3×10^4 BMCs were loaded into the observation area and allowed to adhere for 2 h at 37ºC and 5% CO_2 followed by two rinses with serum/growth factor-free EBM. The adjacent reservoir was filled with serum/growth factor-free EBM and one of the following: (i) SDF-1 (50 ng/mL), (ii) VEGF

(50 ng/mL), or (iii) blood serum collected from diabetic mice sacrificed 3 or 7 days after hindlimb artery ligation surgery to induce ischaemia (as described below). The media contained 30 mmol glucose for diabetic BMCs, or 5 mmol glucose for non-diabetic BMCs, as described above. Slides were incubated for 18 h at 37ºC, and the number of cells that migrated a distance of ≥9 mm towards the chemoattractant was visualized and quantified using microscopy.

Animal model and BM transplant. Mice hemizygous for the hGlo1 transgene were crossed with enhanced green fluorescent protein transgenic mice [eGFP mice; C57BL/6-Tg(CAGEGFP) 1Osb/J; Jackson Laboratories, Bar Harbor, USA], and their progeny were identified by PCR genotyping and used as BM donors ($eGFP^+$ or $hGlo1^+/eGFP^+$ BMCs).

BM transplantation (BMTx) was performed as previously described (Whitman et al., 2004). Briefly, recipient C57BL/6 male mice (8- to 10-week old; Jackson Laboratories) maintained on antibiotic-containing drinking water (NovoTrimel; 1.5 mL/100 mL of water) for 1 week were irradiated with two equal 450 rad doses, 3 h apart, using a caesium source. Donor BMCs ($eGFP^+$ or $hGlo1^+/eGFP^+$) were freshly isolated as described above, but without gradient separation. BMCs (7×10^6 cells/animal) were injected into the tail vein, and 4 weeks later the mice received intraperitoneal injections of STZ (WT-diabetic and GLO1-diabetic groups) or an equal volume of citrate buffer (non-diabetic mice). Four weeks after confirmation of hyperglycaemia, hindlimb ischaemia was induced by ligation of the left proximal femoral artery, under 3% isoflurane (inhaled), as previously described (Kuraitis et al., 2011). Pain was managed pre-operatively and 1 and 2 days after surgery by buprenorphine (subcutaneous).

Glyoxalase activity. GLO1 activity was assayed by measuring the rate of formation of S-D-lactoylglutathione from hemi-thioacetal, as described (Oray and Norton, 1982). Briefly, the assay mixture consisted of 7.9 mM MG, 1 mM glutathione, 14.6 mM $MgSO_4$, and 182 mM imidazole HCl, pH 7.0. Following equilibration, the reaction was initiated by the addition of BMC lysate (20 mg). GLO1 activity is reported as millimolar of S-D-lactoylglutathione formed/min/mg of lysate protein (concentration determined by the Lowry protein assay) (Lowry et al., 1951)

Laser Doppler analysis. Under 3% isoflurane (inhaled), hindlimb blood perfusion was measured using laser Doppler (moorLDI2; Moor Instruments, Axminster, UK), preoperatively, immediately following surgery, and at 7 and 14 days post-ligation, as described (Limbourg et al., 2009). The results are expressed as the ratio of ischaemic to nonischaemic hindlimb blood flow.

Flow cytometry. Flow cytometry was performed on circulating GFP+ cells collected by saphenous vein bleeds pre-operatively and at Days 1, 4, 7, and 14 post-surgery, as described previously (Suuronen et al., 2009). Briefly, the mononuclear cell fraction was labelled with antibodies against the following antigens: c-kit (Southern Biotech, Birmingham, USA), CXCR4 (BD Biosciences, Mississauga, Canada), and flk-1 (mouse vascular endothelial growth factor receptor-2; eBioscience, San Diego, USA), and analysed with a FACSAria flow cytometer (BD Biosciences). The fold-change in the percentage of positive cells for early (Days 1 + 4) and late (Days 7 + 14) response timeswas calculated relative to baseline, as described previously (Kuraitis et al., 2012). For BMC characterization pre-BMTx, the mononuclear cell fraction was labelled with c-kit, CXCR4, flk-1 (same suppliers as above),

and CD34 antibodies (BioLegend, San Diego, USA). The FACSAria flow cytometer was also used for the sorting of $CXCR4^{+}CD34^{+}$ and $CXCR4^{+}CD34^{-}$cells from the BM in order to measure hGlo1mRNAexpression in these BMC populations.

Immunohistochemistry. Two weeks after ligation surgery, mice were sacrificed as described above, and hindlimb muscle tissue distal to the ligation site (medial thigh muscle) was frozen in optimum cutting temperature compound, sectioned, and fixed with methanol. Sections (10 mm) were stained with an a-smooth muscle actin antibody for arterioles, von Willebrand factor (vWF) or CD31 antibodies for endothelial cells, CXCR4 antibody for angiogenic cells, and GFP antibody for recruited BMCs (all antibodies from Abcam). Imaging was performed with a Zeiss Z1 fluorescence microscope and an AxioVision digital image software (Toronto, Canada). To quantify the vascular density and percentage of CXCR4+/GFP^{+} or vWF^{+}/GFP^{+} cells, four random microscopic FOV (0.5 mm^2 for arterioles and 0.15 mm^2 for $CD31^{+}$ cells) per sample (at two depths from ligation site) in a blinded fashion were counted using the ImageJ software (n = 3–7 per treatment group).

Cytokine antibody arrays. Relative cytokine levelswere analysed in hindlimb tissue lysates (100 mg) or blood serum (50 mL) from sacrificed animals (n = 4–6 per treatment group) using RayBio™ Mouse Cytokine Antibody Array Kits (cat# AAM-ANG-G1-8; Raybiotech,Norcross,USA), according to the manufacturer's protocol.

RNA extraction, cDNA synthesis,and quantitative PCR. Total RNA was extracted from muscle or cultured BMCs using Trizol reagent (Invitrogen), following the manufacturer's instructions. First-strand cDNA was synthesized from RNA (2 mg) using GoScriptTM

reverse transcriptase (Promega, Madison, USA) and random hexamer primers (IDT, Toronto, Canada). mRNA levels were assessed by real-time quantitative polymerase chain reaction (RT-qPCR) using BRYT Green GoTaq qPCR Mater Mix (Promega) and a LightCycler 480 Real-Time PCR system (Roche, Mississauga, Canada). Primer pairs (see Supplementary material online, Table S1) were designed using the DNAMAN software (Lynnon Biosoft, Pointe-Claire, Canada) and primer3 (v.0.4.0). Relative changes in mRNA expression of target genes were determined using the Δ-ΔCt method (Pfaffl, 2001), expressed as levels relative to the combined average values of 18S and Gapdh.

Bone marrow and tissue Western blot analysis. BMCs and hindlimb tissue were lysed for protein extraction using RIPA buffer (with protease inhibitor; Roche). Equal amounts of protein (150 mg for BMCs, 40 mg for hindlimb tissue) were loaded into 10% Precise Protein Gels (Fisher, Ottawa, Canada) and resolved by SDS–PAGE. For detection of endothelial nitric oxide synthase (eNOS) dimers, polyacrylamide electrophoresis was performed at low temperature (4ºC), as previously described (Molnar et al., 2005). Protein was transferred onto polyvinylidene fluoride membranes (Immobilon-FL, Millipore, Toronto, Canada), blocked in 5% non-fat dry milk in TBS–Tween 20 buffer for 1 h before probing with primary antibodies. Incubation with anti-cmyc 9E10 (1:1000; ATCC, Manassas, USA), anti-GFP, and anti-eNOS (both 1:100; Abcam, Cambridge, USA) antibodies was performed overnight at 48C. Secondary antibodies were from Cell Signaling Technology (Whitby, Canada). Signal detection was performed with the SuperSignal West Pico chemiluminescence kit (Pierce, Ottawa, Canada). Protein concentration was determined by the BCA assay (Thermo Scientific, Ottawa, Canada). Western blot band intensity was determined using the ImageJ software.

Gene primer	Primer sequence
***Hif1-α* FW**	**5'-CAGTCGACACAGCCTCGATA-3‘**
***Hif1-α* RV**	**5'-CGGCTCATAACCCATCAACT-3'**
***Vegf1* FW**	**5'- CAGGCTGCTGTAACGATGAA-3'**
***Vegf1* RV**	**5'-TATGTGCTGGCTTTGGTGAG-3'**
***eNos* FW**	**5'-TGACCCTCACCGCTACAA-3'**
***eNos* RV**	**5'-CTGCCTTGTCTTTCCACAGG-3'**
***18S* FW**	**5'-CGGCTACCACATCCAAGG-3'**
***18S* RV**	**5’-CTGGAATTACCGCGGCT-3'**
***Gapdh* FW**	**5'-TGAAGGGGTCGTTGATGG-3'**
***Gapdh* RV**	**5'-AAAATGGTGAAGGTCGGTGT-3'**
***Bcl-xl* FW**	**5’-TACAGGCTGGCTCAGGACTAT-3’**
***Bcl-xl* RV**	**5’-CGCAACATTTTGTAGCACTCTG-3’**
***Bcl-2* FW**	**5’-TGGGATGCCTTTGTGGAACT-3’**
***Bcl-2* RV**	**5’-GAGACAGCCAGGAGAAATCAAAC-3’**

TABLE 4.1 Primer sequence

Statistical analysis. All results are expressed as the mean±SEM. Comparisons of continuous data between groups were performed with a one-way analysis of variance, and that between individual groups were performed with a two-tailed Student's t-test using the SigmaStat software. $P \leq 0.05$ were considered statistically significant.

RESULTS

Characterization of hGlo1transgenic mice. The immunoreactive cmyc-hGLO1 was detected by Western blotting in all tissues tested in $hGlo1^{+/-}$ mice (heart, aorta, kidney, eye, and liver, and brain — data not shown). Despite the reported endothelial-specific expression of this promoter, extracts of isolated endothelial, smooth muscle cells, and BM-derived macrophages from $hGlo1^{+/-}$ mice had approximately five-fold greater GLO1 activity than those from nontransgenic littermates (see Supplementary material online, TABLE 4.2). Immunohistochemistry of mouse aortas also indicated that transgene expression was not restricted to the endothelium (not shown). The hGlo1 mRNA expression was confirmed through RT-qPCR in $CXCR4^{+}CD34^{+}$ and $CXCR4^{+}CD34^{-}$ BMCs of transgenic mice. It is, therefore, clear that expression of the hGlo1 transgene is not restricted to endothelial cells.

GLO1 overexpression improves in vitro viability and reduces oxidative stress in BMCs. After 24 h in apoptosis-inducing conditions (serum deprivation, hypoxia, and high glucose), the survival of BMCs from GLO1-diabetic mice (75.4±3.3%) was equivalent to that of non-diabetic WT BMCs (75.2+3.9%; $P = 1.0$); whereas BMCs from WT-diabetic mice had significantly reduced survival (57.7±2.9%; $P \leq 0.024$; FIG 4.1A). Consistent with the increase in survival, mRNA expression of two anti-apoptotic factors, Bcl-2 and Bcl-XL, was

increased in BMCs from GLO1-diabetic mice (5.5±1.1 and 4.7±2.3, respectively) compared with those from WT-diabetic mice (1.3±0.8 and 1.8±0.5, respectively; $P \leq 0.0014$; FIG 4.1B). As a measure of oxidative stress, protein carbonyls in BMCs of GLO1-diabetic mice were reduced by 13-fold compared with non-diabetic WT BMCs, and also by 25-fold compared with WT-diabetic BMCs ($P \leq 0.049$; FIG 4.2).

	S-D-lactoylglutathione formation (mmol / minute / mg cellular protein)	
	hGlo1$^{+/-}$	Non-TG
Aortic endothelial cells	3.33 ± 0.04[1]	0.59 ± 0.03
Aortic smooth muscle cells	2.45 ± 0.04[1]	0.57 ± 0.02
Peritoneal macrophages	0.78 ± 0.02[1]	0.11 ± 0.02
Bone marrow-derived macrophages	0.81 ± 0.07[2]	0.22 ± 0.01
Hepatocytes	3.80 ± 0.08	3.56 ± 0.12

Table 4.2 GLO-1 activity in cells isolated from *hGlo1*$^{+/-}$ mice and non-transgenic littermates; [1]$p<0.001$ and [2]$p<0.002$ versus non-TG.

