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C-peptide Improves Hyperglycemia-induced Endothelial Redox Balance by Preventing Mitochondrial Oxidative Stress and Enhancing NADPH Synthesis

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**C-peptide Improves Hyperglycemia-induced Endothelial
Redox Balance by Preventing Mitochondrial Oxidative Stress
and Enhancing NADPH Synthesis**

Himani Vejandla

Dissertation submitted to the School of Medicine at
West Virginia University in
partial fulfillment of the requirements
for the degree of
Doctor of Philosophy
in

Cellular and Integrative Physiology

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Keywords: C-peptide, Hyperglycemia, Endothelial cells,
mitochondrial oxidative stress

ABSTRACT

C-peptide improves hyperglycemia-induced endothelial redox balance by preventing mitochondrial oxidative stress and enhancing NADPH synthesis

Himani Vejandla

C-peptide, a by-product of insulin biosynthesis, has been shown to ameliorate diabetes-induced renal impairment. Yet, the mechanisms underlying this protective benefit remain unclear. Our studies have shown that C-peptide improved renal peritubular capillary blood flow and reduced vascular oxidants. NADPH (reduced nicotinamide adenine dinucleotide phosphate), appears to be critical to this effect via the actions of numerous vasoprotective systems. Further, our experiments in type I diabetic mice resulted in a significant reduction in renal endothelial NADPH that is subsequently restored with C-peptide. We hypothesized that C-peptide provides protection to renal cortical endothelial cells during type I diabetes by restoring the activity of glucose-6-phosphate dehydrogenase (G6PD), the principal source of NADPH synthesis. Our data show that renal cortical endothelial cells (RCE) from diabetic mice have diminished G6PD activity and their treatment with C-peptide restores its activity back to control levels. These changes in G6PD activity occurred with concomitant alterations in NADPH. Using 2-D gel electrophoresis of RCE lysate coupled with immunoblotting for G6PD, we demonstrate that diabetic RCEs have a significant increase in G6PD post-translational modification (PTM). C-peptide treatment reduced the magnitude of this PTM in diabetic RCEs, which occurred concomitant to restored G6PD activity. These results suggest that improving the activity of endothelial G6PD, by preventing its PTM, may be a potential mechanism by which C-peptide confers protection to renal cortical endothelial cells during type I diabetes. Hyperglycemia-mediated microvascular damage has been proposed to originate from excessive generation of mitochondrial

superoxide in endothelial cells and is the suggested mechanism by which the pathogenesis of diabetes-induced renal damage occurs. To determine whether C-peptide affords protection to renal microvascular endothelial cell mitochondria during hyperglycemia we exposed conditionally immortalized murine renal microvascular endothelial cells (MEC) to low or high glucose (25 mM) media with either C-peptide (6.6 nM) or its scrambled sequence control peptide for 24- or 48-hours. Respiratory control ratio, a measure of mitochondrial electrochemical coupling, was significantly higher in high glucose treated renal MECs treated with C-peptide than those of high glucose alone. C-peptide also restored high glucose-induced renal MEC mitochondrial membrane potential changes back to their basal low glucose state. Moreover, C-peptide prevented the excessive mitochondrial superoxide generation and concomitant reductions in mitochondrial complex I activity that are mediated by the exposure of the renal MECs to high glucose. Together, these data demonstrate that C-peptide protects against high glucose-induced generation of mitochondrial superoxide in renal MECs via restoration of basal mitochondrial function. Although interest in the physiologic benefits of C-peptide has persisted for more than two decades, C-peptide has yet to make its way into standard treatment regimens for various diabetic complications. The findings from our work have provide proof-of-principle evidence in support of the inclusion of C-peptide to the existing therapeutic regimen for treatment of diabetic complications, specifically those related to diabetes-induced renal impairment.

LIST OF ABBREVIATIONS

AGE	Advanced Glycation End-products
AMDCC	American Models of Diabetic Complications consortium
cAMP	Cyclic Adenosine Mono Phosphate
CAT	Catalase
DM	Diabetes Mellitus
DN	Daibetic Nephropathy
EC	Endothelial Cells
eNOS	Endothelial Nitric Oxide Synthase
ERK	Extracellular-Signal Related Kinase
G6PD	Glucose-6-Phosphate Dehydrogenase
GFR	Glomerular Filtration Rate
GSH	Reduced Glutathione
GSSG	Glutathione Disulfide or Oxidized Glutathione
GR	Glutathione Reductase

GPx	Glutathione peroxidase
MAPK	Mitogen-activated Protein Kinases
MEC	Microvascular Endothelial Cell
NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Reduced NADP
NO	Nitric Oxide
NOX	NADPH Oxidase
OH	Hydroxyl Radical
PKA	Protein Kinase A
PKC	Protein Kinase C
PP	Protein Phosphatase
PTM	Post-translational Modification
RCE	Renal Cortical Endothelial Cell
ROS	Reactive Oxygen Species
STZ	Streptozotocin

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SPECIFIC AIMS

Diabetes mellitus (DM) is the leading cause of chronic kidney disease and kidney failure, with nearly 25-40% of patients with diabetes developing end-stage renal failure (21). Even after intensive insulin treatment for optimal glycemic control, patients continue to develop microvascular complications, which are the principal cause of diabetes-induced renal dysfunction (1). Although diabetic nephropathy (DN) is characterized by glomerulosclerosis (4), recent evidence also indicates that glomerular damage causes stagnation of peritubular blood flow and thus development of tubulointerstitial injury and ultimately peritubular capillary loss (15, 16). It has long been accepted that the peptide that connects the A- and B-chains of insulin, known as C-peptide, is biologically inert. However, many studies have demonstrated that replacement doses of C-peptide can ameliorate diabetes-induced renal dysfunction (8-11, 13, 17, 18, 23-25). Yet, the mechanisms underlying the benefits of C-peptide during diabetes are still unclear.

DM has been widely recognized as a state of imbalance between oxidants and antioxidant protective systems (2, 5, 6). Moreover, chronic hyperglycemia associated with diabetes elevates oxidative stress in renal microvasculature (22, 26), directly damages the microcirculation leading to small vessel dysfunction (14), and ultimately manifests as kidney dysfunction (6, 19). **Our central hypothesis is that C-peptide prevents diabetes induced renal microvascular dysfunction by ameliorating oxidative imbalance created within endothelial cells.**

NADPH, a critical cofactor in preserving normal microvascular function through actions of multiple vasoprotective systems like heme oxygenase -1 and nitric oxide synthase (NOS), is depleted in microcirculation owing to increased consumption in multiple pathways including glucose metabolism (7, 20) and NADPH oxidase (NOX) (2, 3). G6PD, the first rate-limiting enzyme of the pentose phosphate pathway, is the major source of NADPH (12, 27).

Our first working hypothesis is that microvascular dysfunction in type I diabetic kidney involves loss of NADPH, due to inhibition of G6PD activity, in renal cortical endothelial cells.

Specific Aim 1: To determine whether C-peptide confers microvascular protection by enhancing renal endothelial antioxidative capacity. To address Aim 1, we characterized renal dysfunction in a streptozotocin (STZ)-induced mouse model of type I diabetes.

1.1: We examined the effect of C-peptide on peritubular capillary perfusion in kidney cortices from STZ- induced type I diabetic mice.

1.2: We examined whether the alterations in microvascular function are reflected as biochemical changes in freshly isolated diabetic RCEs.

1.3: We evaluated the mechanisms responsible for impaired endogenous vasoprotection, NADPH levels, G6PD activity, and phosphorylation of G6PD in RCEs, in the presence and absence of C-peptide.

Our second working hypothesis is that C-peptide independently affords protection to high-glucose exposed renal MECs by diminishing mitochondrial superoxide generation via restoration of basal mitochondrial complex I activity

Specific Aim 2: To determine whether C-peptide ameliorates high-glucose induced microvascular dysfunction by preventing mitochondrial superoxide. To examine this we employed conditionally immortalized renal MECs, exposed to both normal and high glucose conditions, to investigate the effects of C-peptide on mitochondrial parameters.

2.1: We evaluated the effects of C-peptide treatment on renal MEC mitochondrial respiration, membrane potential changes, superoxide production and complex activity under exposure to a high glucose environment.

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Chapter 1:

Literature Review

1.1 Diabetes Mellitus

DM represents a heterogeneous group of metabolic diseases characterized by chronic hyperglycemia resulting from deficiencies in the actions of insulin, either through a lack of production or an insensitivity of its receptors. Depending on the severity, DM may present itself with overt symptoms of hyperglycemia including polydipsia, polyuria, weight loss, sometimes with polyphagia, and vision disturbances. If left uncontrolled, hyperglycemia often leads to biochemical imbalances that progress to acute life-threatening events, such as ketoacidosis, coma and even death (2) Based on the pathogenesis, DM is broadly classified into 1) type I, with an absolute deficiency of insulin secretion, 2) type II, with a combination of resistance to insulin action and an inadequate compensatory insulin secretory response, and 3) gestational DM, any glucose intolerance developed or detected during pregnancy (2). Ultimately, the major causes of morbidity and mortality in diabetic patients are the long-term complications, mainly involving microcirculation of the eyes, kidneys, nerves and heart.

The Diabetes Control and Complications Trial (DCCT) study demonstrated a decreased occurrence of complications in the group of type I DM patients that received intensive insulin therapy and achieved improved blood glucose control (4). This is all in keeping with the view that hyperglycemia is a major culprit in the pathogenesis of microvascular complications. Even with strict glycemic control, a large proportion of these patients developed complications (3). Microvascular complications are devastating; they greatly reduce life expectancy, and adversely

affect the quality of life amongst those affected (98). DM is also associated with an increased incidence of macrovascular diseases (i.e., peripheral arterial and cerebrovascular diseases).

1.1a Global Health Perspective

Widely recognized as one of the leading causes of death and disability worldwide, DM imposes a large economic burden on national health care systems (187). In 2006, DM was the seventh leading cause of death. In most cases DM is reported only as the underlying cause of death, with heart disease, stroke and kidney failure as the attributing causes of death. Accounting for more than 10% of total health care expenditure, DM is emerging as a global health crisis (25). In the US alone, the total costs incurred on diabetes was \$174 billion in 2007 (1).

1.1b Type I Diabetes Mellitus

Previously called insulin-dependent or juvenile-onset, this form of DM develops most often in children but can occur at any age. Resulting from autoimmune-mediated pancreatic β -cell destruction type I DM accounts for 5-10% of the diabetic population. Individuals suffering from this form of DM exhibit varying degrees of insulin dependency and deficiency as well as a risk for ketoacidosis. In the latter stages of disease, many patients encounter an absolute requirement for insulin replacement therapy.

1.1c Type II Diabetes Mellitus

This form of DM accounts for 90-95% of those with diabetes and develops typically after middle age, but may occur in young people. Individuals with type II DM have insulin resistance [with normal or elevated insulin levels] followed by relative insulin deficiency [due to defective insulin secretion]. The risk of developing type II DM increases with age, obesity and lack of physical activity. It frequently goes undiagnosed for many years as the hyperglycemia develops gradually. At its earlier stages it is not often severe enough for the patient to notice any of the classic symptoms of diabetes.

1. 2 Diabetic Kidney Disease

Considered the leading cause of end-stage renal disease, DN manifests within 20-25 yrs of onset of the disease (56). One out of three diabetics worldwide develop DN (129). With recent advances in the management of DM and the consequent decrease in diabetes-associated mortality, patients are living longer and more likely to develop microvascular complications. DN bears a significant burden to the society and this is illustrated by the fact that in 2009 ~6.5% of US Medicare budget is directed to the end-stage renal disease population (5). The earliest clinical evidence of incipient DN is the development and progression of persistent microalbuminuria (urinary albumin excretion rates between 20-200 $\mu\text{g}/\text{min}$), which is also a strong predictor for the future development of end-stage renal disease (115-118). The majority of the type I diabetics progress to overt albuminuria ($>200 \mu\text{g}/\text{min}$) more rapidly than that of rate compared to type II diabetics (56). Other hallmark features of DN include glomerular hyperfiltration, basement membrane thickening,

mesangial extracellular matrix expansion and ultimately glomerulosclerosis with renal dysfunction (50).

Major clinical treatments for DN continue to target hyperglycemia and hypertension (105). Importantly, inhibition of renin-angiotensin-aldosterone axis, and in particular angiotensin II (AngII) has a major impact on slowing the progression of kidney damage. Currently the best approach to treatment of DN includes achievement of optimal glycemic control in combination with tight control of blood pressure (174). Nevertheless, the incidence of DN continues to rise and underscores the compelling need for new treatments.

1.2a Pathophysiology – Structural alterations

Persistent hyperglycemia initiates several complex reactions in the renal vasculature that culminate in DN. Structurally; the kidney is comprised of filter units - nephrons, blood vessels and interstitial tissue. Diabetic kidney damage mainly occurs due to changes in the blood flow through the microcirculation of the glomerular capsule (157). Within the capsule, oxidative remodeling of the vessel walls lead to reduced flow of oxygenated blood and impairment of vessel wall integrity. These changes ultimately lead to irreversible loss of glomerular capillaries, and eventually kidney function (123).

Many studies have shown that hyperglycemia also affects glomerular mesangial cells (66, 158, 170). However, there is a recent shift in the focus to other glomerular cell types and tubulo-interstitial cells (45, 106, 125). Initially, glomerular damage results in post-glomerular ischemia and is associated with downstream

hypoxia, subsequent oxidative stress, tubular injury, and eventually nephron loss. These events, in turn, impose hemodynamic stress in the remaining nephron units (149). Finally, tubulo-interstitial damage results in the loss and distortion of peritubular capillary plexus (123, 124), a network of capillaries that are fed by glomerular efferent arterioles and supply nutrients and oxygen to tubular and interstitial cells.

1.2 b Pathophysiology – Renal hemodynamic alterations

Several lines of evidence indicate that renal hemodynamics are altered in clinical and experimental DM, and that changes in renal hemodynamics play a role in the pathogenesis of the DN (12, 32, 79, 130). Moreover, studies have documented a consistent increase in glomerular filtration rate (GFR) in type I DM during periods of poor metabolic control (12, 23, 38). Conversely, when hyperglycemia is more severe, as in diabetic keto-acidosis, hyperfiltration no longer occurs. Thus, because of the potential importance of alterations in renal microcirculatory dynamics to these functional responses of the glomerulus in DM, it is important to understand the hemodynamic determinants of GFR under normal and diabetic conditions.

