The relationship between glucose metabolism byproduct, D-lactate, and vascular endothelial cell dysfunction and possible role in diabetes

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ABSTRACT

Diabetes mellitus is a chronic disease associated with vascular complications. Vascular endothelial dysfunction caused by increased endothelial cell apoptosis contributes to diabetic cardiovascular complications. The glucose metabolic by-product, D-lactate, is elevated in diabetics and it is unknown whether it contributes to endothelial cell apoptosis. We hypothesized that diabetic D-lactate levels induce apoptosis in human vascular endothelial cells (HUV-EC-C).

HUV-EC-C were incubated with 0.2 mM D-lactate (DLA) and mRNA expression of PI3K/AKT pathway members (AKT1, Bcl-2, BAD, eNOS, PI3K) were measured using Quantitative RT-PCR. DLA downregulated all genes at 6 and 24 hours, followed by increase in expression after 48 hours except PI3K, which remained below control. To further investigate apoptosis, the Human Apoptosis PCR Array was used and expression of all proapoptotic genes (TNF family members) and antiapoptotic genes (IAP family members) were decreased and increased, respectively, at 24 hours followed by an increase and decrease, respectively, at 48 hours. Caspase activity, measured using the Caspase-Glo® 3/7 Assay after HUV-EC-C exposure to 0.2 mM DLA alone or in combination with 20 mM glucose (GLU) or 5 µM methylglyoxal (MG), was increased after 1, 72, and 96 hours. Furthermore, to know whether DLA (0.2 mM) and DLA (0.2 mM), GLU (20 mM) and MG (5 µM) combined cause changes in cellular energy metabolism, creatine (Cr) and high-energy phosphate substrates (CrP, ATP, ADP, AMP) were quantified using HPLC and no changes were observed. We further measured ROS production in HUV-EC-C treated with 0.06-2 mM DLA alone or 0.2 mM DLA with 5-30 mM GLU or 5-160 µM MG. All DLA concentrations increased ROS production by 160% to 216%. DLA with GLU or MG significantly increased ROS production compared to GLU or MG alone. Lastly, D-lactate dehydrogenase (D-LDH) expression was determined using Quantitative RT-PCR and D-LDH was not detected in HUV-EC-C.

In conclusion, DLA altered expression of different pro- and anti-apoptotic genes in HUV-EC-C. Furthermore, exposure of HUV-EC-C to DLA levels typically present in diabetics resulted in time-dependent changes in caspase activity, possibly due to excessive ROS production. Whether these changes eventually lead to endothelial dysfunction in diabetes needs further investigation.

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I would like to dedicate this thesis to

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
AGE	Advanced glycation end-products
AIF	Apoptosis inducing factor
AKT1	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANG-II	Angiotensin II
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAD	Bcl-2 - associated agonist of cell death
Bax	Bcl-2 associated X protein
Bcl-2	Apoptosis regulator Bcl-2 alpha isoform
Bcl-XL	B-cell lymphoma-extra large
Bid	BH3 interacting-domain death agonist
BIRC3	Baculoviral IAP Repeat Containing 3
BIRC8	Baculoviral IAP Repeat Containing 8
Bp	Base pair
Ca^{2+}	Calcium
CASP	Caspase
Cav-1	Caveolin-1
CD27	Tumor necrosis factor receptor CD27
cGMP	Cyclic guanosine monophosphate
Cr	Creatine
CrP	Creatine phosphate
CuZn-SOD	Copper/Zinc superoxide dismutase
CYP450	Cytochrome P450

DHAP	Dihydroxyacetone phosphate
DISC	Death inducing signaling complex
DLA	D-Lactate
DLD	D-Lactate dehydrogenase
DKA	Diabetes ketoacidosis
DCF-DA	2,7-Dichlorofluorescein diacetate
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ET-1	Endothelin-1
FADD	Fas-Associated protein with Death Domain
FasR	Death receptor
GI	Gastrointestinal tract
GIP	Glucose-dependent insulinotropic peptide
GLP-1	Glucagon-like peptide 1
GPX	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HbA1c	Haemoglobin A1c
HPLC	High pressure liquid chromatography
HUV-EC-C	Human umbilical vein endothelial cells
IAP	Inhibitor of apoptosis
ICAM-1	Intracellular adhesion molecule-1
IFG	Impaired fasting glucose
IGT	Insulin glucose tolerance
LAM	Leukocyte adhesion molecule
LLA	L-Lactate
L-LDH	L-Lactate dehydrogenase
LTA	Lymphotoxin alpha

MCP-1	Monocyte chemotactic protein-1
MCTs	Monocarboxylate transporters
MG	Methylglyoxal
mL	Milliliter
mM	Millimolar
Mn-SOD	Manganese superoxide dismutase
mRNA	Messenger ribonucleic acid
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor κ B
NO	Nitric oxide
NOS	Nitric oxide synthase
O_2^-	Superoxide
OD	Optical density
$ONOO^-$	Peroxynitrite anion
oxLDL	Oxidized low density lipoprotein
PAI-1	Plasminogen activator inhibitor-1
PBS	Phosphate buffer saline
PDGF	Platelet derived growth factor
PDH	Pyruvate dehydrogenase
PGI-2	Prostacyclin
pH	Hydrogen ion concentration
PI	Propidium iodide
PI3K	Phosphoinositide kinase-3
PS	Phosphatidylserine
PTP	Permeability transition pores
QRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction

ROS	Reactive oxygen species
Rpm	Revolutions per minute
RT-PCR	Reverse-transcriptase polymerase chain reaction
SBS	Short bowel syndrome
SCFA	Short chain fatty acids
SIVA	CD27-binding protein
SOD	Superoxide dismutase
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA cycle	Tricarboxylic acid cycle
Tm	Annealing temperature
TNFα	Tumor necrosis factor α
TNFRSF10A	Tumor necrosis factor receptor superfamily member 10A
TNFRSF25	Tumor necrosis factor receptor superfamily member 25
TNFSF10/TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
TNFSF15	Monogamous TL1A ligand
t-PA	Tissue-type plasminogen activator
TRAIL	TNF related apoptosis inducing ligand
VCAM-1	Vascular adhesion molecule-1
VSM	Vascular smooth muscle
VSMC	Vascular smooth muscle cell
$\Delta\psi_m$	Mitochondrial membrane potential
μM	Micromolar

1. INTRODUCTION

Diabetes mellitus is a chronic metabolic disease that affects more than 347 million people worldwide today. Two types of diabetes exist; type 1, which occurs in children and young adults, and type 2, which occurs in obese adult patients. This chronic metabolic disease is characterised by hyperglycaemia in circulating blood and impaired ability of cells to utilise glucose as an energy source. Diabetes develops due to defects in insulin production at beta islets of pancreas, or failure in insulin action in insulin sensitive tissues. Diabetes is followed by micro- and macrovascular complications. Macrovascular complications (atherosclerosis, hypertension, stroke, cardiac myopathy) are a leading cause of death. Microvascular complications (nephropathy, retinopathy, peripheral neuropathy) significantly affect the quality of life of diabetic patients and are a leading cause of kidney failure, ocular problems, and lower limb amputation. Early vascular endothelial dysfunction caused by an increased apoptosis of vascular endothelial cells is proposed to be the main cause of diabetic complications.

A mixture of elevated concentrations of glucose and glucose byproducts, methylglyoxal and D-lactate, exist in the blood of diabetic patients. Much research has explored the role of glucose and methylglyoxal in the development of endothelial dysfunction and consequently diabetic complications. It is known that hyperglycemia contributes to increased apoptosis of endothelial cells by the release of mitochondrial cytochrome C, decreased energy production in the cell, as well as activation of the caspase cascade. However, the exact mechanism and initiators of endothelial dysfunction in diabetes have not been explained yet.

One candidate, D-lactate, the detoxification product of methylglyoxal, has received limited attention in the research of endothelial dysfunction in diabetes. It is known that D-lactate concentration in diabetic patients is elevated compared to healthy individuals. In addition, D-lactate causes cellular apoptosis at higher concentrations (condition known as D-lactate acidosis). Whether or not D-lactate contributes to endothelial dysfunction at concentrations normally found in the blood of diabetic patients has not been investigated. Therefore, in my thesis I evaluate whether elevated levels of glucose byproducts can initiate changes that may lead to apoptosis, such as changes in endothelial cell metabolic activity, altered energy production, production of reactive oxygen species (ROS), as well as changes in expression of pro/anti apoptotic proteins in human vascular endothelial cells.

2. LITERATURE REVIEW

2.1. Current state of knowledge in diabetes mellitus and cardiovascular complications

Type 2 Diabetes mellitus (T2DM) is a common chronic metabolic disease that affects more than 347 million people today (Danaei et al. 2011). Often followed by complications, diabetes has raised significant public health concerns (Fowler, 2008). Cardiovascular disease is an important diabetic complication that causes considerable morbidity and mortality (Amos, McCarty, and Zimmet 1997); approximately 70%–80% of people with diabetes die of cardiovascular disease. In addition, for each risk factor present, the risk of cardiovascular mortality is about 2 to 4 times higher in diabetics as compared to people without the condition (Bloomgarden 2003, Garber 1998). Despite the high risk of cardiovascular complications in diabetes, the mechanisms by which diabetic patients develop cardiovascular complications are yet undefined. However, vascular endothelial dysfunction including excessive endothelial apoptosis has been associated with vascular lesion progression, and vascular endothelial dysfunction is considered the underlying cause of cardiovascular disease in diabetes mellitus (Schalkwijk and Stehouwer 2005).

Early and accurate detection and clinical diagnosis of risk factors that contribute to cardiovascular complications in diabetes is important for initiating appropriate treatment to prevent these complications, and reduce overall associated morbidity and mortality. Currently, haemoglobin A1c (HbA1c) is routinely used as a predictive and diagnostic marker for diabetic complications as a measure of the extent of hyperglycemia (Lachin et al. 2008). However, a recent published paper suggested that HbA1c only explained a small percentage of microvascular complication risk while a large proportion (89%) remains uncaptured (Lachin et al. 2008). Therefore, identification of new underlying initiators contributing to endothelial dysfunction and ultimately micro- and macrovascular complications in diabetic patients is vital.

2.2. Diabetes

Diabetes mellitus is one of the most common endocrine disorders affecting approximately 6% of the human population (Meetoo, McGovern, and Safadi 2007). The prevalence of diabetes is on the increase due to population growth, aging, urbanization and increasing obesity rate and physical inactivity. The prevalence of this chronic metabolic disease was estimated to be 2.8% in 2000 and 4.4% in 2030. According to projections (Wild et al. 2004) the total number of people with diabetes will reach 366 million in 2030 in contrast to 171 million estimated for 2010. Diabetic patients have altered metabolism of carbohydrates, protein, fat, water and electrolytes, which ultimately lead to structural changes in tissues of many organ systems, especially the vascular system.

This chronic metabolic disorder is characterized by glucose intolerance and hyperglycemia due to deficiency in insulin secretion or deficiency of insulin action. Two main types of diabetes exist, type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). In addition, gestational diabetes mellitus and impaired glucose intolerance exist as conditions with elevated blood glucose. T1DM usually develops in children and younger adults while T2DM develops later in life, usually in adults over the age of 45 and is associated with obesity. However, the prevalence of diabetes is growing in children and adolescents, which correlates with obesity rates in the population (Rosenbloom et al. 2009, Bloomgarden 2004).

2.2.1. Type 1 Diabetes

Type 1 diabetes mellitus is an autoimmune condition characterized by destruction of insulin producing pancreatic beta cells. As a result of this destruction, the individual no longer produces insulin and has absolute insulin deficiency (Bluestone, Herold, and Eisenbarth 2010). Previously, it was known as juvenile diabetes, commonly diagnosed in children and younger adults. It affects 5% of the overall diabetic population and is believed to be caused by an autoimmune disorder (Daneman 2006). Since the pancreas no longer produces insulin, T1DM patients are absolutely dependent on exogenously administered insulin for survival.

Patients with T1DM are greatly prone to diabetic ketoacidosis. The pancreas does not produce insulin in these patients and glucose cannot enter cells, which leads to increased glucose concentration in the circulating blood. Therefore, cellular energy needs are met in alternative ways. Fat undergoes metabolism through lipolysis to release glycerol and free fatty acids. Glycerol is

further converted into glucose, in the process of gluconeogenesis, for cellular use. The free fatty acids are further metabolized to ketones, resulting in increased ketone concentration in blood and decreased hydrogen ion concentration (pH) (Balasse and Fery 1989). Extra ketones and large amounts of free circulating glucose are eliminated in urine, followed by large amounts of water, leading to dehydration. Finally, high amounts of ketones, large loss of water, decreased pH in body fluids, electrolyte imbalance will result in a condition known as diabetic ketoacidosis (DKA). If it remains untreated, DKA results in coma or death (Savage et al. 2011).

2.2.2. Type 2 Diabetes

Diabetes type 2 includes 90-95% of all diabetic cases (King, Aubert, and Herman 1998). In addition to family history and genetic factors, risk factors that contribute to diabetes are obesity, advanced age, inactive lifestyle, high blood pressure and high cholesterol levels. T2DM is caused by insulin resistance, a condition where peripheral tissues no longer respond to insulin properly. Problems in insulin secretion as well as insulin action cause glucose accumulation in blood. It was proposed that β-cell dysfunction observed in T1DM patients can also occur in T2DM and it was speculated to be an early event that leads to T2DM (Cnop et al. 2005).

Unlike in T1DM, T2DM does not involve autoimmune beta-cell destruction. T2DM is characterized by the following three disorders: 1) Peripheral resistance to insulin; 2) Increased production of glucose by liver; and 3) Altered pancreatic insulin secretion. Increased insulin resistance appears first and is eventually followed by impaired insulin secretion. At first, insulin is being produced by pancreas; however, insulin cannot be properly utilized on the cellular level. Glucose cannot enter the target cell and accumulates in the blood to cause hyperglycemia. High blood glucose levels stimulate high insulin production (hyperinsulinemia). In time, pancreatic insulin production decreases to below normal levels (Burket, Greenberg, and Glick 2003).

Patients with T2DM have minimal insulin production in the body that prevents ketone formation and development of DKA. However, long periods of poorly regulated blood glucose levels may result in hyperosmolar nonketotic acidosis. High levels of blood glucose will lead to increased glucose elimination in urine accompanied by large amounts of water that leads to electrolyte imbalance and acidosis (Burket, Greenberg, and Glick 2003). In addition, diabetes is followed by microvascular and macrovascular complications (Fowler 2008). T2DM progresses slowly and may be undiagnosed for years. Almost half of patients with diabetes are unaware of

the disease. By the time a patient is diagnosed with diabetes, many of complications have already started (Burket, Greenberg, and Glick 2003).

Other conditions with increased blood glucose levels include gestational diabetes, impaired glucose tolerance and impaired fasting glucose. Gestational diabetes usually develops during the third trimester of pregnancy and hyperglycemia is associated with glucose resistance. People with impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) are at high risk of developing T2DM.

2.2.3. Diabetic complications

The group of macrovascular and microvascular complications affecting different organs is the major contributor to high morbidity and mortality rates observed in diabetic patients. Complications that follow diabetes include heart disease, hypertension, stroke, retinopathy, nephropathy and neuropathy. Development of these complications highly depends on the duration of the hyperglycemia. The progression and severity of complications increase with the duration of diabetes. Other disorders often present in diabetic patients, such as hypertension and dyslipidemia, contribute to the risk of microvascular and macrovascular complications (Cade 2008).

The leading cause of mortality and morbidity in diabetic patients is heart failure. Important risk factors for heart failure are vascular complications, such as coronary artery disease (atherosclerosis) and cardiac myopathy (microangiopathy). Atherosclerotic lesions lead to narrowing the diameter of small coronary arteries and increased coronary arterial tension. Lipid deposition and atheroma formation, as well as thickness of arterial walls are seen in larger blood vessels. Abnormal vascular reactivity in the resistant arteries leads to hypertension, stroke or retinopathy (Cade 2008). Endothelial cells play a key role in the development of these complications. Proliferation of endothelial cells and alterations in endothelial function are major contributors to microvascular damage (Choy et al. 2001, Cines et al. 1998, Hadi and Suwaidi 2007, Lorenzi and Cagliero 1991). Prolonged microvascular damage leads to diabetic nephropathy, diabetic neuropathy, and ocular complications. Gangrene of the lower extremities, known as “diabetic foot”, is the leading cause of adult non traumatic limb amputation and it is 30 times higher than in age-matched healthy adults. Peripheral vascular disease followed by peripheral neuropathy and microvascular damage, with loss of both pain sensation and neurogenic inflammatory responses and infection leads to gangrene. Nonenzymatic glycosylation of lens

protein precedes the premature cataracts development in diabetic patients. Diabetic nephropathy develops with the progression of diabetes. It is characterized by proteinuria, and urea and creatinine accumulation in blood (Cade 2008). Complex interaction of hyperglycemia, hyperlipidemia, oxidative stress, accelerated aging, hyperinsulinemia and/or hyperproinsulinemia, and changes in coagulation and fibrinolisis are major events that affect development of atherosclerosis in diabetes.

Severity of complications increases with the duration of diabetes. Early detection and identification of new underlying factors that may contribute to endothelial dysfunction and ultimately micro- and macrovascular complications in diabetic patients is vital.

2.3. The role of the endothelium and endothelial dysfunction in the cardiovascular complications

2.3.1. Endothelial function

Maintaining proper endothelial function is an important factor that prevents development of vascular complications in diabetes mellitus. The endothelium is the active single cell layer that lines the lumen of blood vessels. It forms a barrier between circulating blood in the lumen and the rest of the vessel wall and body tissues. It was previously believed that the endothelium presents only a physical semipermeable barrier between the blood and interstitial layer performing the exchange of water and small molecules (Sharma, Bernatchez, and de Haan 2012). It was recently discovered that the endothelium has a complex role in cardiovascular homeostasis coordinating metabolic, synthetic and regulatory pathways (Cines et al. 1998).

Through synthesis of various bioactive factors in physiological and pathological conditions, the vascular endothelium is responsible for the regulation of vascular tone and blood pressure, blood fluidity, inflammatory activity and cell proliferation (Figure 2.1). In addition, some of the bioactive factors produced by the endothelium affect the activity of other cell types, such as the vascular smooth muscle cells (VSMC's), leukocytes, platelets, renal mesangial cells, retinal pericytes and macrophages (Hadi and Suwaidi 2007, Cubbon, Rajwani, and Wheatcroft 2007, Libby 2002). Under healthy conditions a balance exists between endothelial cell damage and repair. In both experimental diabetic animal models and humans with diabetes, an imbalance in repair and injury has been reported (van den Oever et al. 2010). It is believed that altered endothelial function and loss of modulatory role that can occur as a result of an imbalance in repair and injury may initiate macro- and microvascular complications in diabetes (Cubbon, Rajwani,

and Wheatcroft 2007, Libby 2002, Goldschmidt-Clermont et al. 2005). Previous research showed endothelial cell apoptosis as a significant contributor in the development of early lesions (Behl et al. 2009).

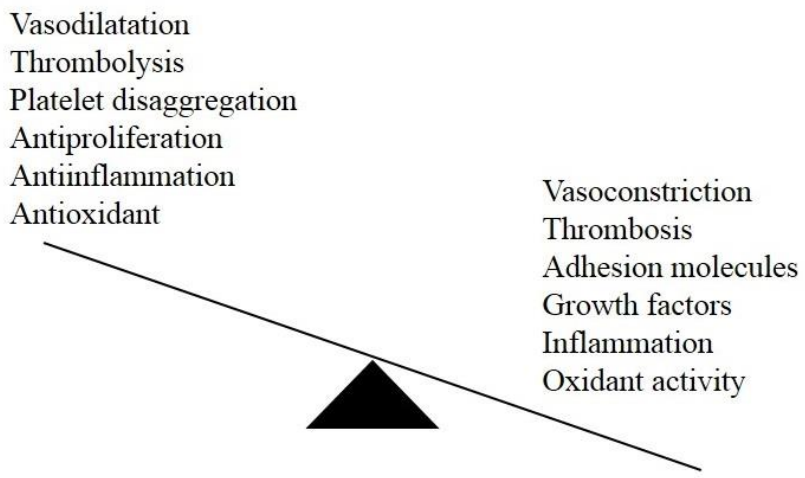

Figure 2.1 Regulatory functions of endothelium. Normal or antiatherogenic vs dysfunction or atherogenic properties (Adapted from Esper et al. 2006).