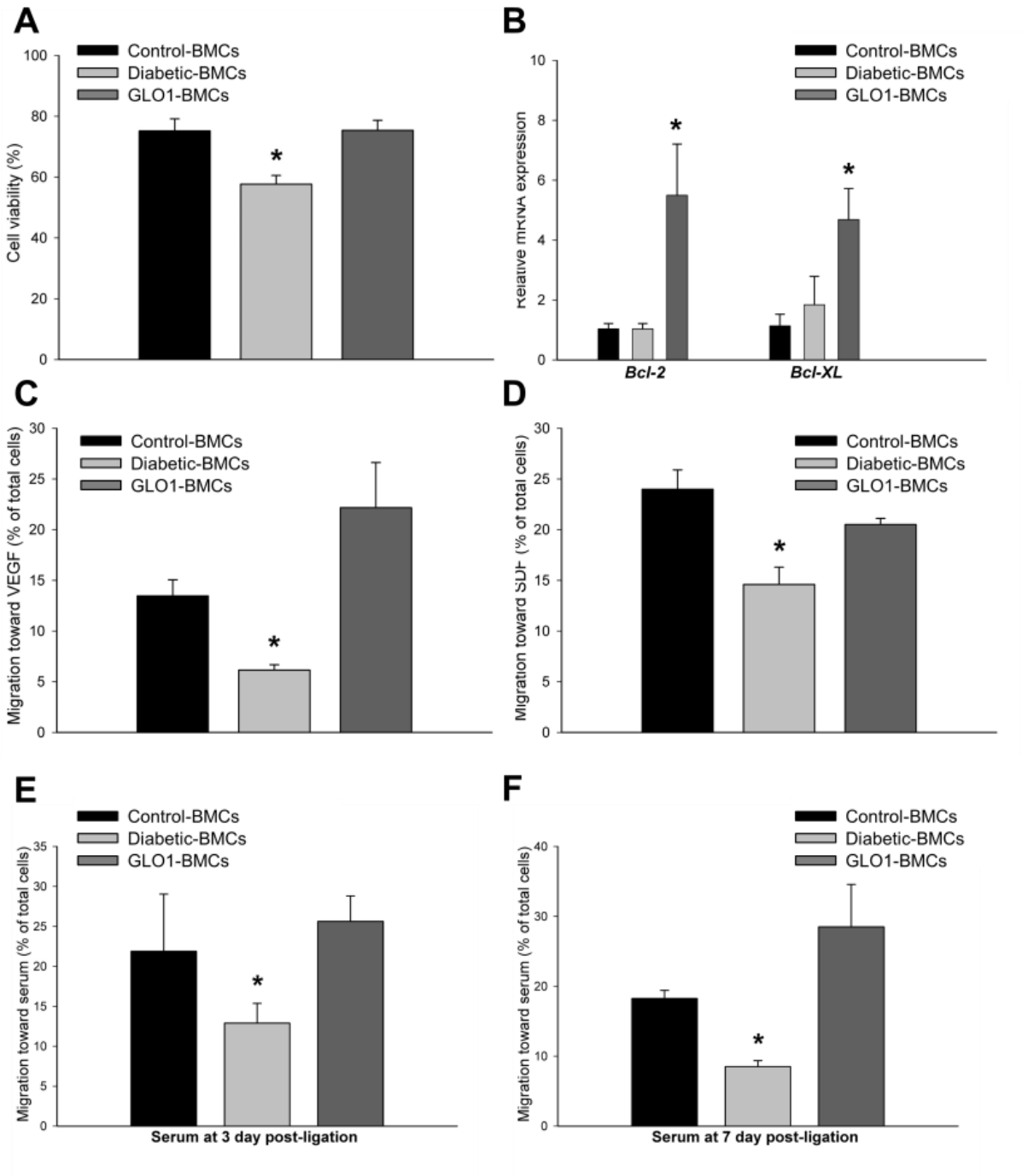

A
Control-BMCs
Diabetic-BMCs
GLO1-BMCs
Cell viability (%)
100
80
60
40
20
0
*
B
Control-BMCs
Diabetic-BMCs
GLO1-BMCs
Relative mRNA expression
10
8
6
4
2
0
*
*
Bcl-2
Bcl-XL
C
Control-BMCs
Diabetic-BMCs
GLO1-BMCs
Migration toward VEGF (% of total cells)
30
25
20
15
10
5
0
*
D
Control-BMCs
Diabetic-BMCs
GLO1-BMCs
Migration toward SDF (% of total cells)
30
25
20
15
10
5
0
*
E
Control-BMCs
Diabetic-BMCs
GLO1-BMCs
Migration toward serum (% of total cells)
35
30
25
20
15
10
5
0
*
Serum at 3 day post-ligation
F
Control-BMCs
Diabetic-BMCs
GLO1-BMCs
Migration toward serum (% of total cells)
40
30
20
10
0
*
Serum at 7 day post-ligation

FIGURE 4.1. In vitro function and viability is improved in GLO1-BMCs. (A) Following 24 h of serum and growth factor starvation in hypoxic conditions, viability was lower in diabetic BMCs compared with control-BMCs and GLO1-BMCs (*P ≤ 0.024, n = 6). (B) Expression of pro-survival genes Bcl-2 and Bcl-XL was reduced in diabetic BMCs compared with GLO1-BMCs (*P ≤ 0.014, n = 5). (C) VEGF-stimulated migration (% of cells that migrated ≥9 mm) was reduced in diabetic BMCs compared with the other groups (P ≤ 0.002). (D) SDF-1-stimulated migration (% of cells that migrated ≥9 mm) was reduced in diabetic BMCs (*P ≤ 0.018 vs. other groups; n = 5). Migration towards blood serum, collected from diabetic mice (E) 3 days and (F) 7 days after hindlimb artery ligation, is greater for GLO1-BMCs and control-BMCs (*P ≤ 0.02).

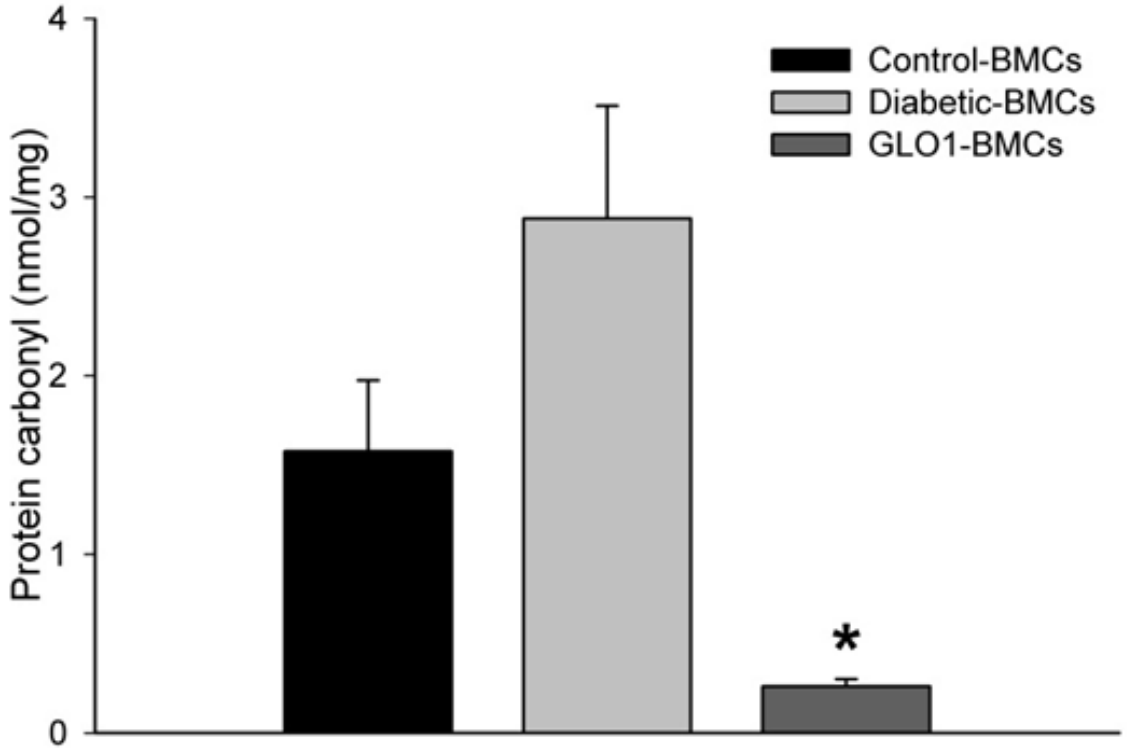


FIGURE 4.2. Levels of dicarbonyl proteins in bone marrow cells (*p≤0.049 vs. other 2 groups; n=3-5).

GLO1 overexpression maintains migratory potential of diabetic BMCs. Testing the ability of BMCs to undergo directional migration along a gradient of VEGF or SDF showed that hGlo1 overexpression increases the number of migrating cells. The percentage of cells responding (by migrating ≥9 mm) to either VEGF or SDF-1 chemo-attractant over an 18 h period was significantly lower for BMCs from WT-diabetic mice (0.3-fold, P≤0.018; FIG 4.1C and D) compared with control-BMCs, while the GLO1-BMC response was similar to that of the control-BMCs. Since pro-angiogenic/BM mobilization cytokine levels are reduced in the circulation of diabetic animals (Mieno et al., 2010), serum that was collected from diabetic mice 3 and 7 days after undergoing hindlimb ligation was used as a chemo-attractant to examine the BMC migratory response towards physiological signals generated by diabetic ischaemic tissue. Day 3 serum attracted approximately two times more GLO1-diabetic BMCs than WT-diabetic BMCs (P=0.02; FIG 4.1E), while Day 7 serum attracted 3.3-fold more BMCs from GLO1-diabetic mice than WT-diabetic mice (P= 0.017; FIG 4.1F).

Generation of hyperglycaemic BMTx mice. For the *in vivo* component of this study, three groups of animals were generated: (i) non-diabetic mice reconstituted with $eGFP^+$ BMCs (nondiabetics); (ii) STZ-treated mice reconstituted with $eGFP^+$ BMCs (WT-diabetics); and (iii) STZ-treated mice reconstituted with $eGFP^+hGlo1^{+/-}$ BMCs (GLO1-diabetics; FIG 4.3A). Hyperglycaemia was detected in 88% of STZ-treated mice, with an average of 20.8±1.1 mmol, compared with 4.8+0.3 mmol in non-diabetic mice (FIG 4.3B). At the end of the study, both groups of STZ-injected mice had significant weight loss compared with the non-diabetic control group (P≤0.01; FIG 4.3C). GLO1 enzymatic activity was increased 1.4-fold in BMCs from GLO1-diabetic mice compared with those from WT-diabetic mice (P=0.005;

FIG 4.3D).Western blot of BMCs extracted from GLO1-diabetic mice showed successful reconstitution of the BM with cmyc-tagged eGFP/hGlo1 BMCs (FIG 4.3E).

GLO1 overexpression in BMCs restores ischaemia-induced CAC mobilization in diabetes. Prior to transplantation, the BMCs from the two donors ($eGFP^+$ or $eGFP^+/hGlo1^+$) did not show any phenotypic differences (16.7±1.2% FLK^+, 2.0±0.3% $CXCR4^+$, 29.2±0.9% $c\text{-}kit^+$, and 5.8±1.0% $CD34^+$ cells). Following ligation, the mobilization of GFP+CACs ($CXCR4^+$, c-kit+, and $VEGFR2^+$) was reduced in WT-diabetic mice compared with the non-diabetic group; whereas GFP+CAC mobilization was maintained in GLO1-diabetic mice (FIG 4.4A). Specifically, during the early response post-ligation (Days 1 + 4), WT-diabetic mice had significantly less circulating $GFP^+VEGFR2^+$ cells than the other two groups ($P \leq 0.048$). Also, at later time points (Days 7 + 14), the number of circulating GFP^+CXCR4^+, $GFP^+VEGFR2^+$, and $GFP^+c\text{-}kit^+$ cells was not different between non-diabetic and GLO1-diabetic mice, but both were greater compared with the WT-diabetic group ($P \leq 0.05$). Analysis of the early serum post-ligation (Days 1 + 4) revealed that both diabetic groups of mice (GLO-diabetic and WT-diabetic) had reduced levels of mobilizing and pro-angiogenic cytokines: SDF-1 was approximately two-fold higher, and VEGF was approximately three-fold higher in the non-diabetic group vs. the two diabetic groups ($P \leq 0.05$; FIG 4.4B). At the later time points (Days 7 + 14), cytokine levels were not significantly different between the three groups (FIG 4.4B).

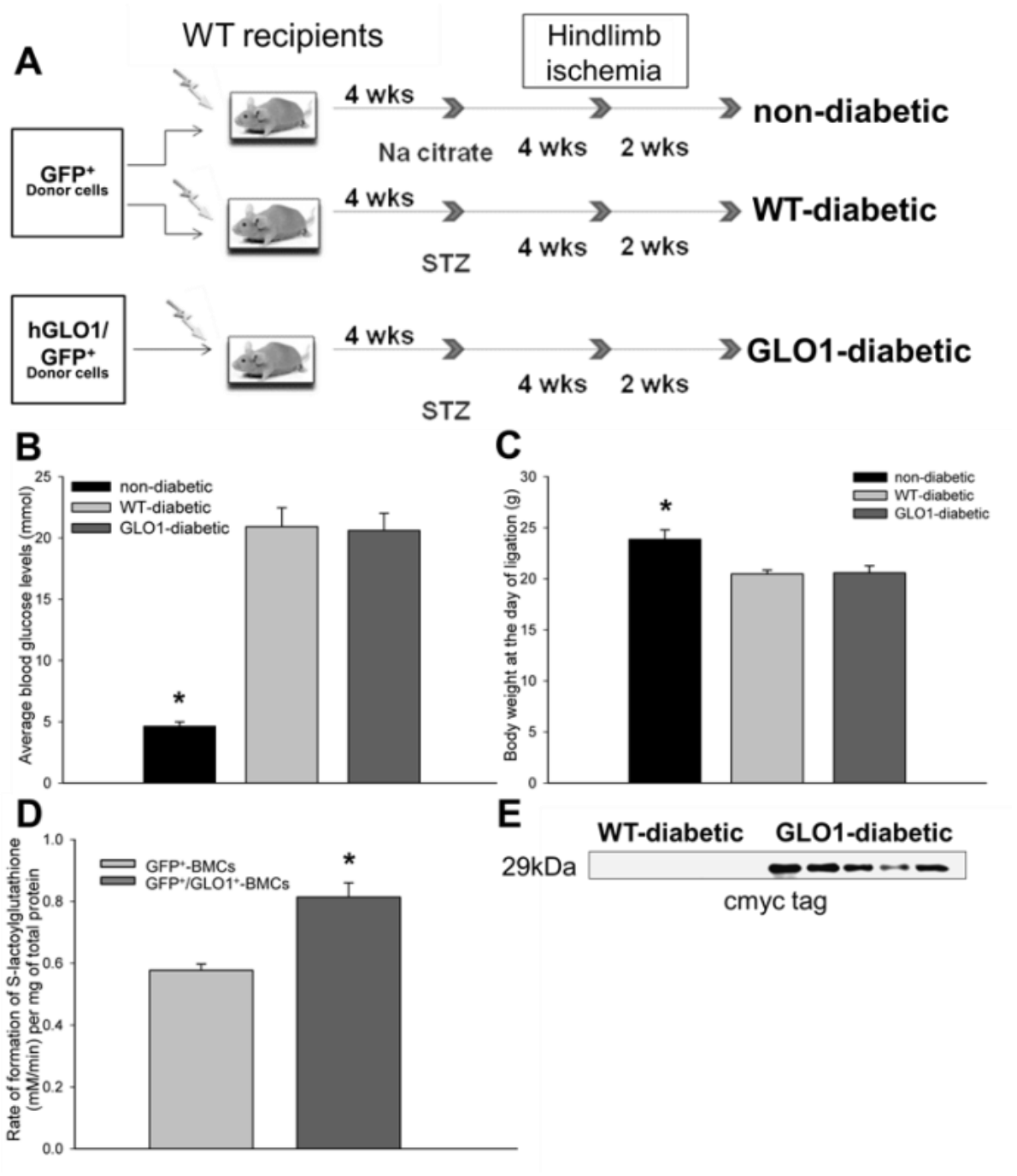

A
WT recipients
Hindlimb ischemia
4 wks
non-diabetic
Na citrate
4 wks
2 wks
GFP+ Donor cells
4 wks
WT-diabetic
4 wks
2 wks
STZ
hGLO1/ GFP+ Donor cells
4 wks
GLO1-diabetic
4 wks
2 wks
STZ
B
Average blood glucose levels (mmol)
non-diabetic
WT-diabetic
GLO1-diabetic
C
Body weight at the day of ligation (g)
non-diabetic
WT-diabetic
GLO1-diabetic
D
Rate of formation of S-lactoylglutathione (mM/min) per mg of total protein
GFP+-BMCs
GFP+/GLO1+-BMCs
E
WT-diabetic
GLO1-diabetic
29kDa
cmyc tag

FIGURE 4.3. hGlo1 overexpressing BM transplant model. (A) After irradiation, donor BMCs was injected via the tail vein. Three donor BM/recipient mouse models were studied: (i) eGFP BM/non-diabetic mice (non-diabetic); (ii) eGFP BM/diabetic mice (WT-diabetic); and (iii) eGFP/hGlo1 BM/diabetic mice (GLO1-diabetic). After 4 weeks, hyperglycaemia (Type 1 diabetes model) was induced by STZ treatment. The non-diabetic group was injected with sodium citrate. Four weeks later, hindlimb ischaemia was induced by femoral artery ligation. (B) Average blood glucose levels of fasting animals ($P \leq 0.001$ vs. other groups). (C) Average body weight (g) on the day of ligation ($P \leq 0.01$ vs. other groups). (D) Glyoxalase activity of BMCs from eGFP/hGlo1 and eGFP donor mice (*$P = 0.005$). (E) Western blot of BMC protein extracts from diabetic mice reconstituted with BMCs from eGFP or eGFP/hGlo1 donors, labelled for cmyc tag protein (used to identify hGlo1 donor cells), demonstrating successful reconstitution of the BM with hGlo1 BMCs in GLO1-diabetic mice.