The balance of vascular tone of the afferent and efferent arteriole is a crucial determinant of glomerular haemodynamics. Despite their intimate anatomical relationship in the juxtaglomerular apparatus, the mechanisms that regulate afferent and efferent arteriolar tone are different (8). In the afferent arteriole, two intrinsic mechanisms, the myogenic response and macula densa-mediated tubuloglomerular feedback (TGF) play a dominant role, maintaining the GFR at a

constant level over a wide range of renal perfusion pressure. Studies have shown that these two mechanisms are modulated by nitric oxide (NO) (90, 172). In addition, an interaction between TGF and AngII seems to be essential to maintaining GFR despite large variations in daily intake of salt and water (172). In the efferent arteriole, neither myogenic response nor TGF seems to be important, while AngII is one major factor involved in the control of vascular resistance. In addition, recent studies have provided evidence that NO and prostaglandins produced by the glomerulus may control resistance of the downstream efferent arteriole (144). As the early segment of the efferent arteriole resides within the glomerulus, various autacoid hormones produced by the glomerulus may reach and directly act on this segment, thereby controlling the glomerular capillary pressure (144).

Decreased renal vascular resistance and diminished responsiveness to numerous vasoconstrictor stimuli are evident during the early stage of DM (78). Indeed, DM impairs afferent arteriolar myogenic and tubuloglomerular feedback responses as well as autoregulation of renal blood flow (78). In rats with STZ-induced DM, the renal vasodilation is localized to the preglomerular vasculature whereas efferent arteriolar resistance is either normal or minimally decreased, a situation that promotes glomerular hypertension and hyperfiltration (7, 12). Further, studies have confirmed that juxtamedullary afferent arterioles are dilated and exhibit reduced constrictor responsiveness to noradrenaline during diabetic hyperfiltration, while these parameters of efferent arteriolar function remain unaltered (130).

1.3 Endothelial Dysfunction in Diabetes

Endothelial dysfunction is the inability of the endothelium to properly maintain vascular homeostasis and results mainly from the reduced bioavailability of NO. The main functional manifestation of this loss of NO bioavailability is impairment of endothelium dependent vasorelaxation (30). Although the underlying mechanisms responsible for enhanced degradation of NO seem to be diverse, during the manifestation and pathogenesis of DM they originate from the effects of hyperglycemia (24), advanced glycation end products (AGE) (28) and oxidative stress (34, 72).

Impaired endothelial function is a fundamental component in the development of diabetic microvascular and macrovascular complications (167). The delicate balance between endothelium dependent relaxation and contraction is altered in diabetes. In addition, DM is associated with increased levels of circulating endothelium-derived adhesion molecules and plasminogen activator inhibitor-I, reflecting a pro-inflammatory and pro-thrombotic endothelial phenotype (114). Onset of endothelial dysfunction is coincident with impaired glucose tolerance, and worsens with the progression of diabetes. Jensen et al., were the first to describe the presence of endothelial injury in type I diabetic patients with incipient nephropathy, as evidenced by an increase in their plasma levels of Von Willebrand factor (83, 84)

DN, a serious complication of DM, can progress to end-stage renal disease. However, the incidence of nephropathy in diabetic patients is variable, with

epidemiological studies showing that only 30% of patients develop overt symptoms (20, 80). Importantly, endothelial function was more severely impaired in patients with overt than incipient nephropathy (83, 84). Similarly, advanced renal lesions do not consistently develop in diabetic animals and require factors in addition to hyperglycemia, with endothelial NO deficiency and subsequent endothelial dysfunction as important contributors (120, 121). Endothelial injury in diabetes is accompanied by structural abnormalities, mainly the loss of endothelial fenestrations (170).

Brodsky and colleagues found that high levels of glucose induce the uncoupling of endothelial NOS (eNOS), which causes a reduction in NO bioavailability and a concurrent increase in superoxide production (24). In brief, the generation of NO from L-arginine by eNOS requires the presence of cofactors that can be inactivated by oxidants; when eNOS is uncoupled, superoxide as opposed to nitric oxide will be generated (69, 162). Even though several studies examined eNOS polymorphisms (40, 102, 147, 160), only some reported positive association of specific eNOS polymorphisms with DN (103, 126, 127, 161, 185). Some studies even reported increases in eNOS enzyme associated with diabetes (77, 166), but it is likely that the increased level of eNOS might be an inactivated form of the enzyme (24, 97). Further, diabetic eNOS knockout mice exhibit higher mortality from progressive renal disease (120), adding strength to the hypothesis that a lack of endothelial NO could be a key factor in the development of advanced DN. Even the failure of rennin-angiotensin system inhibitors to prevent the progression of DN might be accounted for by endothelial dysfunction (18, 113).

The association between diabetes and endothelial dysfunction is particularly true in patients with type I DM who have either early (microalbuminuria) or late (macroalbuminuria) nephropathy (122). What remains a debate is whether endothelial dysfunction is a cause or a consequence of microvascular injury in type I DM.

1.4 Oxidative Stress and Diabetes

1.4a Sources of ROS in Diabetes

A wealth of evidence largely from the last 15 years suggests increased oxidative stress in diabetes (19, 33, 135, 150). There are a number of sources for generation of ROS in diabetes including auto-oxidation of glucose, transition metal catalyzed Fenton reactions, mitochondrial respiratory chain deficiencies, augmented xanthine oxidase activity and activation of enzymes such as peroxidases, NOS and NOX (39). In DM, many studies have shown that NOX is the major source of reactive oxygen species (ROS) in cardiac, vascular and renal tissue (58, 134, 159, 164) with the expression of NOX subunits elevated in micro- and macrovascular tissues of diabetic animals (43, 48). Endothelial dysfunction linked to NOX-induced ROS generation has been observed in animal models, as well as in patients with diabetes (6, 70). Importantly, sustained increases in NOX activity deplete cellular NADPH an essential cofactor for the proper function of major antioxidant enzyme systems (159). Glucose auto-oxidation generates OH radicals (135) and is the major non-enzymatic source of ROS generation. In its enediol form, glucose is oxidized in a

transition metal-dependent reaction to an enediol radical anion that is converted into reactive ketoaldehydes and to superoxide anion radicals. Further, glucose oxidation can also lead to NOX activation via accumulation of AGEs and their receptor activation (61, 156, 177). Overproduction of ROS leads to the oxidation of tetrahydrobiopterin, a cofactor for NOS (162). If NOS lacks its substrate L-arginine or one its cofactors, it may produce superoxide instead of NO and this is referred to as NOS uncoupling (69, 162).

1.4b Deficiency in Antioxidant Protective Systems

Data implicating changes in antioxidant enzyme systems in diabetes are conflicting (13, 19). These differences likely reflect heterogeneity of models (including cell type studied, animal model used, and time tissues were examined).

The following studies indicate reductions in antioxidant capacity in diabetic settings.

a) The activity and expression of catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and superoxide dismutase (SOD) are decreased in various tissues (13) with long-term DM, as well as in a transgenic mouse model of DN (55).

b) Reduced glutathione (GSH) concentration is decreased in the liver, kidney and pancreas of chemically induced diabetic animals (111).

c) Elevated oxidative stress is linked to decreased GSH and NADPH levels (37, 63) in diabetic rodent tissues.

1.4c Oxidative Stress and Diabetic Kidney Disease

Of particular interest in diabetic kidney disease is the superoxide production from mitochondria (159) and NOX (53, 159). It has been suggested that

mitochondrial superoxide is the initiating event in diabetes that turns oxidative stress into an evolving cascade of subsequent insults by stimulating more ROS production via downstream activation of nuclear factor-kappaB mediated cytokine production, protein-kinase C (PKC) activation and NOX (42, 43, 58, 85)

Elevated glucose levels associated with DM increased the expression and/or activation of NOX (77, 166). Renal expression of NOX is enhanced in STZ-induced diabetic rats (10, 48, 131). *In vitro* high glucose has been shown to activate NOX subunits in mesangial cells through PKC activation (71, 81). The inhibition of a NOX subunit, gp91phox, retarded mesangial matrix expansion in rats with DN and NOX-dependent ROS generation in renal cortical and glomerular homogenates, with concomitant reductions in whole kidney glomerular hypertrophy (10). Particularly, elevated levels of nitrotyrosine were observed with increases in gp91phox expression (52). Either direct inhibition of the assembly of NOX by treatment with apocynin (10) or indirect inhibition of NOX activity by angiotensin converting enzyme inhibition and angiotensin receptor blockade (52) resulted in significantly less diabetes-induced renal damage. These reports highlight the importance of NOX as a major source of oxidative stress in diabetic kidney.

1.5 NADPH and G6PD

1.5a Significance of NADPH

Several enzymes that catalyze substrate reduction generate nicotinamide adenine dinucleotide phosphate (NADP⁺). As such NADP and NADPH are the classic molecules involved in energy metabolism, antioxidant potential, and reductive

biosynthesis (15-17). The major biological impact of NADPH are three-fold: the first is to act as a key component in cellular antioxidant systems (184); the second is to act as an electron source for reductive synthesis of fatty acids, steroids and DNA (136); and the third is to act as the substrate for NOX, which plays a key role in many biological and pathological processes by generating ROS (184).

1.5b Sources of NADPH

The NADPH generated via mitochondrial enzymes likely contributes to mitochondrial antioxidant potential and biosynthesis (100, 175), while NADPH generated by cytosolic sources not only permit it to serve as an intracellular reductant, but also as a substrate for NOX-dependent ROS generation. There are four known enzymes that catalyze the formation of NADPH from NADP⁺ in cells. Firstly, there is G6PD and 6-phosphogluconate dehydrogenase - two enzymes in the pentose phosphate pathway; secondly, there are the cytosolic and mitochondrial NADP⁺ dependent isocitrate dehydrogenases; thirdly, there are the cytosolic and mitochondrial NADP⁺ dependent malic enzymes; and lastly, the mitochondrial transhydrogenase. However, the majority of these enzymes do not appear to be adequate sources of NADPH as much as G6PD (184). 6-phosphogluconate dehydrogenase produces as much NADPH as G6PD, but the NADPH produced by the former is entirely dependent on G6PD activity (184).

1.5c Role of NADPH in Cellular Antioxidant Potential

NADPH plays an important role in cellular antioxidant protection. To begin, NADPH is required for generation of GSH from GSSG (oxidized glutathione) through the

action of GR. GSH is the principal intracellular antioxidant in most mammalian cells, participates in the removal of H_2O_2 and toxic end-products of lipid peroxides (184); its depletion renders cells susceptible to oxidative damage. Secondly, a large portion of NADPH binds to the important allosteric binding site of CAT (93), which reactivates CAT when it is inactivated by H_2O_2 and maintains its most active tetrameric form (93). In red blood cells, NADPH plays a significantly more important role than GSH in defending oxidative insults, owing to its capacity in reactivating catalase (57). Moreover, NADPH is an essential component in the thioredoxin system (9). It also binds directly to the reductase domain of eNOS (186) and is required to maintain tetrahydrobiopterin stores via *de novo* synthesis. Another important antioxidant enzyme, heme oxygenase is also dependent on NADPH. It acts as a backbone for generation of antioxidants, such as biliverdin and bilirubin, through degradation of the highly oxidative heme. Biliverdin and bilirubin are considered potent antioxidants because of their ability to directly scavenge ROS and peroxynitrite (108-110). In addition, bilirubin has been shown to act as a potent suppressor of NOX, through unknown mechanisms, independent of its ROS scavenging effect (109).

Seemingly paradoxical, increasing evidence has suggested that NADPH could also significantly contribute to generation of oxidative stress through the activity of NOX (58, 67, 68, 134). Since NADPH has been shown to play both beneficial and detrimental roles in maintaining cellular oxidant status, one could speculate the importance of maintaining NADPH as a favorable endeavor.

Figure 1.1: Schematic depicting the effects of NADPH on cellular antioxidant capacity. Adapted from Ying W, 2008 (184). NADPH can be generated by G6PD, 6GPDH, NADP+ dependent isocitrate dehydrogenases(IDPs), NADP+-dependent malic enzymes(MEPS) and transhydrogenase (TH). NADPH can increase cellular antioxidant capacity by acting as a substrate for glutathione reductase (GR) to reduce GSSG to GSH that is required for activities of the antioxidant enzymes glutathione peroxidase (GPx) and glutathione-S-transferases (GST). NADPH can also increase antioxidant capacity by reactivating H₂O₂-inactivated catalase (CAT) and by promoting thioredoxin reductase (TrxR)-mediated generation of thioredoxin. Adapted from Ying *et al.*, 2008 (185)

1.5d NADPH Depletion During Diabetes

DN involves a state of imbalance between the increased production of oxidants and decreased antioxidant defenses. An important, but often overlooked, consequence of type I DM is the net depletion of NADPH (11). This net loss is owed to its increased consumption in multiple pathways including glucose metabolism (76, 142) and NOX (14, 31). With moderate-to- severe hyperglycemia, the

NADPH/NADP⁺ ratio, GSH concentration and G6PD activity are diminished in the liver and pancreas of diabetic rats (41) with concomitant increases NADP concentration.

1.6 Glucose-6-Phosphate dehydrogenase

1.6a Significance of G6PD

G6PD, a key enzyme for NADPH generation (96), has long been recognized as the only antioxidant enzyme in erythrocytes, the cell type with no alternative source of NADPH. G6PD is also the most studied among the enzymes that synthesize NADPH and is the main source of mammalian NADPH (101, 182). More importantly, G6PD is critical for cell survival via its essential role in normal cell growth and in preventing cell death (168).

G6PD was shown to be critically essential for defense against oxidative stress, although its role was found to be dispensable for pentose phosphate synthesis (133). For example, when fibroblasts are exposed to an oxidative insult, G6PD deficiency has been shown to manifest as premature senescence and diminished cell viability (75). Not surprisingly, G6PD deficient mice exhibit increased oxidative stress, reduced NADPH and GSH levels, and elevated markers of lipid peroxidation (182). Attempts to produce G6PD knockouts were unsuccessful mainly because complete knock-outs were embryonically lethal (104, 133). In vascular endothelium, G6PD is the principal source of NADPH (101) and regulates the activity of enzymes requiring NADPH as a cofactor, including eNOS (101). Thus,

in endothelial cells, G6PD is the critical determinant of intracellular redox state and creates an environment that favors the generation of NO. In response to agonists, G6PD-deficient endothelial cells produce less NO, as a result of eNOS uncoupling (179, 180). Further, G6PD over-expression results in lower endothelial oxidative stress and elevated NO levels (101, 112).