In order to control vascular homeostasis, numerous regulatory bioactive substances require synthesis by endothelial cells. These cells are also capable of sensing alterations in blood constituents and may respond directly to such changes or transmit reactive signals in a paracrine fashion to nearby cells (Brutsaert 2003). Regulation of vascular tone and vascular pressure requires a balanced release of vasoconstrictors such as endothelin-1 (ET-1), prostaglandins and angiotensin II (ANG-II), and vasodilators such as nitric oxide (NO) and prostacyclin (PGI2), by endothelial cells (Tan et al. 2002). When altered endothelial function exists, the balance is moved towards increased synthesis of vasoconstrictors. In addition, the vascular endothelium regulates blood fluidity and restoration of vessel wall integrity to avoid bleeding, by supporting anticoagulant, antiatherosclerotic and antithrombotic pathways (Haller 1997). Fibrinolysis is regulated by endothelial production of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1), while deactivation of the coagulation cascade by thrombomodulin/protein C, heparin sulphate/antithrombin and tissue factor/tissue factor inhibitor interactions exists simultaneously (Cowan and Langille 1996). Endothelial cells also influence vascular remodeling through synthesis of promoters and inhibitors of VSMC growth and differentiation (PDGF –

platelet-derived growth factor and ANG-II – angiotensin II) (Cowan and Langille 1996). Lastly, endothelial cells promote anti-inflammatory activity through synthesis of adhesion molecules such as leukocyte adhesion molecule (LAM), intracellular adhesion molecule (ICAM), and vascular adhesion molecule (VCAM), which attract and capture inflammatory cells and reduce their inflammation potential (Tracy et al. 1997, Biegelsen and Loscalzo 1999).

2.3.2. Role of nitric oxide in endothelial function

Endothelial-derived nitric oxide (NO) is important mediator that is considered the most significant representative of endothelial function (Haller 1997, Furchgott and Zawadzki 1980). NO has vasodilatory, antiplatelet, antiproliferative, permeability-decreasing, anti-inflammatory and antioxidant properties (Kawashima 2004). NO may reduce inflammation through the inhibition of the nuclear factor κ B (NFκB) transcriptional regulation (Tan et al. 2002, Janssen-Heininger, Poynter, and Baeuerle 2000, Jansson 2007). NO inhibits leucocyte rolling and adhesion along with cytokine induced expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemotactic protein-1 (MCP-1) (Khan et al. 1996). NO is synthesized by the enzyme NO-synthase (NOS) through transformation of the amino acid l-arginine to l-citruline. Several isoforms of this enzyme exist in different tissues: NOS1 in brain, NOS2 or iNOS in macrophages, and NOS3 or eNOS in endothelial cells. Pulsatile blood flow and shear stress activate eNOS. In order to become active, eNOS needs to dissociate from its endogenous binding protein, caveolin-1 (Cav-1) (Alderton, Cooper, and Knowles 2001). In addition, eNOS can be activated through protein kinase B/AKT (PI3K/AKT) pathway, after phosphorylation at serine 1177 (Ming et al. 2002). NO regulates the diameter of blood vessels and maintains optimal blood flow. After synthesis NO diffuses to the vascular smooth muscle (VSM) and stimulates the enzyme guanylate cyclase to synthesize cyclic GMP (cyclic guanosine monophosphate) which, in turn, regulates cytosolic Ca^{2+} and activates relaxation of VSM (Loscalzo and Welch 1995). Decreased eNOS activity and NO bioavailability have an important impact on endothelial dysfunction and development of vascular complications (Sharma, Yu, and Bernatchez 2010).

2.3.3. Endothelial dysfunction

Dysfunctional endothelium has an important role in the pathogenesis of diabetic vascular disease. Changes in proliferation, barrier function, adhesion of other circulating cells, and sensitivity to apoptosis are present during endothelial dysfunction in diabetes mellitus (Lorenzi

and Cagliero 1991). Moreover, it is believed that diabetes mellitus has an effect on angiogenic and synthetic properties of endothelial cells (Favaro et al. 2008). Endothelial dysfunction further leads to a variety of pathological conditions such as atherosclerosis and heart failure (Cines et al. 1998, Brutsaert 2003). Numerous factors can contribute to endothelial dysfunction, such as excessive apoptosis of endothelium due to the downstream effects of high levels of glucose, fatty acids, and reactive oxygen species.

Oxidative stress is a major contributor to the decrease in endothelial NO production and may be caused by an increase in oxidant generation, a decrease in antioxidant protection, or a failure in the repair of oxidative damage (van den Oever et al. 2010). When the endothelium functions properly, it regulates the balance between production of prothrombotic and anthitrombothic components, and fibrynolitics and antifibrynolitics. In addition it controls cell proliferation and migration, leukocyte adhesion and activation, and immunological and inflammatory processes (Esper et al. 2006). When endothelial function is disrupted, the capacity to manage one or more of these functions is also impaired. Reduced anticoagulant effects, increased adhesion molecule expression, cytokine release and reactive oxygen species production in the endothelium play an important role in the development of atherosclerosis. Failure of endothelial function enables the endothelium to be invaded by lipids and leukocytes, which initiate the inflammatory response that leads to fatty streak, the initial step in atherosclerosis plaque formation (Esper et al. 2006). With time, atherosclerosis plaque accumulates and the possibility of rupture increases that may lead to thrombogenesis and vascular occlusion. It is known that increased glucose concentration contributes to apoptosis and endothelial dysfunction is present in several conditions, such as diabetes, metabolic syndrome, hypertension and physical inactivity. However, the exact mechanism as well as cause of endothelial dysfunction is not completely explained yet.

2.4. Carbohydrate metabolism

Regulation of blood glucose levels depends on the tight balance between the rate of glucose entering the circulation and glucose utilization. The amount of glucose entering the circulating blood depends on carbohydrate dietary intake and internal glucose production through glycogenolysis and gluconeogenesis during fasting. Once it enters the blood, glucose is transported into cells via various pathways. Within cells glucose may undergo further conversion to and storage as glycogen or undergo glycolysis or glucose may be transported back out into the

circulating blood by the liver and kidneys (enzyme glucose-6-phosphatase necessary for the discharge of glucose into the blood is located in these organs) (Aronoff et al. 2004).

In the healthy body, blood glucose stays within a narrow range and is tightly controlled by various hormones. Two main glucoregulatory hormones are insulin and glucagon. In addition, other hormones such as amylin, glucagon-like peptide 1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), catecholamines, growth hormone, thyroid hormone and glucocorticoids influence glucose homeostasis (Aronoff et al. 2004). Insulin and amylin are produced in beta cells of the pancreas; glucagon is derived from alpha cells of the pancreas, while GLP-1 and GIP originate from the L-cells in the intestine. Insulin and amylin, GLP-1 and GIP lower blood glucose levels, while glucagon, catecholamines, growth hormone, thyroid hormone and glucocorticoids act the opposite way and increase blood glucose levels (Aronoff et al. 2004).

During short term fasting (8-12h), glucose is produced by glycogenolysis, degradation of glycogen in the liver under the influence of glucagon. In long term fasting, glucagon stimulates glucose production through gluconeogenesis (Unger 1971). After a meal, GLP-1 and GIP release is stimulated in intestine. Their release results in insulin release from pancreas. Early insulin release keeps the blood glucose levels constant and prevents hyperglycemia (Yabe and Seino 2011). After dietary glucose absorption from the gastrointestinal tract (GI) blood glucose levels rise. This additionally stimulates release of insulin from beta cells in the pancreas. Insulin stimulates glucose utilization in insulin sensitive tissues, such as muscle, liver and adipose tissue. Insulin initiates uptake of glucose by muscle, promotes glycogenesis in liver, and inhibits secretion of glucagon from alpha cells of the pancreas. In addition, it activates fat synthesis, triglyceride deposition in fat cells, and stimulates cell growth (Gerich et al. 1974). All these actions lead to reduced glucose levels in the circulating blood. Amylin works simultaneously with insulin and keeps the normal glucose level by two mechanisms. It inhibits postprandial glucagon secretion and slows the rate of gastric emptying, hence the speed at which nutrients are transported from the stomach to intestine for absorption (Gedulin, Rink, and Young 1997, Samsom et al. 2000).

2.4.1. Metabolism of glucose

Once glucose enters the cell, it undergoes glycolysis. Glycolysis is a metabolic pathway that converts glucose into pyruvate. The free energy produced during this pathway is in the form of the high-energy compounds ATP (adenosine triphosphate) and NADH (reduced nicotinamide adenine dinucleotide). Under aerobic conditions, pyruvate dehydrogenase (PDH) metabolizes

pyruvate to acetyl-CoA. Acetyl-CoA is a substrate for the TCA cycle (tricarboxylic acid cycle) where energy in the form of ATP is produced. In anaerobic conditions, lactate dehydrogenase catalyzes interconversion of pyruvate to L-lactate (Figure 2.2). During fasting, pyruvate can be converted back to glucose as a substrate for gluconeogenesis (Berg, Tymoczko, and Stryer 2002).

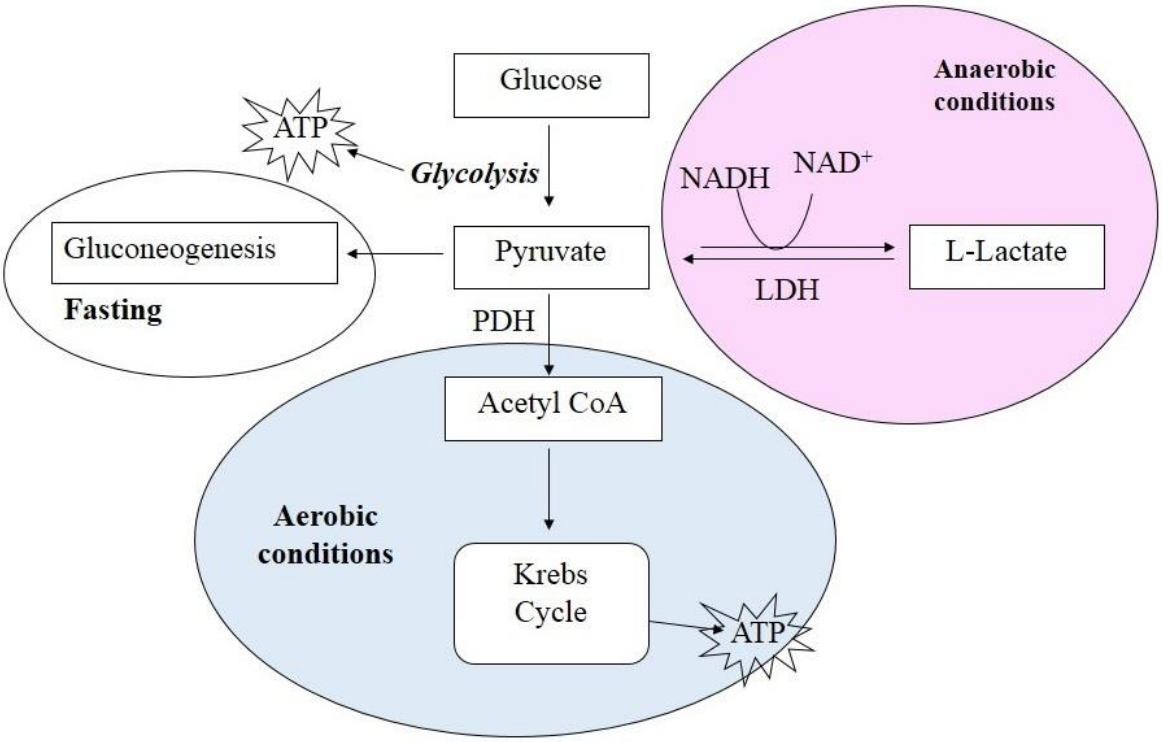

Figure 2.2 Schematic presentation of glucose metabolism in the body in aerobic and anaerobic conditions. PDH: pyruvate dehydrogenase; LDH: lactate dehydrogenase; ATP: adenosine triphosphate; NADH/NAD^+: nicotinamide adenine dinucleotide.

2.4.2. Carbohydrate metabolism in diabetes

Carbohydrate metabolism is dysfunctional in diabetes mellitus. Improper function of pancreatic islets, as well as altered insulin sensitivity of peripheral tissues is present in diabetes. In addition, failure in suppression of postprandial glucagon secretion resulting in increased catabolism and hepatic glucose synthesis also exists in diabetes. Consequently, rise of postprandial glucose concentrations due to deficit in insulin synthesis, altered hepatic glucose production, and irregular gastric emptying after a meal is present in diabetes. All of these result in high free glucose levels in circulating blood (Aronoff et al. 2004).

A portion of the excess glucose undergoes metabolism by glycolysis to pyruvate, as previously described (Chapter 2.4.1.). The remainder undergoes metabolism via the methylglyoxal pathway. In contrast to glycolysis, the methylglyoxal pathway does not produce ATP as a final product. Methylglyoxal is produced from dihydroxyacetone phosphate (DHAP), an intermediate of glycolysis, by the enzyme methylglyoxal synthase. Methylglyoxal is further metabolized to D-lactate via the intermediate *S*-D-lactoylglutathione by the enzymes glyoxalase I and glyoxalase II. On the other hand, methylglyoxal reductase and aldehyde dehydrogenase convert methylglyoxal to L-lactate via the intermediate lactaldehyde. Both D- and L-lactate are further metabolized to pyruvate through the mitochondrial enzyme D- and L-lactate dehydrogenase, respectively (Figure 2.3) (Thornalley 1990, Weber, Kayser, and Rinas 2005).

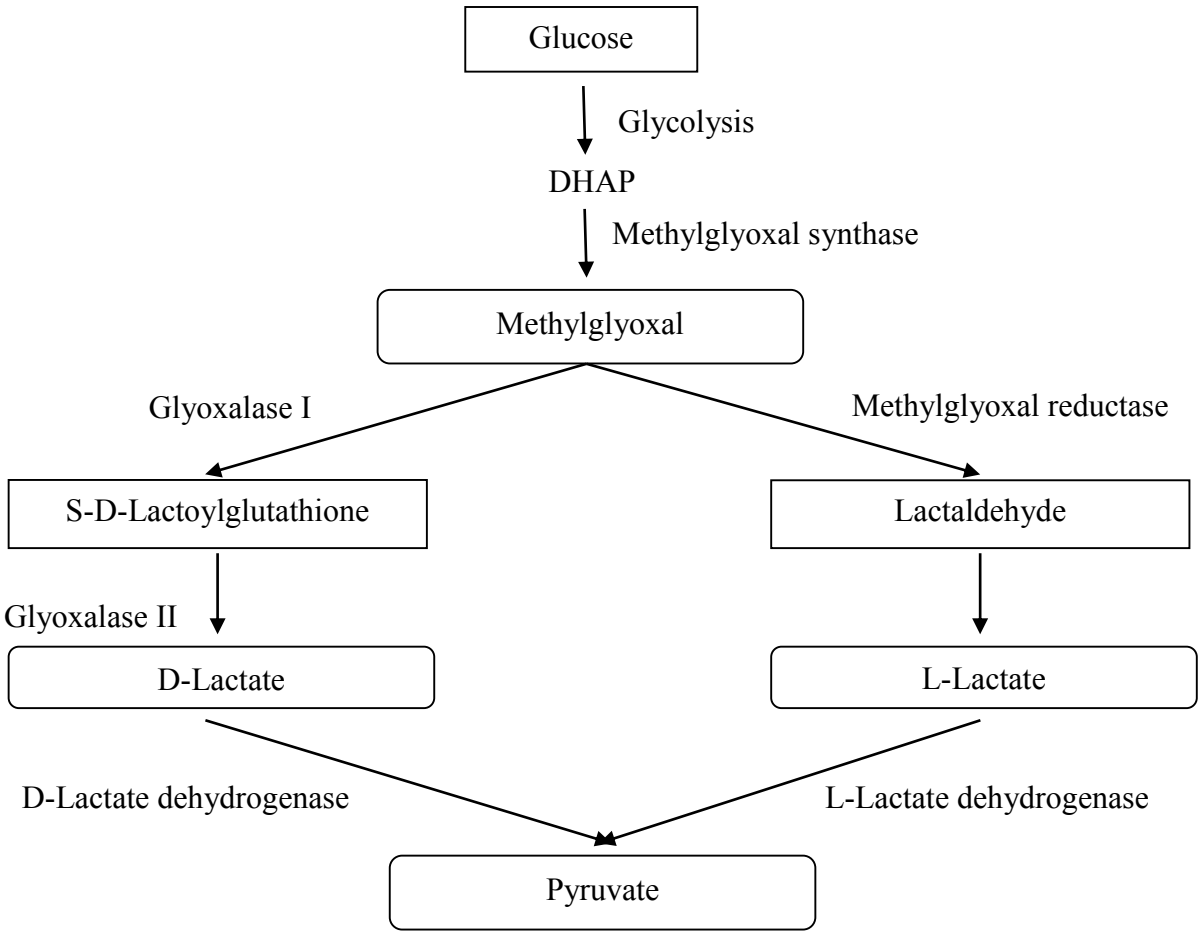

Figure 2.3 Schematic presentation of methylglyoxal pathway.

2.4.2.1. Glucose metabolism byproduct methylglyoxal

Many molecular and cellular factors have been studied for their contribution to vascular endothelial dysfunction associated with diabetes. One candidate, methylglyoxal (MG), a reactive alpha-dicarbonyl, has been identified as an important contributor to vascular complications of diabetes following investigations using animal and human vascular endothelial cells (Bourajjaj et al. 2003), although the exact role played by MG is not clear. MG is produced from various sources in the body, such as glucose, acetone and amino acids. Production of MG from glucose is previously described (Chapter 2.4.2). Acetone is metabolized by cytochrome P450 2E1 via an acetol intermediate and utilizes NADPH + H^+, while amino acids are metabolised by amine oxidase(s). MG is considered toxic and may cause oxidative stress and glycation processes in tissues. The reactive and toxic nature of MG requires the body to eliminate it through the glyoxalase system (Chapter 2.4.2.) (Kalapos 1999).

Increased production of MG in hyperglycemic conditions is associated with elevated D-lactate production in diabetes mellitus (McLellan et al. 1994), and both of these glucose metabolites may contribute to metabolic derangements and possibly to endothelial dysfunction (Oh et al. 1985). In addition, the concentrations of D-lactate are also positively correlated with glucose and HgA1c levels in diabetic patients (McLellan et al. 1994). It is previously mentioned that MG can be produced from ketone bodies and it is suggested that increased D-lactate production is associated with ketoacidosis rather than hyperglycemia (Christopher et al. 1995). Being an end product of the methylglyoxal pathway, D-lactate is considered non-toxic. However, D-lactate effects on the development of diabetic complications have not been investigated. To date, no reports have linked D-lactate (or its related metabolic intermediates including MG, L-lactate and pyruvate) with diabetic complications or in predicting diabetic vascular damage.

2.4.2.2. D-Lactate in diabetes: metabolism and toxicity

Lactate (2-hydroxypropanoate) is a hydroxycarboxylic acid and can be found in the human body as two stereoisomers (Figure 2.4), L-lactate and D-lactate (Ewaschuk, Naylor, and Zello 2005). Normal serum lactate concentrations are 1-2 mmol/L, where the majority is in the form of L-lactate. D-Lactate is present in the human body in small amounts, only 1%-5% relative to L-lactate (McLellan, Phillips, and Thornalley 1992). L-Lactate is formed in the body from pyruvic acid in the process of anaerobic glycolysis while D-lactate is formed through the glyoxalase

pathway from methylglyoxal. In addition, lactate may originate from exogenous sources such as sauerkraut, yogurt, pickles or certain medications (Ewaschuk, Naylor, and Zello 2005, Christopher, Eckfeldt, and Eaton 1990). GI lactate is produced by bacteria of the gastrointestinal tract (lactobacilli and bifidobacteria). D-lactate is normally found at low levels in healthy patients (Ewaschuk, Naylor, and Zello 2005).

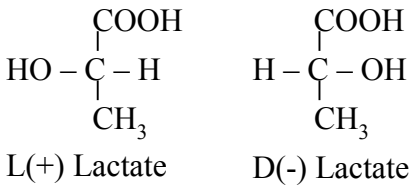

Figure 2.4 Chemical structure of L- and D-lactate.