Neovascularization and perfusion is restored in GLO1-diabetic mice. Vascular density was assessed by α-SMA staining for arterioles and CD31 staining for capillaries in hindlimb tissue 2 weeks post-ischaemia (FIG 4.5A–H). The number of arterioles was greater in GLO1-diabetics (1.7±0.3 arterioles/0.5 mm^2) compared with WT-diabetic mice (0.5±0.1; $P \leq 0.01$), but not different from non-diabetics (1.3±0.3; $P \leq 0.4$; FIG 4.5G). Also, the number of CD31+ cells was greater in GLO1-diabetics (13.4±2/0.15 mm^2) compared with WT-diabetic mice (8.75+0.7; P= 0.038), but not different from non-diabetics (14.7±0.5; $P \leq 0.45$; FIG 4.5H). Femoral artery ligation reduced perfusion in all groups (ischaemic/non-ischaemic hindlimb ratio of 0.5±0.1; p= 0.001 compared with pre-op baseline; FIG 4.5I and J). In WT-diabetic animals, perfusion of the hindlimb distal to the ligation site showed no signs of recovery either at 7 or 14 days. At 7 days, blood flow in GLO1-diabetic mice (1.0±0.2) was greater than in WT-diabetic mice (0.5±0.1; $P = 0.015$). After 2 weeks, more blood flow was observed for GLO1-diabetic (1.1±0.1) and non-diabetic (1.1±0.2) mice compared with WT-diabetic mice (0.4±0.01; $P \leq 0.012$).

Gene expression of angiogenic factors in the ischaemic tissue. At Day 3 post-ligation, the transiently increased mRNA expression of Hif1-α and Vegf-A in non-diabetic mice was greater compared with both diabetic groups ($P \leq 0.046$), while eNos mRNA was not significantly different between the groups (FIG 4.6A). The level of *eNos* mRNA from the hindlimbs was significantly increased in WT-diabetic mice compared with non-diabetics at 2 weeks post-ischaemia ($P \leq 0.04$; FIG 4.6B). A trend for increased expression of *Vegf-a* mRNA was observed in the WT-diabetic group (vs. non-diabetic mice), while the expression

of *Hif1-α* mRNA was higher in WT-diabetic mice than in the other two groups ($P \leq 0.05$; FIG 4.6B).

Cytokine and eNOS analysis in ischaemic tissue The protein level of tumour necrosis factor-α (TNF-α), an inflammatory cytokine, was elevated in the hindlimb of WT-diabetic mice at 2 weeks post-ligation compared with the other two groups ($P \leq 0.0014$; FIG 4.6C). Cytokine analysis of the tissue also revealed that VEGF content was significantly lower in WT-diabetic hindlimb muscle than in non-diabetic mice ($P \leq 0.05$; FIG 4.6C), while SDF-1 was reduced in WT-diabetic andGLO1-diabetic mice compared with non-diabetic controls ($P \leq 0.02$; FIG 4.6C). Although the level of *eNos* mRNA was significantly increased in WT-diabetics at 2 weeks post-ligation, the protein detected was mainly in the monomeric form. The ratio of metabolically active eNOS dimer to non-functional eNOS monomer in WT-diabetic and GLO1-diabetic mice was significantly lower than in non-diabetic mice ($P \leq 0.05$; FIG 4.6D and E).

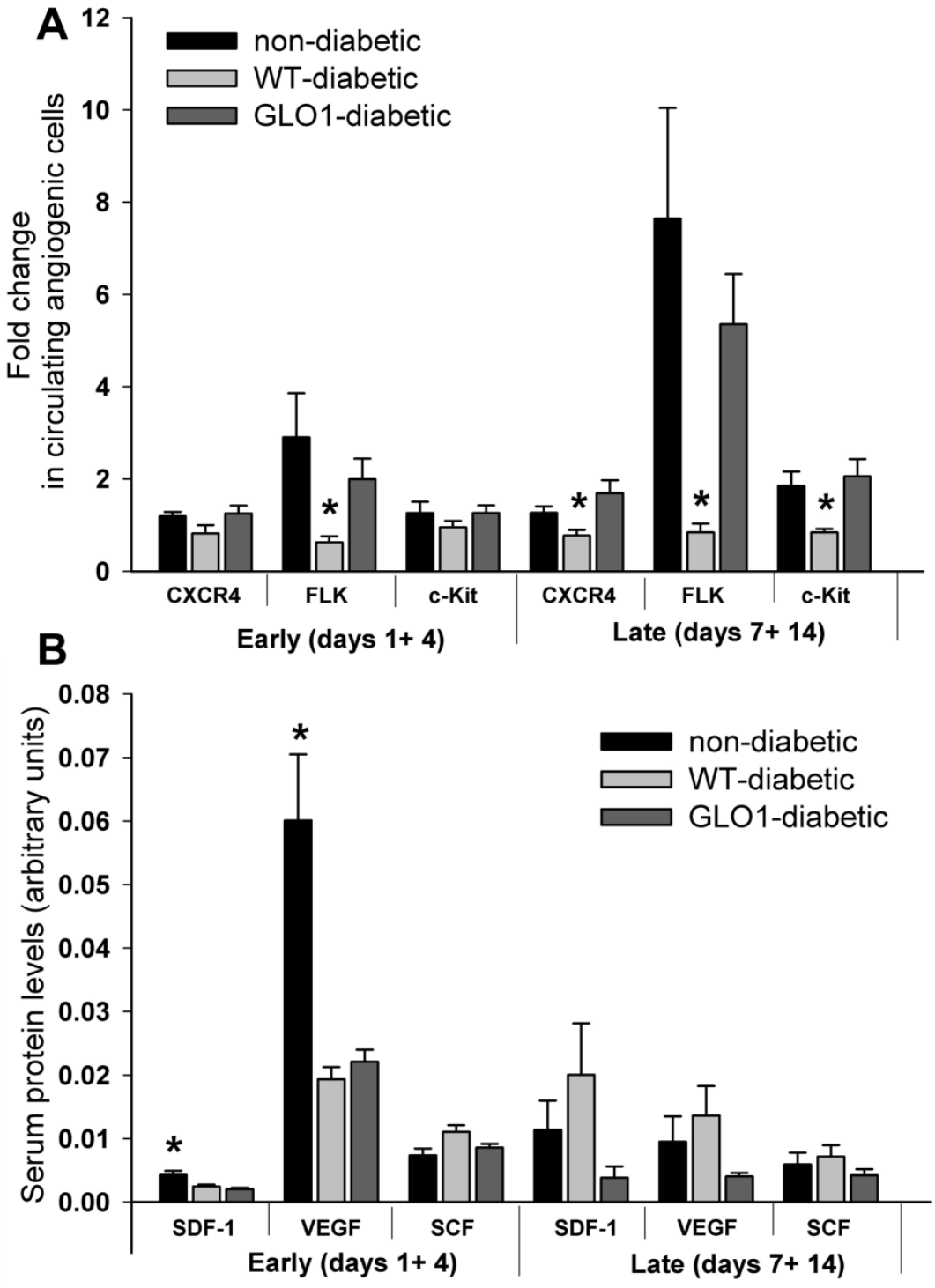

A
Fold change in circulating angiogenic cells
non-diabetic
WT-diabetic
GLO1-diabetic
CXCR4
FLK
c-Kit
Early (days 1+ 4)
Late (days 7+ 14)
B
Serum protein levels (arbitrary units)
SDF-1
VEGF
SCF

FIGURE 4.4. Overexpression of hGlo1 in BMCs enhances ischaemia-induced BM-CAC mobilization despite poor tissue signaling. (A) The number of circulating GFP^+CXCR4^+, GFP^+Flk^+, and $GFP^+c\text{-}kit^+$ cells, measured early (Days 1 + 4) and late (Days 7 + 14) after hindlimb ischaemia, is expressed as a fold-change relative to its pre-operative baseline (*$P \leq 0.048$ *vs*. other groups; n=6–8). (B) Analysis of mobilizing and pro-angiogenic cytokines in the serum at early (Days 1 + 4) and late (Days 7 + 14) time points post-ligation ($P \leq 0.05$ *vs.* other groups; n =3).

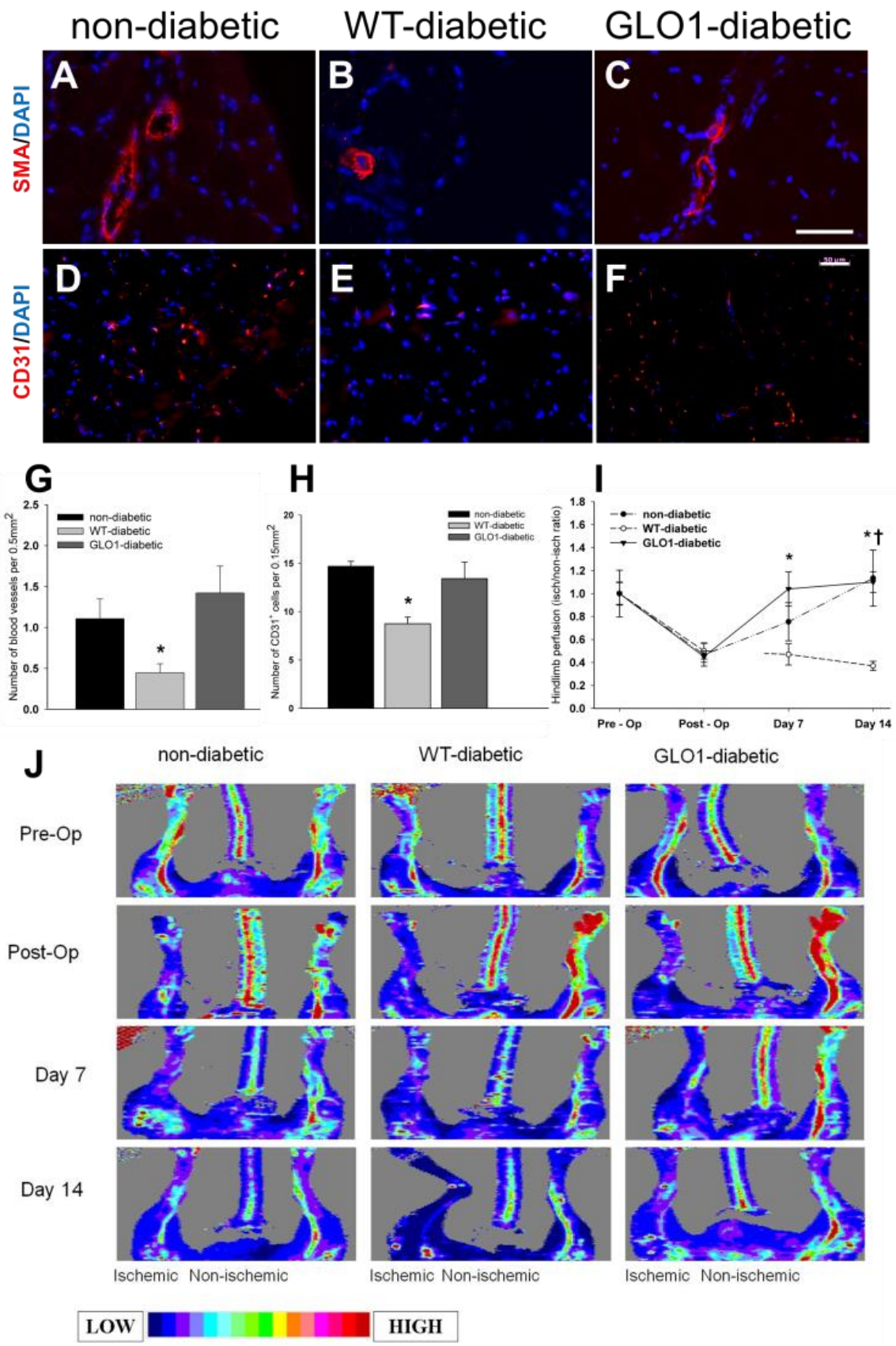

non-diabetic
WT-diabetic
GLO1-diabetic
SMA/DAPI
CD31/DAPI
A
B
C
D
E
F
G
H
I
J
Number of blood vessels per 0.5mm2
Number of CD31+ cells per 0.15mm2
Hindlimb perfusion (isch/non-isch ratio)
Pre - Op
Post - Op
Day 7
Day 14
Pre-Op
Post-Op
Day 7
Day 14
Ischemic
Non-ischemic
LOW
HIGH

FIGURE 4.5. Recovery of neovascularization and blood flow in GLO1-diabetic mice. (A–H) Representative images of a-SMA staining (A–C) for arterioles (red), and of CD31 staining (D–F) for capillaries (red); nuclei stained with DAPI (blue); scale bar = 50 mm. (G) The average number of blood vessels per 0.5 mm2 (*P ≤ 0.02 for WT-diabetic *vs.* other groups; n=5-6). (H) The average number of CD31+ cells per 0.15 mm^2 (*P ≤ 0.038 for WT-diabetic vs. other groups; n=3). (I) Perfusion was measured by laser Doppler analysis, and data are presented as the average ratio of ischaemic to non-ischaemic limb blood flow (*P ≤ 0.0015 for GLO1-diabetic vs. WT-diabetic; †P = 0.012 for non-diabetic vs. WT-diabetic). (J) Representative images of perfusion over a period of 2 weeks post-ligation (red= highest perfusion; blue= lowest).