1.6b G6PD in Diabetes

Several studies have shown that G6PD activity is decreased in the liver, pancreas and other tissues of diabetic animals and patients (140, 141, 152, 171, 178). In addition to deficient G6PD activity, reductions in NADPH and GSH have been reported in diabetic conditions both *in vivo* and *in vitro* (171, 181). This inhibition of G6PD activity was attributed to cyclic Adenosine Mono Phosphate (cAMP)-mediated phosphorylation in high glucose exposed cultured endothelial cells, as well as in diabetic rat kidney cortex (181, 188). Interestingly, G6PD deficient mice exhibit renal damage as evidenced by increased urinary albumin, similar to those seen in experimental diabetic animals (182). Many even contend that diabetes is an acquired form of G6PD deficiency leading to decreased G6PD activity, NADPH levels and damage to kidney tissue and endothelium (164, 178, 182).

1.7 C-Peptide

1.7a History

In their quest to understand insulin biosynthesis, Steiner and colleagues (165) discovered pro-insulin. Pro-insulin is the intact parent molecule of insulin with the connecting peptide, C-peptide, serving the structural role of folding the A and B chains of insulin into a favorable conformation. C-peptide is subsequently cleaved from the pro-insulin molecule and released in an equimolar ratio with insulin. The functional impact of the action of C-peptide was confined to only a structural/stereometric role owing to data demonstrating the failure of C-peptide to have any influence on glucose metabolism or the lipolysis of isolated fat cells (95).

With the advent of advanced immunological methods, in the early-to-mid 1970s, a growing number of studies were aimed at measuring C-peptide concentrations in several diabetic settings, or compared portal and peripheral blood

Figure 1.2: Schematic representation of human proinsulin,. Adapted from Brandenburg, 2008. (22)

levels (95, 137, 151, 169). C-peptide, unlike insulin, undergoes negligible first-pass metabolism (151). This led to the journey of C-peptide as a diagnostic marker in DM (151). Therefore, peripheral C-peptide concentrations are now used as a valuable semi-quantitative marker of beta-cell function as well as insulin secretion under normal and diabetic conditions (137, 138, 169). Very careful studies by Kitabchi *et*

al., demonstrated that C-peptide possessed no insulin-like physiological activity (94).

Chance *et al.*, were the first to unravel the amino acid sequence of C-peptide in the process of isolating and characterizing proinsulin from crystalline porcine insulin (35). Intensive protein-chemical work conducted by several other groups on pro-insulin and C-peptide across several species helped determine and confirm the sequence of C-peptide (62, 183). Surprisingly, this work demonstrated the lack of structural homogeneity/ conservation among C-peptides isolated from different species, and further corroborated the previous work showing lack of functional significance. The highly conserved region amongst mammalian sequence patterns of C-peptide is only four amino acids long and includes four non-consecutive positions with glutamic acid, an interspaced glycine-rich segment and some other residues (62, 183).

The question as to whether C-peptide had a physiological role was re-visited by John Wahren's group from the Karolinska Institute in the early 1990's (176). Since then, interest into the physiological impact of C-peptide has persisted, and garnered significant support from the clinical observations that patients with type I DM, with a small level of endogenous beta cell activity, are less prone to develop long term complications (176). Replacement of physiological concentrations of C-peptide in type I diabetic patients that lack C-peptide resulted in significant amelioration of diabetes-induced abnormalities of regional blood flow, as well as improvements in peripheral nerve conduction velocity and kidney function (46, 86,

88, 89) Further, combined replacement of insulin and C-peptide was also deemed beneficial (87). These findings prompted the general hypothesis that C-peptide deficiency in type I DM may contribute to the development of microvascular complications, and ultimately its associated complications.

1.7b C-peptide and kidney dysfunction

The work of Johansson *et al.*, demonstrated that the administration of C-peptide for short term as well as long-term (3 months) to type I diabetic patients receiving insulin, reduced glomerular filtration rate and urinary albumin excretion (87). Net dilation of the efferent arteriole, as well as prevention of mesangial matrix expansion, were proposed as the underlying mechanisms in this 3-month double blind study (87). However the patients in these studies also exhibited better glycemic control, hence their claims suggesting the specific C-peptide related effects lacked support and warranted further studies.

The hypothesis that C-peptide has a renal protective effect started gaining more strength due to the data obtained from animal models of experimental diabetes. These studies exhibited similar beneficial renal outcomes, such as decreased GFR and proteinuria (107, 154, 155). The glomerular microcirculation is regulated, to a great extent, through alterations in the vascular resistance of the afferent and efferent arterioles (145). The constriction of afferent arterioles lowers glomerular filtration pressure, and thus decreases GFR. C-peptide constricts isolated renal afferent arterioles from diabetic mice, but not from normoglycemic mice with no changes in renal blood flow (155). However, the afferent constriction caused by

C-peptide was demonstrated in isolated arterioles, where the arteriole is not set up with an intact tubulus and thus is independent of TGF. In addition, it was recently shown by Sallstrom et al that TGF does not mediate diabetes-induced hyperfiltration, since diabetes-induced glomerular hyperfiltration occurs in adenosine A1-receptor-deficient mice known to lack a functional TGF mechanism (153). If TGF is not the mediator of diabetic hyperfiltration, it is unlikely that C-peptide would exert its effect on filtration via a TGF-dependent mechanism. Therefore, a possible conclusion is that C-peptide exerts its effect directly on the glomerular afferent arterioles. Yet another study reported that acute administration of C-peptide reduced GFR in diabetic rats via seemingly two different mechanisms, by causing a net dilation of efferent arteriole and by inhibition of tubular sodium reabsorption (128). Taken together, these studies suggest that C-peptide reduces diabetes-induced glomerular hyperfiltration via constriction of glomerular afferent arteriole, but with a simultaneous dilation of the efferent arteriole that counterbalances the effect of renal blood flow.

1.7c C-peptide - Cellular and Molecular effects

The cell signaling elicited by C-peptide, and its subsequent transduction pathways, were the subject of research mainly because C-peptide exhibited beneficial renal, neural and microvascular effects. Due to the behavior of c-terminal pentapeptide (akin to a peptide ligand interacting with its specific receptor), some studies postulated the existence of a C-peptide receptor (139, 146). Indeed, specific and displaceable binding of ¹²⁵I-labelled C-peptide was first demonstrated by Flatt

et al. (51) who derived a curvilinear Scatchard plot of specific C-peptide binding to pancreatic islet beta-cells. Surprisingly, the mid-region segment was reported to mediate non-specific interactions by Ido's group (82). However, the balance of activity provided by these two regions of the C-peptide molecule *in vivo* is not well understood. The general consensus about the structure-activity relationship of C-peptide suggests that it exists as a tripartite structure with its terminal sections responsible for functional interactions and the mid-portion forming a joining segment (189). Interactions of human C-peptide with plasma membranes were reported, with the following binding characteristics (139, 146): high affinity specific binding with an association rate constant of ~ 3 nM; half maximal occupation of binding sites at ~ 0.3 nM C-peptide; and full occupation at ~ 0.9 nM C-peptide. Importantly, the same study reported that the binding of C-peptide was inhibited by pertussis toxin. Overall, these results strongly support the existence of a specific GTP-binding protein-coupled receptor (GPCR) for C-peptide, linked either to the G-protein α -subunit $G\alpha_i$ or $G\alpha_o$, which interacts with the C-terminal pentapeptide region of the C-peptide molecule.

C-peptide has salutary effects on organ physiology and microvasculature of type I diabetic animals as well as diabetic animals. Consequently there is a clear need for molecular studies evaluating the effects of C-peptide in various microvascular cell types to delineate mechanisms that are the basis for the observed clinical effects.

1.8 Mitochondrial Oxidative Stress and Complex I

1.8a Mitochondrial Oxidative Stress

Both insulin-deficient and insulin-resistant diabetic states have been associated with mitochondrial dysfunction since the 1950s. There is an ongoing controversy as to whether mitochondrial dysfunction is a cause or consequence of diabetes and/or its complications. Despite several studies using the phrase “mitochondrial dysfunction”, it must be acknowledged that beyond dysfunction, evidence also exists for defects in mitochondrial biogenesis, number, morphology, and dynamics (29, 36, 92). Reductions in mitochondrial respiration were found for the skeletal muscle mitochondria of type II diabetic patients (92, 148). Conflicting reports also exist reporting a lack of change to heart and brain mitochondria, with apparent increases in renal mitochondrial respiration on succinate or complex I substrates from STZ diabetic rats (91).

Much of the work linking mitochondria to long-term diabetic complications is focused in cells that are insulin-independent, such as kidney podocytes and mesangial cells, capillary endothelial cells of the retina and neurons/Schwann cells of peripheral nerves, and vascular endothelial cells (27). DM, and therefore hyperglycemia, results in a constant exposure of this particular subset of cells exposed to excess glucose. This causes selective damage because of their inability to regulate glucose transport. The excess glucose entering these cells is fed into the tricarboxylic acid cycle leading to increased generation and channeling of electron donors (NADH_2 and FADH_2) into the electron transport chain. As a consequence,

Figure 1.3: Mitochondrial overproduction of superoxide activates four major pathways of hyperglycemic damage by inhibiting GAPDH. Adapted from Brownlee *et al.*, 2001 (26)

voltage across the mitochondrial membrane increases and electrons are transferred to molecular oxygen leading to the production of superoxide. Brownlee *et al.*, proposed this superoxide generation as a common element linking the damage pathways underlying the pathogenesis of diabetic complications (27). As indicated previously, superoxide reacts with NO to form peroxynitrite. This will induce lipid peroxidation and consume NO, which can impair endothelium-mediated vasodilation (74). Superoxide can also damage iron-sulfur centers, reducing catalysis by enzymes such as aconitase (173). Moreover, hydrogen peroxide, produced from superoxide by MnSOD, can react with iron to form the very reactive OH.

Several reports suggest that the diabetic state increases mitochondrial superoxide generation both in *in vitro* high glucose conditions (44, 49, 64, 143), as well as in diabetic animal models (29, 73). Furthermore, this excessive mitochondrial superoxide generation leads to the subsequent accumulation of other free radicals, thereby imparting diffuse damage to proteins and mitochondrial DNA, and ultimately leading to mitochondrial dysfunction (132, 163). Finally, mitochondrial damage and consequent dysfunction will disrupt calcium transit and induce the mitochondrial permeability transition, leading to apoptosis (54).

1.8b Complex I

Although all of the complexes of the mitochondrial electron transport chain contribute to total mitochondrial superoxide generation, growing evidence now suggests that complex I produces most of the superoxide generated within intact mammalian mitochondria (21). It is well known that complex II also has the capacity to produce superoxide, but this only occurs while in the presence of specific inhibitors and is not a physiologically relevant site of generation (65). Complex III, on the other hand, has been shown to produce large amounts of superoxide, at least in the presence of antimycin (65). Complex I is the first member of the mitochondrial membrane-bound electron transport chain and its substrates/co-factors have the lowest potentials in the chain (47). Inhibition of the electron transport chain during pathological conditions and/or the build-up of NADH (and subsequent elevation in the NADH/NAD⁺ ratio) have been shown to contribute significantly to superoxide generation by complex I (99). Under physiological

conditions, mitochondrial superoxide originates predominantly from complex I and complex III (59, 60), yet growing evidence suggests that complex I alone is responsible for the majority of the superoxide produced within intact mammalian mitochondria (119). Further, Lambert and Brand (99) have shown that high

Figure 1.4: Hyperglycemia-induced production of superoxide by the mitochondrial electron transport chain. Adapted from Brownlee et al., 2005 (27).

superoxide generation by the mitochondria requires inhibition of complex I activity. In conclusion, complex I appears to be the major source and site for superoxide generation.

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Chapter 2:

C-peptide Confers Anti-oxidative Protection in

Renal Cortical Endothelial Cells during

Type I diabetes by Restoring

Glucose-6-Phosphate Dehydrogenase Activity

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ABSTRACT

Previously we have shown that C-peptide, a by-product of insulin biosynthesis, improves renal peritubular capillary blood flow and reduces vascular oxidants. NADPH appears to be critical to this effect via the actions of numerous vasoprotective systems. We have found that diabetes results in a significant reduction in endothelial NADPH that is subsequently restored with C-peptide. We hypothesize that C-peptide provides protection to RCEs during type I diabetes by restoring the activity of G6PD, the principal source of NADPH synthesis. Our data show that RCEs from diabetic mice have diminished G6PD activity ($p < 0.05$) and their treatment with C-peptide restores its activity back to control levels ($p < 0.05$). These changes in G6PD activity occurred with concomitant alterations in NADPH. Using 2-D gel electrophoresis of RCE lysate coupled with immunoblotting for G6PD, we demonstrate that diabetic RCEs have a significant increase in G6PD PTM. C-peptide treatment reduced the magnitude of this PTM in diabetic RCEs, which occurred concomitant to restored G6PD activity. These results suggest that improving the activity of endothelial G6PD, by preventing its PTM, may be a potential mechanism by which C-peptide confers protection to RCEs during type I diabetes.

Keywords: C-peptide, Glucose-6-Phosphate Dehydrogenase (G6PD), Endothelial cells, Diabetic Nephropathy.

INTRODUCTION

DN is the leading cause of chronic kidney disease and end-stage renal failure in type I diabetic patients. Even with optimal glucose control, many type I diabetic patients develop end-stage renal failure. Although DN is characterized by glomerulosclerosis (14), recent evidence also indicates that glomerular damage causes stagnation of peritubular blood flow and thus development of tubulointerstitial injury and ultimately peritubular capillary loss (36, 37).

DN involves a state of imbalance with increased production of oxidants and decreased antioxidant defenses (57). An important, but often overlooked, consequence of type I diabetes is the net depletion of NADPH (3), the principal intracellular reductant for all cells. Moreover, NADPH is a critical cofactor in preserving normal microvascular function through actions of multiple vasoprotective systems like heme oxygenase -1 and NOS. There is a net loss of NADPH in microcirculation owing to increased consumption in multiple pathways including glucose metabolism (17, 42) and NOX (5, 8). G6PD, the first rate-limiting enzyme of the pentose phosphate pathway, is the major source of NADPH (30, 58). In fact, when G6PD activity is reduced to 10% of normal, the pentose phosphate pathway fails to generate any NADPH (1). Recent reports show that hyperglycemia causes inhibition of G6PD activity, thus NADPH depletion, in diabetic rat kidney cortex and bovine aortic endothelial cells (57, 60). Further, in endothelial cells, hyperglycemia-induced inhibition of G6PD appeared to occur via PKA mediated

phosphorylation (60). We hypothesize that microvascular dysfunction in type I diabetic kidney involves loss of NADPH, due to inhibition of G6PD activity, in RCEs.