Elevated lactate levels in the body lead to a pathologic condition known as lactic acidosis. D-Lactate acidosis is a metabolic acidosis; a condition characterized by increased serum D-lactate levels above 3 mmol/L (Uribarri, Oh, and Carroll 1998). D-Lactic acidosis causes severe neurotoxicity and encephalopathy, leading to neurological symptoms such as ataxia, slurred speech, and coma (McLellan, Phillips, and Thornalley 1992, Uribarri, Oh, and Carroll 1998). The mechanism of D-lactate neurotoxicity is not known yet. This metabolic condition is rare in humans, but is sometimes noted in patients with short bowel syndrome (SBS). These patients have increased gastrointestinal fermentation of carbohydrate by lactobacilli as well as impaired ability of the body to properly eliminate D-lactate (Ewaschuk, Naylor, and Zello 2005).

Previous research showed increased D-lactate concentration in the blood of diabetic animals and humans. Various reports of D-lactate concentration in diabetic patients exist. The D-lactate levels reported in diabetic patients vary from 0.028 (Hasegawa et al. 2003) up to 0.47 mM. (Lu et al. 2011). However, significantly higher D-lactate levels of 3.8 mM were reported in patients with diabetic ketoacidosis (Lu et al. 2011).” ncreased serum D-lactate is associated with increased production through the methylglyoxal pathway. Ketoacidosis and ketone metabolism by hepatic cytochromes P450 enzymes (CYP450s) are suggested to be a main source of methylglyoxal in diabetic patients (Christopher et al. 1995). Increased D-lactate production in tissues with insulin independent glucose uptake is present in hyperglycemic conditions. In addition, methylglyoxal levels are significantly higher in plasma, while being lower in liver and muscles of starved diabetic animals, compared to healthy individuals (Kondoh et al. 1992). Also, enzymes involved in

methylglyoxal metabolism, such as aldose reductase, glyoxalase I, and glyoxalase II, are elevated in diabetic patients (Ratliff et al. 1996).

Under normal physiological conditions (non-diabetic states), D-lactate can be efficiently metabolized by hepatic mitochondrial D-lactate dehydrogenase (DLD) to pyruvate, which is then used for further energy metabolism (Oh et al. 1985, Hove and Mortensen 1995, Ewaschuk, Naylor, and Zello 2005). Therefore, this pathway has been proposed as a detoxification pathway to reduce MG levels. It was believed that the liver enzyme D-α-hydroxy acid dehydrogenase metabolizes D-lactate to pyruvate at one-fifth the rate at which L-lactate dehydrogenase (L-LDH) metabolizes L-lactate (Beisswenger et al. 1999). Previously thought to be only in lower organisms, mitochondrial D-lactate dehydrogenase (EC 1.1.1.28) has been recently discovered in higher organisms. It metabolizes D-lactate to pyruvate and can be found in heart, skeletal muscle, liver and kidney. D-Lactate dehydrogenase is located on the inner side of inner mitochondrial membrane. In order to be metabolised, D-lactate needs to be transferred from the cytosol to the mitochondrial matrix via three transporters: the D-lactate/H^+ symporter, the D-lactate/oxoacid antiporter and the D-lactate/malate antiporter (Flick and Konieczny 2002, de Bari et al. 2002). D-Lactate is extensively metabolised in the body (~90%) and only a small amount is excreted in urine (~10%) (Oh et al. 1985). However, the increases in MG levels in DM2 may overwhelm the clearance of blood D-lactate concentrations in diabetic patients as D-lactate levels are significantly higher when compared to those without diabetes (Beisswenger et al. 1999, Talasniemi et al. 2008, Lu et al. 2011). Such elevations in D-lactate have not been considered as a significant contributor to diabetic complications since it does not cause acute toxicity and D-lactate is efficiently eliminated in healthy individuals. It is unknown if the capacity to metabolize and eliminate D-lactate in DM2 is compromised. However, D-lactate infusion at high levels saturate hepatic D-lactate metabolism and result in a prolonged half life in healthy humans (de Vrese, Koppenhoefer, and Barth 1990).

D-Lactate plasma membrane transport is mediated by the proton-dependent monocarboxylate transporters (MCTs) (Enerson and Drewes 2003). MCTs are expressed ubiquitously in the body including retina, muscle, kidney, brain capillary endothelial cells, cardiac myocytes, enterocytes, erythrocytes, hepatocytes, placenta and nervous tissue (Enerson and Drewes 2003). D-lactate is absorbed by epithelial MCT1 in small intestine and colon (Ding and Xu 2003, Preston and Noller 1973). MCTs are responsible for the transport of other important molecules including pyruvate, L-lactate and short chain fatty acids (SCFA), which are vital

substrates for cellular bioenergetics (Garcia et al. 1994, Poole et al. 1990). Consequently, D-lactate can competitively inhibit the transport of these important cellular energy substrates via MCTs at the cellular level. In fact, D-lactate inhibits L-lactate uptake into the erythrocytes and pyruvate into cardiac myocytes (Poole et al. 1990, Trosper and Philipson 1987, Ros et al. 2001). MCTs are also involved in mitochondrial transport of energy substrates for ATP. Interestingly, intracerebral D-lactate exposure causes energy deficiency in the brain and memory loss in chickens (Gibbs and Hertz 2008). Furthermore, interferences of D-lactate with pyruvate and/or L-lactate reduce brain cell survival (Ros et al. 2001).

2.5. Apoptosis and possible underlying mechanism of endothelial dysfunction

2.5.1. Apoptosis

Under normal physiological conditions, the balance between cell proliferation and cell apoptosis is tightly maintained to ensure correct cellular development and function (Dowsett et al., 1999). Apoptosis is an actively regulated process of cell suicide that is morphologically and biochemically distinct from necrosis. It involves individual cells and requires energy to occur. Apoptosis is characterized by a series of biochemical and morphological changes in cells, such as blebbing, cell shrinkage, nuclear fragmentation, and chromatin condensation (Strasser, O'Connor, and Dixit 2000). On the contrary, necrosis is a process of traumatic cell death that causes destruction of tissues and organs through uncontrolled cell lysis and can lead to serious health problems (Alison and Sarraf 1994). Apoptosis is a crucial physiological process and continues throughout the life of all multicellular organisms (embryonic development, adult tissue homeostasis, removal of damaged or infected cells) (Vaux and Korsmeyer 1999). Uncontrolled apoptosis can result in inappropriate cell functions that ultimately lead to disease development and progression (i.e. tumor development, neurodegenerative diseases) (van den Oever et al. 2010). Increased apoptosis of pancreatic beta cells and vascular endothelial cells can be found in diabetes (Robertson, 2004).

Apoptosis can be initiated by extrinsic and intrinsic signals. Extrinsic signals are usually toxins, hormones, growth factors, nitric oxide or cytokines (Popov et al. 2002, Brune 2003). In order to initiate response, these signals initiate binding of the death inducing ligand for the death receptors located on the outer cell membrane (van den Oever et al. 2010). Apoptosis can also be triggered by intrinsic signals as a response to a cellular stress. Nutrient deprivation, hypoxia,

oxidative stress, chemicals, heat, radiation, etc. can trigger intrinsic response in damaged cells (Kroemer 2003). In addition to the severity of the stimulus, the balance of pro- and anti-apoptotic proteins in the cell, as well as the stage of the cell cycle will determine whether the cell undergoes apoptosis. In order for apoptosis to occur, apoptotic signals must induce regulatory proteins to initiate the apoptosis pathway.

2.5.1.1. Extrinsic apoptosis pathway

Fas ligand, TNFα and TRAIL (TNF related apoptosis inducing ligand) are three essential death inducing ligands. Their connection to the specific death receptor on the cell membrane activates apoptotic signals in the cell, which results in caspase cascade activation. After death ligand/receptor binding, generation of ceramide starts. Ceramide further stimulates clustering of death receptors through lipid raft fusion, which enables exposure of the death domain of the receptor and results in an increase of the apoptotic signaling (Carpinteiro et al. 2008). This structural rearrangement is known as the death inducing signaling complex (DISC). DISC activates transformation of procaspase 8 into caspase 8 that activates execution of apoptosis (Khosravi-Far and Esposti 2004, van den Oever et al. 2010) (Figure 2.5).

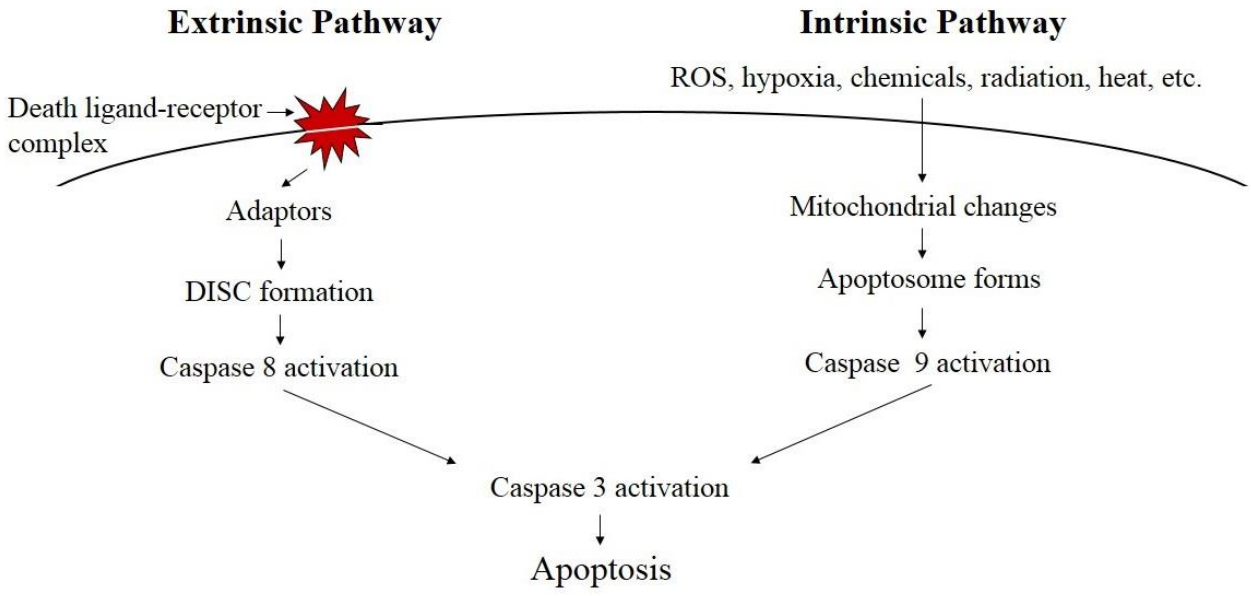

Figure 2.5 Schematic presentation of the two main pathways of apoptosis, extrinsic and intrinsic pathway (Adapted from (Elmore 2007)).

2.5.1.2. Intrinsic apoptosis pathway

Cellular stress initiates mitochondrial discharge of proteins involved in the intrinsic apoptosis pathway. Mitochondrial discharge of both pro- (Bad, Bax, Bid) and anti-apoptotic (Bcl-2, Bcl-XL) proteins occurs after cellular damage occurs (Tan et al. 2006, Yang et al. 2008). During cellular stress or damage, proapoptotic Bcl-2 proteins, which are normally found in cytosol, travel to the surface of mitochondria where antiapoptotic proteins are normally located. This process initiates development of permeability transition pores (PTP) in mitochondrial membranes (Zamzami et al. 1998). Once formed, PTP enable release of mitochondrial proapoptotic proteins such as Apoptosis Inducing Factor (AIF), Smac/DIABLO, and cytochrome C. Once released into cytosol, these proteins generate development of the apoptosome and activation of caspase cascade (Kluck et al. 1997, Nakagami et al. 2001) (Figure 2.5).

2.5.1.3. Caspase cascade and execution of apoptosis

Caspases are cysteine-dependent aspartate proteases and belong to a family of cysteine proteases that are crucial in the execution of apoptosis. The caspases involved in apoptosis are caspase 2, 3, 6, 7, 8, 9 and 10. After initiation of apoptosis by extrinsic signals, DISC activates upstream caspases (caspase 2, 8, and 10). Caspase 8 and 10 further activate executioner caspase 3, 6 and 7, which initiate cleavage of proteins involved in programmed cell death. In addition, caspase 8 and 10 activate pro-apoptotic protein BID that stimulate release of mitochondrial cytochrome C and formation of the apoptosome. In addition, formation of the apoptosome and cytochrome C release occurs during intrinsic signals initiation. Cytochrome C activates caspase 9, which further activates downstream executioner caspase 3 and 7 (Janicke et al. 1998, Khosravi-Far and Esposti 2004, Thornberry et al. 1997).

2.5.1.4. Apoptosis in diabetes

An accumulation of partly damaged and dysfunctional cells is likely expected in the vasculature during diabetic conditions. It could rapidly lead to severe defects in the regulation of endothelial function. High glucose concentrations found in diabetic patients trigger cellular apoptosis (Baumgartner-Parzer et al. 1995). It was previously reported that D-lactate interference with pyruvate and/or L-lactate reduces brain cell survival (Ros et al. 2001). In addition, intracerebral D-lactate exposure causes energy deficiency in the brain and memory loss in chickens (Gibbs and Hertz 2008). Increased apoptosis of endothelial cells and disturbed endothelial function

correlates with an impaired thromboresistance and vasodilation of the vessel wall and may be one significant risk factor for diabetic vascular complications (Risso et al. 2001).

2.5.2. Mitochondria in endothelium

In many cell types mitochondria are small organelles in the cytosol which play an important role in cell energy production via the electron transport chain (Bayir and Kagan 2008). In endothelial cells, mitochondria account for only 5% of the cell volume (Blouin, Bolender, and Weibel 1977). Endothelial cells obtain energy through non-mitochondrial pathways, primarily through glycolytic metabolism of glucose. It is believed that 99% of the glucose in endothelial cells is metabolized into L-lactate through anaerobic glycolysis, which significantly minimizes endothelial oxygen consumption (Mertens et al. 1990, Krutzfeldt et al. 1990). As a result, mitochondrial function was not considered significant in endothelial cells until recently, when its signaling function was discovered. Endothelial mitochondria act as important sensors of the local environment through regulation of the production of reactive oxygen species (ROS), nitric oxide (NO), and calcium (Ca^{2+}) (Davidson and Duchen 2007). These mediators regulate proliferation, barrier function, inflammatory response, vasorelaxation, and vascular remodeling. In addition to cell signaling, mitochondrial functional and morphological changes can induce cell apoptosis (increase in mitochondrial ROS production, mitochondrial fission, mitochondrial calcium overload, and the opening of mPTP) (Pangare and Makino 2012).

2.5.2.1. Mitochondrial ROS production

ROS are produced regularly in all cells as a side product during mitochondrial energy production by the electron transport chain. Electron transport chain consists of four protein complexes located on mitochondrial inner membrane. Complex I (NADH-ubiquinone oxidoreductase) and complex II (succinate dehydrogenase) reduce ubiquinone (coenzyme Q), which transfers electrons to complex III (ubiquinol-cytochrome c oxidoreductase). Cytochrome C transports pairs of electrons to complex IV. Complex IV (cytochrome c oxidase) is used for reduction of molecular oxygen to water. During this process, one part of oxygen (1-4%) is partially reduced to superoxide, which further initiates formation of other ROS. Superoxide (O_2^-) is formed at complexes I and III and can be distributed into the mitochondrial matrix and inter- membrane space, as well as transported into cytosol (Zhang and Gutterman 2007). Superoxide anion can be further metabolized to hydrogen peroxide (H_2O_2) by various superoxide dismutases (SOD),

including manganese SOD (Mn-SOD) in the mitochondrial matrix and copper/zinc SOD (CuZn-SOD) in the cytosol and intermembrane space (Okado-Matsumoto and Fridovich 2001). Further on, matrix enzyme glutathione peroxidase (GPX) converts H_2O_2 into water and molecular oxygen using glutathione (Rhee et al. 2005). Mitochondrial ROS production leads to disruption of mitochondrial membrane potential and further release of pro/anti apoptotic factors and lead to caspase cascade activation and initiation of apoptosis. Mitochondria are not the only source of ROS, and mitochondrial ROS initiate ROS production from other sources in the cell. Superoxide anion contributes to formation of nitrosylation byproducts. Superoxide anion reacts with NO and forms peroxynitrite. Superoxide anions, peroxynitrite and other ROS are potent chemical oxidants.

2.5.2.2. Role of calcium in mitochondria

Calcium regulates important mitochondrial enzymes and changes in mitochondrial Ca^{2+} levels cause disturbances in mitochondrial function. Extracellular stimuli initiate release of Ca^{2+} from endoplasmic reticulum (ER) where Ca^{2+} is normally stored, which then enters mitochondria (Lawrie et al. 1996). In pathophysiological conditions, increase in mitochondrial Ca^{2+} alters mitochondrial function and leads to fragmentation of mitochondria. High levels of intramitochondrial Ca^{2+} induce mPTP opening and cell death (Pangare and Makino 2012). Mitochondria regulates VSMC by keeping the balance between Ca^{2+}, NO and ROS. Shear stress causes ROS release from endothelial mitochondria. Mitochondrial ROS cause Ca^{2+} increase in mitochondria and activation of NF-κB. Initiated by shear stress, NF-κB and increased Ca^{2+} levels activate eNOS to synthesize NO (Donato et al. 2009). mNOS activation presents a negative feedback on increased mitochondrial Ca^{2+} levels and it has a protective role. As a protective mechanism, NO inhibits the mitochondrial respiration and reduces the mitochondrial membrane potential ($\Delta\psi_m$) (Dedkova et al. 2004). In addition, increase in Ca^{2+} contributes to increased ATP production (Lawrie et al. 1996).

2.5.2.3. Mitochondrial fission and fusion

In normal physiological conditions a balance between mitochondrial fission and fusion exists. Mitochondrial fission is the separation of mitochondrion in the cell to form two or more separate compartments, while mitochondrial fusion is merging of mitochondria in the cell to form a single compartment. A balance in the fusion/fission dynamics is important for proper cell function. In cells that undergo apoptosis, the balance is moved in favor of mitochondrial fission

(Pangare and Makino 2012). It was previously reported that hyperglycemic environment increase mitochondrial fission (Wang et al. 2012). In addition, increased Ca^{2+} level in mitochondria decreases mitochondrial membrane potential and initiates mitochondrial fission (Pangare and Makino 2012). Mitochondrial fragmentation is elevated by excess ROS production in pathophysiological conditions.

2.5.2.4. Mitochondria and energy production

Endothelial mitochondria have high reserve capacity. Substrate depletion causes decreased energy production through glycolysis in endothelial cells. As a response mechanism, energy is produced through mitochondrial oxidative phosphorylation using fatty acids as a substrate (Dagher et al. 2001). Decreased ATP levels in the cell activate AMPK (AMP-activated protein kinase) that further triggers endothelial cells to switch ATP production from non-oxidative (glycolysis) to mitochondrial oxidative pathways (oxidative phosphorylation) (Dagher et al. 2001). In conditions such as diabetes, where normal energy production through glycolysis is interrupted, mitochondria may become significant energy source (Davidson and Duchen 2007).

2.5.3. Energy homeostasis of vascular endothelial cells

One potential mechanism that may contribute to apoptosis is disturbance in energy homeostasis. Endothelial cells provide energy through anaerobic glycolysis and utilization of L-lactate (Krutzfeldt et al. 1990). ATP concentrations in endothelial cells can be affected if there is an interruption in glycolysis due to substrate depletion. Ensuring energy substrate supply to the vascular endothelial cells will be essential to maintain ATP levels in the system.

Decreases in cellular ATP content can proceed towards apoptosis (van den Oever et al. 2010). In fact, vascular cell apoptosis is a significant contributor to diabetes complications in vascular system (van den Oever et al. 2010). Apoptosis has been observed in vascular cells, and myocardium of diabetic humans and experimental animals although whether it contributes to or is a marker of complications in these tissues is unclear. Incubation of human umbilical vein endothelial cells (HUV-EC-Cs) with high levels of glucose also caused a significant increase in apoptosis (Ido, Carling, and Ruderman 2002). Interestingly, D-lactate has been related to cellular apoptosis in other tissues including liver and lung. For instance, resuscitation with racemic lactated Ringer's solution induces cellular apoptosis in rat liver, which may be related to the D-isomer

(Jaskille et al. 2006). Also, D-lactate increases pulmonary apoptosis by restricting phosphorylation of BAD and eNOS in a rat model of hemorrhagic shock (Jaskille et al. 2004).