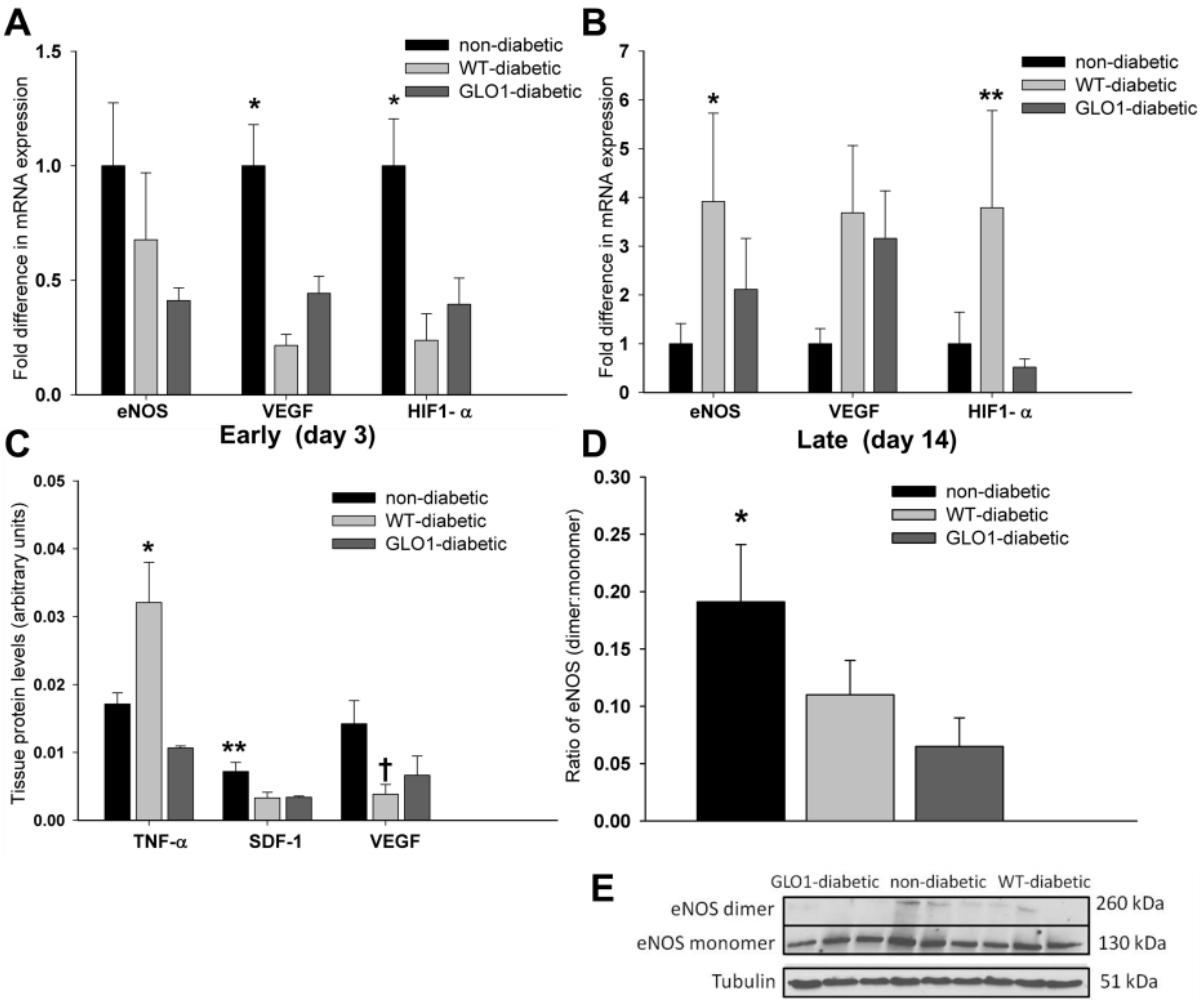

A
non-diabetic
WT-diabetic
GLO1-diabetic
Fold difference in mRNA expression
1.5
1.0
0.5
0.0
*
*
eNOS
VEGF
HIF1- α
Early (day 3)
B
non-diabetic
WT-diabetic
GLO1-diabetic
Fold difference in mRNA expression
7
6
5
4
3
2
1
0
*
**
eNOS
VEGF
HIF1- α
Late (day 14)
C
non-diabetic
WT-diabetic
GLO1-diabetic
Tissue protein levels (arbitrary units)
0.05
0.04
0.03
0.02
0.01
0.00
*
**
†
TNF-α
SDF-1
VEGF
D
non-diabetic
WT-diabetic
GLO1-diabetic
Ratio of eNOS (dimer:monomer)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
*
E
GLO1-diabetic
non-diabetic
WT-diabetic
eNOS dimer
eNOS monomer
Tubulin
260 kDa
130 kDa
51 kDa

FIGURE 4.6. Gene and protein expression of angiogenic factors in hindlimb tissue. mRNA levels of *eNos, Vegf,* and *Hif-1α* in hindlimb tissue (A) 3 days and (B) 2 weeks after ischaemia was induced, expressed as a fold-difference relative to the non-diabetic group at its respective time point (in A: *P ≤ 0.046 vs. other two groups; n =3–5; in B: *P = 0.04 vs. non-diabetic; **P ≤ 0.05 vs. other two groups; n= 3–5). (C) Relative protein levels of pro-inflammatory (TNF–α) and angiogenic (SDF-1 and VEGF) cytokines in hindlimb tissue 2 weeks after ischaemia (*P≤0.0014 vs. other two groups; **P ≤ 0.02 vs. other two groups; †P ≤ 0.05 vs. non-diabetic; n= 4–6). (D) Ratio of eNOS (dimer/monomer) in hindlimb tissue, 2 weeks after ischaemia, was induced (*P ≤ 0.05 vs. other two groups; n=4–6). (E) Representative Western blots for the analysis of eNOS monomer and dimer in hindlimb tissue lysates, 2 weeks after ischaemia was induced.

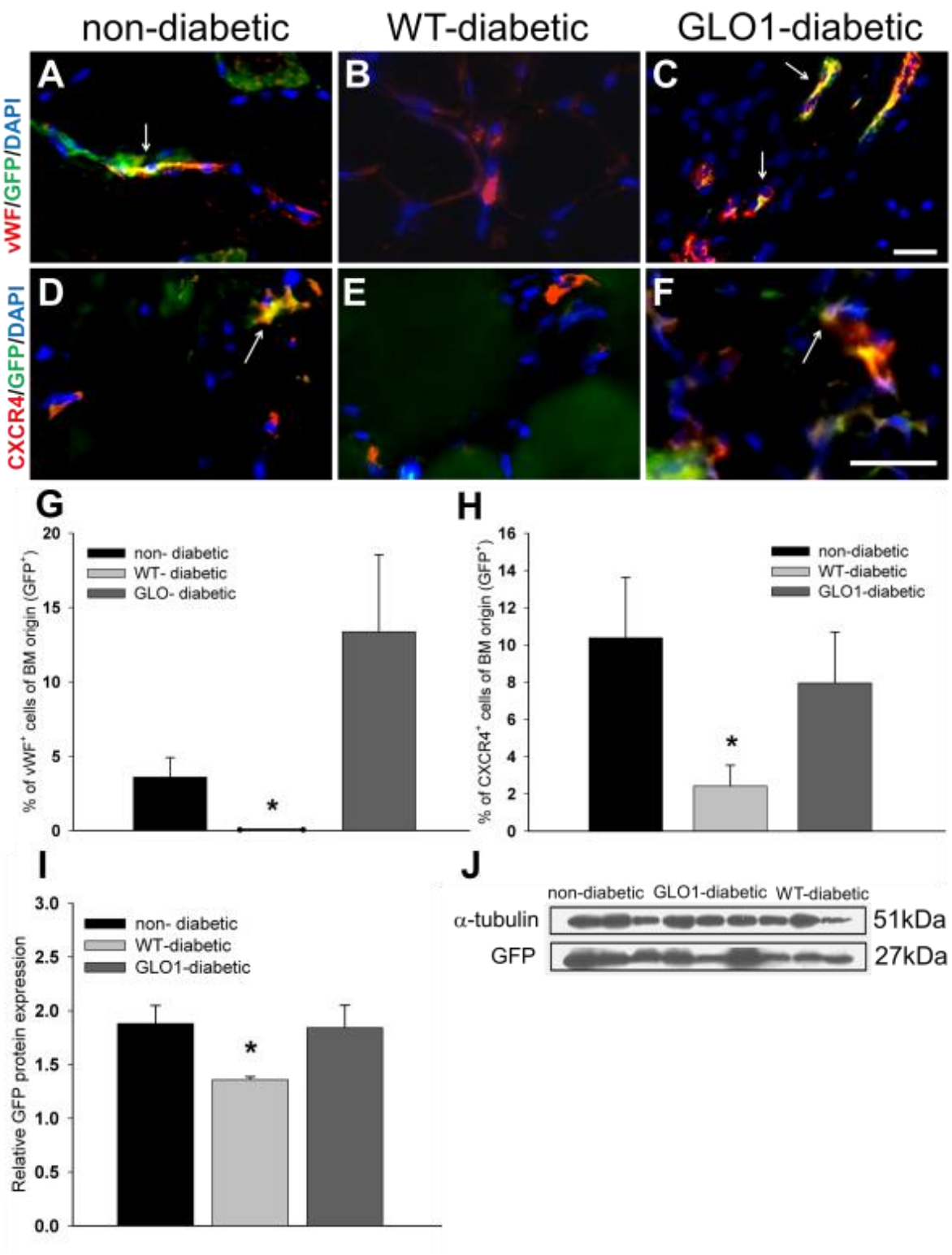

non-diabetic
WT-diabetic
GLO1-diabetic
A
B
C
D
E
F
vWF/GFP/DAPI
CXCR4/GFP/DAPI
G
% of vWF+ cells of BM origin (GFP+)
non- diabetic
WT- diabetic
GLO- diabetic
H
% of CXCR4+ cells of BM origin (GFP+)
non-diabetic
WT-diabetic
GLO1-diabetic
I
Relative GFP protein expression
non- diabetic
WT-diabetic
GLO1-diabetic
J
non-diabetic GLO1-diabetic WT-diabetic
α-tubulin
GFP
51kDa
27kDa

FIGURE 4.7. Increased recruitment of angiogenic BMCs in GLO1-diabetic hindlimbs. (A–C) Representative images of ischaemic hindlimb tissue sections stained for vWF (endothelial cells; red), GFP (marker for recruited BMCs; green), and DAPI (nuclei; blue); scale bar = 20 mm. (D–F) Representative images of ischaemic hindlimb tissue sections stained for CXCR4 (angiogenic cell marker; red), GFP (marker for recruited BMCs; green), and DAPI (nuclei; blue); scale bar = 20 mm. Arrows indicate examples of GFP^+vWF^+ or GFP^+CXCR4^+ cells. (G) Percentage of vWF^+ cells in hindlimb tissue that are of BM origin (GFP^+ BMCs) (per 0.5 mm2; *P $\leq$ 0.034 vs. other groups; n=5–7). (H) Percentage of $CXCR4^+$ cells in hindlimb tissue that are of BM origin (GFP^+ BMCs) (per 0.5 mm^2; *P $\leq$ 0.049 vs. other groups; n =5–7). (I) Quantification of GFP protein expression (relative to a-tubulin) in the ischaemic hindlimb tissue 2 weeks after ligation (*P $\leq$ 0.03 vs. other groups, n=3). (J) Representative Western blot for the analysis of GFP expression in hindlimb tissue lysates.

Increased homing and engraftment of GLO1 overexpressing BMCs in ischaemic hindlimbs. The presence of GFP^+ cells in tissue sections confirmed the recruitment of BMCs to the ischaemic muscle (FIG 4.7A–F). There were no detectable GFP^+vWF^+ cells in the hindlimbs of WT-diabetic mice, but these were found in non-diabetic mice (3.6±1.3 GFP^+vWF^+ cells/FOV) and GLO1-diabetic mice (13.3±5.2 GFP^+vWF^+ cells/FOV. FIG 4.7G). The percentage of GFP^+CXCR4^+ cells in ischaemic tissue was higher in GLO1-diabetic (8.0±2.7% cells/FOV) and non-diabetic (10.4+3.3%) mice than in WT-diabetic mice (2.4±1.1%; $P \leq 0.049$; FIG 4.7H); and no difference was observed between non-diabetic and GLO1-diabetic mice ($P \leq 0.6$). Little evidence of recruited GFP^+ cells incorporating into arterioles was observed for any of the groups. The recruitment of GFP^+ BMCs to hindlimb tissue was confirmed by the Western blot. GFP protein was increased in the hindlimb tissue of GLO1-diabetic (1.8±0.1) and non-diabetic mice (1.9±0.2) compared with WT-diabetic mice (1.4±0.0; $P \leq 0.03$; FIG 4.7I and J).

DISCUSION

An impaired angiogenic response to ischaemia contributes to the poor clinical outcomes observed in diabetic patients with coronary or peripheral artery disease (Issan et al., 2012). The present study demonstrates that overexpressing Glo1 in BMCs could reverse the diabetes-induced impairment of BM-derived CACs in vitro and in vivo. To our knowledge, this is the first study to show that increased GLO1 activity, restricted to bone marrow and bone marrow-derived cells, can preserve CAC mobilization and recruitment, and restore neovascularization to ischaemic hindlimb in a model of Type 1 diabetes, despite the diabetic/glycating environment.

BMCs extracted from GLO1-diabetic mice migrated towards SDF-1 and VEGF as effectively as BMCs from non-diabetic mice. More importantly, GLO1-diabetic BMCs migrated more than WT-diabetic BMCs towards stimuli present in serum taken from diabetic mice with hindlimb ischaemia. This suggests that BMCs overexpressing hGlo1 are more likely to respond to mobilization and homing signals generated by ischaemic tissue in the diabetic setting, which we did, in fact, observe in vivo. Early after ischaemia (1–4 days), serum levels of VEGF and SDF-1 pro-angiogenic cytokines in GLO1-diabetic and WT-diabetic mice were reduced compared with non-diabetics. Despite this, the mobilization of BM-CACs inGLO1-diabetic mice was equal to non-diabetic mice, whereas the numbers were reduced in the WT-diabetic group.