C-peptide, a by-product of insulin biosynthesis (50), had long been thought to be physiologically inert (26). However, several studies have reported beneficial effects of exogenous C-peptide on renal function in type I diabetic patients (11, 22-24). In experimental diabetes, short-term infusions of C-peptide have been shown to improve glomerular hyper-filtration, reduce albuminuria whereas long-term substitution improved overall renal function in diabetic patients (18, 21, 46-48). Despite numerous reports indicating that C-peptide replacement is beneficial to the state of renal function in type I DM, the exact nature of its bioactivity and physiological effect on the renal microcirculation requires further clarity.

We examined the effect of C-peptide on peritubular capillary perfusion in kidney cortices from experimental diabetic animals. Further we correlated the alterations in microvascular function with changes in biochemistry of freshly isolated diabetic RCEs. To evaluate the mechanisms responsible for impaired endogenous vasoprotection, NADPH levels, G6PD activity, and phosphorylation of G6PD were examined in RCEs in the presence and absence of C-peptide.

MATERIALS AND METHODS

Murine Model of Type 1 diabetes:

Male C57BL/6 mice (Jackson Laboratories) were maintained on standard rodent chow with free access to water. Mice weighing 20-25 g were randomly assigned to control and diabetic groups. Type I diabetes was induced using NIDDK-sponsored Animal Models of Diabetic Complications Consortium (AMDCC) low-dose STZ regimen. Briefly, mice received daily STZ (Sigma, St. Louis, MO) at a dose of 50 mg/kg BW, freshly made in 0.1 M sodium-citrate buffer, pH 4.5, for 5 consecutive days whereas control mice received only the vehicle. Body weight and fasting blood glucose level (AccuSoft Advantage(R) glucose meter, Roche Diagnostics, Indianapolis, IN, USA) were examined weekly in all animals (19). Mice were considered diabetic if their fasting blood glucose exceeded 200 mg/dL after four weeks.

Biosynthetic Rat C-peptide II:

Rat C-peptide II (NH₂ EVEDPQVAQLELGGPGAGDLQTLALQVARQ-COOH) with purity greater than 90% was obtained from Primm biotech, Inc., Cambridge, MA, USA). Briefly, the peptide was dissolved in 0.5 M acetic acid and further dilutions made with saline. After 5-weeks of untreated diabetes, mice were anesthetized with inhalational isoflurane and an Alzet micro-osmotic pump (DURECT Corporation, Cupertino, CA, USA) was implanted subcutaneously. The pump was filled with rat C-peptide II and delivered 50 pmol/kg/min of C-peptide

for 24 hours. At the end of 24 hr, animals were anesthetized for either *in vivo* digital microscopy or for tissue harvest and processing.

Urinary Albumin and Creatinine Excretion:

The excretion of urinary albumin was determined using the albumin-to-creatinine ratio on morning spot urine as endorsed by the AMDCC. Spot urine collection was conducted using a custom-made collection cage that is constructed with a 96-well assay plate as the floor. Mice were permitted to roam over the plate until spontaneous urination. Only the urine without contamination was used for assay. The urinary albumin and creatinine concentrations were determined using commercially available immunoassay kits (Albuwell M and The Creatinine Companion, Exocell)(43)

***In Vivo* Digital Microscopy:**

To properly visualize the microcirculation for perfusion analysis, prior to microscopy, a Fluorescein isothiocyanate-conjugated, high molecular weight (500 kDa; Invitrogen) neutral dextran was injected via tail vein. With an anesthetized mouse (isoflurane; 5% induction, 2% maintenance) lying on the stage of an inverted microscope (Axiovert 200; Zeiss), the left kidney was exposed and imaged through a retro-peritoneal left flank incision(51, 54). The left kidney surface was moistened with warmed saline; heat lamps were used to preserve body and bath temperatures at 37° C. Following preparation of the kidney for imaging, 10 random high magnification fields of view (Plan-Neo 20x/0.5 and 40x/0.75; Zeiss) were projected

onto a 1/2" CCD video camera (C2400-75E; Hamamatsu) and digitally recorded for later analyses.

Peritubular Perfusion:

To determine peritubular perfusion, a stereological point-counting grid was used to provide a greater than 95% confidence that the density of points counted is proportional to the density of peritubular capillaries within the area of interest. A capillary was only counted if a point from the pre-determined array lands within its dimensions. Continuously perfused capillaries are defined as vessels that possess continuous red cell perfusion throughout the entire 1-minute observation period. Intermittently perfused capillaries are vessels that possess red cell perfusion that stopped at least once during the observation period. A non-perfused capillary was defined as any vessel that is devoid of red cell perfusion throughout the observation period. Peritubular perfusion was expressed as a proportion of the total number of capillaries (%).

Immunomagnetic Separation of Renal Cortical Endothelial Cells:

For sufficient yields, a pool of 15 kidney cortices (5 mice) was used. Briefly, the cortices were pressed through a 250 μm stainless steel sieve using a flattened pestle and the slurry transferred for digestion in liberase (1 mg/ml liberase blendzyme 3, Roche corporation, Indianapolis, IN, USA, in HBSS) at 37°C for 45 minutes with gentle agitation (15, 52). The liberase-digested slurry was then filtered through a 100 μm cell strainer and washed with 5 ml of ice-cold HBSS. The remaining cell suspension was centrifuged at 600 g for 10 minutes. Subsequently,

the supernatant was discarded and the pellet resuspended. RCEs were isolated and purified from this cell suspension using immunomagnetic beads coated with sheep anti-rat IgG (Dynabeads M-450)(13, 35). The endothelial cell-specific rat anti-mouse CD31 antibody (Pharmingen) was bound to the beads as per manufacturer's instructions. After incubation of the cortical cell suspension with the magnetic beads at 4°C. CD31-positive cells were captured with the aid of a magnet and washed. The identity and purity of the CD31-positive cells was initially verified by immunocytochemistry for endothelial-specific Factor VIII and Flk-1 (Pharmingen).

Assay for G6PD Activity

RCE lysates were used for the quantitative determination of G6PD activity using a spectrophotometric assay from Oxis Research. In principle, the assay would measure the production of NADPH by detecting the increase in absorbance at 340 nm (from the reduction of NADP⁺). The minimal detectable limit was 0.024 mU enzyme/cuvette. One unit of activity was defined as the quantity of enzyme needed to produce 1 μ M NADPH per minute (U/mg protein).

Assay for NADPH

NADPH levels in RCEs were determined spectrophotometrically based on the absorbance of the reduced coenzyme at 340 nm, as described previously (61). An initial reading was taken from lysates, representing the total amount of pyridine nucleotides in the sample ($A_{1} = \text{NADPH and NADH}$). A second sample (50 μ l) was incubated with glutathione reductase (5 U) for 5 minutes to convert all of the NADPH to NADP⁺. Glutathione (0.005 M) was added for an additional 5 minutes to

initiate the reaction. Because the oxidized nucleotide NADP⁺ does not have absorbance at 340 nm, the absorbance that occurs strictly represents NADH (A₂=NADH). As such, subtraction of A₂ from A₁ equals the total amount of NADPH (M).

Two-Dimensional Gel Electrophoresis:

Sample Preparation: The RCEs obtained after immunomagnetic separation were washed with cell wash buffer (10 mM Tris pH 8.0, 5 mM magnesium acetate) followed by suspension in cell lysis buffer (30 mM Tris, 2 M thiourea, 7 M urea, 4% CHAPS (w/v) at pH 8.5). RCE lysates were prepared by brief sonication followed by centrifugation at 5000 rpm for 5 min. Protein content was determined using 2D quant assay kit (GE Health Care Ltd).

Two-dimensional gel electrophoresis was performed using Zoom IPG Runner system (Invitrogen, Carlsbad, CA). Briefly RCE lysates (equivalent to 50 µg of protein) were solubilized in rehydration buffer (100 mM DTT, Zoom carrier ampholytes 0.5% and a trace of bromophenol blue) to a total of 140 µl and loaded on Zoom Narrow range strips (6.1 - 7.1). After 1.5 hr rehydration, strips were loaded onto mini-cell chamber of the Zoom IPG runner. Isoelectric focusing was carried out using voltage ramp protocol (175 V for 15 min, 175-2000 V ramp for 45 min and 2000 V for 120 min) followed by equilibration by reduction (using Nupage sample reducing agent) and alkylation (using 125 mM Iodoacetamide) in Nupage sample buffer. Equilibrated strips were loaded into Nupage 4-12% Bis-Tris gels and electrophoresis was performed for ~1 hr at 200 V. Focused and separated proteins

were transferred onto nitrocellulose membrane followed by blocking (5% Nonfat dry milk (NFDM) in 0.1% Tween in PBS), overnight incubation with primary antibody to G6PD (Bethyl research labs) (1:2500 in 5% NFDM in 0.1% Tween/PBS), 1 hr incubation with Protein A conjugated HRP (Amersham Biosciences) (1:2500 in 5% NFDM in 0.1% Tween/PBS). Blots were developed using ECL advance (Amersham Biosciences) and chemiluminescence was visualized and quantified by using G-Box (Syngene Ltd.)

RESULTS

Animal Characteristics (Table 1):

Compared to non-diabetic groups, diabetic mice had significantly higher blood glucose levels with concomitant significant reductions in serum C-peptide levels and elevations in urinary albumin excretion with no change in body weight. C-peptide administration did not show any significant effect on body weight and blood glucose in diabetic mice with significant improvement in diabetes-induced reductions in ACR.

Renal Peritubular Capillary Perfusion :

As depicted in Figure. 1, in control and diabetic mice treated with C-peptide, majority of the renal peritubular capillaries were continuously perfused. Compared to non-diabetic controls the number of continuously perfused renal capillaries was significantly lower in diabetic mice whereas the number of intermittently and non-

perfused capillaries was significantly higher . C-peptide treatment normalized perfusion of peritubular capillaries.

NADPH Bioavailability:

We determined whether RCEs from type 1 diabetic mice exhibit decreased NADPH levels. As shown in Figure 2, RCEs isolated from diabetic mice have significantly reduced NADPH levels compared to non-diabetic RCEs. C-peptide treatment restored the NADPH to more than control levels. Notably, there was significant elevation in endothelial NAPDH with C-peptide.

G6PD activity:

Studies were performed to determine the G6PD activity in all the groups. As shown in Figure 3, G6PD activity was significantly reduced in diabetic RCEs compared to their non-diabetic counterparts. C-peptide treatment augmented the G6PD activity in RCEs of diabetic mice.

G6PD Post-translational Modifications:

We examined the effect of C-peptide treatment on phosphorylation status of G6PD in type I diabetic mouse RCEs. The Figure 4 depicts that type I diabetes caused PTMs of G6PD. RCEs isolated from type I diabetic treated with C-peptide, have shown reductions in number of trailing spots as well as decrease in spot intensity, suggesting a decrease in PTMs of G6PD.

DISCUSSION:

Many investigators have challenged the notion that C-peptide is physiologically inert (25, 26). Both short and long term infusions of C-peptide in type I diabetic patients and experimental animals have proven beneficial not only in terms of diabetes-induced functional changes (i.e., improved glomerular filtration rate and decreased urinary albumin excretion (11, 18, 21-24, 46-48) but also on structural changes i.e., glomerular hypertrophy and mesangial matrix expansion (34). Since there has been a shift in focus from the glomerular to the tubular segment during the progression of diabetic nephropathy, tubulointerstitial injury has been broadly recognized as the final common pathway for kidney failure (10, 37, 38). This injury is extensively associated with distortion and loss of peritubular capillaries(6, 9, 33). Although numerous reports suggest that C-peptide prevents diabetes-induced changes in vascular perfusion in peripheral vascular beds (11, 24), opposing or no effects have been reported in renal and retinal vessels (4, 27, 55). Our results clearly indicate that C-peptide improves peritubular perfusion in the kidney cortices of type I diabetic mice. Yet, mechanisms underlying this biological effect are not fully understood.

The role of the endothelium in micro-vascular blood flow regulation has been extensively investigated. Endothelial cells not only serve as a barrier between circulating red blood cells and vascular smooth muscle cells but also play a central role in maintaining vascular tone and remodeling. Therefore, it is not surprising that early pathological changes induced by hyperglycemia are first evident in endothelial

cells. It is commonly accepted that the imbalance between oxidants and antioxidants caused by hyperglycemia leads to the pathogenesis of microvascular complications (7, 39). Disturbance in antioxidant status within endothelial cells causes the redox imbalance (16). Importantly, NADPH is an essential cofactor for the proper function of many antioxidative enzymes like GPx, glutathione-s-transferase, CAT (56) as well as the vasoprotective systems involving hemeoxygenase and NOS. Diabetes results in a net depletion of NADPH mainly due to the increased flux through polyol pathway (17, 42) in addition to increased production of reactive oxygen species through NOX (5, 8). Previous studies have shown diabetes decreased NADPH levels in rat kidney cortices and in bovine aortic endothelial cells (3, 57, 60). We have shown, for the first time, that there is a net loss of NADPH in renal cortical endothelial cells of type I diabetic mice. C-peptide treatment restored the diabetic RCE NADPH to more than control levels. Notably, there is significant elevation in endothelial NADPH with C-peptide. This unanticipated rise may be a consequence of decline in NADPH consumption as a result of reduced oxidant stress and/or increased synthesis due to elevated G6PD activity. These results suggest that type I DM leads to a loss in endothelial NADPH in mouse kidney cortex and provide the first indication that treatment of diabetic mice with C-peptide enhances endothelial NADPH. This suggests that loss of NADPH may be an important factor by which endothelial dysfunction, and the consequent impairment in vasoprotection, might occur.

Our results also demonstrate that C-peptide may confer antioxidant benefit by preventing the loss of NADPH during type I DM. Notably, with C-peptide

treatment, there is a significant increase in diabetic endothelial NADPH above non-diabetic levels. This interesting and unexpected finding demonstrates that C-peptide can be acting by enhancing the synthesis of NADPH and/or decreasing its consumption. To explore the first possibility we examined the effect of C-peptide on the systems that synthesize NADPH, mainly G6PD.