AMP-activated protein kinase (AMPK) is a serine/threonine protein kinase that acts as a sensor of cellular energy status, regulating metabolism of cells and organisms (Zou and Wu 2008). AMPK is activated in response to various environmental stressors to restore cellular and whole body energy balance, influencing their survival. Changes in AMP/ATP ratio determine whether an AMPK activity is in favour of anabolism or catabolism. AMPK is activated by high levels of substrate adenosine monophosphate (AMP) under energetic stress including nutrient deprivation (Bungard et al. 2010). AMPK activation initiates a program of metabolic adaptation to preserve cellular energy via ATP production and maintain cellular viability. Once activated, AMPK inhibits acetyl CoA carboxylase and fatty acid synthesis. In addition, AMPK has a protective role in response to oxidative stress. Different physiological stimuli, decreased energy production, and oxidants increase AMPK activity, leading to the conclusion that AMPK tends to protect cells from apoptosis (Ido, Carling, and Ruderman 2002, Zou and Wu 2008).

2.5.4. Production of reactive oxygen species

Another potential mechanism that may contribute to apoptosis is production of reactive oxygen species (ROS). ROS are chemically reactive molecules containing oxygen, usually oxygen ions and peroxides. They are potent agents, which initiate oxidation and damage of other physiologically relevant molecules in the body. ROS are regularly produced in the body as a natural byproduct of the metabolism of oxygen and have important role in cell signaling and homeostasis (Szocs 2004). In the cardiovascular system, ROS modify smooth muscle cell growth and motility by oxidizing cysteine residues of target proteins, such as transcription factors and protein phosphates (Chiarugi and Cirri 2003). Under pathological conditions, excessive ROS production leads to the damage of cells and tissues, a condition known as oxidative stress. Oxidative stress has been associated with the progression of many cardiovascular diseases, such as diabetes mellitus, atherosclerosis, coronary artery disease, and hypertension, through the development of endothelial dysfunction (Kaneto et al. 2010, Rocha et al. 2010).

2.5.4.1. ROS production and antioxidative defense

Increased production of ROS or failure in intracellular antioxidative mechanisms results in a development and progression of various diseases. Nonenzymatic glycosylation reactions,

electron transport chain in mitochondria, and membrane bound NADPH oxidase are defined as intracellular sources of ROS (Basta, Schmidt, and De Caterina 2004, Sauer, Wartenberg, and Hescheler 2001). In diabetic patients, high glucose levels initiate nonenzymatic glycation reactions in different tissues and organs, and numerous proteins such as glycosylated hemoglobin, albumin and lens crystalline. During the glycation reaction ROS are produced as a byproduct (Basta, Schmidt, and De Caterina 2004). In diabetics, glucose metabolism is disturbed and energy production is compensated through mitochondrial energy production (TCA cycle). Mitochondrial electron transport chain is involved and excessive amounts of ROS are generated (detailed mechanism described in the Chapter 2.5.2.1.). Another source of ROS is NADPH oxidase located on the cell membrane (Ushio-Fukai et al. 1996). It forms superoxide anion by transferring electrons from NADPH inside the cell across the membrane and coupling these to molecular oxygen. This enzyme is activated by different stimuli increasingly produced in diabetes, such as AGEs (advanced glycation end-products), insulin, and angiotensin II (Ushio-Fukai et al. 1996). In addition, NADPH oxidase appears to have an important role in the development of atherosclerosis in mice and humans (Kaneto et al. 2010).

In addition to antioxidative cellular enzyme defense (SOD and GPX, described in the chapter 2.5.2.1.), cells contain different DNA repair mechanisms that are responsible for repair of oxidant-induced defects of DNA. A tight balance between ROS production and antioxidative defense exists in the healthy body. Overproduction of ROS that exists under pathologic conditions or inadequate intracellular defense can initiate cellular dysfunction that leads to a disease. DM2 is associated with increased ROS production that exceeds the normal antioxidant defense mechanisms of the cell resulting in oxidative stress (Rachek et al. 2007).

2.5.4.2. ROS and cellular oxidative damage in diabetes

It was previously described that diabetic vascular complications occur due to oxidative stress. ROS reacts with vasoactive substances and alter their vasodilator effect. ROS may oxidize and damage DNA, proteins, and lipids that may directly lead to insulin resistance (Houstis, Rosen, and Lander 2006) and may contribute to endothelial dysfunction and late diabetic complications (Rosen et al. 2001). In addition to increased ROS production, antioxidant defenses may be lower in diabetes due to reduced activity of antioxidant enzymes (Laight, Carrier, and Anggard 2000). The endothelium is in direct contact with the circulating blood, hence in direct contact with toxic

substances in blood such as oxLDL (Oxidized low density lipoprotein), which can damage endothelium through oxidative processes.

Increased oxidative stress contributes to atherosclerosis in several ways. ROS adversely affects endothelial function through inactivation of endothelial nitric oxide synthase (eNOS) and decreased nitric oxide (NO) production (Madamanchi, Vendrov, and Runge 2005). Superoxide anion reacts with NO to form peroxynitrite radical ($ONOO^-$) that further contributes to oxidative stress. NO has important antioxidant activities in the vessel wall, including the direct scavenging of superoxide anion and the inhibition of lipid peroxidation (Pacher, Beckman, and Liaudet 2007). ROS induced expression of adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) result in inflammatory cell recruitment and lipid disposition in the intimal layer (Kaneto et al. 2010). Changes in NO, cytokines, acute-phase reactants and cellular adhesion molecules caused by hyperproduction of ROS precede atherosclerosis (Figure 2.6).

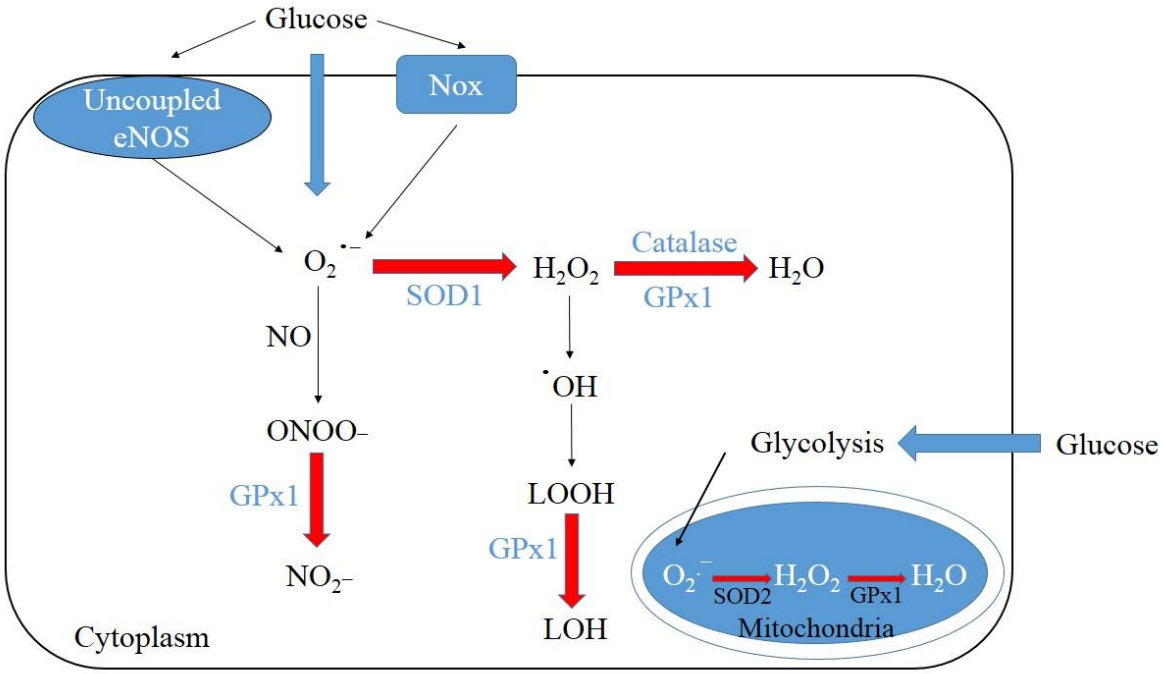

Figure 2.6 Antioxidant pathways for the neutralization of ROS produced by hyperglycemia in the cytoplasm and mitochondria of endothelial cells. Nox: NAD(P)H oxidase; $O_2^{\cdot-}$: superoxide anion; $ONOO^-$: peroxynitrite; NO_2^-: nitrite; H_2O_2: hydrogen peroxide; $^{\cdot}OH$: hydroxyl radicals; LOOH: lipid hydroperoxides; LOH: lipid alcohol. (Adapted from (Sharma, Bernatchez, and de Haan 2012))

2.5.5. PI3K Pathway

Whether a cell undergoes apoptosis depends on the tightly controlled balance between the levels and activation status of pro- and antiapoptotic proteins. An important pathway that has been associated with antiapoptotic signaling in different cell types is the Phosphoinositide Kinase-3 (PI3K) system (Fujio and Walsh 1999, Nor et al. 1999). A well-recognized downstream component of survival signalling through PI3K is the serine/threonine kinase, termed Akt, or protein kinase B (Akt/PKB). The PI3K/Akt pathway, in turn, exerts its antiapoptotic properties through multiple mechanisms; the most well described being the phosphorylation of certain key proteins, particularly BAD and endothelial nitric oxide synthase (eNOS). In its unphosphorylated state, BAD induces apoptosis through heterodimerization with key antiapoptotic proteins (eg, bcl-2), neutralizing their function. Once phosphorylated by the PI3K/Akt pathway, BAD protein is sequestered, allowing antiapoptotic proteins, such as Bcl-2, to exert their prosurvival effect. Another specific downstream target of the PI3K/Akt cascade is endothelial nitric oxide synthase (eNOS), which is also involved in apoptosis. Akt stimulates the phosphorylation and activation of eNOS, and formed nitric oxide promotes cellular survival by inhibiting caspases through nitrosylation (Choy et al. 2001, Fujio and Walsh 1999, Nor et al. 1999) (Figure 2.7).

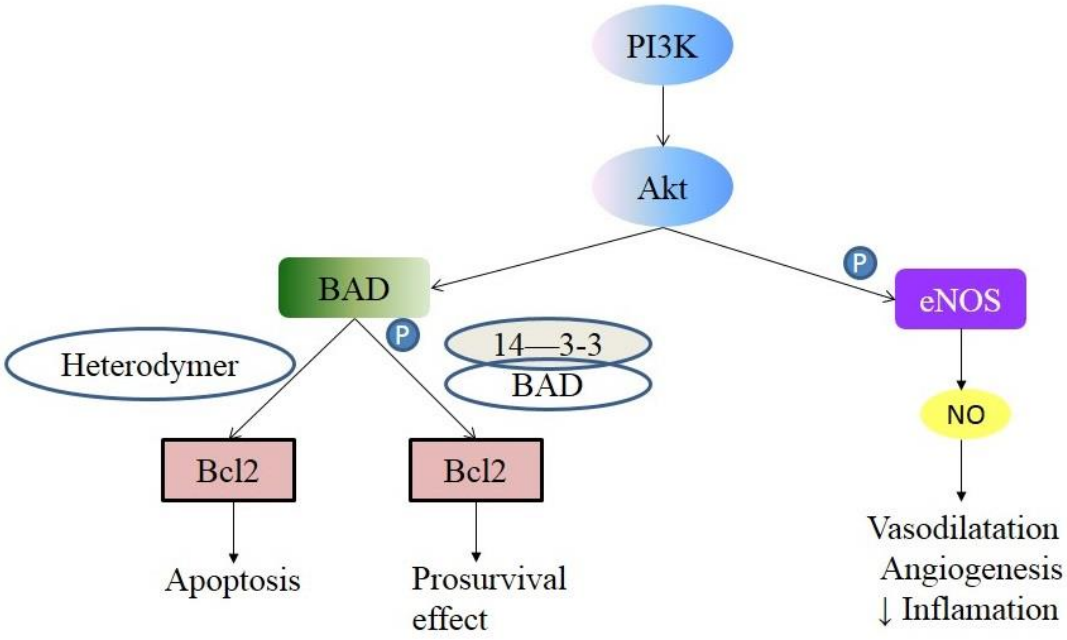

Figure 2.7 Schematic presentation of PI3K pathway. PI3K: Phosphoinositide Kinase-3; Akt: protein kinase B; Bcl-2: apoptosis regulator Bcl-2 alpha isoform; BAD: Bcl-2 associated agonist of cell death; NO: nitric oxide; eNOS: endothelial NO synthase; P: phosphorylation.

2.6. Rationale

The effects of constant sub-clinical levels of D-lactate on apoptosis of vascular endothelial cells have not been investigated. An increased apoptosis after resuscitation with racemic lactated Ringer's has previously been described (Jaskille et al, 2006), and we want to determine the effect of D-lactate on endothelial apoptosis. Once we confirm that apoptosis does occur, my research will proceed to a greater understanding of how D-lactate induces increased apoptosis in vascular endothelial cells. It was previously described that D-lactate may interfere with pyruvate and/or L-lactate to cause reduced brain cell survival (Ros et al, 2001). These data lead us to speculate that once D-lactate has entered the endothelial cells, it can affect the transport of L-lactate, pyruvate and/or SCFA into the mitochondria and thus affect the usage of these substrates and alter mitochondrial function (Gibbs and Hertz, 2008). We speculate that the interaction between D-lactate and these molecules may inhibit the utilization of these substrates in the cardiovascular system and therefore compromise cardiovascular energy status and/or mitochondrial function.

2.6.1. Hypothesis

1. Elevated levels of D-lactate or D-lactate in combination with methylglyoxal and glucose increase apoptosis of vascular endothelial cells.
2. Elevated levels of D-Lactate or D-lactate in combination with methylglyoxal and glucose interfere with energy homeostasis of vascular endothelial cells.

2.6.2. Objectives

The overall objective of my research is to investigate whether elevated levels of D-lactate alone and in combination with other glucose by-products typically associated with diabetic patients cause vascular endothelial cell apoptosis and the underlying mechanisms involved. For this purpose, *in vitro* experiments will be conducted using human umbilical vein endothelial cells (HUV-EC-C).

1. To determine the effects of elevated levels of D-lactate alone and in combination with high glucose and methylglyoxal levels on human vascular endothelial cell apoptosis. The pre- and apoptotic changes in endothelial cells are assessed by evaluating changes in cell metabolism, quantitative RT-PCR analysis of pro/antiapoptotic gene expression levels, and caspase 3/7 activities.

2. To identify the potential underlying mechanisms of human vascular endothelial cell apoptosis induced by elevated levels of D-lactate alone and in combination with glucose and methylglyoxal. Cellular energy metabolism is assessed by evaluating high energy phosphates production, reactive oxygen species production and D-lactate dehydrogenase activity.

3. MATERIALS AND METHODS

3.1. Materials

T-75 and T-150 flasks, sterile 15 mL and 50 mL polypropylene centrifuge tubes, 6-well plates, 96-well plates, eppendorf tubes, cell scrapers, Multiply® PCR® strips – with anti-contamination shield and writing space on cap, were purchased from Sarstedt (Newton, North Carolina, USA). Black 96-well flat bottom microplate was ordered from Greiner Bio-One GmbH (Frickenhausen, Germany). Mixture of Kaighn's Modification of Ham's F-12 Medium (F-12K) was purchased from American Type Culture Collection (ATCC) (Rockville, Maryland, USA). High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor and Power SYBR® Green PCR Master Mix two-step SYBR green RT-PCR, TaqMan Gene Expression Assays, and TaqMan Mastermix Gene Expression kits were acquired from Applied Biosystems (Foster City, California, USA). Ribonucleic acid (RNA) isolation mini kit and QuantiTect® reverse Transcription Kit were purchased from Qiagen Inc. (Toronto, Ontario, Canada). The CellTiter 96® AQueous One Solution Cell Proliferation Assay and the Caspase-Glo® 3/7 Assay were purchased from Promega (Madison, Wisconsin, USA). Endothelial cell growth supplement from bovine neural tissue (ECGS), Heparin Sodium Salt from Porcine Intestinal Mucosa (Heparin), Fetal Bovine Serum (FBS), Trypsin-EDTA 1×, phosphate buffered saline, D-Lactate (DLA), Methylglyoxal (MG), Glucose (Glu) 2,7-Dichlorofluorescein diacetate (DSF-DA), Adenosine triphosphate (ATP), Adenosine diphosphate (ADP), Adenosine monophosphate (AMP), Creatine (Cr), Creatine Phosphate (CrP), were purchased from Sigma-Aldrich (Toronto, Ontario, Canada. High performance liquid chromatography (HPLC) grade methanol was purchased from Caledon Laboratories (Georgetown, Ontario, CA). Highly purified deionized water was obtained from a MilliQ Synthesis water purification system (Millipore, Bedford, MA). All other solvents and reagents used were of the highest analytical grade available.

3.2. Cell culture

HUV-EC-C cell line, an immortalized human umbilical vein endothelial cell line that has phenotypic characteristics of endothelial cells *in vivo*, was purchased from ATCC at passage number 13. Cells are cultured in ATCC formulated F-12K medium with 10% fetal bovine serum, 25 µg/mL endothelial cell growth supplement, and 100 µg/mL heparin. The cells were maintained in raw plastic T-75 and T-150 flasks at 37°C under an atmosphere of 95% O_2 and 5% CO_2 in a

humidified incubator. Cell culture media was changed every 2-3 days. At approximately 70-80% confluency cells were subcultured using 0.25% 1× Trypsin-EDTA solution.

3.3. Effects of glucose byproducts on apoptosis of HUV-EC-C

Changes in cell metabolism, mRNA expression levels of genes involved in pro/anti-apoptotic pathways, and caspase 3/7 activity were measured to determine whether elevated D-lactate (DLA) levels cause pre- and apoptotic changes in endothelial cells. Three different methods were used; i.e., the CellTiter 96® AQueous One Solution Cell Proliferation Assay; measurement of mRNA expression levels of genes involved in pro-/anti-apoptotic pathways using QRT-PCR and the Caspase-Glo® 3/7 Assay.

3.3.1. Determination of changes in cell metabolism in HUV-EC-C caused by elevated levels of D-lactate

The CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega) is a commercially available kit that measures the ability of cells to reduce the MTS tetrazolium compound into a water-soluble, colored formazan product. Active cell metabolism that results in the generation of reducing equivalents such as NADH is necessary for the tetrazolium to be reduced. Increase in cell metabolism is consistent with apoptosis being an active energy-requiring process.

HUV-EC-C cells were grown in T-75 cell culture flasks and passaged once before treatment. Cells were plated in 96-well cell culture plates at density of 2×10^4 cells/mL. 100 µL of cell suspension were added to each well in 96-well cell culture plates. The cells were incubated at 37°C in a 5% CO_2 and 95% air gas mixture and grown to 70-80% confluence. Cells were treated with different concentrations of D-lactate (0.0125 mM, 0.025 mM, 0.05 mM, 0.1 mM, 0.2 mM, 0.5 mM and 2 mM) and the effects of different concentrations of D-lactate on HUV-EC-C cell metabolism activity were measured at different time points (1, 3, 6, 12 and 24 hours). CellTiter 96® AQueous One Solution Reagent was added and absorbance at 490 nm was recorded using a UV/VIS spectrophotometer (8453E, Agilent Technologies, Palo Alto, CA).

3.3.2. Effects of D-lactate on mRNA expression of genes involved in pro/anti- apoptotic pathways in HUV-EC-C

Previous experiments showed that 0.2 mM of DLA cause the most significant increase in cell metabolism of HUV-EC-C. In addition, our lab previously evaluated blood samples obtained from diabetic patients and showed that DLA levels were 0.2 mM on average (results are preliminary and not published). From this point forward 0.2 mM of DLA was taken as an experimental concentration.

mRNA expression levels of several genes involved in PI3K/AKT apoptosis pathway were measured and included the protein kinase B (AKT1); apoptosis regulator Bcl-2 alpha isoform (Bcl-2); Bcl-2 - associated agonist of cell death (BAD); endothelial nitric oxide synthase (eNOS) and phosphoinositide kinase-3 (PI3K).