The viability of BMCs from GLO1-diabetic mice was greater than those from WT-diabetic mice when they were exposed to apoptosis-inducing conditions. Notably, GLO1-BMCs viability was equivalent to that of BMCs from non-diabetic mice. This may be explained by the higher level of Bcl-2 and Bcl-XL mRNA in GLO1-BMCs compared with WT-diabetic BMCs. Bcl-2 and Bcl-XL are anti-apoptotic factors (Youle and Strasser, 2008), and MG has been shown to reduce Bcl-2 expression in retinal endothelial cells (Bento et al., 2010). Unlike BMCs from GLO1-diabetic mice, Bcl-2 and Bcl-XL expression were not increased in non-diabetic BMCs, possibly explained by the lack of chronic stress in non-diabetic mice (no STZ-induced diabetes in vivo and no hyperglycaemia during hypoxia in vitro). Therefore, preserved up-regulation of this pro-survival pathway is likely involved in conferring resistance to GLO1-BMCs from cell death under hyperglycaemic/hypoxic conditions, and it may also contribute to the increased BM-CAC engraftment observed in ischaemic hindlimbs of GLO1- vs. WT-diabetic mice.

It is noticed that the positive effects of BM reconstitution with hGlo1 overexpressing BMCs were obtained with a moderate 1.4-fold increase inGLO1activity, measured in vitro. A consideration is that STZ administration may irreversibly damage some BMC subpopulations through DNA alkylation (Nichols et al., 1981, Weiss, 1982). While we cannot formally exclude that increased GLO1activity through unknown mechanisms may lessen STZ-induced BMC cytotoxicity, it is more probable that the beneficial effects of hGlo1 over-expression in BMCs result from reduced hyperglycaemia-induced dicarbonyl stress. The reduction in oxidative stress (protein carbonyl levels) measured in GLO1-diabetic BMCs compared with WT-diabetic BMCs supports this.

High glucose in diabetes impairs HIF-1α expression leading to reduced expression of angiogenic factors (Botusan et al., 2008, Thangarajah et al., 2010). This was demonstrated early after induction of ischaemia (Day 3), where hindlimb expression of Hif-1α mRNA increased in non-diabetic mice, compared with the two diabetic groups. The low level of Hif-1α in the tissues of GLO1- diabetic and non-diabetic mice after 2 weeks likely reflects the restoration of blood flow and the absence of hypoxia. In contrast, ischaemia persisted in the WT-diabetic mice at Day 14, and Hif-1α mRNA was elevated compared with the other two groups. Despite the increased Hif-1α mRNA in WT-diabetic mice, this did not lead to elevated protein levels of HIF-1α-regulated cytokines SDF-1 and VEGF. At 2 weeks, pro-inflammatory TNF-α expression was higher in the WT-diabetic group, suggesting the persistence of local inflammation, which was not observed in GLO1-diabetic mice. The level of eNos mRNA in WT-diabetic mice was also increased compared with the other two groups, probably driven by the lack of oxygen. The paradoxical increase of eNos expression during hypoxia in diabetic mice has previously been reported (Ding and Triggle, 2005),

arguing that chronic elevated expression of eNos mRNA does not result in production of functional eNOS dimer protein, which was also observed in this study.

In conclusion, our findings show that overexpression of GLO1 uniquely in the BM can protect BM-CACs' viability and function, and is sufficient to overcome the defective neovascularization that is characteristic of diabetes. This may prove to be a stepping stone for developing strategies aimed at improving the efficacy of revascularization therapies in diabetic patients

CHAPTER 5 – DISCUSSION

5.0 GENERAL DISCUSSION

Most of the morbidity and mortality observed in diabetic patients can be attributed to vascular complications. The primary cause of this is still considered to be the prolonged exposure to hyperglycemia and its harmful effects on EC function (Brownlee, 2001b). The purpose of this thesis was to examine the relationship between MG accumulation in ECs and BMCs and the development and progression of diabetic cardiovascular complications – namely heart failure and loss of neovascularization in DM.

The highly reactive dicarbonyl compound MG is believed to be the major source of intracellular AGEs, its presence increases ROS formation and it is possibly one of the biggest contributors to cell inflammation, loss of function and subsequent cell death (Rabbani and Thornalley, 2012, Allaman et al., 2015, Bourajjaj et al., 2003, Vasdev and Stuckless, 2011, Adriana Adameova, 2014). By making use of a mouse model that overexpresses GLO1 under the control of pro-endothelin, we were able to investigate the contribution of MG to the development of *in vivo* diabetic complications like diabetic heart failure (Chapter 2) and altered angiogenesis (Chapter 4). In Chapter 3, using human ECs, we confirmed that MG augments TNF-α apoptotic signaling in part through increased oxidative stress. The studies presented here provide new insights into the effects of MG on the function of ECs and BMCs, but also highlight the importance of ECs and BMCs for overall function of the heart and blood vessels in diabetes.

5 .1 USE OF THE PRO-ENDOTHELIN GUIDED hGLO1 MOUSE MODEL

In order to study the effect of the glyoxalase system on cardiovascular pathology it was necessary to use two mouse models: the STZ diabetic mouse and the GLO-1 overexpressing mouse.

The rat / mouse model of DM induced by STZ is one of the most widely used models of type I diabetes and mimics the human pathological situation of decreased insulin production (untreated type I diabetes) or decreased tissue responsiveness to insulin (if combined with high fat diet) (Bugger and Abel, 2009). It is especially convenient for use with our transgenic mouse model, as it does not require breeding manipulation. STZ injections are also time-controlled, and can be used on mice of different ages. Most importantly for the studies in this thesis, the STZ model has prominent vascular ED (Ding et al., 2005, Nacci et al., 2009). STZ accumulates in the pancreatic β-cells via the GLUT2 glucose transporter and destroys them, causing insulin-dependent like diabetes (Lenzen, 2008). STZ treatment results in hyperglycemia which is the main source of MG – an element most important for the studies described herein.

The GLO-1 overexpressing rodent model was previously used to investigate the contribution of the GLO-1 substrate - MG and, indirectly, MG-derived AGEs, in the development of *in vivo* diabetic complications like nephropathy, cardiomyopathy and atherosclerosis (Brouwers et al., 2013, Brouwers et al., 2011, Brouwers et al., 2010). The GLO1 transgenic mouse model used in this thesis had the vasculature protected from MG by GLO1 overexpression under the control of pro-endothelin. Endothelin is a peptide hormone, originally identified in 1988 as an endothelium-derived factor that produces prolonged vasoconstriction and increases arterial blood pressure (Yanagisawa et al., 1988). To establish

that the GLO1 transgenic mice have hGLO1 expression limited to vasculature and hematopoietic cell lines, we confirmed that both the localization of *hGlo1* and the associated increase in GLO1 activity were present in the ECs and BMCs. This allowed us to distinguish the role of endothelium in the development of diabetic heart failure from the overall effect of MG on the whole heart. The study on function of BMCs alone on revascularization in diabetes was even more stringent, using bone marrow transplantation to completely isolate GLO1 overexpressing cells from the rest of the body.

Overall, our mouse models of GLO1 overexpressing DM proved to be a useful tool for studying the pathophysiology of the role of MG in development of cardiovascular complications of DM. Further studies may include a GLO1 knock down (GLO1 KD) mouse (see appendix 1), or floxed STOP *hGLO1* knock-in mice which permit the generation of cell-type specific hGLO1 over-expression after crossing with the cardiomyocyte-promoter driven Cre mice (available from Dr Brownlee's laboratory). Using these mice, the effects of MG on the survival, function and signaling of cardiomyocytes alone in the myocardium can be examined.

5.2 Role of small blood vessels in CAD

Microangiopathy is a direct result of chronic hyperglycaemia and the main cause of major clinical microvascular complications, such as nephropathy, cardiomyopathy, retinopathy and neuropathy (Fowler, 2008, Vithian and Hurel, 2010, Adriana Adameova, 2014). Dysfunction of the endothelium is an important factor in both the initiation and progression of vascular complications. Healthy blood vessels not only provide a good supply of oxygen and nutrients, but also play a role in homoeostasis and support cardiomyocyte functions (Hsieh et al., 2006, Favero et al., 2014, Félétou, 2011, van Hinsbergh, 2012).

Two principal types of ECs are present in the heart: the endocardial and the vascular ECs. Both types can affect cardiac performance because of their close proximity to adjacent cardiomyocytes (Verma and Anderson, 2002, Tirziu et al., 2010). ECs exposed to HG are in danger of functional loss and also, through passive diffusion, are a source of MG or glycation products for surrounding cells (Sena et al., 2012, Thornalley, 2008a). Under normal physiological conditions, ECs are well protected against glycation; however the defense mechanisms of ECs can be impaired by MG as a result of ROS, AGEs or direct reactions of MG with regulating proteins or DNA (Rabbani and Thornalley, 2012, Rabbani and Thornalley, 2015, Tikellis et al., 2014, Tu et al., 2013). The changes that follow can promote inflammation and cell death. EC apoptosis contributes to cardiac pathophysiology, as repeated episodes of myocardial microvascular ischemia and small infarcts ultimately contribute to heart failure (Kehat and Molkentin, 2010).

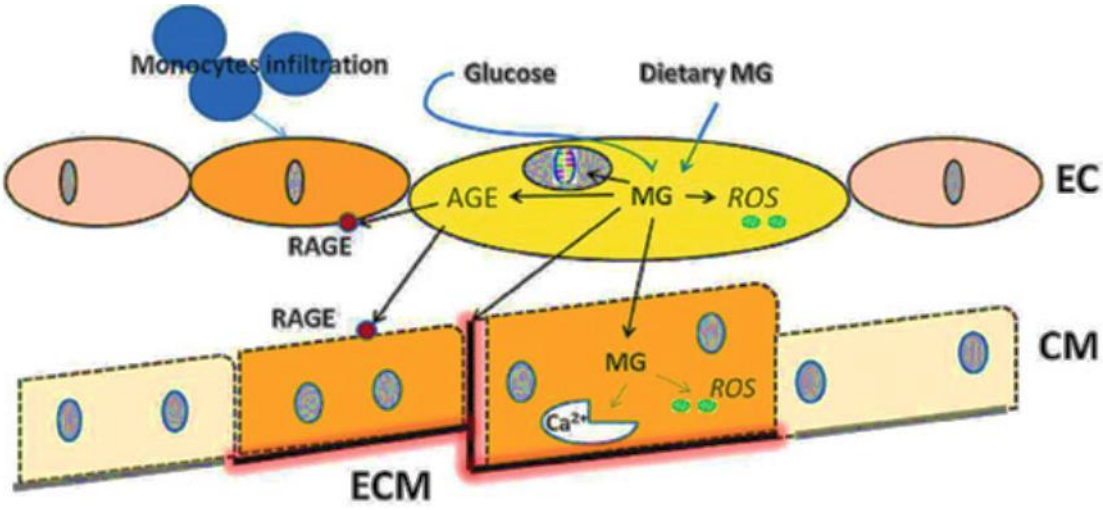
5.2.1 In vivo detection of ECs dysfunction and inflammation

Increased levels of the soluble adhesion molecules VCAM, ICAM and E-selectin in blood serum are good indicators of endothelial dysfunction *in vivo* (Hwang et al., 1997, Blankenberg et al., 2001). VCAM, ICAM and E-selectin regulate leukocyte adhesion and migration across the vascular endothelium, and their presence in the circulation is a reliable sign of vascular inflammation and EC death (Okouchi et al., 2002, Chen et al., 2011). We have shown that GLO1 overexpression could protect ECs in diabetic mice as the formation and release of soluble VCAM, ICAM and E-selectin in blood serum was reduced. The relation between ICAM, VCAM and MG was also confirmed by examining the serum of GLO KD mice (see Appendix 1), and in other studies (Yao et al., 2007, Brouwers et al., 2014).

5.2.2 ECs can affect inflammation in the heart

In chapter 2 we showed that MG caused EC dysfunction and this contributed to heart failure development in the STZ-induced diabetes model. MG-induced inflammation (increased RAGE and TNF-α expression) and cell death, seen through reduced capillary numbers, were associated with decreased paracrine support of ECs to cardiomyocytes. Vascular GLO1 overexpression in hyperglycemic mice prevented the increase of soluble adhesion molecules in blood serum, preserved cardiac EC viability and signaling (eNOS and neuregulin), reduced cell death and delayed and limited the loss of cardiac function.

Damage to the small blood vessels, in which oxidative stress and a proinflammatory state play an important part, may further deteriorate the function and structure of cardiomyocytes (FIG 5.1). In diabetic patients, signs of activation of the inflammatory response in the heart's endothelium is often followed by dysfunction of ECs and cardiomyocytes and, eventually, early stage cardiac failure (Westermann et al., 2007, Wang et al., 2006, Bando and Murohara, 2014). MG-formed AGEs can activate RAGE and with it other inflammatory responses in cells (Yan et al., 2010, Ramasamy et al., 2011). Moreover, MG induces the recruitment of monocytes in the vasculature, again causing an inflammatory condition (Su et al., 2012). As seen in Chapter 2, although hGLO1 over-expressing mice had increased carbonyl stress, the inflammatory cytokine production (TNF-α) was reduced compared to the WT-diabetic mice, which also likely contributed to postponed signs of heart failure.



FIGS 5.1 MG-formed AGEs activate RAGEs and the inflammatory response in cells. MG-AGEs of mitochondrial proteins lead to the formation of ROS. MG also affects the DNA in ECs. MG increases oxidative stress in cardiomyocytes (CM), modifies Ca^{2+} handling and augments fibrosis of the extracellular matrix (ECM). Furthermore, MG induces the recruitment of monocytes in the microvasculature, causing an inflammatory condition related to diabetic vascular complications. (Vulesevic et al., 2014b)

5.2.3 EC interaction with cardiomyocytes has a role in heart failure

ECs in the heart can directly regulate cardiomyocyte apoptosis induced by ROS, through neuregulin-1β/ErbB4 signaling (Gui et al., 2012). The direct effect of MG on neuregulin has been further examined (see Appendix 1), but no conclusive results were seen. The most probable cause for deficiency of neuregulin in diabetic mice is the reduced number of ECs that produce it. This is then followed by loss of cardiomyocytes, and progressive decline in left ventricular function and finally heart failure. The results described in Chapter 2 highlighted the importance of the endothelium and capillaries in protecting the heart from the effects of MG and preservation of heart function.