G6PD is a critical participant antioxidant protection because it produces NADPH, the essential intracellular reductant (53). Reductions in NADPH bioavailability, attributed to PKA mediated phosphorylation of G6PD activity, have been reported both in *in vitro* and *in vivo* diabetic settings (57-59). Previously, G6PD is thought to be minimally effected by post-translational modifications, and was only thought to be regulated by specific signal transduction molecules (40, 49, 53, 57, 58). Moreover, among all the enzymes that produce NADPH, only G6PD is activated during oxidative stress (12). It has also been shown that deficiency in G6PD activity increases the susceptibility of endothelial cells to oxidant (29, 30). Recent work from Stanton's group (57, 59) demonstrated that, in rat kidney cortices and bovine aortic endothelial cells, hyperglycemia causes inhibition of G6PD activity (by activating PKA via c-AMP) with concomitant decreases in G6PD expression and NADPH.

Previously other labs have demonstrated that diabetes causes inhibition of G6PD activity in rat kidney cortex and this effect being mediated by serine phosphorylation of G6PD via PKA activation (57). Our findings showing the decrease in G6PD activity in RCEs of type I diabetic mice reinforced the reports from

Robert Stanton's group. Using antibodies against phosphoserine, they also reported that the inhibition of G6PD activity is by PTMs, i.e. via phosphorylation of key serine residue/s on G6PD. Our attempt to identify phosphorylation of G6PD using phosphoserine specific antibodies (data not shown) produced ambiguous results. In spite of advances in detecting phospho-proteins by western blotting using phospho-specific antibodies, phospho-serine and phospho-threonine antibodies lack considerable specificity. Moreover our focus on mouse renal cortical endothelial cells, as opposed to Stanton et al., study in diabetic rat kidney cortex, may have contributed to differences in identification of G6PD phosphorylation using phosphoserine specific antibodies. Another recent study by Pan et al.,(40) demonstrated c-Src mediated tyrosine phosphorylation of G6PD in bovine aortic endothelial cells. These seemingly contradictory results highlight the existence of a post-translational mechanism regulating G6PD at the tissue level, countering the popular notion of G6PD as a "house-keeping" enzyme (32, 41). Hence further work is warranted to delineate mechanisms by which G6PD is regulated under normal as well as pathophysiological conditions.

Our work resorted to more sophisticated detection technique for pinpointing post-translational modifications, using two-dimensional gel electrophoresis. Our 2D-gel electrophoresis data indicates that G6PD is post-translationally modified in diabetic RCEs. Unique resolving power of 2D-gel electrophoresis allows for separation of different modified forms of the same protein into distinct spots on the gel (2). Heterogeneous phosphorylation of a protein gives a series of spots with same molecular weight but different pI values (44). The shift of G6PD immuno-

reactive spots towards cathodic end denotes a change of the pI towards more acidic values and may be an indication of the predominant PTM i.e. phosphorylation. In addition to 2D-gel electrophoresis, further studies using mass-spectrometry are needed for confirming the phosphorylation of G6PD.

Treatment with C-peptide ameliorated the diabetes-induced reductions in G6PD activity by preventing its PTM, potentially phosphorylation, in RCEs. Preventing the PTMs of G6PD thereby enhancing the synthesis of NADPH may be a major mechanism by which C-peptide improves endogenous vasoprotection. The exact intracellular mechanism underlying such an event needs to be investigated. In fact, C-peptide has been shown to interact (20) and attenuate the activity of protein tyrosine phosphatase (31). Similar interactions with other phosphatases, specifically serine/threonine phosphatases, may be occurring physiologically. One such Ser/Thr phosphatases is protein phosphatase 2A(PP2A) and has been widely implicated in regulating the activity of several protein kinases. Specifically, PP2A has been shown to dictate the functional status of the calcium channel protein in β -cell by fine-tuning the balance between protein kinase A mediated phosphorylation (thereby cAMP activation) and serine/threonine dephosphorylation (28). The exact role of C-peptide in modulating PP2A mediated dephosphorylation, and possibly protein kinase A mediated phosphorylation, needs to be examined and is beyond the scope of this paper.

In conclusion, C-peptide has been shown to improve anti-oxidant status of the diabetic renal cortical endothelial cells by preventing the PTMs of anti-oxidative

enzyme G6PD, and thereby enhancing the synthesis of NADPH. This anti-oxidant benefit can complement the repertoire of other beneficial effects reported for C-peptide. This study adds more substantial evidence to include C-peptide in the therapeutic regimen for treatment of type I diabetes-associated microvascular complications.

Table 1.

Table 1. Characterization of Low-Dose Streptozotocin (STZ) and 24 Hour C-peptide on C57BL/6 Mice

	Body Weight, g	Glucose, mg/dl	C-peptide, ng/ml	ACR, μ g/mg
Non-Diabetic	21.5 $\pm$ 1.6	74.7 $\pm$ 3.3	2.6 $\pm$ 0.3	18.2 $\pm$ 6.1†
Diabetic	20.0 $\pm$ 0.5	394.1 $\pm$ 20.4*	0.4 $\pm$ 0.1†	153.4 $\pm$ 36.0‡
Diabetic + C-peptide	19.9 $\pm$ 0.6	411.6 $\pm$ 21.2*	4.8 $\pm$ 1.2	78.0 $\pm$ 11.3

ACR, Urinary Albumin:Creatinine Ratio. Data are expressed as means $\pm$ SEM. *, significantly greater than Non-Diabetic ($p<0.05$); †, significantly less than all groups ($p<0.05$); ‡, significantly greater than all groups ($p<0.05$).

Figure 1.

Figure 2:

Figure 3.

Figure 4.

FIGURE LEGENDS:

Table 1: Body weight, fasting blood glucose, serum C-peptide, and urinary Albumin Creatinine Ratio (ACR) levels of Non-Diabetic, Diabetic, and Non-Diabetic + C-peptide mice 5 weeks after STZ injections are shown in Table 1.

Figure 1: Perfusion of peritubular capillaries of type I diabetic mice was improved by C-peptide. Data were expressed as Mean $\pm$ SEM. (n=5 in each group). *, significantly less than Non-diabetic + C-peptide ($p<0.03$); †, significantly greater than Non-Diabetic + C-peptide ($p<0.02$).

Figure 2: Reductions in NADPH of RCEs from STZ-induced type I diabetic mice were reversed by 24 h of C-peptide treatment. Data expressed as mean $\pm$ SEM (n=5 in each). *, significantly less than all groups ($p<0.05$); †, significantly greater than all groups ($p<0.02$).

Figure 3: RCEs from type I diabetic mice have lower G6PD activity than non-diabetic mice. Treatment with C-peptide restores its activity. Data expressed as mean $\pm$ SEM (n=5). * $p<0.05$ less than all groups.

Figure 4: Representative western blot images of 2D gels probed for G6PD using anti- G6PD antibody as described in methods section. Notice the presence and intensity of more G6PD-immunoreactive spots in chemiluminescent G6PD signal obtained from RCE cell lysate of type I diabetic mice, compared to non-diabetic and diabetic mice treated with C-peptide for 24 h.

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Chapter 3:

C-peptide Reduces Mitochondrial Superoxide Generation by Restoring Complex I Activity in High Glucose-Exposed Renal Microvascular Endothelial cells.

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ABSTRACT

Hyperglycemia-mediated microvascular damage has been proposed to originate from excessive generation of mitochondrial superoxide in endothelial cells and is the suggested mechanism by which the pathogenesis of diabetes-induced renal damage occurs. C-peptide has been shown to ameliorate diabetes-induced renal impairment. Yet, the mechanisms underlying this protective benefit remain unclear. The objective of this study was to determine whether C-peptide affords protection to renal microvascular endothelial cell mitochondria during hyperglycemia. Conditionally immortalized murine renal microvascular endothelial cells (MEC) were exposed to low (5.5 mM) or high glucose (25 mM) media with either C-peptide (6.6 nM) or its scrambled sequence control peptide for 24- or 48-hours. Respiratory control ratio, a measure of mitochondrial electrochemical coupling, was significantly higher in high glucose renal MECs treated with C-peptide than those of high glucose alone. C-peptide also restored high glucose-induced renal MEC mitochondrial membrane potential changes back to their basal low glucose state. Moreover, C-peptide prevented the excessive mitochondrial superoxide generation and concomitant reductions in mitochondrial complex I activity that are mediated by the exposure of the renal MECs to high glucose. Together, these data demonstrate that C-peptide protects against high glucose-induced generation of mitochondrial superoxide in renal MECs via restoration of basal mitochondrial function.

Keywords: C-peptide, diabetes, renal microvasculature, kidney dysfunction, endothelial cells, hyperglycemia, mitochondria, superoxide.

INTRODUCTION:

Diabetic renal impairment, a serious and frequent complication of both type I and type II diabetes (32, 42), is a leading cause of chronic renal disease (34, 35). The pathobiology of diabetic renal impairment is founded in the microcirculation. It is well established that the chronic hyperglycemia associated with diabetes directly damages the microcirculation, leading to small vessel dysfunction (33). More specifically, hyperglycemia has been shown to selectively damage endothelial cells because their glucose transport rate is not diminished, leading to an excessive accumulation of glucose within them (4, 5). As a consequence, the elevated intracellular glucose that ensues leads to increased generation of mitochondrial superoxide, proposed as a central player in the pathogenesis of all diabetic complications (4). Several reports contend that the diabetic state increases mitochondrial superoxide generation both in *in vitro* high glucose conditions (8, 10, 14, 44), as well as in diabetic animal models (6, 17). Furthermore, this excessive superoxide generation leads to the subsequent accumulation of other free radicals, thereby imparting diffuse damage to proteins and mitochondrial DNA, and ultimately leading to mitochondrial dysfunction (43, 51).

Complex I is the first member of the mitochondrial membrane-bound electron transport chain and its substrates/co-factors have the lowest potentials in the chain (9). Inhibition of the electron transport chain during pathological conditions and/or the build-up of NADH (and subsequent elevation in the NADH/NAD⁺ ratio) has been shown to contribute significantly to superoxide generation by complex I (29). Under physiological conditions, mitochondrial superoxide originates predominantly from complex I and complex III (12, 13), yet growing evidence suggests that complex I alone is responsible for the majority of the superoxide produced within intact mammalian mitochondria (39).

C-peptide, a by-product of insulin biosynthesis (52), had long been thought to be physiologically inert. However, several studies have reported the beneficial effects of exogenous C-peptide on renal function in type I diabetes (22, 23). In fact, short-term infusions of C-peptide have been shown to improve glomerular hyperfiltration and reduce albuminuria (19, 40, 41), whereas long-term administration improved overall renal function in diabetic patients (46, 47). Not surprisingly, many *in vitro* studies have been aimed at delineating the molecular and cellular basis for the observed actions of C-peptide (1, 49, 56). Despite these reports, the exact nature of its bioactivity and physiologic impact on the renal microcirculation during diabetes needs further clarification. Given that C-peptide, administered in the absence of insulin, provides benefits to general renal and microvascular function during type I diabetes, our central hypothesis follows that C-peptide independently affords protection to high-glucose exposed renal MECs by diminishing mitochondrial superoxide generation via restoration of basal

mitochondrial complex I activity. To examine this, we evaluated the effects of C-peptide treatment on renal MEC mitochondrial respiration, membrane potential changes, superoxide production and complex activity while exposed to a high glucose environment.

MATERIALS and METHODS:

Biosynthetic C-peptide (NH₂-EVEDPQVAQLELGGGPGAGDLQTLALQVARQ-COOH) and its scrambled sequence peptide control (NH₂-LGGGPQVGTLAQVEQEAEDGLDVRLAQQPA-COOH), both with a purity greater than 90% (HPLC), was synthesized by PrimmBiotech, Inc. (Cambridge, MA). The scrambled sequence peptide control has the same composition as the native C-peptide, but with the amino acid residues in random order. Cell culture media was supplied by Gibco (Invitrogen, Carlsbad, CA). All other chemicals were from Sigma Chemical (St. Louis, MO), with the exception of MitoSOX Red [3,8-phenanthradinediamine, 5-(6' triphenylphosphoniumhexyl)-5,6 dihydro-6-phenyl], which was purchased from Molecular Probes (Invitrogen, Carlsbad, CA).

Cell Culture: Conditionally immortalized MECs were obtained from Dr. Isaiah J. Fidler (MD Anderson Cancer Center). Cells were derived from the kidneys of transgenic mice harboring the temperature-sensitive SV-40 large T antigen, referred to as *H-2Kb-tsA58* mice(30). Cells were either grown in a T75 flask or 100 mm plates in a humidified incubator gassed with 5% CO₂ and 95 % O₂ in DMEM containing 5% fetal bovine serum and supplemented with non-essential amino acids, penicillin and streptomycin. Cells were used between passages 3-10 and renal MECs were grown

and maintained at 33°C. Once the cells reached 50-60% confluence, they were shifted to 37°C to permit activation of the temperature sensitive antigen and retention of the original endothelial cell phenotype. After 48-hours at 37°C, cells were serum deprived for 24-hours to allow for synchronization of cell cycles prior to treatments. Cells were subsequently divided into the following groups and medium was supplemented with the following agents: A) low-glucose - 5.5 mM D-glucose (LG); B) low-glucose with C-peptide - 6.6 nM (LG + C-pep); C) high-glucose - 25 mM D-glucose (HG); D) high-glucose with C-peptide - 6.6nM (HG + C-pep); E) Low-glucose with scrambled peptide, 6.6 nM and F) high-glucose with scrambled peptide for either 24- or 48-hours. Low glucose cells were also treated with 19.5 mM raffinose to serve as an osmotic control. We chose to treat cells with 6.6 nM of C-peptide because that is the physiological postprandial concentration observed in humans (18) and this concentration is comparable to the previous literature involving *in vitro* C-peptide studies (54). For the sake of clarity and brevity, we did not present data on the high-glucose/low-glucose + scrambled peptide control groups since they did not differ from their respective non-treatment conditions for any of the parameters assessed. Renal MECs treated with high-glucose did not exhibit any changes in cell viability (as assessed by trypan blue exclusion), nor did they demonstrate changes in their characteristic cobblestone morphology (data not shown).