HUV-EC-C cells were seeded in triplicates on 6-well plates at a density of 1×10^5 cells/mL. 3 mL of cell suspension were added to each well in 6-well cell culture plates and incubated at 37°C. The cells were grown to 70-80% confluence. The cell culture media was replaced with fresh media and cells were treated with 0.2 mM of DLA solution for 1, 6, 24 and 48 hours. Cells not treated with DLA were considered as controls. At a given incubation time the media was aspirated and the cells were washed once with 1 mL of RNA-ase/DNA-ase free water. The water was aspirated and 350 µL of buffer RLT containing β-ME was added for cell lysis. Lysed cells were collected using a cell scraper and transferred by pipetting into 1.5 mL eppendorf tubes. Cell lysate was homogenised using a pellet pestle mixer and stored at -80°C for mRNA extraction followed by QRT-PCR.

3.3.2.1. RNA isolation

Total RNA was extracted using RNeasy Mini Kits according to the manufacturer's instructions. Frozen homogenised cell lysate was thawed for 10 min at room temperature. To this lysate, one volume of 70% ethanol was added to precipitate the nucleic acids. The cell lysate was applied to the Mini column and a series of buffers were added to remove the cellular contaminants according to the manufacturer's instructions. The purified RNA was isolated from the column using RNase-free water (30 µL).

The quality and quantity of the extracted mRNA was determined using a UV/VIS spectrophotometer (8453E, Agilent Technologies, Palo Alto, CA). Total RNA was quantified by measuring the optical density (OD) of a diluted RNA (RNA:RNase-free water) at 260 nm according to the following formula:

$$\text{Concentration of RNA} = 40\ \mu g/mL \times A260 \times \text{Dilution factor}$$

RNA purity was assessed by measuring absorbance ratio (A260/A280) of a diluted sample of RNA (RNA:10 mM TrisCl (pH-7.5)). Pure RNA has a ratio between 1.9 and 2.1. All samples used for QRT-PCR had high purity. Isolated RNA was stored at -80°C until further analysis.

3.3.2.2. Primer Design

Gene sequences for AKT1, Bcl-2, BAD, eNOS, PI3K and β-actin were obtained from the National Center for Biotechnology Information Gene bank (NCBI) and specific primers were designed using Primer3 software (http://frodo.wi.mit.edu/). The forward and reverse sequences and amplicon size are given in Table 3.1.

Table 3.1 Primer pairs for real-time RT-PCR with accession number.

Genes	Forward primer	Reverse Primer	Accession No.
β-actin	ttgctatccaggctgtgc	atgtcacgcacgatttcc	NM_001101.3
AKT1	ccacacactcaccgagaacc	tctccgagtgcaggtagtcc	NM_001014431
Bcl-2	ggtggaggagctcttcagg	atcccagcctccgttatcc	NP_000624
BAD	aggacgacgaagggatgg	caccaggactggaagactcg	NM_004322
eNOS	agcaggatggctctgtgc	agggctgcaaaccactcc	NG_011992
PIK3CA	gatgcccccaagaatcc	gccgaaggtcacaaagtcg	NM_006218

3.3.2.3. Quantitative real time-polymerase chain reaction (QRT-PCR)

The relative expression of apoptosis regulator proteins was determined using QRT-PCR. Two-step QRT-PCR was performed using an Applied Biosystems 7300 Real-Time PCR system (Foster City, California). High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor was used for reverse transcription (RT) step, where messenger RNA (mRNA) is reverse

transcribed into its complementary DNA (cDNA). 2× RT master mix was prepared according to manufacturer instructions. All RT reactions were performed in a final volume of 20 μL as per Table 3.2.

Table 3.2 Components of 2× RT master mix. Adapted from Applied Biosystems. (http://www.appliedbiosystems.com)

Component	**Volume (μL)/Reaction**
10× RT Buffer	2.0
25× dNTP Mix (100 mM)	0.8
10× RT Random Primers	2.0
MultiScribe™ Reverse Transcriptase	1.0
RNase Inhibitor	1.0
Template (mRNA)	10.0
Nuclease free water	3.2
Total	**20**

Programmed thermal cycler conditions used in RT step are obtained from manufacturer protocol and shown in Table 3.3.

Table 3.3 Thermal cycler conditions optimized for use with the High Capacity cDNA Reverse Transcription Kits. (http://www.appliedbiosystems.com)

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

Power SYBR® Green PCR Master Mix was used for polymerase chain reaction (PCR) according to manufacturer instructions. All RT reactions were performed in a final volume of 25 μL as per Table 3.4.

Table 3.4 Components of PCR Master Mix. Adapted from Applied Biosystems. (http://www.appliedbiosystems.com)

Component	**Volume (µL)/Reaction**
Power SYBR Green PCR master Mix (2×)	12.5
Forward Primer	2.0
Reverse Primer	2.0
Water	6.5
Template (cDNA)	2.0
Total	**25**

The initial activation step (1 cycle at 95ºC for 10 min) was followed by a three step thermal cycling (40 cycles; denaturing at 94ºC for 15 s, annealing at 60ºC for 30 s, and extension at 60ºC for 30 s). Finally, a melt curve analysis from 65ºC to 95ºC at 0.5ºC/s was performed.

Primers were optimized and validated for its most favorable annealing temperature and highest primer efficiency. The optimal annealing temperature produces a single melting peak for a specific product, which is not identified in a blank sample containing no RNA (a negative control). QRT-PCR products were further resolved by horizontal 2% (w/v) agarose gel-electrophoresis for a single band at the specified amplicon size correlating to the single, specific product melt peak, to assure a single PCR product as identified by a single band on the gel. Primer efficiency was calculated from the slope of a 3-point standard curve and efficiencies of 1.8 to 2.2 were considered as optimal. Primers giving closer efficiency to the efficiency of β-actin (internal gene) were used for QRT-PCR reaction. The reactions were quantified following determination of the threshold cycle (C_T; the amplification cycle when PCR products are first detected above baseline fluorescence) and fluorescence was measured from the intercalation of SYBR green dye into the double standard product after the primer elongation phase. A non-template negative control was incorporated into all analysis runs. PCR products were analyzed using comparative C_T or $2^{-\Delta\Delta CT}$ method.

3.3.3. Human Apoptosis PCR Array

As above, HUV-EC-C cells were incubated and treated with 0.2 mM of DLA and mRNA was extracted after 24 and 48 hours. Samples were analyzed following the previous method (Section 3.3.2.).

Genomic DNA elimination step was performed using DNA Elimination Mixture that was prepared according to manufacturer instructions as per Table 3.5. Samples were incubated at 42^{o}C for 5 minutes.

Table 3.5 Components of the Genomic DNA Elimination Mixture. Adapted from Qiagen. (http://www.sabiosciences.com)

Component	**Volume (µL)/Reaction**
Total RNA	180 ng
GE** (5X g DNA Elimination Buffer)	2.0 µL
H_2O to a final volume of	10.0 µL

The RT step was completed using RT^2 First strand kit (Qiagen, Maryland, US) and the protocol suggested by the manufacturer was followed. All RT reactions were performed in a final volume of 20 µL as per Table 3.6.

Table 3.6 Components of the RT cocktail. Adapted from Qiagen. (www.sabiosciences.com)

Component	**Volume (µL)/Reaction**
BC3 (5X RT Buffer 3)	4.0
P2 (Primer and External Control Mix)	1.0
RE3 (RT Enzyme Mix 3)	2.0
H_2O	3.0
Genomic DNA Elimination Mixture	10.0
Total	20.0

Programmed thermal cycler conditions used in the RT step were obtained from the manufacturer protocol. Samples were incubated at 42ºC for 15 minutes and the reaction was stopped by heating at 95ºC for 5 minutes. Ninty one microliter of water was added to each 20 µL synthesis reaction and samples were stored on ice for the next step.

The RT^2 SYBR® Green ROX^{tm} qPCR Mastermix and RT^2 Prolif PCR Array (Qiagen, Maryland, US) were used for polymerase chain reaction (PCR) and expression of 96 genes was measured for each sample. Reactions were performed according to manufacturer instructions in a final volume of 25 µL as per Table 3.7.

Table 3.7 Components of PCR Master Mix. Adapted from Qiagen. (www.sabiosciences.com)

Component	**Volume (µL)/Reaction**
RT^2 SYBR® Green qPCR Mastermix	12.5
ddH_2O	10.5
Template cDNA	1.0
Gene-specific 10 µM PCR primer pair stock	1.0
Total	25.0

Initial activation step (1 cycle at 95ºC for 10 min) was followed by a three step thermal cycling (40 cycles; denaturing at 94ºC for 15 s, annealing at 60ºC for 30 s, and extension at 60ºC for 30 s). As in section 3.3.2., PCR products were analyzed using comparative C_T or $2^{-\Delta\Delta CT}$ method.

3.3.4. Measurement of changes in caspase-3 and -7 activities caused by elevated levels of glucose byproducts (DLA, GLU and MG)

The Caspase-Glo® 3/7 Assay is a commercially available kit that measures caspase-3 and -7 activities (Promega). The assay consists of luminogenic caspase-3 and -7 substrate that contains the tetrapeptide sequence DEVD in a reagent optimized for caspase activity. Caspase cleavage of the substrate will result in the generation of a "glow type" luminescent signal, produced by luciferase. The amount of caspase activity present is proportional to luminescence.

HUV-EC-C cells were grown in T-75 cell culture flasks and passaged once before treatment. Cells were plated in black 96-well cell culture plates at density of 5×10^4 cells/mL. 100 µL of cell suspension were added to each well in 96-well cell culture plates. The cells were incubated at 37°C in a 5% CO_2 and 95% air gas mixture and grown to 70-80% confluence. Cells were treated with 0.2 mM of D-lactate, 20 mM of glucose, 5 µM of methylglyoxal or a combination of all three together. Effects of D-lactate, glucose and methylglyoxal on caspase-3/7 activity were measured at different time points (1, 6, 12, 24, 48, 72 and 96 hours). The Caspase-Glo® 3/7 Assay was added and luminescence was recorded using a Multi-Detection Microplate Reader Synergy HT (Biotek, Winooski, VT).

3.4. Changes in HUV-EC-C that may contribute to apoptosis

3.4.1. Measurement of high energy phosphate (ADP, AMP and ATP) and creatine (Cr and CrP) compounds in HUV-EC-C cells caused by elevated levels of glucose byproducts (DLA, GLU and MG)

HPLC-UV was used to measure high energy phosphate substrates (AMP, ADP and ATP) and creatine compounds (creatine (Cr) and creatine phosphate (CrP)). The liquid chromatography instrumentation consisted of an Agilent 1200 series HPLC with a pump, an injector, an autosampler, a variable wavelength detector and diode array detector (Aglilent 1200) and Chemstation software, all from Agilent (Mississauga, ON). The column was a Varian Pursuit XRs 5C18 (250 mm × 3.0 mm) (Varian Inc - Chromatography, Walnut Creek, CA). The mobile phase

was filtered through a 0.45 μm nylon filter (Pall Scientific, Mississauga, ON). The analytes were eluted under gradient conditions (Table 3.8.) at a flow rate of 0.7 mL/min and absorbance was monitored at 210 nm. The column was maintained at 20°C. A rinse phase was run during each sample and consists of 95% MilliQ water and 5% methanol (MeOH) filtered through a 0.45 μm nylon filter (Pall Scientific, Mississauga, ON) and degassed in an ultrasonic bath for 30 min prior to use. None of the compounds of interest eluted during this wash portion of the cycle. Total run time for each sample was 40 min.

Table 3.8 Gradient assay of HPLC-UV method to measure Cr, Cr P, ATP, ADP and AMP levels in HUV-EC-C cells.

Time (min)	20 mM Phosphate buffer (pH-5.5)	Methanol	Water	Flow rate (mL/min)
0.0	100	0	0	0.7
17.0	100	0	0	0.7
17.5	0	95.0	5.0	0.4
19.5	0	95.0	5.0	0.4
20.0	0	5.0	95.0	0.4
22.0	0	5.0	95.0	0.4
23.0	100	0	0	0.5
28.0	100	0	0	0.7

3.4.1.1. Standards preparation

A validation of the HPLC method was run prior to sample analysis. Primary stock solutions for Cr, CrP, ATP, ADP and AMP were prepared at a concentration of 1 mg/mL by dissolving in phosphate buffer (pH-5.5). A standard mixture was prepared using each of the compounds of interest at different concentrations – Cr (20 μg/mL), CrP (20 μg/mL), ATP (20 μg/mL), ADP (20 μg/mL) and AMP (80 μg/mL). This standard mixture then underwent 5 serial dilutions, to give a total of 6 standard solutions (including the initial undiluted standard mixture, 20 to 0.625 μg/mL for Cr, Cr P, ATP, ADP and 80 to 2.5 μg/mL for AMP). High quality control (HQC), middle

quality control (MQC) and low quality control (LQC) samples at 15, 4, and 1 µg/mL for Cr, Cr P, ATP, ADP and 30, 15, and 5 µg/mL for AMP were prepared using the stock solution independent of those concentrations used for the standard curve. Primary stock solutions were stored at -20°C. 10 µL aliquots of each standard mixture were separately injected into the HPLC system, and run using the same method, sequence and mobile phase as each of the samples. The peaks on the chromatograph from each standard mixture were integrated, and the amounts of each compound present were calculated.

3.4.1.2. Sample preparation

Human umbilical vascular endothelial cells (HUV-EC-C) were plated and grown according to manufacturer's instruction (ATCC'S). Cells were then trypsinized, re-suspended in media and plated in 6-well plates. Cells were treated with 0.2 mM D-lactate or a combination of 0.2 mM D-lactate, 25 mM glucose and 5 µM methylglyoxal, prepared in the phosphate buffer. Cells used as a control received only phosphate buffer. Cells were kept in incubation at 37°C with 5% CO_2 for the duration of the study. Cells were periodically (at 24 h, 48 h) removed from incubation for sample collection.

At the appropriate time points (24 h and 48 h), media from each well of incubated endothelial cells was aspirated off, and cells were subsequently washed twice with Phosphate Buffered Saline (PBS). 400 µL of 0.7 M percholoric acid was applied to lyse cells and a cell scrapper was used to detach the cells from the surface of the well. Cell lysate was collected, homogenized using pellet pestle mixer, frozen and stored at -80°C until analysis. Cell lysate was removed from -80°C and centrifuged for 10 min at 12000 rpm Eppendorf microcentrifuge (Accuspin Micro 17, Fisher Scientific) for 7 min. Lysate pH was adjusted to 7.0 using 0.25 M potassium hydroxide solution (KOH), and 0.7 M percholoric acid. Samples were filtered through 0.45 µm filter (Pall life sciences, Mississauga, ON) and then centrifuged at 12000 × *g* for an additional 10 minutes. 10 µL aliquots of each sample were injected separately onto the column and run for 40 minutes. As all of the compounds of interest were eluted by a time point of 15 minutes, a wash phase consisting of filtered MilliQ water and methanol followed the buffered mobile phase.

3.4.2. Effects of glucose byproducts (DLA, GLU and MG) on production of reactive oxygen species

Production of reactive oxygen species caused by different concentrations of D-lactate was evaluated using 2,7-Dichlorofluorescein diacetate (DCF-DA) as a probe. DCF-DA (Sigma) is a nonfluorescent dye when chemically reduced, but after cellular oxidation and removal of acetate groups by cellular esterases it becomes fluorescent. The fluorescence was measured using Multi-Detection Microplate Reader Synergy HT (Biotek, Winooski, VT).

Human umbilical vascular endothelial cells (HUV-EC-C) were plated and grown according to manufacturer's instruction (ATCC'S). Cells were then trypsinized, re-suspended in media and plated on a black flat bottom 96-well microplates at 10,000 cells per well and were incubated at 37°C in a 5% CO_2 and 95% air gas mixture.

After incubation, cells were treated with different concentrations of D-lactate (2.0, 1.0, 0.5, 0.25, 0.125, 0.06 and 0.03 mM), glucose (30, 25, 20, 15, 10 and 5 mM) and methylglyoxal (160, 80, 40, 20, 10 and 5 µM). An additional 0.2 mM of D-lactate was added in some wells, which were previously treated with glucose and methylglyoxal, respectively. Cells not treated were considered as controls. After 24 hours production of reactive oxygen species was measured.

The DCF-DA probe is a photosensitive compound and the experiment was conducted in the dark. Stock solution of DCF-DA concentration (10 mM) was prepared using DMSO (Sigma). A working solution was prepared using medium F12K (0.1 mM). Half an hour after DCF-DA solution was added in each well, media was aspirated and cells were washed twice with 1× PBS (phosphate buffer saline). 100 uL of 1× PBS was added into each well and fluorescence was recorded at excitation 485 nm and emission 528 nm using fluorometer.

3.5. D-Lactate dehydrogenase expression

In order to determine whether HUV-EC-C cells have the ability to metabolize D-lactate, mRNA expression levels of D-lactate dehydrogenase (D-LDH), a mitochondrial enzyme involved in metabolism of D-lactate, were measured. HepG2 (Hepatocellular carcinoma cells, human) were used as positive control for D-LDH mRNA expression. HUV-EC-C and HepG2 cells were seeded in triplicate on T-75 flasks at a density of 3×10^4 cells/mL. The cells were grown to 70-80% confluence. At collection time, the media was aspirated and cells were trypsinized using 3 mL of

0.25% trypsin/flask. After a few minutes, 5 mL of cell culture media was added to each flask in order to stop the action of trypsin. Cells along with the media were then collected in 15 mL centrifuge tubes and centrifuged at 180 rpm for 5-7 min. The media was aspirated out and 350 µL of buffer RLT containing β-ME was added to the cell pellet for cell lysis. Cell lysates were transferred to 1.5 mL eppendorf tubes and were homogenised using a pellet pestle mixer. Samples were stored at -80°C for mRNA extraction followed by QRT-PCR.

3.5.1. RNA isolation

Total RNA was extracted using RNeasy Mini Kits as described in section (3.3.2.1.). mRNA concentration and purity were measured using a GELifeSciences NanoVue Plus Spectrophotometer (Upsala, Sweden).

3.5.2. Primers

TaqMan Gene Expression Assay (Applied Biosystems) was used for D-lactate dehydrogenase analysis. Pre-validated sequences for β-actin and D-lactate dehydrogenase (D-LDH) (Table 3.9.) were obtained from Applied Biosystems (http://www.appliedbiosystems.com). β-actin was used as internal control.

Table 3.9 Pre-validated TaqMan Gene Expression Assays. (http://www.appliedbiosystems.com)

Gene Symbol	Gene Name	Assay ID
LDHD	D-Lactate dehydrogenase	Hs00544860_m1
ACTB	β-actin	Hs99999903_m1

3.5.3. Quantitative real time-polymerase chain reaction (QRT-PCR)

The relative expression of D-LDH and β-actin was determined using QRT-PCR. Two-step QRT-PCR was performed using an Applied Biosystems 7300 Real-Time PCR system (Foster City, California). QuantiTect® Reverse Transrciption Kit was used for reverse transcription (RT) step, where messenger RNA (mRNA) was reverse transcribed into its complementary DNA (cDNA). Genomic DNA elimination reaction was performed according to manufacturer instructions as per Table 3.10. The mixture was incubated at 42°C for 3 min.

Table 3.10 Components of Genomic DNA elimination reaction. Adapted from Qiagen. (www.qiagen.com)

Component	Volume (µL)/Reaction
gDNA Wipeout Buffer, 7x	2.0
Template RNA	1 µg
RNase-free water to a final volume of	14

Reverse transcription reaction components were prepared according to manufacturer instructions. All RT reactions were performed in a final volume of 20 µL as per table 3.11.

Table 3.11 Components of reverse transcription reaction. Adapted from Qiagen. (www.qiagen.com)

Component	Volume (µL)/Reaction
Reverse-transcription master mix	1.0
Quantiscript RT Buffer, 5x	4.0
RT Primer Mix	1.0
Template RNA	14.0
Total	**20.0**

Programmed thermal cycler conditions used in RT step were obtained from manufacturer protocol and shown in the Table 3.12.

Table 3.12 Thermal cycle conditions optimized for use with the QuantiTect® Reverse Transciption Kit. (http://www.qiagen.com)

	Step 1	**Step 2**
Temperature (ºC)	42	95
Time	25 min	3 min

The Taqman Mastermix Gene Expression and TaqMan Gene Expression Assays (Applied Biosystems, Foster City, California) were used for polymerase chain reaction (PCR) and expression of D-lactate dehydrogenase was measured for HUV-EC-C and HepG2 cells. Reactions were performed according to manufacturer instructions in a final volume of 25 μL as per Table 3.13.