The data shown contribute to the premise that oxidative stress effects on the vasculature may regulate inflammation in the diabetic heart (Case et al., 2008, Jay et al., 2006, Pitocco et al., 2013, Basta et al., 2004). Protection of the heart's vasculature in diabetes is crucial: healthy blood vessels not only provide a good supply of oxygen and nutrients, but also play a role in homoeostasis and support cardiomyocytes.

5.3 EFFECT OF MG ON ECs

To understand the involvement of MG and MG-derived AGEs in hyperglycaemia induced vascular damage, several studies have investigated cellular pathways in cultured endothelial or other vascular cells directly stimulated by MG (Nigro et al., 2014, Li et al., 2013, Cai et al., 2010, Chan and Wu, 2008). The overall results of these studies reveal that MG has cytotoxic properties, which are exerted due to oxidative stress, DNA damage and apoptosis, which are discussed in the following sections.

5.3.1 MG in vitro studies

The *in vivo* concentration of MG is under debate, and the relevance of the dose in the *in vitro* cell studies therefore remains unclear. In general, MG levels increase two- to four-fold in diabetic patients compared with controls (Lu et al., 2011, Lapolla et al., 2003, Ogawa et al., 2010). It should also be emphasized that extracellular and intracellular levels of MG could be rather different, because of intracellular formation of MG increased by oxidative stress. A study by Randell et al. showed that tissue MG levels in rats were an order of magnitude higher compared with their plasma levels (Randell et al., 2005). Lack of consensus on the physiological concentration range of MG is likely a consequence of different methods used for measuring MG (i.e. different pre-analytical processing of the sample, direct HPLC method or measurements of MG-H1 adduct) (Rabbani and Thornalley, 2014).

In our *in vitro* experiments, to confirm that the MG added to our cell cultures was absorbed by the ECs, we used an MBo probe (methyl diaminobenzene-BODIPY, kindly provided by Dr Spiegel, Department of Chemistry, Yale University, USA), which was developed to label and detect free MG in live cells (see Appendix 2). Also, all the experiments were repeated with 30mmol glucose (similar concentration of glucose that we recorded in blood in our *in vivo* studies), and they gave comparable results. The advantage of adding MG only to the cell media is in the reduction of the other effects of HG (increased ROS production, induction of PKC, sorbitol and hexosamine pathways), allowing us to better isolate the MG effects.

5.3.2 MG-induced apoptosis

The mechanism of MG-induced apoptosis is cell type-dependent, MG induces apoptosis in Jurkat cells (immortalized line of human T lymphocyte cells) through JNK (c-Jun N-terminal kinase) with cells showing typical apoptotic morphology (e.g. DNA fragmentation) (Du et al., 2000). Administration of MG to bovine retinal pericytes induces apoptosis through the formation of ROS (Kim et al., 2004). In HUVECs, MG triggers several pathways (Akhand et al., 2001). A first pathway involves protein-tyrosine kinase (PTK) activation for tyrosine phosphorylation of several cellular proteins, leading to the activation of ERK and a pro-survival response. A second MG-induced pathway in these cells involves ROS and the activation of JNK, p38 kinase and c-Jun leading to apoptosis (Akhand et al., 2001). Interestingly, glyoxal, which differs from MG only by a methyl group, is only capable of inducing the first signal, but unable to induce the second. This suggests the specificity of MG and its possible role as a signaling molecule. It is notable that different studies also used very different doses of MG, which may explain variations in results and conclusions.

In Chapter 3 we demonstrated that the incubation of ECs with 5µM MG alone resulted in increased oxidative stress (measured by carbonyl stress). By an Extracellular Flux Analyzer (Seahorse Bioscience), it was determined that mitochondrial respiration was reduced in cells treated with HG or MG. These results are consistent with a study by Miyazawa et al. showing that MG incubation resulted predominantly in mitochondrial-derived superoxide production which further reacted with peroxynitrite and hydrogen-peroxide (Miyazawa et al., 2010). Therefore, it appears that the loss of mitochondrial function is associated with increased ROS leading to apoptosis.

5.3.3 Defense system against MG-induced cell death

Under physiological conditions, the cell combats reactive dicarbonyls like MG by the up-regulation of defense mechanisms such as glutathione production, and specific enzymes like glutathione-S-transferases or the glyoxalase system (Finkel and Holbrook, 2000). Doing so returns its level into the initial steady-state range. In this system, short-term MG-induced stress may have no serious consequences for living organisms, and even can be beneficial, as pre-conditioning has been shown to increase the capability to eliminate reactive carbonyls (Ruhs et al., 2010). On the other hand, prolonged exposure to a high concentration of glucose or MG can result in severe changes in proliferation and adhesion and induced inflammation signals in cells (Giacco and Brownlee, 2010, Ceriello et al., 2009, Zhang and Wu, 2014).

Besides directly reducing MG, the defense mechanisms confer protective effects by sheltering anti-apoptotic molecules from interaction with dicarbonyls. In Chapter 4 we showed that GLO1 overexpression resulted in preserved mRNA expression of anti-apoptotic *Bcl-2* and *BcXl* mRNA. The connection between MG and Bcl2 regulation has also been shown in studies done by the Brownlee group (Giardino et al., 1996).

Data presented in Chapter 3 gave evidence of connection between TNF-α and MG in cell death induction. Since carbonyl stress was increased in cells exposed to HG or MG even without TNF-α, but there was no recorded cell death, it is more likely that MG through increase of oxidative stress switches the TNF-α inflammatory role into pro-apoptotic. The death-inducing capability of TNF-α *in vivo* is often prevented by simultaneous activation of NF-κB. To further elucidate MG's function as a signaling molecule for cell death in ECs it would be important to examine the regulation of NF-κB nuclear translocation and possible cytochrome C expression and caspase activation. With high ROS, cytochrome c is released

from mitochondria and subsequently forms caspase-3-inducing apoptosome complex. MG has been shown to prevent transcription of *Bcl-2* – a protein that regulates cytochrome C release. This too can be one of the possible pathways for increased apoptosis in cells under hyperglycemia. Another important factor that should be considered is GLO1 protection against cell death is regulation of the HIF1-α gene. HIF1-α plays a role in pro-survival pathways by transcriptionally regulating protective genes such as *EPO*, *Glut1* and *VEGF* (Ke and Costa, 2006). These pathways can be activated by reduced O_2, but can also be abolished through increased ROS (Qutub and Popel, 2008). MG alone has been shown to interfere with HIF1-α's regulation of genes (Ceradini et al., 2008).

5.3.4 MG-TNF-α interaction

The increase in MG-induced oxidative stress, as described in Chapter 3, leads to decreased cell viability after 24 hours, only when cells were incubated with TNF-α, demonstrating a synergistic effect between these two molecules. Other studies have also demonstrated a similar synergy - in the mouse fibrosarcoma cell line L929, TNF-α induced cell death is also dependent on the accumulation of MG (Laga et al., 2007). The possible synergistic TNF-α/MG effect was confirmed in Chapter 2. GLO1-diabetic mice had less cell death in their hearts compared to WT-diabetic mice, despite similar levels of TNF-α between the groups. One explanation is that the reduction of MG by GLO1 overexpression prevented the TNF-α/MG induction of cell death seen in WT-diabetic mice that had increases of both MG and TNF-α.

It is very likely that increased ROS production with the accumulation of MG leads to the activation of several different routes all resulting in cell death. The results presented in

Chapter 2 and 3 suggest the increased ROS as a main pathway in TNF-α/MG induced cell death through effects on mitochondria and total carbonyl stress, which is illustrated in FIG 5.2.

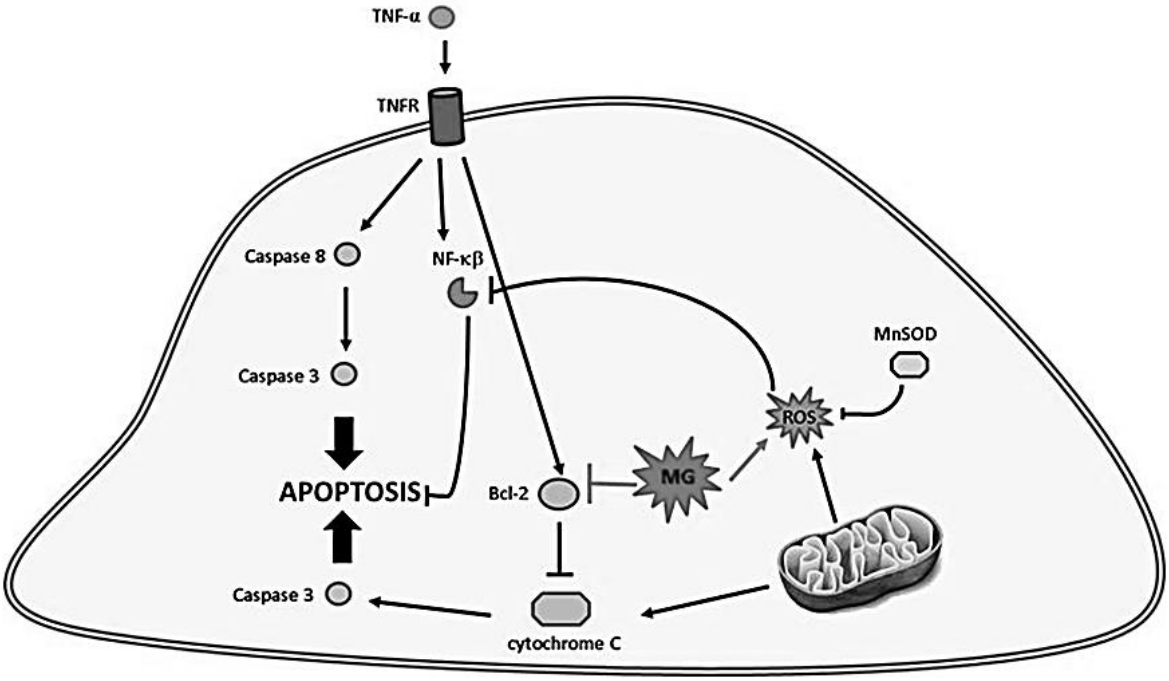


FIGURE 5.2 Proposed mechanism of methylglyoxal (MG) role in TNF-α-induced pathways. TNF-α activates cell death (caspase-8, -3), and also anti-apoptotic signaling pathways through transcription factor nuclear factor-kappa B (NFκB) and/or Bcl-2. Through formation of advanced glycation end products (AGE), MG induces ROS accumulation, inflammation, adhesion molecule expression and apoptosis, while reducing Bcl-2 expression, shifting the balance to apoptosis and cell loss.

5.4 GLO1 AND BM FUNCTION

An impaired angiogenic response to ischemia contributes to the poor clinical outcomes observed in diabetic patients with coronary or peripheral artery disease (Capla et al., 2007, Howangyin and Silvestre, 2014, Xu et al., 2012). Angiogenesis, defined as the formation of new blood vessels out of pre-existing capillaries, plays a crucial role in maintaining vascular health. Impaired angiogenesis contributes to delayed wound healing, impaired recovery from peripheral limb ischemia, and also to cardiac morbidity by reduction of capillary turnover. Mechanisms underlying diabetic angiogenesis impairment are complex. One of them is directly linked to MG accumulation. Covalent modification of HIF1α by MG at Arg-17 and Arg-23 domains reduces both heterodimer formation with ARNT and HIF1α binding to the promoters of SDF-1 and VEGF (Ceradini et al., 2008). In hypoxic mouse CACs, the same HIF1α modification reduces binding to the CXCR4 promoters (Ceradini et al., 2008). Several studies have examined the mechanisms of reduced angiogenic response caused by diabetes, but the study described in Chapter 4 is the first to focus on the role of BMCs alone.

5.4.1 GLO1 overexpression preserves BMC function

Chapter 4 demonstrated that overexpressing GLO1 in BMCs could reverse the diabetes-induced impairment of BM-derived CACs *in vitro* and *in vivo*. BMCs extracted from GLO1-diabetic mice migrated towards SDF-1 and VEGF as effectively as BMCs from non-diabetic mice. More importantly, GLO1-diabetic BMCs migrated more than WT-diabetic BMCs towards stimuli present in serum taken from diabetic mice with hindlimb ischemia. These *in vitro* data suggest that BMCs overexpressing *hGlo1* are more likely to

respond to mobilization and homing signals generated by ischemic tissue in the diabetic setting, which we did, in fact, observe *in vivo.*

Decreased HIF-1α function impairs both ischemic cell signaling and the BM-CACs response, but our findings show that overexpression of GLO1 solely in the BM can protect BM-CACs' viability and function, and prevented diabetes-induced post-ischemic defects in neovascularization and normalized tissue survival.

5.4.1 BMCs function in angiogenesis

Circulating BM-derived cells have potent activity to repair endothelial injury and promote angiogenesis (Asahara et al., 1999). BMCs exhibit paracrine effects but can also differentiate and incorporate directly into the vessel wall (Rajantie et al., 2004, Uemura et al., 2006, Shi et al., 1998). DM impairs the ability of ischemic tissue to direct the molecular and cellular signals leading to restoration of tissue perfusion, affecting the BM–derived cell mobilization and angiogenic response.

The results presented in Chapter 4 may also suggest that the preservation of vasculature in the heart described in Chapter 2 could be due, at least partially, to GLO1 expression in BMCs. This offers another target for post ischemia and heart failure therapy, focusing on the improvement and protection of bone marrow function.

5.5 GLO1 AS A THERAPEUTIC TARGET

Several studies have shown that MG levels are elevated in diabetic patients' plasma compared to healthy subjects. More and more studies focus on understanding how advanced glycation by MG may have a part in inflammation related to vascular dysfunction (Price et al., 2010, Rabbani and Thornalley, 2011). An interesting case study by Miyata et al. described a 69 year old woman who suffered from recurrent cardiovascular complications, despite the absence of significant risk factors. They observed very low levels and activity of GLO1 in this patient, which was associated with higher levels of MG and their derived AGEs (Miyata et al., 2001).

Diabetic kidney disease increases the risk and severity of CAD dramatically and the glyoxalase system is an important protective mechanism against the formation and subsequent accumulation of AGEs in kidneys (Schiffrin et al., 2007). GLO1 is also a possible candidate to be a contributing CAD gene. A recent genomic study proposed the glyoxalase system as a possible common pathway that could be responsible for both the microvascular and macrovascular complications observed in subjects with diabetes. The connection was suggested because many of the same genes were affected by CAD-associated SNPs in the human GWAS (Mäkinen et al., 2014). The authors of this conclude by encouraging the development of GLO1 inducing or AGE reducing treatments for reducing the effects of both diseases.