Mitochondrial Respiration Assays: Mitochondrial respiration (a marker for mitochondrial electrochemical coupling) was measured by oxygen consumption rates in digitonin-permeabilized renal MECs using a Clark-type oxygen

electrode(43). Cells were trypsinized, centrifuged at 1000 g for 10 min and resuspended in 10 ml of respiration buffer containing (in mmol/l) 20 HEPES, 10 MgCl_2 , and 250 sucrose with 100-150 μg of digitonin. Cells were then incubated on ice for 10-minutes, washed three times with respiration buffer containing 0.5% bovine serum albumin, followed by resuspension at 5-8 million cells per 1.5 ml of respiration buffer into a Gilson chamber (Gilson; Middleton, WI). The chamber was maintained at 30°C and attached to YSI 5300 Biological Oxygen Monitor (YSI; Yellow Springs, OH)(7). After an initial period of stabilization, the substrates glutamate and malate (100mM of each) were added followed by ADP. Protein content was determined using the Bradford method (BCA Protein assay, Pierce), and values were expressed as nmol O_2 consumed/min/mg protein. State 3 respiration is an active state of maximal respiration with ADP and substrates, while state 4 respiration is a slow respiration state with low ADP and substrates (11). The respiratory control ratio (RCR), a ratio of the state 3 to state 4 respiration rates, is a measure coupling the transfer of electrons driving oxygen consumption and proton transfer driving ATP synthesis.

Mitochondrial Membrane Potential Measurements: Assessments of mitochondrial membrane potential ($\Delta\Psi_m$) were performed using a previously described method (38), which employs the J-aggregate lipophilic cationic dye, JC-1 (Molecular Probes; Eugene, OR). Briefly, renal MECs were incubated in the dark with JC-1 (7.5 μM , 30 min at 37 °C) following either the 24- or 48-hour treatments. After incubation, renal MECs were measured for fluorescence emission at 530 nm (monomer = green) and at 590 nm (J-aggregates = red) in a F-2500 Fluorescence

Spectrophotometer (Hitachi High Technologies America; Pleasanton, CA) equipped with a cover slip holder and using an excitation wavelength of 488 nm. Relative $\Delta\Psi_m$ changes were calculated using the ratio of the J-aggregate to monomer (590 nm/530 nm). Values were expressed as fold increase in J-aggregate/monomer fluorescence over control cells. As a complement, cells were grown on six-well dishes and observed using an Eclipse 800 Fluorescence Microscope (Nikon; Melville, NY) equipped with a dual filter for fluorescein and rhodamine.

Mitochondrial Superoxide Measurements: Mitochondrial superoxide was measured using a previously described flow cytometry method (37). Briefly, after the 24- or 48-hour treatments, MitoSOX Red was added to fresh media to a final concentration of 5 μ M according to manufacturer's instructions. The renal MECs were incubated with the MitoSOX for 30-minutes in the dark, washed two times with PBS containing calcium and magnesium. Antimycin (100 μ M), a complex III inhibitor, was used as a control. Flow cytometry measurements were carried out using a FACScalibur (BD Biosciences; San Jose, CA). A laser at 488 nm excited MitoSOX Red, and data was collected at forward and side scatter, 582/42 nm (FL2) channels. Data were presented in the FL2 channel. Cell debris, represented by a distinct low forward scatter, was gated out of the analyses.

Mitochondrial Complex Activity Measurements: Mitochondrial complex I, III, and IV activities were assessed using the previously described method of Trounce *et al.* (53). Complex activities were expressed as nmol/min/mg protein.

Western blotting: Cell supernatants were obtained from the renal MECs grown in 100 mm plates and subjected to 24-hour treatments. While adherent, cells were washed twice with ice-cold PBS followed by treatment with ice-cold 1X RIPA Buffer (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na₃VO₄, 1 µg/ml leupeptin) supplemented with protease and phosphatase inhibitor cocktails, for 5-minutes on ice. Renal MECs were scraped gently using a rubber policeman, collected into a 1.5 ml centrifuge tube and sonicated briefly to ensure complete lysis of cells. The extract is then centrifuged for 10-minutes at 14,000 g to obtain the supernatants. Protein concentration was determined using Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL)

Cell supernatants (equivalent to 40 mg of protein) were separated on a 4-12% Novex NuPAGE Bis-Tris gels using Novex system (Invitrogen, Carlsbad, CA) and then transferred on to nitrocellulose membrane. Membranes were then blotted for Extracellular-Signal Regulated Kinase (ERK)1/2 (p44/p42 [ERK1/2] 137F5 Rabbit mAb), Phospho ERK1/2 (p44/p42 [ERK1/2] (Thr202/Tyr204) Rabbit mAb), p38 Mitogen-Activated Protein Kinase (MAPK) mAb, and phospho p38 MAPK (Thr180/Tyr182) Rabbit mAb (Cell signaling, Danvers, MA), followed by incubation with peroxidase conjugated anti-rabbit secondary antibody. Membranes were developed with ECL advance detection reagent (Amersham, GE Healthcare, Piscataway, NJ) followed by detection of chemiluminiscent signal using G:Box, Gel Imager (Syngene, Synoptics Ltd.). Densitometric analysis was performed using

Gene Tools (Syngene) software and data were calculated as arbitrary units. Phosphorylated MAPK phosphorylations were normalized to total MAPK levels.

Statistical Analysis: Statistical significances were determined using standard ANOVA procedures. If significance was found, the effects were tested further by using the Student Newman-Keuls post hoc comparison. A probability of 0.05 was accepted as statistically significant, and the sample sizes for each experimental group provided a statistical power of greater than 80%. Data are expressed as means $\pm$ SEM.

RESULTS:

C-peptide ameliorated defects in mitochondrial electrochemical coupling during high glucose exposure of renal MECs. State 3 mitochondrial respiration is the slope of measured oxygen consumption in the presence of glutamate-malate and ADP. State 3 respiration rates were significantly decreased in high glucose cells at both 24- and 48-hours. C-peptide treatment restored state 3 respiration to basal levels (Figure 1A; $p < 0.02$). State 4 respiration is the slope of measured oxygen consumption after ADP depletion. State 4 respiration was increased in high glucose cells at both 24- and 48-hours while C-peptide treatment restored state 4 respiration (Figure 1B). RCR, the ratio of state 3-to-state 4 respiration, is a measure coupling the transfer of electrons driving oxygen consumption and proton transfer driving ATP synthesis. RCRs were significantly decreased in high glucose cells at both 24- and 48-hours and were restored with C-peptide (Figure 1C; $p < 0.0001$).

These data demonstrate that C-peptide abrogates mitochondrial respiratory defects caused by high glucose.

C-peptide restored the mitochondrial membrane potential ($\Delta\Psi_m$) of renal MECs during high glucose exposure. Changes in $\Delta\Psi_m$ were measured in renal MECs using JC-1 staining. At 24-hours, high glucose exposure resulted in significant hyperpolarization of the mitochondrial membrane (Figure 2; $p < 0.009$). Subsequently, endothelial mitochondria were depolarized after 48-hour (Figure 2; $p < 0.002$). Treatment with C-peptide resulted in restoration of $\Delta\Psi_m$ at both 24- and 48-hours of exposure to a high glucose stress.

C-peptide reduced the excessive mitochondrial superoxide levels observed in high glucose exposed renal MECs. Using MitoSOX Red and measuring fluorescence by flow cytometry, the exposure of renal MECs to high glucose significantly elevated mitochondrial superoxide generation (Figure 3b; $p < 0.0001$) compared to low glucose at both 24- and 48-hours. C-peptide treatment restored mitochondrial superoxide generation to basal levels at both time points.

C-peptide improves mitochondrial complex I activity in high glucose exposed renal MECs. Renal MECs exposed to high glucose exhibited significant reductions in complex I activity at 24- and 48-hours (Figure 4A; ($p < 0.002$ at 24 hr and $p < 0.0009$ at 48 hr), with no change to complex III and IV (Figure 4B and 4C). C-peptide restored complex I activity back to levels observed with low glucose.

C-peptide activates Extracellular Signal Regulated Kinase (ERK) 1/2 in high glucose exposed renal MECs. C-peptide treatment resulted in activation of

ERK 1/2 (Fig 4A; $p < 0.005$) in high glucose exposed renal MECs, however failed to induce phosphorylation of p38 MAPK.

DISCUSSION:

Chronic hyperglycemia is the main trigger initiating diabetic renal impairment. Although to varying degrees, chronic hyperglycemic injury affects all of the cell types within the glomerular, vascular and tubulo-interstitial compartments of the kidney. However, the initial stages of diabetic renal impairment can be ascribed to dysfunction of glomerular capillaries and barrier function, which clinically manifest as hyperfiltration and microalbuminuria. The glomerulus is composed of endothelial cells, podocytes and mesangial cells, all of which play a crucial role in maintaining barrier function. Furthermore endothelial cells, both from glomeruli and the tubulo-interstitium, play an important role in the overt hyperglycemic damage to the kidney due to the previously discussed deficiency in glucose transport. The murine renal microvascular endothelial cells utilized in this study are an ideal cell type to study the effects of high glucose-induced deleterious changes that ultimately lead to renal microvascular endothelial dysfunction.

This study demonstrates, for the first time, that C-peptide confers protection against hyperglycemia-mediated mitochondrial dysfunction in endothelial cells. We show that C-peptide inhibits high glucose-induced changes to mitochondrial respiration, membrane potential, superoxide generation and complex I activity. Several studies suggest that C-peptide prevents diabetes-induced renal microvascular complications, and subsequently improves renal function (19, 40, 41,

46, 47). Although mitochondrial superoxide generation has been widely implicated in the pathogenesis of diabetic microvascular complications (5, 33), there is a dearth of knowledge regarding the impact of C-peptide on diabetes-induced mitochondrial dysfunction. This is the first investigation addressing whether the protection rendered by C-peptide during diabetes involves the mitochondria.

Our studies examining the effect of C-peptide on hyperglycemia-mediated mitochondrial dysfunction suggest that C-peptide improved mitochondrial respiration in high glucose-exposed renal MECs. Mitochondrial respiration is a reflection of its capacity to synthesize ATP, which occurs by means of the electron transport chain and oxidative phosphorylation. The process of electron transfer and ATP generation are tightly coupled, with respiratory control ratio used as an indicator of the extent of electrochemical coupling. Recent reports using the mitochondria isolated from diabetic animals demonstrated compromised mitochondrial respiration (6, 17, 50). Recently, Palmeira *et al.* (43), reported impairment of mitochondrial respiration rates under similar high-glucose conditions in HepG2 cells. Clearly, there is a need to delineate the mechanisms underlying high glucose-mediated damage to microvascular endothelial cells, the main cell type associated with diabetic end-organ complications. Since it has already been shown that C-peptide prevents diabetes-induced renal microvascular complications (40, 41), restoration of mitochondrial respiration and attenuation of mitochondrial superoxide generation in high glucose-exposed renal MECs is a legitimate channel by which C-peptide can exert these beneficial renal effects during diabetes.

It is well recognized that the major pathways involved in the pathogenesis of diabetic microvascular dysfunction are activated by hyperglycemia-mediated superoxide generation by the mitochondria (4). Hyperglycemia results in the increased availability of reducing equivalents NADH and FADH₂, which in turn lead to increased electron transfer through the mitochondrial electron transport chain. The overall rate of this electron transport is governed by mitochondrial respiratory control, which ultimately is the amplitude of the electrochemical trans-membrane proton gradient. In our studies, we believe that the high glucose environment generates a high trans-membrane proton gradient and high $\Delta\Psi_m$, thereby prolonging the presence of the superoxide generating intermediate – ubiquinone (28, 45). This is in agreement with the findings of others demonstrating changes in $\Delta\Psi_m$ in high glucose-exposed cells(24, 38). We also showed that C-peptide treatment prevented both the hyperpolarization, and the subsequent depolarization, in high glucose-exposed renal MECs. We propose that the increases in $\Delta\Psi_m$, described as hyperpolarization, may result in the partial inhibition of electron transport chain complexes leading to a large stimulation of mitochondrial superoxide generation. Our studies also show increased superoxide generation by the mitochondria in high glucose-exposed renal MECs, which was abrogated by treatment with C-peptide. A similar increase in mitochondrial superoxide generation upon exposure to high glucose has also been reported in various other cell types (8, 14, 24, 38, 44).

Although all of the complexes of the mitochondrial electron transport chain contribute to total mitochondrial superoxide generation, growing evidence now

suggests that complex I produces most of the superoxide generated within intact mammalian mitochondria (3). It is well known that complex II also has the capacity to produce superoxide, but this only occurs while in the presence of specific inhibitors and is not a physiologically relevant site of generation (15). Complex III, on the other hand, has been shown to produce large amounts of superoxide, at least in the presence of antimycin (15). To characterize the role of each individual complex in the generation of excessive mitochondrial superoxide in our high glucose model, we assessed the activities of complex I, III and IV. As expected in our high glucose exposed renal MECs, we found a significant reduction in complex I activity, with no apparent changes to the activities of complex III and IV. This is in accordance with the observations of others (3, 7, 15, 24). Further, Lambert and Brand (29) have shown that high superoxide generation by the mitochondria requires inhibition of complex I activity. Surprisingly, C-peptide prevented the reduction in complex I activity caused by the high glucose exposure, in the absence of any effect on basal complex III and IV activities. Moreover, increased mitochondrial superoxide generation exacerbates mitochondrial oxidative stress further and leads to conversion of the mitochondrial glutathione pool to glutathione disulfide (21). Glutathionylation of complex I has been shown to contribute to oxidative damage to its subunits and loss of activity (2, 20).

C-peptide has been shown to be a potent stimulant of MAPKs (25-27). Activation of ERK by C-peptide has been shown in numerous cell types (16, 31, 55, 57). Our experiments to study the effect of C-peptide on MAPKs yielded similar results demonstrating the activation of ERK1/2. This is especially important because

several recent studies indicate that ERK 1/2 may affect the mitochondrial activities by regulating the expression of mitochondrial proteins in the nucleus in addition to having intrinsic mitochondrial activities (48). Specifically, ERK1/2 has been shown to localize to mitochondria in human alveolar macrophages and control mitochondrial membrane potential as well as ATP production (36). However, the exact mechanisms by which ERK1/2 are translocated and regulated in the mitochondria are still obscure and are beyond the scope of our study.