Table 3.13 Components of TaqMan cocktail. Adapted from Qiagen. (www.qiagen.com)

Component	**Volume (μL)/Reaction**
TaqMan Master Mix	10.0
Primer	1.0
Template	1.0
Water	8.0
Total	**20.0**

Programmed thermal cycler conditions used in PCR step were obtained from manufacturer protocol and shown in the table 3.14. Relative quantification was performed using FAM detector.

Table 3.14 Thermal cycle conditions used in PCR step.

	Step 1	**Step 2**	**Step 3**	
No. of cycles	1	1	40	
Temperature (ºC)	50	95	95	60
Time	5 min	10 min	15 s	1 min

3.6. Statistical Analysis

Caspase 3/7 activity, high energy phosphate compounds and reactive oxygen species production data were analyzed using two-way ANOVA analysis followed by Tukey's multiple comparison test. A P-value of less than 0.05 was taken to indicate a significant difference between the means of sets of data. All data were expressed as mean ± standard error of mean (SEM).

4. RESULTS

4.1. D-lactate has an effect on cell metabolism in HUV-EC-C

To determine whether D-lactate has an effect on cell metabolism HUV-EC-C cells were exposed to different concentrations of D-lactate (0.0125, 0.025, 0.05, 0.1, 0.2, 0.5 and 2 mM) for 1, 3, 6, 12 and 24 hours and metabolism was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay. Data represent percent of control (Figure 4.1).

D-Lactate at 0.2 mM had the greatest effect on cellular metabolism. After 1 hour of D-lactate treatment, HUV-EC-C cells showed an increase in their ability to reduce the tetrazolium compound, suggesting an increase in cell metabolism consistent with apoptosis being an active energy-requiring process. After 24 hours of D-lactate treatment, cell metabolism, as indicated by the tetrazolium assay, dropped to 81% of control (Phosphate buffer (vehicle)) (Figure 4.1).

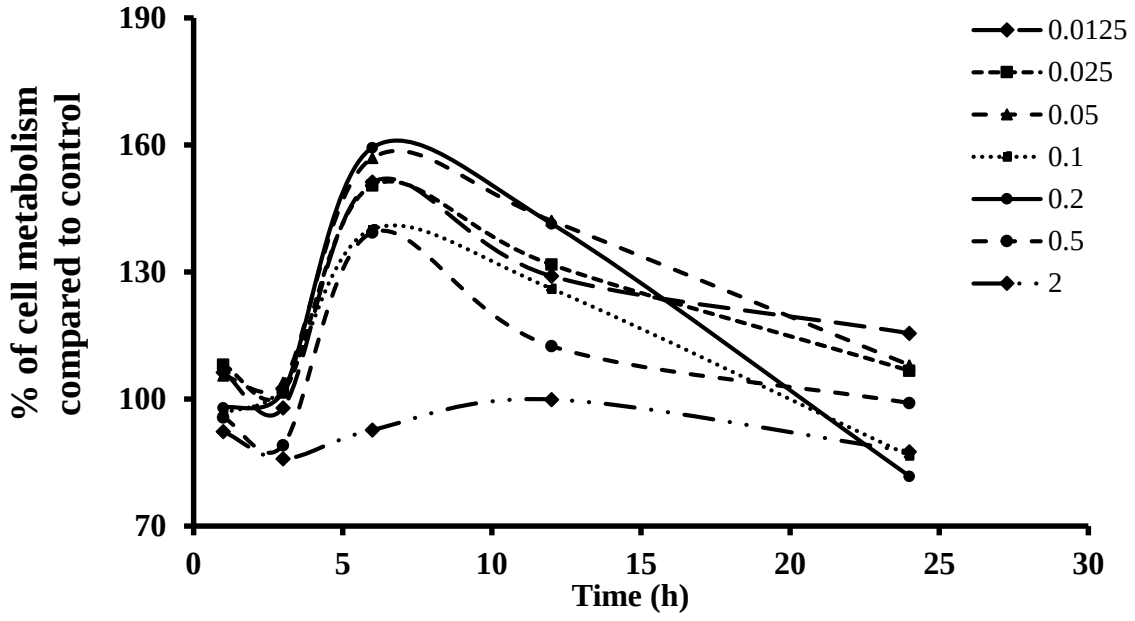

Figure 4.1 Cell metabolism (% of control) of HUV-EC-C treated with different concentrations of D-lactate (0.0125, 0.025, 0.05, 0.1, 0.2, 0.5 and 2 mM) for 1, 3, 6, 12 and 24 hours. (n=1)

4.2. D-Lactate alters mRNA expression of genes involved in pro/anti-apoptotic pathways in HUV-EC-C

To determine whether elevated levels of D-lactate affect expression of genes involved in pro/anti-apoptotic pathways, HUV-EC-C cells were exposed to 0.2 mM of D-lactate for 1, 6, 24 and 48 h and mRNA expression of genes involved in PI3K/AKT pathway was measured (AKT1, Bcl-2, BAD, eNOS, PI3K). Exposure of HUV-EC-C cells to 0.2 mM D-lactate caused down regulation of mRNA expression levels of all selected genes at 6 hours (50-70% of control) with further reduction at 24 hours (20-40% of the control). Expression levels of AKT1, BAD, eNOS and Bcl2 were upregulated (150 to 170% of control) after 48 hours of D-lactate exposure, while PI3K was still lower compared to control (72%) (Figure 4.2).

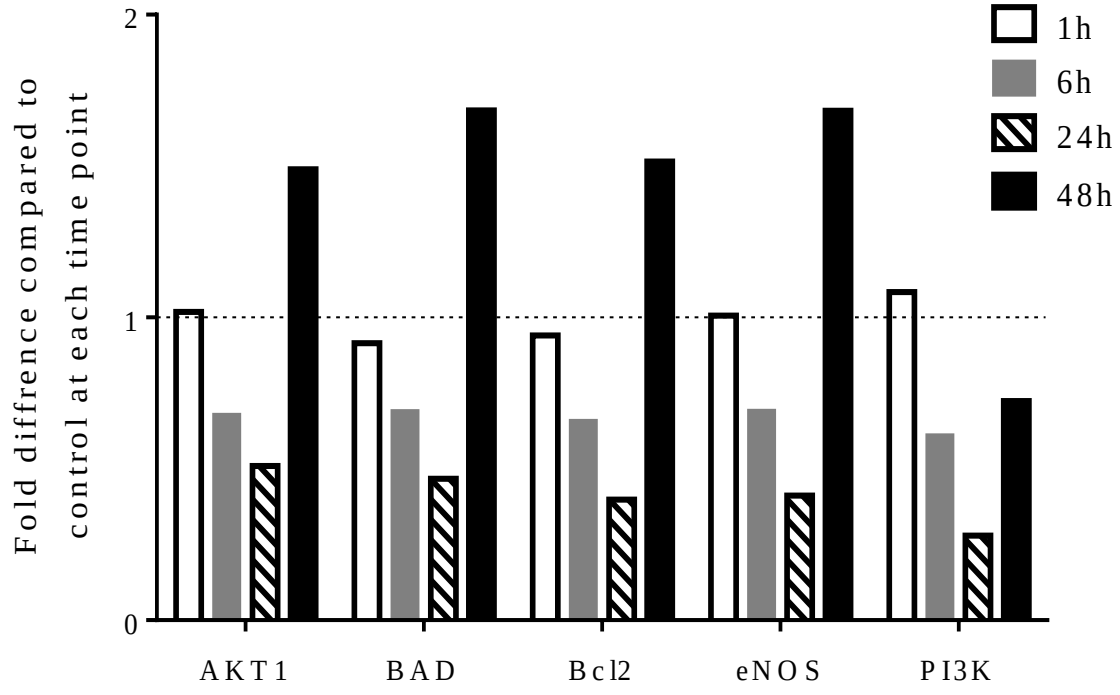

Figure 4.2 Fold Difference (FD) of mRNA expression of AKT1, BAD, Bcl-2, eNOS and PI3K compared to control in HUV-EC-C cells when incubated with 0.2 mM D-lactate for 1, 6, 24 and 48 hours. mRNA expression was normalized to ß-actin and fold difference (FD) determined by using $2^{-\Delta\Delta CT}$ method. (n=1)

4.2.1. Human Apoptosis PCR Array

In addition to the previous experiment, expression of 84 key genes involved in cell death was tested using the human apoptosis PCR Array. HUV-EC-C cells were exposed to 0.2 mM of D-lactate and changes in mRNA expression were measured at 24 and 48 hours. Expression levels of proapoptotic genes BAD, LTA, TNFRSF10A, CD27 and TNFSF25 were downregulated (150 to 340% of control) after 24 hours of D-lactate exposure, while expression levels of antiapoptotic genes BIRC3 and BIRC8 were upregulated (330% and 440% of control) after 24 h of D-lactate exposure. After 48 h of D-lactate exposure expression levels of proapoptotic genes were upregulated (130% to 680% of control), while expression levels of antiapoptotic genes were downregulated (450% of control) (Figure 4.3). Opposite to the previous trend, expression level of proapoptotic CASP5 gene was upregulated at 24 hours (260% compare to control) and downregulated at 48 hours (190% compare to control) of D-lactate exposure.

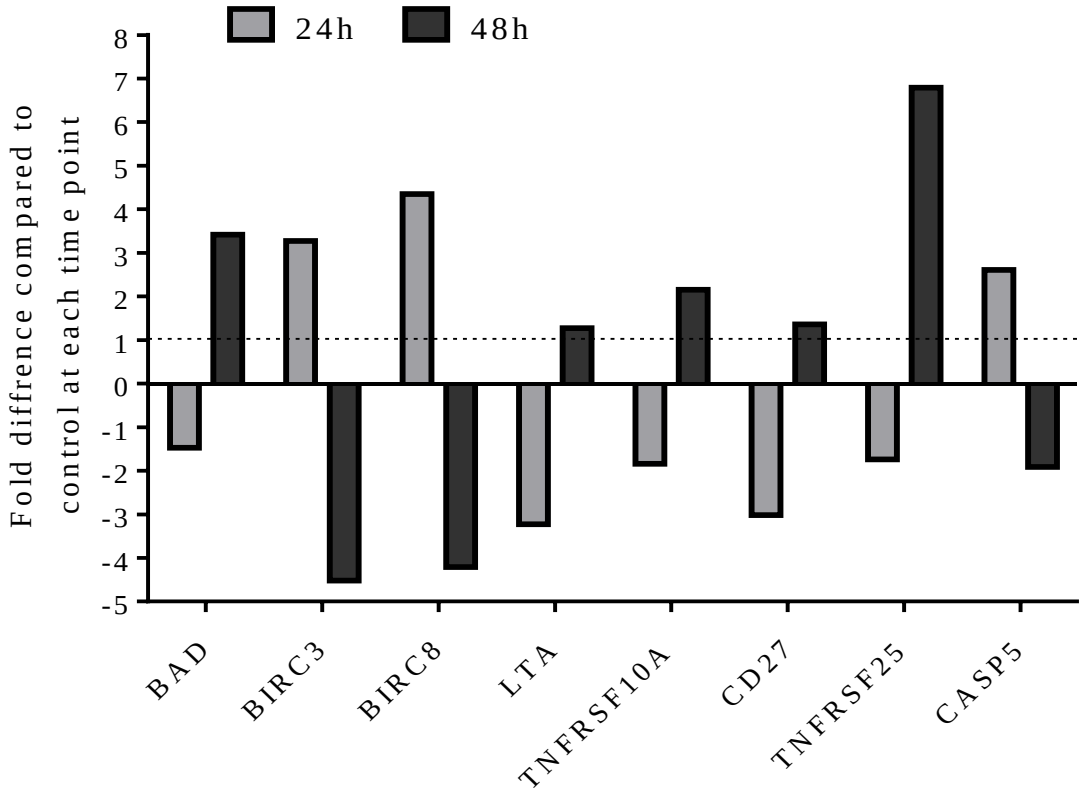

Figure 4.3 Fold Difference (FD) of mRNA expression of BAD, BIRC3, BIRC8, LTA, TNFRSF10A, CD27, TNFSF25 AND CASP5 compared to control in HUV-EC-C cells when incubated with 0.2 mM D-lactate for 24 and 48 hours. mRNA expression was normalized to ß-actin and fold difference (FD) determined by using $2^{-\Delta\Delta CT}$ method. (n=1)

4.3. Measurement of changes in caspase-3 and -7 activities caused by elevated levels of glucose byproducts (DLA, GLU and MG)

To determine whether supra physiological level of D-lactate and levels of glucose and methylglyoxal similar to those found in blood samples of diabetic patients can contribute to apoptosis, HUV-EC-C cells were exposed to 0.2 mM of D-lactate, 20 mM of glucose, 5 µM of methylglyoxal and combination of all three for 1, 6, 12, 24 and 48 hours. In addition, cells were exposed to D-lactate for 72 and 96 hours. Changes in caspase activity were measured using the Caspase-Glo® 3/7 Assay. Data represent percent of control. (Figure 4.4 and Figure 4.5)

With D-lactate treatment, HUV-EC-C cells showed an increased caspase 3/7 activity at different time points (Figure 4.4). Enhanced caspase 3/7 activity was observed at 1, 72 and 96 hours (24.0%, 18.1% and 70.4% increase compared to control, respectively).

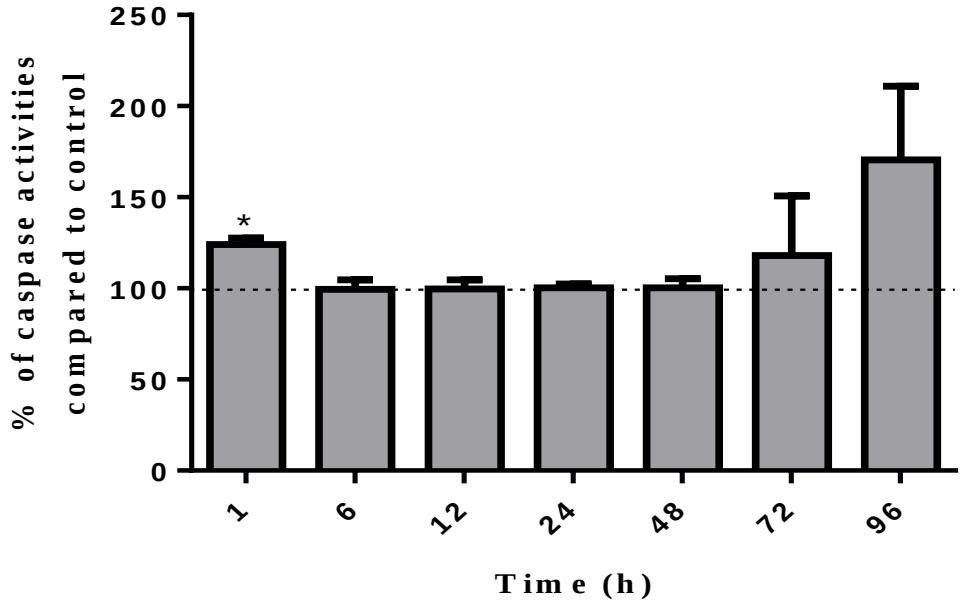

Figure 4.4 Mean ± SEM of the effects of 0.2 mM of D-lactate on caspase 3/7 activity in HUV-EC-C cells at 1, 6, 12, 24, 48, 72 and 96 hours. The Caspase Glo® 3/7 Assay kit was added and luminescence was recorded. Data represent % caspase activities compared to control. Means (n=3) were compared with control (*) using two-way ANOVA ($P < 0.05$) followed by Tukey's Multiple Comparison test.

With glucose, methylglyoxal and combination treatment, HUV-EC-C cells showed an increased caspase 3/7 activity at different time points (Figure 4.5) With glucose treatment,

enhanced caspase 3/7 activity was observed at 1 and 12 hours (19.2% and 59.8% increase compared to control, respectively). With methylglyoxal treatment increase of caspase activity was recorded after 1 hour (13.5%). With combination treatment increase of caspase activity was noted after 12 hours (44.2%).

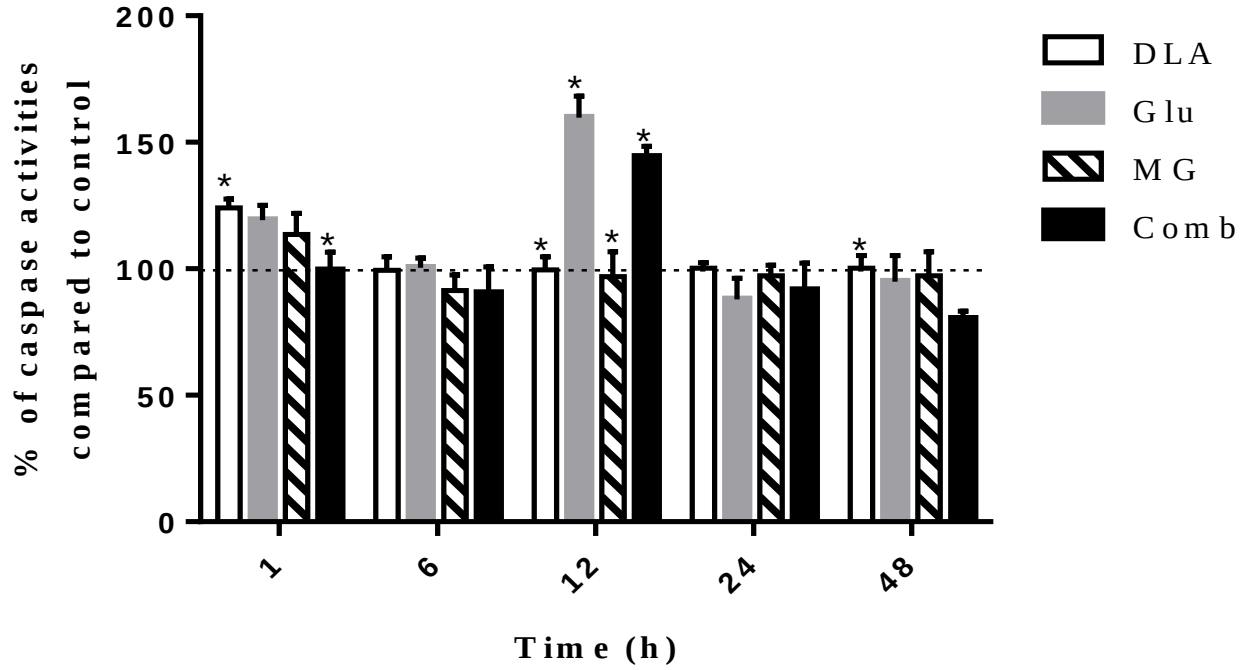

Figure 4.5 Mean ± SEM of the effects of 0.2 mM of D-lactate (DLA), 20 mM of glucose (GLU), 5 µM of methylglyoxal (MG) and combination (Comb) on caspase 3/7 activity in HUV-EC-C cells at 1 h, 6 h, 12 h, 24 h and 48 h. The Caspase Glo® 3/7 Assay kit was added and luminescence was recorded. Data represent % of caspase activities compared to control. Means (n=3) were compared with control (*) using two-way ANOVA ($P < 0.05$) followed by Tukey's Multiple Comparison test. Significant differences between groups are identified in table 4.1.

Table 4.1 Mean ± SEM % of caspase activities compared to control in HUVEC cells incubated with 0.2 mM of D-lactate (DLA), 20 mM of glucose (GLU), 5 µM of methylglyoxal (MG) and combination for 1 h, 6 h, 12 h, 24 h and 48 h (n=3).

Time	DLA	GLU	MG	Combination
1 h	124 ± 3.5[*a]	119 ± 5.8	113 ± 8.4	99 ± 7.2[a]
6 h	99 ± 5.3	100 ± 3.8	91 ± 6.2	90 ± 10.2
12 h	99 ± 5.0[a]	160 ± 8.5[*b]	97 ± 9.8[a]	144 ± 4.1[*ab]
24 h	100 ± 2.2	88 ± 8.3[d]	97 ± 4.3	91 ± 10.7
48 h	78 ± 13.6[*b]	95 ± 10.3[d]	97 ± 9.5	80 ± 3.1[d]
72 h	118 ± 32.7			
96 h	170 ± 40.5			

*Multiple comparisons for % of caspase activities compared to control caused by DLA, GLU, MG and combination of all three with different incubation times were analyzed using two-way ANOVA with Tukey's Multiple Comparison test. Means with (*) are significantly different from control. Fold differences within the treatment were determined by using One-way ANOVA with Tukey's Multiple Comparison test. Significance level was set at $P<0.05$.
[a]Means with (a) were significantly different between the treatment with single chemical and combination.
[b,c,d,e]Means with (b,c,d,e) were significantly different when compared to 1 h, 6 h, 12 h and 24 h of exposure within the treatment, respectively.