Interestingly, there is apparent dichotomy in the effects of GLO1 overexpression / knockdown on macrovascular disease (Geoffrion et al., 2014, Hanssen et al., 2014a) and microvascular disease (Giacco et al., 2014). In ApoE(-/-) mice with or without diabetes, GLO1 overexpression did not lead to decreased atherosclerotic lesion size or systemic inflammation (Hanssen et al., 2014a). Although higher levels of AGEs in human carotid

atherosclerotic plaques are associated with a rupture-prone phenotype (Hanssen et al., 2014b), increasing GLO1 levels does not seem to be an effective strategy to reduce glycation in atherosclerotic lesions, likely due to increased AGE formation through GLO1-independent mechanisms. Of course this may just be a particular result seen in ApoE knockdown mice and requires further investigation.

5.5.1 Development of MG reducing therapeutics

Anti-AGE therapeutics can work at multiple levels to reduce diabetic complications either by inhibiting the formation of AGEs or by reducing the toxic products of AGEs once formed. Pharmacologic agents that can breakdown and/or prevent AGE crosslinking may have benefits as clinical interventions. The best approach, however, would be to reduce the formation of AGEs. Aminoguanidine, the best characterized MG scavenger compound to-date, acts as a non-specific inhibitor of MG (Lo et al., 1994). While early clinical trials showed promising therapeutic potential of aminoguanadine, the ACTION II trial was terminated early due to lack of efficacy and for safety concerns (toxic vitamin B6 metabolism, inhibiting pyridoxal phosphate dependent enzymes and inhibition of diamine oxydase) (Freedman et al., 1999). Other compounds and drugs have shown promise, both experimentally and clinically, in protecting against and/or reducing diabetic complications. Metformin, a common treatment for type 2 diabetes, was able to reduce levels of serum reactive dicarbonyls and AGEs in type 2 diabetic patients, probably by increasing cell sensitivity to insulin and reducing glucose levels, but also through direct interaction with MG (Beisswenger and Ruggiero-Lopez, 2003, Detaille et al., 2005, Ota et al., 2007). Treatment with soluble RAGE (sRAGE) has shown promising results, acting as a decoy receptor for AGEs, and preventing the development of diabetic neuropathy in mice. However, this

method seems inapplicable clinically for now as limited effects were seen on the regulation of circulating esRAGE and sRAGE in humans (Koyama et al., 2007).

5.5.2 Regulation of GLO1 function

Pharmacologic agents that increase GLO1 activity might have unique clinical efficacy in the prevention and treatment of diabetic conditions. Age-related decrease in GLO1 activity in nematode *C. elegans* caused increased mitochondrial ROS production, which in turn limited the lifespan of the organism (Morcos et al., 2008b). Consistently, increasing expression of GLO1 reduced ROS production and increased lifespan (Rabbani and Thornalley, 2008). A recent study using Akita mice, a spontaneous type 1 diabetic model, found that fisetin, a naturally-occurring flavonoide, increased GLO1 expression and activity and the synthesis of glutathione (Maher et al., 2011).

Upregulation of GLO1 after increased dicarbonyl presence is part of the Nrf2 stress-responsive system that protects protein and DNA from increased damage and preserves cell function (Xue et al., 2012). Thus, prolonged Nrf2–ARE–GLO1 interaction would be beneficial in conditions of extended HG and/or MG exposure. Accordingly, the development of molecules that target GLO1 and increase its activity could have therapeutic benefit in diabetic patients.

Increased GLO1 activity has different consequences in different cells. While beneficial effects were seen in ECs, other studies found a connection between GLO1 and anxiety disorders (Distler and Plant, 2012). Since ECs are the main target of pathology caused by increased blood glucose levels, protecting these cells alone may be a very specific way of preserving blood vessels' health and protecting the affected organs. But, there is currently no gold standard treatment for endothelial cells dysfunction in the diabetic or non-

diabetic setting. Investigation into other compounds like fisetin that increase GLO1 levels or activity in ECs only, could have profound clinical implications by reducing the incidence of diabetic complications.

Given the important contribution of EC function to vascular and cardiac cell biology, hopefully future research will develop methods towards directly treating ECs in diabetes, subsequently decreasing the morbidity and mortality caused by cardiovascular complications in diabetic patients.

5.6 SUMMARY

This thesis examined just a few possible links between diabetic complications in cardiovascular disease (defects in EC function and lack of blood vessel growth) and the accumulation of MG. Our results may provide a basis for the rational design of drugs that target the modification/reduction of MG, or enhancing GLO1 expression and activity. It still remains to be determined if the increase of GLO1 by influencing natural regulatory systems would be capable of preventing increased concentrations of reactive dicarbonyls and accumulation of AGEs in DM. Unfortunately, drug discovery has mainly focused on inhibitors of GLO1, because of its necessary role in cancer cells' survival and in the development of tumor multi-drug resistance. Hopefully, more and more interest will develop in targeting this enzyme system as a solution to cardiovascular complications caused by DM.

The conclusions drawn from the 3 studies suggest an underlying mechanism for several pathophysiological conditions *in vivo*. Namely, HG leads to accumulation of MG, and the subsequent MG modification of various target molecules then results in inflammation and cell death, finally leading to failed organ function. Also, this thesis underlines the importance of ECs and BMCs in the overall pathology of DM. Overall,

hopefully this work will contribute to better understanding of the mechanisms leading to vascular dysfunction and heart disease and will inspire new strategies to reduce cardiovascular morbidity and mortality in patients with DM.

APPENDIX I

KNOCKDOWN OF GLYOXALASE 1 DOES NOT AFFECT THE NEUREGULIN EXPRESSION IN NON-DIABETIC MICE

Contributions of Authors

All experiments performed in the two appendixes were performed by me.

AI-1.0 INTRODUCTION

In chapter 2 of this thesis, it was shown that hGLO1 overexpression prevented the loss of ECs and reduced neuregulin production in ECs. In order to further examine if this deficit of neuregulin is caused by MG modification of the protein itself or if it reflects the loss of ECs, non-diabetic Glo1-knockdown (GLO1-KD) were used (Giacco et al., 2014). The reduction of GLO1 activity by GLO1-KD increases MG modification of proteins and oxidative stress, causing alterations in kidney morphology identical to those caused by diabetes (Giacco et al., 2014). The study done by our collaborators from Albert Einstein Institute, Bronx, NY, confirmed that GLO1 activity regulates the sensitivity of the kidney to hyperglycemic-induced renal pathology. Using the same mouse model, we assessed if the same is true for the heart and the development of heart failure caused by MG accumulation.

AI- 2.0 RESEARCH DESIGN AND METHODS

GLO1-KD mice were generated in Dr. Brownlee's lab for studies on diabetic nephropathy as previously described (Queisser et al., 2010). Reduced *Glo1* mRNA levels were confirmed by

quantitative PCR. Hearts from heterozygous offspring had a 30% decrease in GLO1 activity, determined by GLO1 activity assay performed as described before (FIG AI-1). For most of the experiments, 3 groups of mice were examined: GLO-KD mice 1 year old, their WT littermates' of the same age and 12 week old GLO-KD mice. This study conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Serum levels of 3 soluble adhesion molecules (E-selectin, ICAM and VCAM) were assessed as described in chapter 2. Protein expression of neuregulin in the hearts was determined as described in chapter 2. Hearts were collected, perfused with saline, and then fixed in 4% formalin and paraffin-embedded, sectioned and stained with the CD31 (Abcam) EC marker antibody. Visualization was completed using a Zeiss Axiophot microscope equipped with a Hamamatsu C5985 chilled CCD camera, and Metamorph imaging software 4.01 (Molecular Devices).

AI- 3.0 RESULTS

One year old GLO-KD mice were used since the previous study done in Dr. Brownlee's lab showed that GLO-KD mice of that age had increased MG modification of proteins and oxidative stress in the kidneys (Giacco and Brownlee, 2010). We observed elevated serum levels of 2 ED markers (ICAM and VCAM) in both GLO1-KD groups (young and old), partially confirming a pro-inflammatory state of blood vessels caused by MG accumulation. E-selectin was not affected and GLO1-KD control mice had lower levels of this ED markers than all other groups (TABLE AI-1). Western blot analysis of heart tissue from the 1-year old mouse groups did not show any difference in the levels of neuregulin or in MG-H1

accumulation (FIGURE AI-2). There was also no change in the number of ECs per field-of-view in the heart sections (data not shown).

These data suggest that neuregulin is not directly affected by MG accumulation, and that the pro-inflammatory state in ECs alone does not lead to the loss of ECs or reduced neuregulin production. Future experiments can use a protein specific pull-down assay for neuregulin from GLO1-KD mice and examine possible protein glycation. Also, treatment of WT-diabetic mice with neuregulin may better elucidate the role of neuregulin in diabetic heart failure. This may also confirm neuregulin as one of the therapeutic targets for maintenance of heart function.

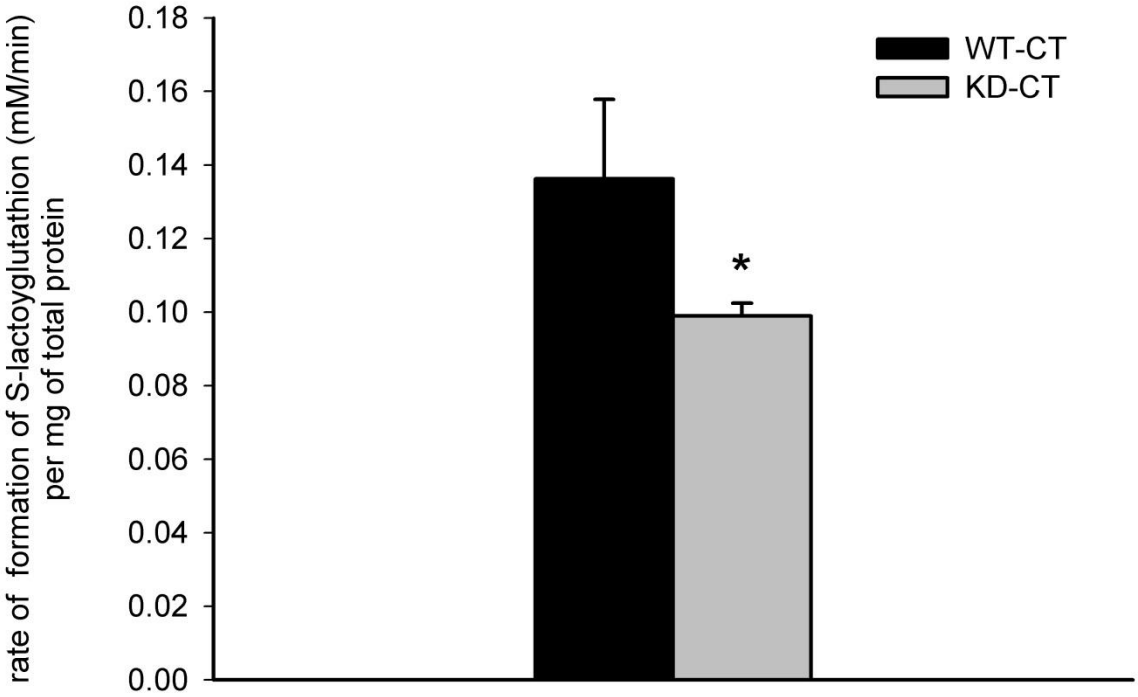

rate of formation of S-lactoyglutathion (mM/min)
per mg of total protein
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
WT-CT
KD-CT
*

FIGURE AI-1. Glyoxalase activity in the heart tissue measured as rate of formation of S-D-lactoglutathione was significantly reduced in GLO1-KD (*p=0.038).

	GLO1-KD CONTROL	GLO1-KD 1 YEAR OLD	WT 1 YEAR OLD
sEselectin	0.76	0.97	1.00
sICAM	1.19*	1.23*	1.00
sVCAM	1.14*	1.12*	1.00

TABLE AI-1 Serum levels of endothelial inflammation markers observed in GLO1-KD groups normalized to values of 1 year old WT mice (* $p \leq 0.045$).

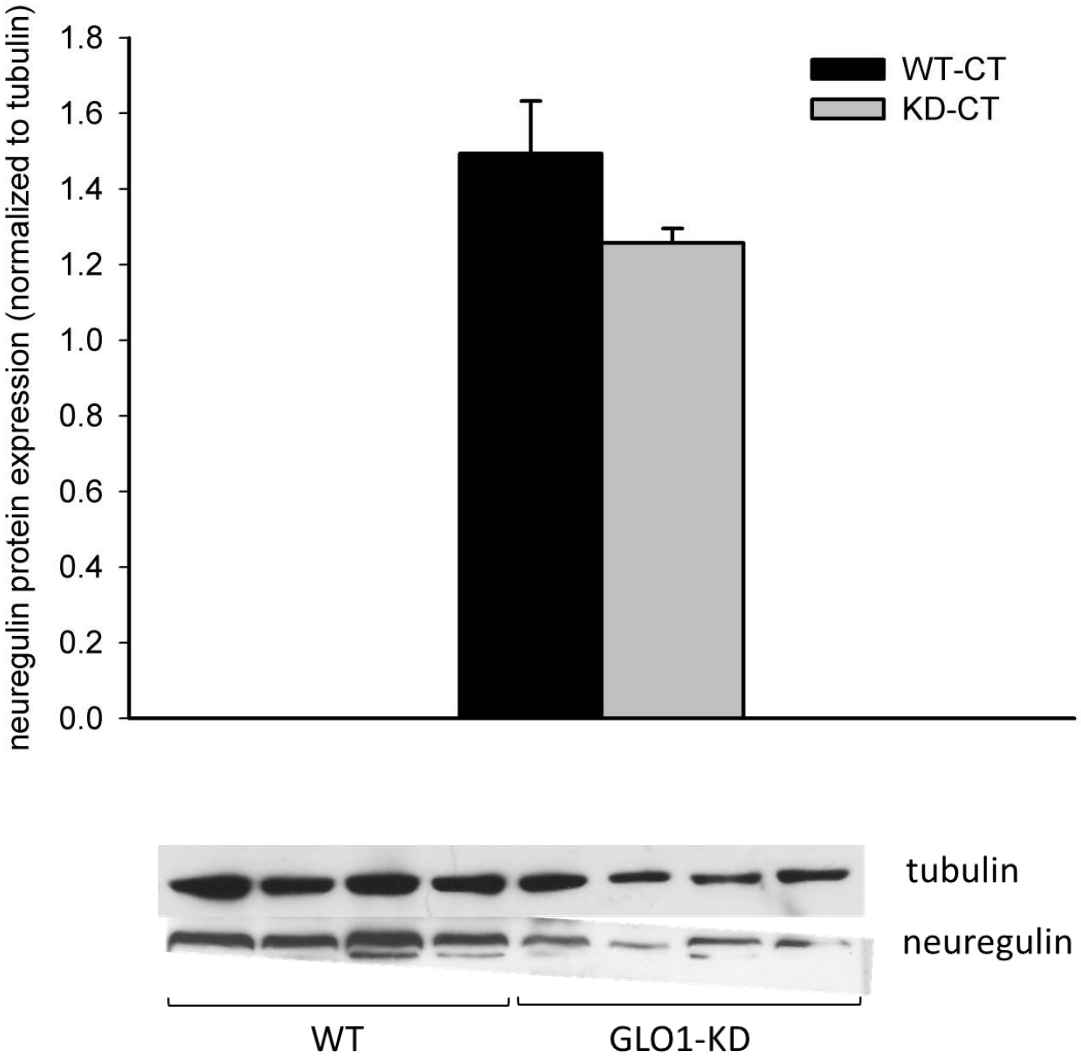


FIGURE A1-2 Levels of neuregulin assessed using Western blot were not significantly different between two groups (WT and GLO-KD, p=0.069, n=4)

APPENDIX II

DETECTION OF MG IN LIVE CELLS USING MBO FLUORESCENCE PROBE

AII-1.0 INTRODUCTION

Up to this day, there is a lack of a suitable and simple assay to monitor the intracellular trafficking and reactivity of MG *in vivo*. This is due in part to the high reactivity of MG with proteins, nucleic acids, and lipids in living organisms. Thus, most studies report the total content of MG in a given tissue, in terms of a derived adduct, commonly prepared with ophenylenediamine (OPD) or similar molecules, followed by HPLC or LC−MS analysis (Rabbani and Thornalley, 2014). These methods, however, require tissue homogenization and tedious protocols, many of them under harsh conditions, which could render additional oxidative stress and leading to an overestimation of MG content. Thus, as a result, the measurement of MG levels using the different methodologies can differ in several orders of magnitude.