In this work, we have shown that C-peptide protects renal MECs exposed to a high glucose environment from mitochondrial dysfunction via improvements to mitochondrial electrochemical coupling and reductions in mitochondrial superoxide generation through restoration of mitochondrial respiratory complex I activity. Together, these data not only support the continued thought that C-peptide treatment affords protection against high glucose environments, but that its unique impact on the mitochondria should be a mechanism pursued in more detail.

Conclusions: Our work demonstrates that C-peptide curtailed high glucose-induced mitochondrial functional impairments in renal MECs. Although interest in the physiologic benefits of C-peptide has persisted for more than two decades, C-peptide has yet to make its way into standard treatment regimens for various diabetic complications. The findings from our work have to be confirmed *in vivo*, but provides proof-of-principle evidence in support of the inclusion of C-peptide to the existing therapeutic regimen for treatment of diabetic complications, specifically those related to diabetes-induced renal impairment.

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Figure 1

Figure 2.

Figure 2. B.

Figure 3

A1.

A2.

Figure 3

Figure 4

Figure 5A.

Figure 5B.

FIGURE LEGENDS:

Figure 1: A) State 3 respiration is the slope of measured O₂ consumption in the presence of glutamate-malate and ADP. ★, significantly different than low-glucose and high-glucose + C-peptide ($p < 0.02$). B) State 4 respiration is the slope of measured O₂ consumption after ADP depletion. C) RCR is the ratio of state 3 to state 4 respiration. ★, significantly less than low-glucose and high-glucose + C-peptide (★ $p < 0.0001$). $n = 8$ experiments. Data represent mean $\pm$ SEM.

Figure 2: A) At 24-hours, high glucose resulted in significant mitochondrial membrane hyperpolarization. Subsequently, endothelial mitochondria were depolarized (after 48-hours). Treatment with C-peptide resulted in restoration of $\Delta\Psi_m$ at both 24- and 48-hours of exposure to a high glucose stress. ★, significantly greater than low-glucose and high-glucose + C-peptide ($p < 0.009$); #, significantly less than low-glucose and high-glucose + C-peptide ($p < 0.002$). Data represent means $\pm$ SEM and $n = 6$ experiments. B) Representative fluorescence micrographs of renal MECs treated with JC-1.

Figure 3: A) Representative histograms of MitoSOX Red flow cytometry data at 24 hrs. Y-axes represent the number of counts of cells that emit fluorescence. X-axes represent the average fluorescence intensity of cells. A1 represents treatment conditions; A2 represent vehicle control and positive control. B) Fold-change of mean fluorescence intensity of cells, including the control of low-glucose + antimycin. ★, significantly different than low-glucose and high-glucose + C-peptide

($p < 0.0001$). #, significantly different than high-glucose and low-glucose + antimycin ($p < 0.0001$).

Figure 4: Mitochondrial complex activity, as measured by spectrophotometry. A)

There is a significant decrease in complex I activity in high-glucose exposed renal MECs compared to low-glucose and high-glucose + C-peptide at both 24- and 48-hours. *, significantly less than low-glucose and high-glucose + C-peptide ($p < 0.002$ at 24 hr and $p < 0.0009$ at 48 hr). #, significantly greater than high glucose at 48 hr ($p < 0.05$). B) and C) Both complex III and IV activities are not different among the treatment conditions. Data represent mean $\pm$ SEM; n=16 experiments.

Figure 5: Cell supernatants obtained after 24 hr treatments were subjected to

western blotting analysis for detection of phosphorylated ERK isoforms (p44 kDa/pERK1 and p42 kDa/pERK2) and Phosphorylated p38 MAPK isoforms. A) Phosphorylated MAPK band intensities were normalized to total MAPK levels. Data are expressed as percentage change relative to control low glucose treatment. Data represent mean $\pm$ SEM, n=6. B) Representative western blot images

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Chapter 4:

General Discussion

It had long been accepted that the peptide that connects the A- and B-chains of insulin, termed C-peptide, was biologically inert. However, many studies have demonstrated that replacement doses of C-peptide can ameliorate diabetes-induced renal dysfunction (35, 40-42, 61, 69, 70, 89, 90, 92). Yet, the mechanisms underlying the benefits of C-peptide during DM are still unclear. DM has been widely recognized as a state of imbalance between oxidants and antioxidant protective systems (7, 26, 29). Moreover, the chronic hyperglycemia associated with DM causes elevated oxidative stress in the renal microcirculation (87, 99), directly damaging the microvessels leading to dysfunction (64), and ultimately manifesting as kidney damage and dysfunction(29, 79). **Our central hypothesis is that C-peptide prevents diabetes-induced renal dysfunction by ameliorating oxidative imbalance within the microcirculation, and specifically renal microvascular endothelial cells.**

STZ-induced type I DM has been widely used as model for DN, however interpreting results in this model can be confounded by the non-specific toxicity of STZ (10, 100). To mitigate this non-specific cytotoxicity, we employed the AMDCC model of multiple low-dose STZ injections – daily *i.p.* injections of 50mg/kg STZ for five days (10, 11). This protocol has been shown to cause repetitive low-grade β -cell damage accompanied by lymphocytic infiltration of the pancreatic islets (57, 88). In our hands, the AMDCC multiple low-dose STZ protocol provided a mouse model with high fasting blood glucose levels that lacks the catabolic state that commonly accompanies the single high-dose STZ protocol (10).

Our evaluation of the short-term effects (24-hours) of C-peptide were based on three factors/previous findings: 1) the half-life of C-peptide is 30-minutes (63); 2) its mechanism of action, at least in part, is mediated by rapid G-protein-coupled receptor signaling and changes to protein activation/expression (31, 80, 85), and 3) the replacement of physiological levels of C-peptide on a short-term basis (1–3 hours) in diabetic patients lacking C-peptide has provided significant protection against diabetic complications (39, 42). Moreover, the treatment with C-peptide did not alter fasting blood glucose or bodyweight (refer to table 1 in chapter 2). As such, we believe that these variables did not confound our evaluations of C-peptide and its effects on the renal microcirculation.

Our studies to examine the effect of C-peptide on early renal dysfunction in mice due to type I DM demonstrate that short-term infusion of C-peptide improved renal function. Our results are in good agreement with other studies that examined the benefits of short-term administration of C-peptide in experimental animal models of diabetes (91, 98). Several studies reported that C-peptide reduces diabetes-induced glomerular hyperfiltration via constriction of renal afferent arterioles (35, 41, 42). However, renal vasoconstrictive effect of C-peptide takes considerably longer time to develop compared to well-known constrictors such as angiotensin II or norepinephrine (70). An explanation of this could be a sequential cascade of intracellular events leading to vasoconstriction.

It has been suggested that alterations in renal proximal Na^+/K^+ -ATPase activity may in itself regulate GFR (106). Indeed, Na^+/K^+ -ATPase activity in the

kidney is elevated during the early onset of diabetes, probably due to the increased tubular electrolyte load resulting from the elevated GFR (106). Recently, C-peptide has been recently shown to alter renal proximal tubular Na^+ load by inhibiting Na reabsorption, thereby suggesting that C-peptide may decrease diabetic hyperfiltration through inhibition of Na^+/K^+ -ATPase activity (69). In this study, the diabetic rats displayed reduced proximal tubular Na^+ reabsorption after C-peptide administration, and it is possible that the reduced GFR observed in these animals is caused by TGF activation.

Although C-peptide clearly possesses a reducing effect on diabetic hyperfiltration, by constricting afferent arteriole and dilating efferent arteriole there is still no effector, no receptor, and no downstream signalling cascade reported for this phenomena. Thus, regulation of GFR, tubular electrolyte handling and renal blood flow are interconnected via several mechanisms which need to be investigated separately in order to fully understand how C-peptide might influence kidney function. Even though our studies did not include GFR measurements, we measured urinary albumin creatinine ratio, an indicator for microalbuminuria (95), which in turn is a sign of generalized microvascular dysfunction. C-peptide administration for 24 hours decreased albumin creatinine ratios in type I diabetic mice, suggesting that C-peptide has a salutary effect on renal microvasculature at initial stages of DN.

Although numerous reports suggest that C-peptide prevents diabetes-induced changes in the perfusion of peripheral vascular beds (18, 42), opposing or

no effects have been reported in the kidney (6, 48, 107). With diabetic kidney disease, despite important structural changes within the glomerulus, tubular and interstitial lesions are ultimately an outcome that occurs with the progression of type I DM (21, 86, 101). In fact, tubulointerstitial injury, as opposed to glomerular injury, is the predominant form of renal damage/dysfunction that occurs, and develops independently in type I diabetic patients (67). Human tubulointerstitial injury is associated with decreases in the number and area of post-glomerular capillaries as the fibrosis of the renal cortical interstitium increases (113). During the early stages of injury, the peritubular vasculature is damaged by pro-apoptotic stimuli, particularly transient ischemia (86). Consequently, rarefaction of the peritubular microvasculature with ensuing chronic hypoxia is a hallmark of tubulointerstitial fibrosis (101). Concomitantly, work has also shown that there is a loss of endothelial cell staining correlated to areas of tubulointerstitial injury, further suggesting a loss of peritubular capillaries (21). Our results clearly indicate that C-peptide improves peritubular perfusion in the kidney cortices of type I diabetic mice. C-peptide treatment not only improved perfusion, but also improved renal function, as indicated by improved albumin creatinine ratios, similar to other reports in diabetic animal models (61, 68, 70).

Hyperglycemia preceding DM alters microvascular perfusion in different vascular beds and can result in heterogeneous flow patterns (43, 44, 49, 74). We examined the effect of C-peptide on type I diabetes-induced heterogeneity in flow pattern within renal peritubular capillaries. For the purposes of our study, we focus on one aspect of Ht in peripheral vasculature: flow heterogeneity (Ht). Flow Ht

simply means that total inflow is not distributed identically among the perfused vessels (17). Flow Ht is controlled within microcirculation and is essential to our understanding of tissue function and vascular perfusion. . One of the caveats in our discussion of flow Ht is that some of the key measurements, such as RBC flux and velocity, are lacking and is mainly based on perfusion times obtained from visual observation of flow patterns. A quotient of mean flow (an index of central tendency of the flow) and standard deviation (an index of dispersion) is the coefficient of variation, is a convenient measure of the relative dispersion or the true heterogeneity of the flow. At a constant level of mean flow, redistributing flow among vessels might alter fractional flow distribution, and the coefficient of variation will show the change in relative dispersion (17).

Important information regarding flow dispersion may be gained through an analysis of histograms describing the sample distribution (34, 60, 93). A change in the shape of sample distribution is indicative of a redistribution of flow. This is often manifested as a change in the shape of histograms (34, 60, 93). Our analysis of frequency distribution histograms, as shown in Figure 4.1, depicts a left shift in the flow pattern and shorter perfusion times in intermittently perfused capillaries of diabetic kidney, indicating a heterogeneous flow pattern. Similar heterogeneous flow patterns were described for other vascular beds in diabetic animal models as well as DM patients (43, 44, 49, 74).

Figure 4.1 : Frequency distribution histogram depicting blood flow in individual renal peritubular capillaries. Capillary perfusion was determined using stereological point-counting grid (see Methods section under Chapter 2 for detailed description of analysis). A capillary was counted if a point landed within its dimensions. 10-15 fields of view per animal were used for analysis. X- axis represents the perfusion times binned as 3 second groups, with ticks representing center of each bin. Y- axis represents percentage of capillaries. A continuously perfused capillary (open bars) was defined as one which possessed continuous red blood cell flow (RBC) for the 20 sec video period. If a perfused capillary demonstrated RBC flow that stopped at least once during the 20 sec period, it was deemed intermittently perfused capillary (hatch bars). Non-perfused capillaries (filled bars) were any capillaries that did not have RBC flow through out the entire 20 sec observation period. Both Diabetic and Diabetic + C-pep mice have higher number of non-perfused and intermittent capillaries compared to the Non-diabetic mice. There is a right shift in the distribution of perfusion times of intermittently-perfused capillaries in Diabetic + C-pep mice compared to that of Diabetic mice.

Even though C-peptide treatment for 24 hours did not alter the percentage of non-perfused and continuously perfused capillaries compared to that in diabetic mice, it caused a right shift in the distribution histogram indicating that more intermittent vessels were perfused for longer periods of time. This was expected with a shorter treatment period and we believe that the 24- hour duration of C-peptide treatment utilized in our study is not long enough to cause structural changes. As such, our study focuses on early renal functional abnormalities caused by DN at around 6-7 weeks, where in structural abnormalities are still evolving within the diabetic kidney. Moreover our frequency distribution analysis includes perfusion data collected from only one animal per group. Further analysis utilizing a larger sample size is needed to understand the effect of C-peptide on flow Ht observed in peritubular capillaries of type I diabetic kidney.

In small capillaries, microvascular blood flow is increasingly determined by rheological properties of RBC (78). Studies of the rheological behavior of RBCs in the capillary network clearly demonstrated that capillary flux and velocity are strongly dependent on the ability of RBCs to deform on entry into the capillaries (78). Thus reductions in RBC deformability may adversely affect capillary perfusion and reduces flow in microcirculation, if the capillary diameter and blood pressure remain constant (16). Microvascular network may passively compensate for increasing RBC stiffness by shunting RBC within the capillary network through pathways of lesser resistance (58). However, such ability to recruit additional capillaries by normal compensatory mechanisms may be compromised in disorders manifest by diminished RBC deformability, notably DM (78). Na^+/K^+ -ATPase activity

was found to be decreased in many tissues, especially RBC membrane, of type I diabetic patients as well as animal models (1, 104). This impairment could be at least partly responsible for development of diabetic complications. It has been shown that early type I DM is characterized not only by functional disturbances such as increased shear stress and tangential pressure on the microvascular endothelium, increased leukocyte-endothelial interactions but also hemorheological disturbances such as increased blood viscosity, increased RBC aggregation and changes in the hemodynamic properties of the RBCs (24). These disturbances affect microvascular blood flow and precede structural alterations.

Figure 4.2: Schematic presentation of the molecular mechanism of C-peptide activity on endothelial cells and microvascular blood flow. Adapted from Forst *et al.*, 2008 (24)

C-peptide supplementation has been shown to restore Na⁺/K⁺-ATPase activity in different cell types during *in vitro* and *in vivo* investigations (22, 23, 30, 51, 71, 73, 117). C-peptide has also been shown to stimulate endothelial NO secretion by activation of eNOS via Ca²⁺ calmodulin regulation. Thus, there is strong evidence that C-peptide improves RBC deformability and microvascular blood flow in type I DM. As illustrated in Figure 4.2, the underlying mechanisms involve at least the activation of eNOS and Na⁺/K⁺-ATPase.