4.4. Creatine and high energy phosphate substrate levels in HUV-EC-C cells caused by elevated levels of D-lactate, glucose and methylglyoxal

To determine whether elevated levels of D-lactate and D-lactate in combination with glucose and methylglyoxal alter high energy substrate levels in HUV-EC-C cells, the concentration of high energy phosphate substrates (Cr, CrP, ATP. ADP, AMP) was measured in HUV-EC-C cells after 24 and 48 hours of 0.2 mM D-lactate and combination of 0.2 mM of D-lactate, 25 mM glucose and 5 uM of methylglyoxal. Figure 4.6 represents the HPLC chromatograms of blank (mobile phase) (A) and the standards of creatine compounds (Cr and CrP) and high energy phosphate substrates (ATP, ADP and AMP) spiked in mobile phase (B). Figure 4.7 represents the changes in creatine compounds and high energy phosphate substrate levels due to D-lactate and combination of D-lactate, glucose and methylglyoxal exposure in HUV-EC-C cells. Figure 4.8

represents AMP/ATP ratio due to D-lactate and combination of D-lactate, glucose and methylglyoxal exposure in HUV-EC-C cells.

D-Lactate and combination treatment significantly increased ADP levels (174.3% and 126.6% of control, respectively) at 24 hours. No significant changes were found in other energy phosphate substrates or creatine compounds. With D-lactate treatment an increase in AMP/ATP ratio at 24 hours (147.4%) and decrease at 48 hours (52.1%) was demonstrated. With combination treatment a decrease was observed at both 24 and 48 hours (74.5% and 76.9% of control, respectively).

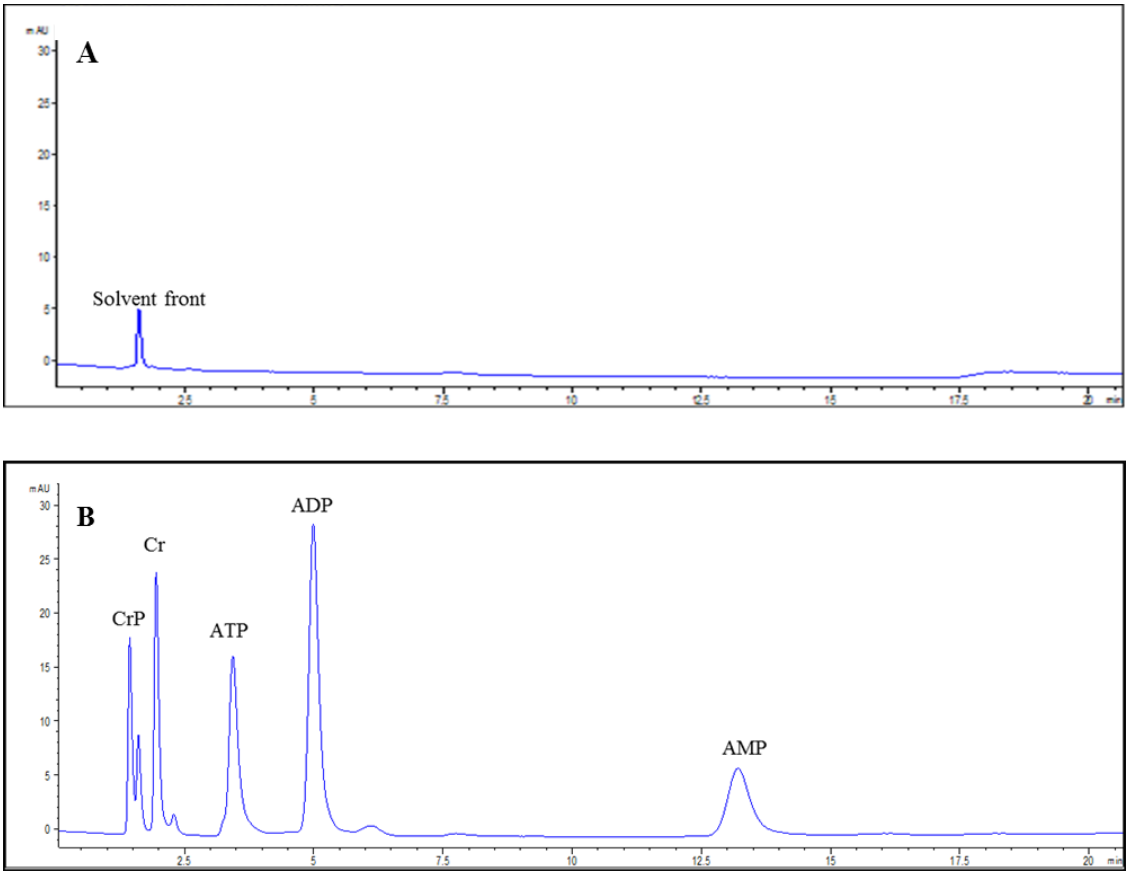

Figure 4.6 HPLC chromatograms of mobile phase (phosphate buffer, pH-5.5) (A), and phosphate buffer spiked with CrP, Cr, ATP, ADP (10 µg/mL) and AMP (20 µg/mL) (B).

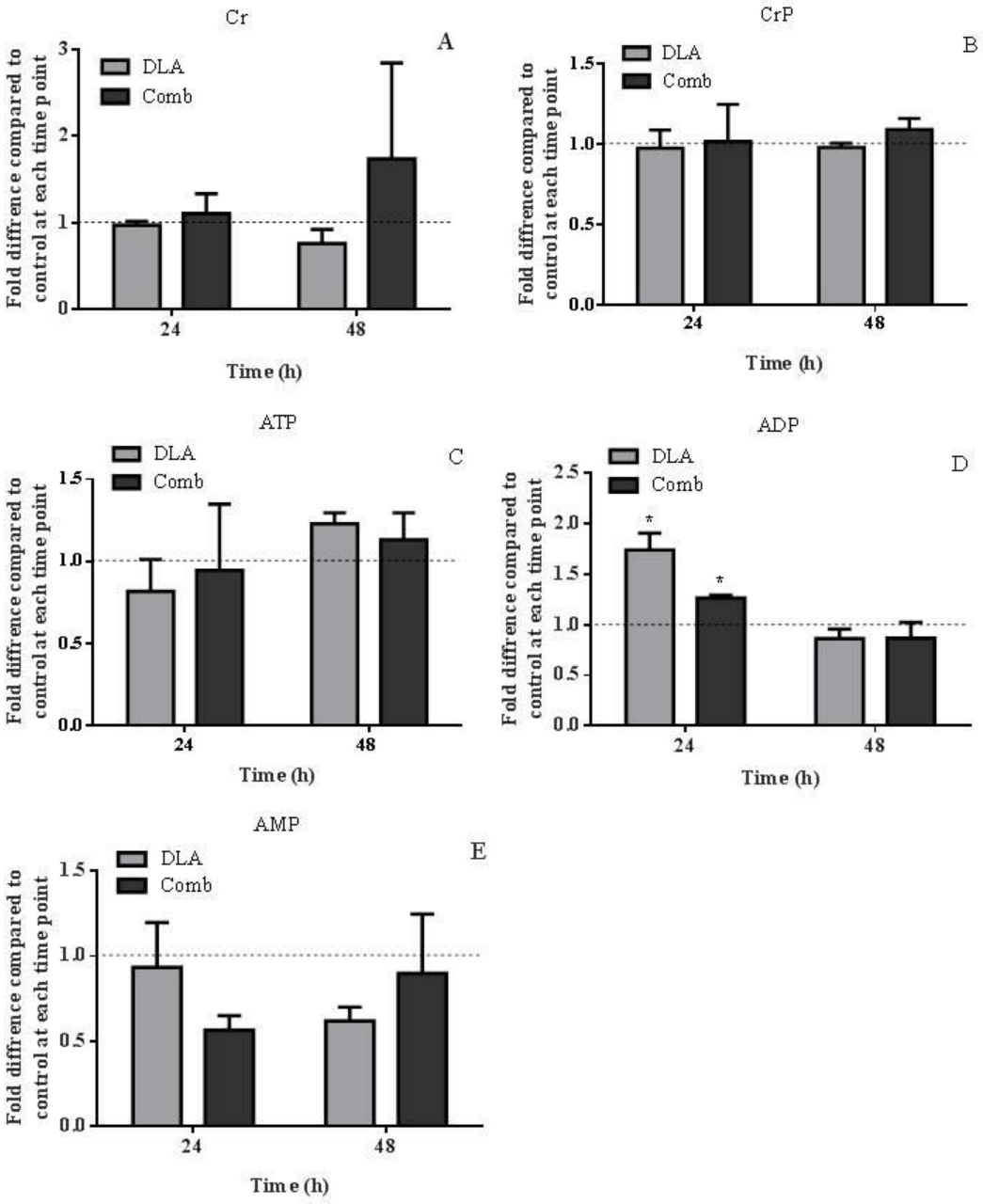

Figure 4.7 Mean ± SEM of fold differences compared to control of Cr, CrP, ATP, ADP and AMP (Figure A, B, C, D, E respectively) in HUV-EC-C cells incubated with 0.2 mM D-lactate (DLA) and combination (Comb) of 0.2 mM D-lactate, 20 mM glucose and 5 µM methylglyoxal and cell culture media (control) for 24 and 48 hours. Means were compared using two-way ANOVA analysis ($P < 0.05$) followed by Tukey's Multiple Comparison test.

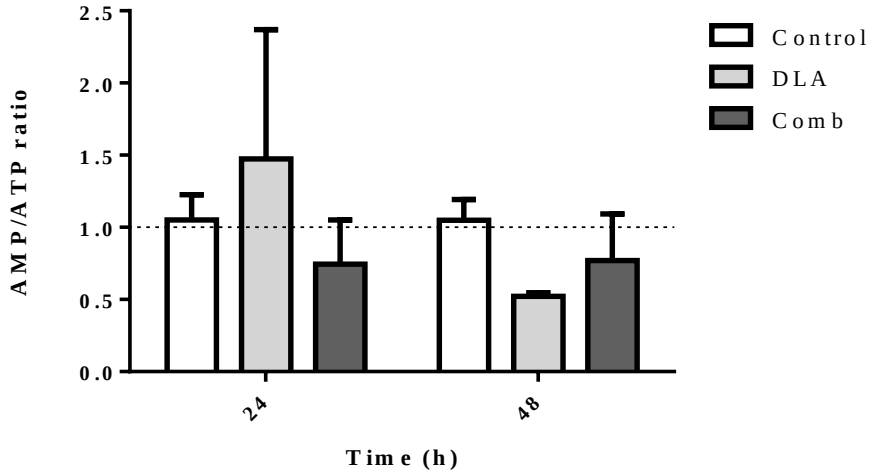

Figure 4.8 Mean ± SEM of AMP/ATP ratios in HUV-EC-C cells incubated with 0.2 mM D-lactate (DLA) and combination (Comb) of 0.2 mM D-lactate, 20 mM glucose and 5 µM methylglyoxal and cell culture media (control) for 24 and 48 hours. Means were compared using two-way ANOVA analysis ($P < 0.05$) followed by Tukey's Multiple Comparison test.

4.5. Reactive oxygen species production

In order to find an underlying mechanism of apoptosis of endothelial cells, reactive oxygen species production of HUV-EC-C cells treated with D-lactate and combination of D-lactate with glucose and methylglyoxal was measured, respectively. Figure 4.9 represents the increase in reactive oxygen species production when HUV-EC-C cells were treated with different concentrations of D-lactate (0.03-2.0 mM). Figure 4.10 represents reactive oxygen species production compared to control in HUV-EC-C cells when treated with glucose (5-30 mM) (A) and methylglyoxal (5-160 µM) (B) and combination with 0.2 mM D-lactate, respectively.

All D-lactate concentrations caused a significant increase in ROS production (160% to 215%) in HUV-EC-C cells (Figure 4.9). In addition, D-lactate with glucose and D-lactate with methylglyoxal significantly increased ROS production compared to glucose or methylglyoxal alone ($p<0.05$) (Figure 4.10 A&B). Means ± SEM % of increase of ROS production after different treatments (DLA, GLU, MG and combination) at 24 hours compared to control in HUVEC cells were shown in Table 4.2.

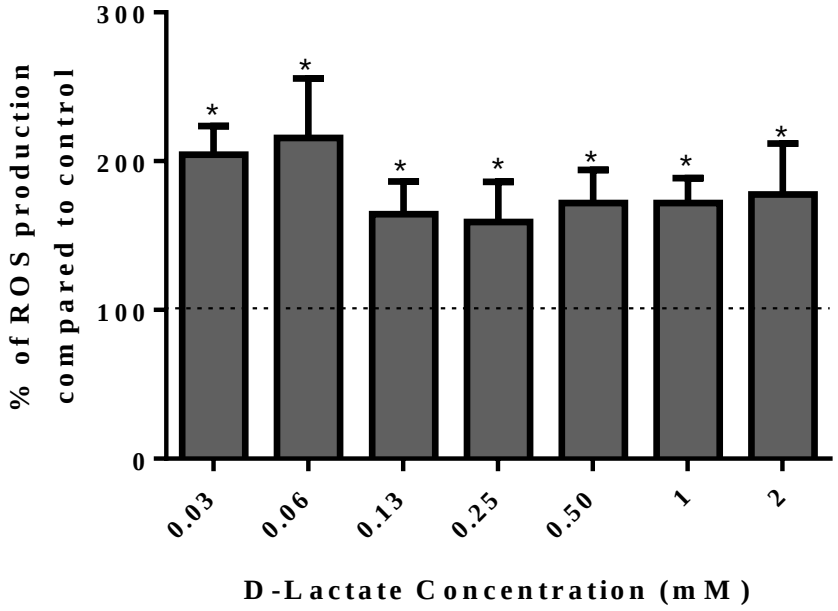

Figure 4.9 Mean ± SEM of the effects of different concentrations of D-lactate (0.03-2 mM) on HUV-EC-C cell ROS production at 24 hours. Means (n=3) were compared with control (*) using two-way ANOVA ($P < 0.05$) followed by Tukey's Multiple Comparison Test. Significant differences between groups are identified in table 4.2.

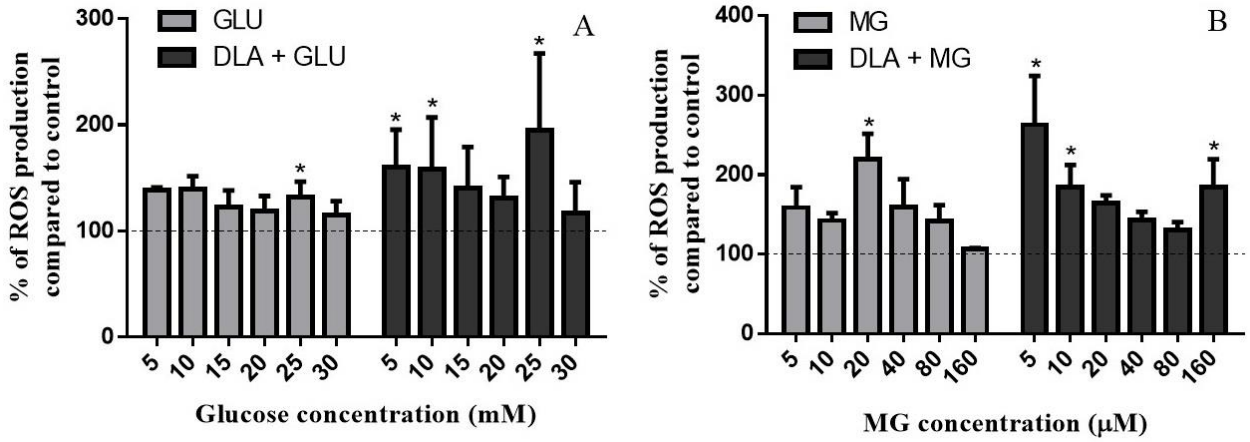

Figure 4.10 Mean ± SEM of the effects of different concentrations of glucose (GLU) (5-30 mM) alone and glucose with 0.2 mM D-lactate (DLA) (Fig 2A); methylglyoxal (MG) (5-160 μM) alone and methylglyoxal with 0.2 mM D-lactate (Fig 2B) on ROS production in HUV-EC-C cells at 24 hours. DSF-DA (2,7-Dichlorofluorescein diacetate) was added and fluorescence was recorded. Means (n=3) were compared with control (*) using two-way ANOVA ($P< 0.05$) followed by Tukey's Multiple Comparison Test. Significant differences between groups are identified in table 4.2.

Table 4.2 Mean ± SEM % of ROS production compared to control in HUVEC cells incubated with 0.03 – 2.0 mM of D-lactate (DLA), 5 – 30 mM of glucose (GLU), 5 – 160 µM of methylglyoxal (MG) and combination of glucose and methylglyoxal with 0.2 mM D-lactate respectively for 24 h (n=3).

D-Lactate		Glucose				Methylglyoxal			
		GLU		GLU + 0.2 mM DLA		MG		MG + 0.2 mM DLA	
Conc mM	% control	Conc mM	% control	Conc mM	% control	Conc µM	% control	Conc µM	% control
0.03	204 ±19*	5	138 ± 2	5	160 ± 20*	5	159 ± 25b	5	263 ± 62*b
0.06	216 ± 40*	10	139 ± 7	10	158 ± 28*	10	143 ± 9	10	184 ± 28*
0.13	164±22*	15	122 ± 9	15	141 ± 22	20	220 ± 32*e	20	165 ± 10*
0.25	159±27*	20	118 ± 8	20	131 ± 12	40	159 ± 35	40	143 ± 10d
0.50	172±22*	25	132 ± 8ac	25	195 ± 42*a	80	142 ± 20	80	131 ± 10d
1.0	172±17*	30	115 ± 7	30	117 ± 16	160	107 ± 1	160	184 ± 35*
2.0	177±34*								

*Multiple comparisons for ROS production compared to control caused by various concentrations of DLA, GLU, MG and combination at 24 hours incubation time were analyzed using two-way ANOVA with Tukey's Multiple Comparison test. Means with (*) are significantly different from control. Fold differences within the treatment were determined by using one-way ANOVA with Tukey's Multiple Comparison test. Significance level was set at $P<0.05$.

[a,b]Means with (a,b) were significantly different between the treatment with GLU or MG alone or in combination with 0.2 mM DLA, respectively.

[c]Means with (c) were significantly different when compared to different concentrations up to 30 mM within the glucose treatment.

[d,e]Means with (d,e) were significantly different when compared to different concentrations of 5 µM – 160 µM within the methylglyoxal treatment, respectively.

4.6. D-Lactate dehydrogenase expression

To determine whether HUV-EC-C cells have the ability to metabolize D-lactate, mRNA expression levels of D-lactate dehydrogenase were measured. HepG2 cells were used as a positive control. mRNA expression for D-LDH was not detected in HUV-EC-C, while HepG2 had a C_T value of 38.1 ± 0.53 (Mean ± SEM) (data not shown).

5. DISCUSSION

Micro- and macrovascular complications, caused by increased apoptosis of endothelial cells and consequently endothelial dysfunction in diabetes, significantly affect quality of life of diabetic patients. Previous studies confirmed that high glucose and methylglyoxal levels contribute to endothelial dysfunction through increased apoptosis (Risso et al. 2001, Mukohda et al. 2013). In addition, it was previously shown that D-lactate in higher concentrations causes apoptosis in lungs and memory loss in chickens (Gibbs and Hertz 2008). Even though apoptosis is a normal homeostatic process, an imbalance in apoptosis and increase in apoptotic cell death can be a sign of cellular injury that has been proposed as a cause of multiple organ dysfunction (Papathanassoglou et al. 2003, Cobb et al. 2000). My study evaluated the time dependent effects of D-lactate alone and different combinations of glucose, methylglyoxal, and D-lactate on endothelial cell function using the immortalized human vascular endothelial cell line, HUV-EC-C. I demonstrated that *in vitro* D-lactate may alter normal physiological functions of HUV-EC-C. My data suggest that D-lactate causes time-dependent changes in endothelial cell metabolism, mRNA expression levels of genes involved in regulation of apoptosis, and caspase 3/7 activities. In addition, D-lactate alone and in combination with glucose and methylglyoxal increase ROS production in endothelial cells. These data provide a link between increased D-lactate concentration, increased ROS production, and probable vascular complications.