In a recent study, the pre-fluorescent (*i.e.* it becomes fluorescent upon reaction with a radical, in our case MG) methyl diaminobenzene-BODIPY (MBo) probe was presented as a candidate for the visualization of MG in living cells (Wang et al., 2013). MBo showed specificity for MG detection over other biological dicarbonyls and its *in vitro* performance was tested in cultures of human cervical cancer HeLa cells. In this thesis, we have further optimized probe concentration and the limit of detection for MG in ECs and human skin fibroblasts. Further, we have also tested the potential toxic effect of the probe using propidium iodine (PI) staining for necrotic cells.

AII – 2.0 RESEARCH DESIGN AND METHODS

Fibroblasts were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. Human Cardiac Endothelial Cells (HCECs) were grown in EBM media supplemented with 1% heparin and 10% of FBS. Both cell types were plated on regular tissue culture plates (BD bioscience). For fluorescence microscopy experiments, cells were seeded overnight in culture media (2 ml) on Nunc(R) Lab-tek(R) II chamber slide and grown to ~50% confluence. The media was then replaced with 1 ml of HEPES-Krebs-Ringer's buffer (HKR) (140 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES pH 7.4), and 0.5 μM of MBo fluorophore (from 1uM stock in DMSO) was added. The cells were incubated with the probe for 1h. Cells were then washed once with 1 ml HKR, and MG was added (0 and 5 μM final concentrations). Cells were incubated for an additional 30 min to an hour prior to imaging. To confirm false positive signals from interaction of MBo with nitric oxide (NO), 100 μM of NO synthase inhibitor L-N-Methylarginine (L-NME) was added to the cells prior to incubation with the probe and MG. In order to determine the time during which free MG can be detected in the cell, MG and MBo were added in reverse order. For testing of possible toxic effects after incubation with MBo, MG cells were stained with PI for another 30 minutes to label apoptotic cells. Live cell imaging studies were performed on a Zeiss Axiovert 200M Fluorescence microscope with a 20X regular or 63X oil immersion lens and an AxioCam mRM camera. Excitation was at 470 ± 40 nm. Emission corresponds to 540 ± 50 nm. The use of these two cell types was chosen since ECs present the most used cell types for studies on MG effect on tissue in diabetes. We have chosen the dermal fibroblast as control, knowing that their production of NO is minimal compared to ECs. Both cell types have been used in MG *in vitro* studies with different MG doses applied.

AII – 3.0 RESULTS

Our data confirm that MG freely permeates the cell membrane, and that it can be found free for up to 30 minutes (FIGURE AII-1). Unfortunately, upon reaction with MBo, MG cannot perform its normal biological functions. Thus, labeled MG is incapable of binding to proteins, lipids or DNA which makes it impossible to track its natural pathways in the cell. Also, the probe does not account for the "ghost" MG, which corresponds to those molecules of MG that have reacted with cell areas inaccessible for MBo. Furthermore, the addition of L-NME reduced significantly the signal from the probe, indicating that some of the fluorescence observed is originating by interaction with NO produced by the cells. This was more pronounced in ECs, probably because of their more ample NO production, especially under oxidative stress. Note that MG-MBo was not toxic for the cells as the number of PI positive cells was not different in the cells treated with MG and MBo compared to the un-treated cells (FIGURE AII-2).

In summary, our results indicate that MG permeates the cell membrane of ECs. Further, we have significantly lowered the amount of MBo needed for detecting MG (down to 0.5 µM) compared to the original protocol. Furthermore, we have shown for the first time that MG does have a "half-life" in the cell while it can still be freely detected with the probe (with assumption that the dye does not decompose after 90 min). However, MBo has intrinsic limitations for MG detection since this merely acts as a positive/negative reporter for the presence of MG. Room for improvement of fluorescent dyes for MG detection includes the development of dyes with higher hydrophobicity, anchored to phospholipids like structures, that could be used for monitoring MG trafficking through the cell membrane and

MBo like dyes linked to specific organelle targeting molecules, which will facilitate a more ubiquitous evaluation of the distribution of MG within the cell.

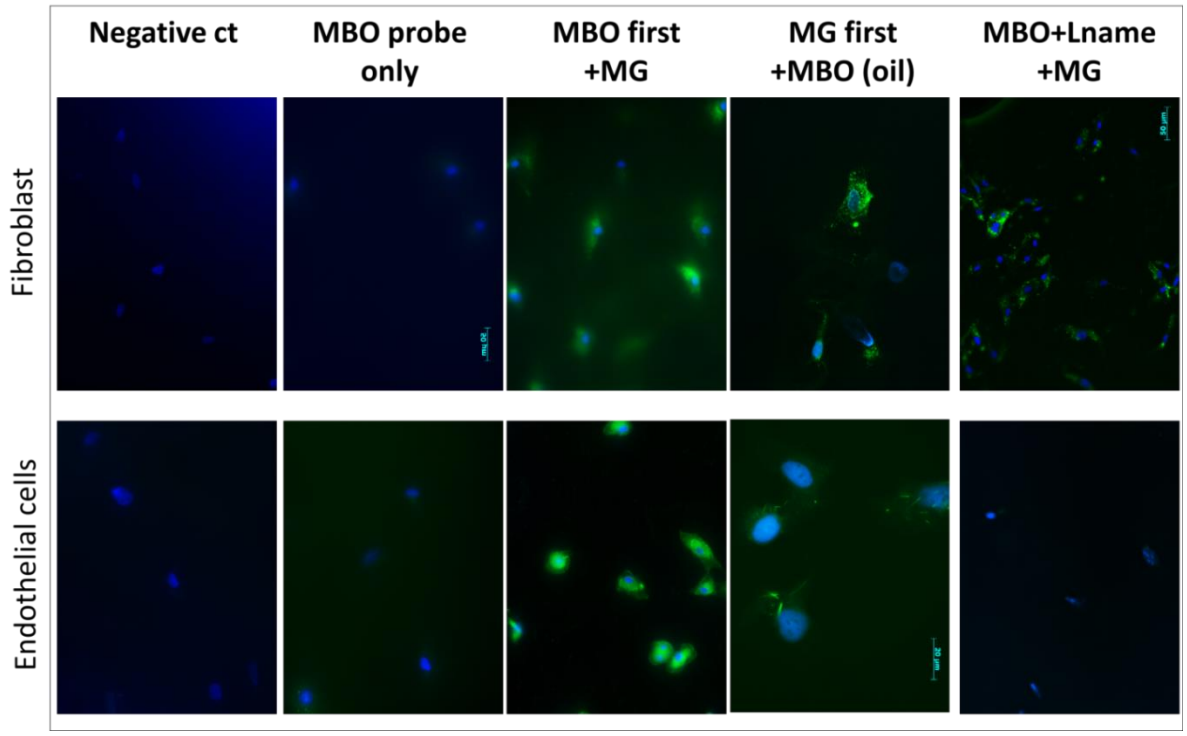


FIGURE AII-1. Labeling of MG (5 µM) in ECs and fibroblasts using MBo probe (0.5 µM) with L-NAME as a control for non-specific NO binding. MBO+MG (green) and DAPI (blue)

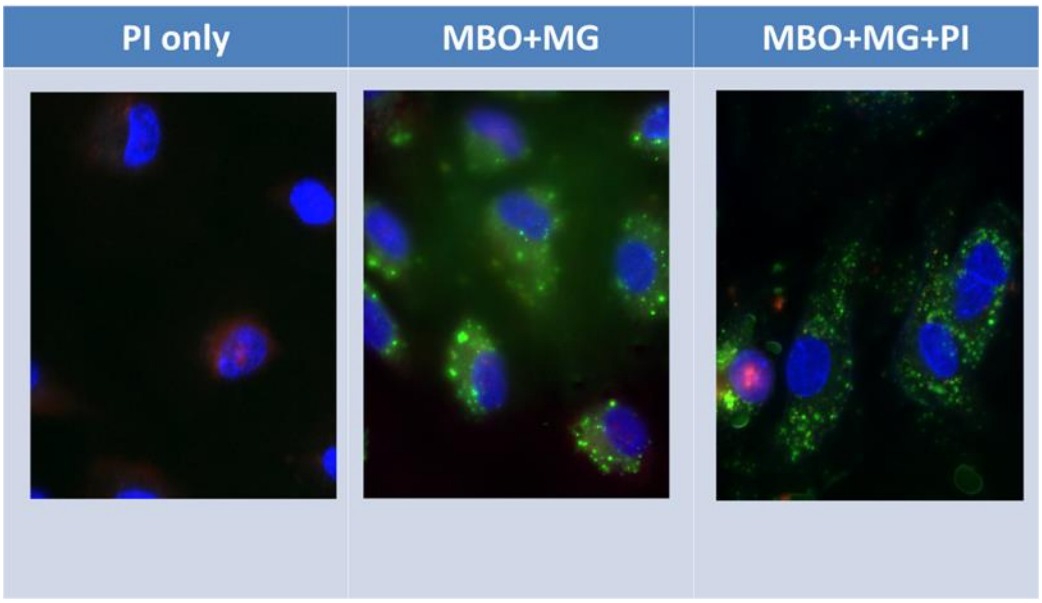


FIGURE AII-2. Detection of apoptotic fibroblasts exposed to MG+MBo complex using PI. There was no increase in cell death in fibroblasts treated with MBO+MG (p=0.9)

APPENDIX III

AUTHORIAZATIONS

Authorization for Chapter #1 – INTRODUCTION

Chapter 1. Figure 1.1

Dear Branka

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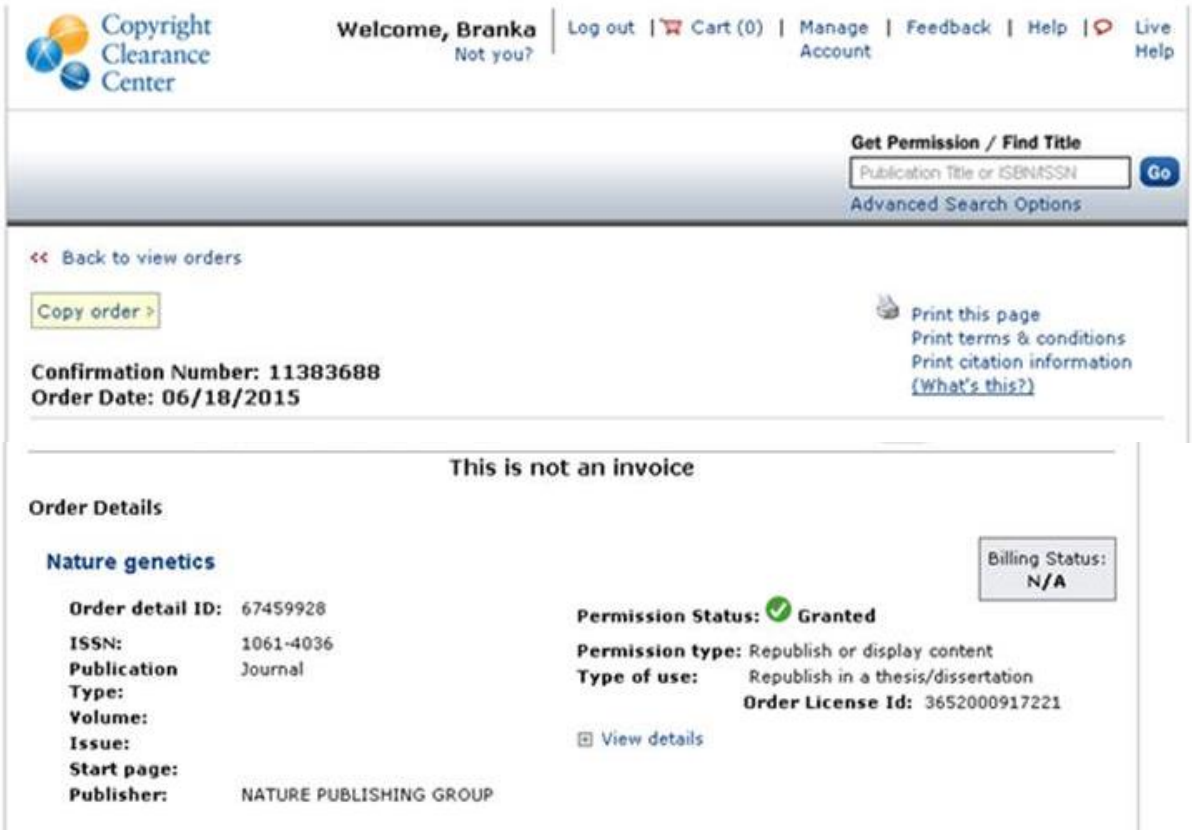

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