The HUV-EC-C cell line was used for my studies, as the immortalized human umbilical vein endothelial cell line is known to have similar phenotypic characteristics of vascular endothelial cells *in vivo.* According to previous reports, HUV-EC-C cells are widely used as an *in vitro* model in analysis of endothelial cell function and studies confirm their ability to maintain endothelial cell characteristics with culture (Gifford et al. 2004). Endothelial cells act as a primary interface between chemicals in the circulating blood and the rest of the vessel wall and body tissues, and have an important role in cardiovascular homeostasis. Experimental conditions were created to simulate the exposure of endothelial cells in blood vessels of diabetic patients. To the best of our knowledge, it has not been investigated whether D-lactate (at concentrations lower than levels resulting in D-lactic acidosis) contribute to endothelial apoptosis, and consequently to cardiovascular dysfunction in diabetic patients. Considering this, optimization experiments that measure changes in cellular metabolism were carried out in order to determine the most appropriate

concentration of D-lactate to employ in subsequent experiments. The optimization studies involved a cellular cytotoxicity kit that measures increases in cellular metabolism, since an increase in cellular metabolism is consistent with apoptosis being an active energy-requiring process.

A small pilot study was performed to determine whether different levels of D-lactate may have different effects on cellular metabolism of endothelial cells. Variations in cellular metabolism caused by different levels of D-lactate were determined by analysis of changes in cellular NADH levels. The highest increase in cellular metabolism activity was noted in the first 6 hours at 0.2 mM of D-lactate concentration, followed by 0.5 mM of D-lactate. A peak at 6 hours was followed by the decrease in cellular metabolism activity by 24 hours. At 2 mM of D-lactate concentration the cellular metabolism remained below control levels. It is unknown whether lower cellular metabolism activity at 2 mM is due to decreased number of living cells at 2 mM as cell viability was not determined in this optimization experiment. Previous research confirmed reduction in cell proliferation of HUV-EC-C cells after exposure to higher concentrations of glucose (Varma et al. 2005). Whether lower number of cells is due to reduced proliferation rate or increased cell apoptosis was questioned by other authors (Davidson and Yellon 2006). More research is needed to investigate what happens long term and whether or not changes in metabolism activity caused by D-lactate can affect cell proliferation or cell apoptosis long term (up to 15 days).

According to previous reports, high glucose, methylglyoxal and D-lactate levels cause changes in expression levels of genes involved in regulation of apoptosis in some tissues (Risso et al. 2001, Riboulet-Chavey et al. 2006, Jaskille et al. 2006). Apoptosis is a highly regulated process and can be initiated by two important pathways, intrinsic and extrinsic pathway. In my study, I measured time dependent D-lactate effects on the expression levels of well-known pro-apoptotic (BAD) and anti-apoptotic (Bcl-2) genes, as well as expression levels of upstream pro-survival PI3K and AKT genes, components of the intrinsic apoptotic pathway. In addition, expression levels of pro-survival eNOS were evaluated. To further investigate apoptotic effects of D-lactate, Human Apoptosis Array was used for evaluation of 84 pro/anti apoptotic genes. My data suggest time dependent differences in expression levels of relevant pro/anti apoptotic genes. Levels of all genes (AKT, BAD, Bcl-2) were decreased up to 24 hours and then increased at 48 hours of exposure. Activation of phosphatidylinositol 3-kinase (PI3K) system followed by stimulation of AKT, is suggested to be the dominant pro-survival signaling pathway (Song, Ouyang, and Bao

2005). Activation of AKT pathway allows the cell to survive even after caspase cascade activation, altering apoptotic transcription response and directly targeting components of the caspase cascade (BAD, caspase 9) (Datta, Brunet, and Greenberg 1999). It is now well recognized that apoptosis may occur due to activation of the caspase cascade, downregulation of the pro-survival PI3K/AKT pathway, or both. In a lung resuscitation study DL-lactated Ringer's solution increased expression of proapoptotic proteins as well as the less effective PI3K/AKT pathway (Jaskille et al. 2004). In contrast, in a second study where hepatocellular apoptosis was reported in the presence of lactated Ringer's solution, apoptosis was observed without any significant alterations in the PI3K/AKT system (Jaskille et al. 2006). In the same study, after alterations of Ringer's solution, a pro-survival response was reported (decreased BAD and increased Bcl-2 expressions), indicating inhibition of apoptosis through a non-AKT pathway (Jaskille et al. 2006).

Endothelial nitric oxide synthase (eNOS) expression levels are also regulated through phosphorylation by AKT (Shiojima and Walsh 2002), which represents another protective anti-apoptotic mechanism. In our study, expression levels of eNOS were decreased up to 24 hours followed by an increase at 48 hours. eNOS is responsible for the synthesis of NO and has a pro-survival effect on the cell. The events observed in our system cannot be explained with certainty at this point. Proteins in the PI3K pathway have other functions than apoptosis. In addition, Varma et al. (2005) found no correlation between mRNA expression levels of pro/anti apoptotic proteins and their activities in HUV-EC-C after high glucose exposure. Even though mRNA expression levels were not significantly affected, significant changes in enzyme activities was reported. This inconsistency may be explained by the fact that enzyme activity can be affected by post-translational modifications and allosteric regulators. At this point it is not certain whether the observed changes in expression levels of mRNA of pro/anti-apoptotic genes are consistent with changes in enzyme activities and consequently apoptosis.

In our study, expression levels of PI3K remained below control even after 48 hours, alluding to potential downregulation of the PI3K/AKT pathway. In addition to having an effect on apoptosis, dysregulation in PI3K can also affect insulin stimulated glucose uptake in muscle cells (Riboulet-Chavey et al. 2006). If muscle is affected, the overall glucose homeostasis will be disturbed. Furthermore, the PI3K/AKT pathway has an effect on cell survival, migration, and capillary like structure formation by endothelial cells (Shiojima and Walsh 2002). Rather than having a direct effect on apoptosis, we suspect that D-lactate through PI3K might influence other

components of endothelial function such as cell survival, migration, and capillary like structure formation.

An additional experiment that was performed (data not shown) showed that no quantifiable levels of D-lactate were present in media at 48 hours post exposure to D-lactate. The increase in expression levels observed at 48 hours might be due to disappearance of D-lactate in media and return to normal physiological function. More research is needed to further investigate whether the observed changes in mRNA expression levels are due to disappearance of D-lactate in media or quick cell adaptation to D-lactate exposure. The recommendation is that measurement of both mRNA expression levels as well as enzyme activities, should be performed with treatment with D-lactate every 24 hours and measurements determined up to 96 hours of continuous D-lactate exposure.

The Human Apoptosis PCR Array identified significant changes in the expression of the TNF gene family, important components of the extrinsic apoptotic pathway. After D-lactate treatment a decrease at 24 hours with significant increase at 48 hours was observed in lymphotoxin alpha (LTA), tumor necrosis factor receptor superfamily member 10A (TNFRSF10A), tumor necrosis factor receptor superfamily member 25 (TNFRSF25), and tumor necrosis factor receptor CD27. These receptors bind tumor necrosis factor-related apoptosis inducing ligand (TNFSF10/TRAIL), monogamous TL1A ligand (TNFSF15), and CD27-binding protein (SIVA), respectively. These receptors are essential in mediation of TNF-dependent signals from the cell membrane to nucleus and have a role in activation of apoptosis through regulation of NF-κB and FADD (Fas-Associated protein with Death Domain) (Grunert et al. 2012). After activation FADD recruits caspase-8 to the activated receptor, resulting in DISC (Death Inducing Signaling Complex) formation and caspase cascade activation (Grunert et al. 2012). NF-κB has both, pro- and anti-apoptotic functions, which depends on the nature of apoptotic stimulus. After stimulation with TNF family members, pro-apoptotic effects become dominant (Iimuro et al. 1998, Plumpe et al. 2000). In an additional study we found a decrease in NF-κB expression levels after 24 hours and an increase at 48 hours (data not shown). D-Lactate might express apoptotic effects through TNF stimulation of NF-κB. We found significant time dependent changes in genes of inhibitor of apoptosis (IAP) family, BIRC3 and BIRC8. BIRC3 and BIRC8 levels were increased at 24 hours, followed by a decrease at 48 hours. IAP are a family of proteins that act as endogenous inhibitors of apoptosis. They express anti-apoptotic effects by binding and inhibition of caspase (Deveraux

and Reed 1999). Upregulation of pro-apoptotic genes of the TNF family, and downregulation of anti-apoptotic IAP genes might create conditions in endothelial cells that lead to apoptosis. In addition, caspase 5 (CASP5) expression levels were increased at 24 hours (3 fold), with a decrease (2 fold) at 48 hours. CASP5 is a component of the inflammasome and is involved in inflammatory processes. It is inhibited by Bcl-2 anti-apoptotic gene (Martinon, Burns, and Tschopp 2002, Liu, Liu, and Qu 2012).

Evidence for apoptosis was obtained by several methods. Furthermore, caspase 3/7 activities were evaluated. Being a downstream executioner, caspase 3/7 activities were previously determined as solid evidence of apoptosis. In my study, no significant changes in caspase activity compared to control was found in both D-lactate alone and combination treatment up to 48 hours. An increase in caspase activity was observed after exposure to D-lactate at 72 and 96 hours (118 ± 32.7; 170 ± 40.5, % of control respectively). The difference in caspase activity was found at 72 and 96 hours after one single D-lactate exposure and daily D-lactate treatments, with significant increase in caspase activity after continuous treatment. This finding correlates with our results showing disappearance of D-lactate from the media of treated cells after 48 hours. This finding on caspase activity has study limitations. During our measurements, cell morphology was not observed in detail and the number of living/dead cells was not recorded. The large variations in recordings and lack of significant increase in relative caspase activity may be due to decreased signal caused by lower number of living cells. It is recommended for the experiment to be repeated with continuous D-lactate treatment (every 24 hours) for at least 96 hours. During each measurement it is recommended that a picture of the cells (capturing cell morphology) is taken and number of cells in each well counted. In such a controlled experiment, caspase activity per number of living cells should be calculated. This approach will exclude potential error in caspase activity signal caused by lower number of living cells in treated vials compared to untreated vials.

Decreased energy production in the cell can lead to apoptosis. It is hypothesized that once D-lactate enters the cell, it can interfere with the utilization of energy substrates such as L-lactate and pyruvate. Endothelial cells largely meet all energy needs through non-mitochondrial energy production through anaerobic glycolysis and utilization of L-lactate (Krutzfeldt et al. 1990). After treatment with both D-lactate and combination of D-lactate, glucose and methylglyoxal we did not observe any significant changes in high energy phosphate (ATP, ADP, and AMP) and creatine compounds (Cr and CrP) levels in our short term study (up to 48 hours). Previous reports showed

that endothelial cells have high ability to resist changes in energy conditions and maintain cellular ATP levels for prolonged periods of time despite deficiencies in energy substrate levels (Culic, Gruwel, and Schrader 1997). Also, when certain substrates are depleted within the cell such as lactate or pyruvate, endothelial mitochondria play a role in cellular energy production through oxidative phosphorylation (Dagher et al. 2001, Davidson and Duchen 2007). My study investigated energy changes in a short time frame (up to 48 hours) and further research is needed to investigate whether chronic exposure of endothelial cells lead to changes in energy production or results in a switch to mitochondrial ATP production.

Reactive oxygen species are known to cause a series of physiological changes that finally lead to apoptosis. Oxidative stress has a role in endothelial dysfunction associated with various vascular disorders (Cai and Harrison 2000). ROS are important signaling molecules and if ROS are overproduced may interrupt normal cell signaling (Brieger et al. 2012). In addition, ROS initiates free radical formation, a chain reaction that can alter normal cell physiology and lead to cell death. After treatment with different elevated concentrations of D-lactate an increase of ROS production was observed (>160% of control). An increase in ROS was observed after treatment with glucose and methylglyoxal as well, which has been well demonstrated in previous research (Bonnefont-Rousselot 2002, Desai and Wu 2008). Significantly higher increases in ROS production were observed when D-lactate was combined compared to glucose and methylglyoxal and when compared to each of them alone. The increase in ROS production when D-lactate was combined with glucose and methylglyoxal might be due to additive effect of stressors on endothelial cell function. ROS are well known to trigger apoptosis through different mechanisms, one of which is triggering of death receptor TNF family and Fas (Simon, Haj-Yehia, and Levi-Schaffer 2000). We have previously recorded an increase of mRNA expression levels of TNF family after exposure to elevated D-lactate. Increased ROS production by D-lactate might contribute to apoptosis through activation of TNF family. Disruption of the mitochondrial membrane potential by ROS results in oxidation of mitochondrial pores, which may initiate the caspase cascade through mitochondrial cytochrome C release (Zamzami et al. 1995). It was previously reported that ROS have strong interactions with eNOS. Superoxide anions react with NO resulting in peroxynitrile anion formation ($ONOO^-$) formation, and decreased beneficial effects of NO on vascular homeostasis and inducing apoptosis. Furthermore, increased ROS

concentration decreases the ability of eNOS to produce NO and results in superoxide anion production instead, and consequential endothelial dysfunction (Kuzkaya et al. 2003).

In addition to apoptosis, increased ROS concentration causes direct damage of cell structures. ROS leads to a series of reactions that result in oxidative damage of DNA, lipids, proteins and important enzymes (i.e. antioxidative enzymes) and organelles (i.e. mitochondria) that are necessary for maintaining cellular function. Accumulating oxidative damage will decrease efficiency of mitochondria and other defense mechanisms and further increase ROS production and damage of cells and tissues (Kumari et al. 2013). D-Lactate and combination of D-lactate, glucose and methylglyoxal stimulated increase of ROS might interfere with normal cell physiology and contribute to apoptosis of vascular endothelial cells in the long term.

The lack of quantifiable levels of D-lactate in media after 48 hours in cell culture may be the result of cellular uptake and metabolism of D-lactate. Examination of the mRNA expression of the enzyme that metabolizes D-lactate in the cell, D-lactate dehydrogenase, suggested that this enzyme is not expressed in HUV-EC-C. Loss of D-lactate in the cell media remains unknown and requires further investigation. Nonetheless, the body of data does indicate that D-lactate is transported into HUV-EC-C cells where it might interfere with metabolism of other energy substrates (L-lactate and pyruvate) (Poole et al. 1990, Trosper and Philipson 1987, Ros et al. 2001) or can cause other functional changes of the cell (i.e. ROS production).

This study has some obvious limitations. For logistic reasons, we could not have numerous time points and combination of treatments for all experiments. We could not study numerous other pro- and antiapoptotic pathways that may have been involved. We have selected the most commonly described apoptotic pathway PI3K/AKT and have used PCR Array to cover the more relevant apoptotic genes. Many of investigated proteins have other functions, not related to apoptosis. This was short term *in vitro* model, evaluating early D-lactate effects on vascular endothelial cells function and survival. We have shown changes in expression levels of different pro- and antiapoptotic proteins, increase in caspase 3/7 activities, and increase in ROS production. However, it is common for biological systems to be flexible and have the ability for self-repair. Whether changes observed in this study lead to long term apoptosis of vascular endothelial cells and endothelial dysfunction is not clear. More research is necessary to further investigate effects of D-lactate on the function of vascular endothelial cells.

6. FUTURE DIRECTIONS

Effects of D-lactate on endothelial cell function require further investigation. We did not definitively confirm that D-lactate causes endothelial cell apoptosis in our study. However, we suspect that D-lactate is influencing important signaling pathways in endothelial cells that eventually lead to endothelial dysfunction through different mechanisms, including alterations in cell survival and cell migration.

HUV-EC-C's slow rate of cellular proliferation was a significant limitation of my studies. I suggest an alternative cell culture system to overcome this important limitation. A cell line that undergoes more rapid growth characteristics would allow more extensive simultaneous evaluations involving more time point evaluations and combinations of D-lactate with glucose and/or methylglyoxal. Immortalized bovine umbilical vein endothelial cells and primary bovine pulmonary artery endothelial cells are confirmed to have similar phenotypic characteristics of endothelial cells *in vivo* and are used as *in vitro* system for studies of endothelial function and endothelial metabolism (Gospodarowicz et al. 1978, Cajero-Juarez et al. 2002). Bovine endothelial cells grow faster than human cells when cultured (Gospodarowicz et al. 1978), which allows for more productive experiments.

We found that D-lactate disappears from the media of treated cells after 48 hours. I suggest that all experiments conducted in my research such as mRNA expression levels of pro/anti apoptotic proteins, caspase activity, and ROS effects in endothelial cells be repeated with daily media changes to allow for a continuous D-lactate exposure. Previous research found changes in activities of pro/anti apoptotic proteins, even though mRNA expression levels of those proteins were not changed (Varma et al. 2005). Therefore, in addition to mRNA expression levels, measurement of activities of pro/anti apoptotic proteins should be carried out using western blot. Furthermore, activity of an important modulator of endothelial function, eNOS, should be measured to investigate whether it interferes with increased ROS production caused by D-lactate. Additional experiments are required to determine whether recorded changes in mRNA levels of TNF family lead to changes in protein levels. In addition, changes in cell surface expression of the death receptors should be measured.

Chronic complications are the major outcome of diabetes mellitus progress. Therefore, long term effects of D-lactate on endothelial cell function should be investigated. It was confirmed that

high glucose levels affect endothelial cell proliferation long term (Varma et al. 2005). Whether endothelial cells undergo apoptosis or necrosis depends on the amount of glucose present. Long term D-lactate effects on endothelial cell proliferation have not been investigated. Cell proliferation of endothelial cells after long term (up to 15 days) of continuous D-lactate exposure require investigation. It should be determined whether D-lactate causes decreased cell proliferation or increased cell death through apoptosis/necrosis. Therefore, it would be advisable to measure apoptosis/necrosis of endothelial cells in presence of D-lactate using flow cytometry.

7. SUMMARY OF FINDINGS

The principle of aim of this thesis was to provide evidence for the ability of D-lactate to enhance apoptosis of human umbilical vein endothelial cells that may, in part, explain endothelial dysfunction associated with type II diabetic patients. D-Lactate exposure of the human umbilical vein endothelial cell line, HUV-EC-C, showed time dependent changes in expression of different pro/anti apoptotic genes. Upregulation of pro-apoptotic TNF family and downregulation of anti-apoptotic IAP family of genes might suggest that D-lactate has an effect on HUV-EC-C apoptosis through the extrinsic pathway. The lack of association between the mRNA levels of pro/antiapoptotic genes at 48 hours and actual apoptosis was not unexpected because regulation of apoptosis is a complex process. Further research is needed to determine whether pro- or antiapoptotic effects will predominate long term.

The decrease in expression levels at 24 hours may be explained as an effect of D-lactate treatment on apoptosis. The return of expression to control or increased levels at 48 hours may be explained as a result of D-lactate loss from cell culture over time. Alternatively, HUV-EC-C cells may have robust short-term self defense mechanisms with appropriate compensatory responses to D-lactate exposure to assure survival. Caspase activity, a direct measurement of apoptosis, showed increases in HUVEC cells over several days of continuous D-lactate exposure. This correlates with the fact that diabetes mellitus is a chronic metabolic disease that takes years to develop. The diabetic condition forces the body to undergo adaptive changes to address the current anomaly in the system. In time, after the constant presence of different stressors, the body is unable to adequately compensate and vascular complications become noticeable. Sometimes it takes years or decades for complications to develop.

Functional assessments in D-lactate and the combination of D-lactate, glucose and methylglyoxal did not show any significant differences in high energy phosphate levels in my short term study. Endothelial cells are highly resistant to changes in substrate availability for energy production. In case of substrate depletion of L-lactate, necessary for anaerobic glycolysis, mitochondria can become an important source of cellular energy. In addition, when energy levels are decreased, endothelial cells are highly capable of reorganizing energy needs within the cell and decreasing cellular stress. Our study investigated energy changes in a short time frame (up to 48

hours). Endothelial cells can resist short term, but what happens long term while endothelial cells are exposed to constant non-physiological conditions and stress?

HUV-EC-C exposure to D-lactate, glucose and methylglyoxal resulted in a significant increase in ROS production, with significantly higher ROS production when substrates are combined. Increased ROS concentration leads to a series of reactions that culminate in damage of DNA, lipids, proteins and important enzymes that are necessary for maintaining cell function. In addition, ROS contributes to apoptosis through activation of TNF family. We propose that increased D-lactate concentrations lead to ROS overproduction that further lead to apoptosis through activation of TNF family.

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