The endothelial dysfunction that occurred in STZ-induced diabetic rats was reported to elicit an inflammatory tubulointerstitial response and plasma leak through injured peritubular capillaries (97). Moreover, diabetic mice lacking eNOS were also shown to develop tubulointerstitial injury (66). Type I diabetic patients exhibit a strong correlation between the ratio of asymmetric dimethyl arginine to L-arginine (a marker of endothelial dysfunction) and tubulointerstitial injury (5). Based on these reports and our studies, wherein C-peptide improved peritubular perfusion, we chose to investigate the molecular mechanisms of C-peptide in RCEs isolated from kidneys of type I diabetic mice.

Disturbances in the antioxidant capacity of endothelial cells contribute to the redox imbalance that exists during DM (26). Important to this redox status is the bioavailability of NADPH, an essential cofactor for the proper function of many antioxidant enzyme systems (e.g., GR, GPx, glutathione-s-transferase, CAT (108)) as well as the vasoprotective systems (including heme oxygenase and NOS). DM results in a net depletion of NADPH mainly due to the increased flux through the polyol

pathway (33, 81), in addition to increased NOX activity (7, 14). NOX could act as a double-edged sword, with transient activation providing a feedback antioxidant response to ROS via receptor tyrosine kinases and redox-sensitive transcription factors (25). Prolonged activation, on the other hand may lead to depletion of intracellular NADPH and impaired ROS scavenging associated with eNOS uncoupling and mitochondrial dysfunction (25).

Previous studies have shown DM decreased NADPH levels in rat kidney cortices and in bovine aortic endothelial cells (3, 111, 115). As such, loss of NADPH is likely an important factor by which endothelial dysfunction, and the subsequent impairment in vasoprotection, might occur. Although others have demonstrated similar reductions in NADPH within the diabetic kidney (111), these studies were of the whole organ. We have shown, for the first time, that there is a net loss of NADPH in renal cortical endothelial cells of type I diabetic mice. C-peptide treatment restored the diabetic endothelial NADPH levels to more than basal levels. This suggests that type I DM leads to a loss in endothelial NADPH in mouse RCEs, and provides the first indication that treatment of diabetic mice with C-peptide enhances endothelial NADPH. We demonstrate that C-peptide confers antioxidant benefit by preventing the loss of NADPH during type I DM. Notably, with C-peptide treatment, there is a significant increase in diabetic endothelial NADPH above non-diabetic levels. This interesting and unexpected finding suggests that C-peptide may act by enhancing the synthesis of NADPH and/or decreasing its consumption. It is generally accepted that improvements to NADPH bioavailability in a diabetic setting is related to its diminished consumption. However, information on the impact of

treatments on its synthesis is limited. To explore the role of C-peptide in the augmentation of NADPH synthesis, we examined its effect(s) on the rate-limiting enzyme of NADPH synthesis, G6PD.

G6PD is a critical participant in cellular antioxidant protection because it produces NADPH, the essential intracellular reductant (102). Among the enzymes that generate NADPH, only G6PD was found to be activated during oxidative stress (20). It has also been shown that a deficiency in G6PD activity increases the susceptibility of endothelial cells to oxidants (54, 55). Superoxide production by neutrophil NOX is decreased in granulocytes suggesting that NOX dependent superoxide production may be regulated by NADPH derived from G6PD driven pentose phosphate pathway (103). Interestingly, a recent observation indicates G6PD can localize to mitochondria and serve as an important source of mitochondrial NADPH (62). They have shown that, under high glucose conditions, muscle cells relied heavily on matrix associated G6PD as a source of NADPH.

PKA mediated phosphorylation of G6PD, and consequently diminished G6PD activity, during exposure to *in vitro* and *in vivo* hyperglycemia has been shown to reduce NADPH bioavailability (111, 112, 114). Previously, G6PD was thought to be minimally affected by PTMs, and only regulated by specific signal transduction molecules (76, 99, 102, 111, 112). Recent work from Stanton's group (111, 114) demonstrated that, in rat kidney cortices and bovine aortic endothelial cells, hyperglycemia caused inhibition of G6PD activity (by activating PKA via c-AMP) with concomitant decreases in G6PD expression and NADPH bioavailability. The

diminished endothelial G6PD activity we show in our studies reinforces these reports. Using antibodies against phosphoserine, Stanton's group reported that the inhibition of G6PD activity is by PTMs, via phosphorylation of key serine residue/s on G6PD (111, 114). Our attempt to identify phosphorylation of G6PD using phosphoserine specific antibodies (data not shown) produced ambiguous results. In spite of advances in detecting phospho-proteins by Western blotting using phospho-specific antibodies, phospho-serine and phospho-threonine antibodies lack considerable specificity (84). Moreover our focus on mouse renal cortical endothelial cells, as opposed to Stanton group's study in diabetic rat kidney cortex, may have contributed to differences in identification of G6PD phosphorylation using phospho-serine specific antibodies (111, 114). Moreover, a recent report by Pan et al., demonstrates that G6PD translocates to the plasma membrane and is tyrosine phosphorylated by c-Src, a non-receptor tyrosine kinase (76). Using G6PD tyrosine mutants and protein phosphate inhibitors, they show that tyrosine phosphorylation of G6PD plays a key role in vascular endothelial growth factor mediated endothelial cell responses, mainly angiogenesis. One more recent report highlights that activation of G6PD in diabetic livers may involve phosphorylation via a signaling cascade that involves PI-3 kinase and/or Src kinase (28). Given such contradicting reports, it is obvious that G6PD is subject to differential regulation based on the tissue observed and disease condition. More importantly, these reports also highlight the existence of a post-translational mechanism regulating G6PD at the tissue level, countering the popular notion of G6PD as a "house-keeping" enzyme

(59, 77). Hence further work is warranted to delineate mechanisms by which G6PD is regulated under normal as well as pathophysiological conditions.

Our work relied on more sophisticated detection technique for pinpointing post-translational modifications, using two-dimensional (2D) gel electrophoresis. Our 2D-gel electrophoresis data indicates that G6PD is post-translationally modified in diabetic RCEs. Unique resolving power of 2D-gel electrophoresis allows for separation of different modified forms of the same protein into distinct spots on the gel (2). Heterogeneous phosphorylation of a protein yields a series of spots with same molecular weight but different pI values (83). The shift of G6PD immuno-reactive spots towards cathodic end denotes a change of the pI towards more acidic values and may be an indication of the predominant PTM i.e. phosphorylation. In addition to 2D-gel electrophoresis, further studies using mass-spectrometry are needed for confirming the phosphorylation of G6PD.

Treatment with C-peptide ameliorated the diabetes-induced reductions in G6PD activity by preventing its PTM, potentially phosphorylation, in RCEs. We believe that preventing the PTMs of G6PD, thereby enhancing the synthesis of NADPH, may be a major mechanism by which C-peptide improves endogenous vasoprotection. In fact, C-peptide has been shown to interact (38) and attenuate the activity of protein tyrosine phosphatase (56). Similar interactions with other phosphatases, specifically serine/threonine phosphatases, may be occurring physiologically. One such serine/threonine phosphatase is protein phosphatase 2A (PP2A) and has been widely implicated in regulating the activity of several protein

kinases. Specifically, PP2A has been shown to dictate the functional status of a calcium channel protein in β -cell by fine-tuning the balance between PKA mediated phosphorylation (thereby cAMP activation) and serine/threonine dephosphorylation (50). The exact role of C-peptide in modulating PP2A mediated dephosphorylation, and possibly PKA mediated phosphorylation, needs to be examined further.

Mitochondrial superoxide generation has been widely implicated in the pathogenesis of diabetic microvascular complications (12, 64). Further, reports using the mitochondria isolated from diabetic animals demonstrated compromised mitochondrial respiration (13, 32, 96). Recently, Palmeira *et al.*(75) reported impairment of mitochondrial respiration rates under high-glucose conditions in HepG2 cells. Clearly, there is a need to delineate the mechanisms underlying high glucose-mediated damage to microvascular endothelial cells, the main cell type associated with diabetic end-organ complications. More importantly, microvascular endothelial cells play an important role in the overt hyperglycemic damage to the kidney due to their deficiency in glucose transport. The murine renal microvascular endothelial cells utilized in this study are an ideal cell type to study the effects of high glucose-induced mitochondrial impairments that ultimately lead to renal microvascular dysfunction.

In our study, we attempted to fill the dearth of knowledge regarding the impact of C-peptide on diabetes-induced mitochondrial dysfunction. We examined the influence of C-peptide on mitochondrial function in high glucose-exposed renal

microvascular endothelial cells. We show that C-peptide inhibits high glucose-induced changes to mitochondrial respiration, membrane potential, superoxide generation and complex I activity. Our investigation is the first study to demonstrate that the protection rendered by C-peptide during diabetes likely involves the mitochondria.

Although all of the complexes of the mitochondrial electron transport chain contribute to total mitochondrial superoxide generation, growing evidence now suggests that complex I produces most of the superoxide generated within intact mammalian mitochondria (9). Further, Lambert and Brand (52) have shown that elevated superoxide generation by the mitochondria requires inhibition of complex I activity. Increased mitochondrial superoxide generation exacerbates mitochondrial oxidative stress and leads to conversion of the mitochondrial glutathione pool to glutathione disulfide (37). As a consequence, glutathione depletion has been shown to cause mitochondrial dysfunction and inhibition of complex I activity. Moreover, glutathionylation of complex leads to contribute to oxidative damage to its subunits and loss of activity (8, 36) as well as increased mitochondrial superoxide formation (8). As discussed previously, NADPH acts as a backbone for regeneration of GSH from oxidized glutathione and can serve as an important contributor in maintaining mitochondrial glutathione pool as well. Figure 4.3 illustrates the proposed mechanism by which C-peptide may ameliorate mitochondrial dysfunction through an indirect effect on mitochondrial GSH via increasing cellular NADPH bioavailability.

Figure 4.3: Schematic illustrating the speculated mechanism by which C-peptide affords protection to hyperglycemia exposed endothelial cells. C-peptide binds and acts through a putative G-protein coupled receptor (GPCR), followed by subsequent activation of adenyl cyclase, PKA via release of cAMP and Phosphoinositide-3 Kinase (PI-3K). This leads to increase in G6PD activity and NADPH. NADPH plays a pivotal role by acting as a backbone for action of cellular antioxidative enzymes (such as eNOS, heme oxygenase) and by enhancing GSH levels. GSH has been shown to regulate mitochondrial Complex I activity through reversible glutathionylation (via action of glutathione), and thereby acts to reduce mitochondrial superoxide. Additionally cAMP released by C-peptide's binding to membrane GPCR can act as substrate for activation PGC-1a via its binding to cAMP response binding element (CREB). As a result there may be an increase in expression of mitochondrial antioxidative enzymes such as SOD and UCP-2, that can counteract mitochondrial superoxide. Combined, C-peptide can act through the above mentioned pathways and prevent endothelial damage by decreasing cellular ROS

C-peptide has been shown to be a potent stimulant of MAPKs (45-47). Activation of ERK by C-peptide has been demonstrated in numerous cell types (27, 56, 116, 117). Our experiments evaluating the effect(s) of C-peptide on the renal microcirculation yielded similar results, demonstrating the activation of ERK1/2. This is especially important because several recent studies indicate that ERK1/2 may affect several mitochondrial functions by regulating the expression of mitochondrial proteins in the nucleus (94). ERK1/2 may also have intrinsic mitochondrial activities. Specifically, ERK1/2 has been shown to localize to the mitochondria of human alveolar macrophages and control mitochondrial membrane potential as well as ATP production(65). However, the exact mechanisms by which ERK1/2 translocates to and is regulated in the mitochondria are still obscure.

In conclusion, our study demonstrates that C-peptide improves anti-oxidant status of the diabetic renal cortical endothelial cells by preventing the PTMs of anti-oxidative enzyme G6PD, and thereby enhancing the synthesis of NADPH. This anti-oxidant benefit can complement the beneficial effects of C-peptide on mitochondrial function in high glucose exposed endothelial cells. Briefly, C-peptide ameliorates hyperglycemia-induced endothelial oxidative stress by improving antioxidant defenses and by preventing mitochondrial dysfunction. This study adds more substantial evidence to include C-peptide in the therapeutic regimen for treatment of type I diabetes-associated microvascular complications

4.1 Future Directions

Interactions of C-peptide with protein tyrosine phosphatases has been shown previously (38, (56). There is a potential for physiological interactions with other phosphatases such as serine/threonine phosphatases. Thus C-peptide could be playing an important role in modulating the phosphorylation status of intracellular effectors. A serine/threonine phosphatase, protein phosphatase 2A (PP2A), has been widely implicated in the regulation of the activity of several protein kinases (50), specifically PKA (50). Future work to examine the influence of C-peptide on protein phosphatases may help to shed light on the intracellular mechanisms by which C-peptide alters the phosphorylation status of G6PD and other potential effectors.

Further studies are needed to investigate in more detail how C-peptide signalling is manifested within the mitochondria, and specifically with complex I. To that, recent reports suggest that C-peptide activates cAMP response element binding (CREB) protein through the p38 mitogen-activated protein kinase pathway (82). CREB functions as an upstream regulator of ND6 (4, 109), a mitochondrial-encoded gene product and well-characterized subunit of complex I. ND6 is essential for proper complex I assembly and function (15, 19). CREB also regulates the expression and function of peroxisome proliferator-activated receptor gamma coactivator-1 α (PGC-1 α)(53). PGC-1 α , in turn, regulates the expression of several mitochondrial proteins involved in oxidative phosphorylation (72, 110), as well as several mitochondrial antioxidant enzymes (105) (i.e., manganese superoxide dismutase, peroxiredoxins III and V, mitochondrial thioredoxin and thioredoxin reductase, glutathione peroxidase

and the mitochondrial uncoupling protein 2). Of these, several groups have shown that increased mitochondrial uncoupling protein 2 expression and activation results in significantly reduced superoxide production. We believe that C-peptide has salutary effects on endothelial redox balance by preventing mitochondrial superoxide generation and enhancing antioxidant defenses mentioned above.

4.2 References

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EDUCATION

Ph.D. Candidate, Center for Research in Cardiovascular and Respiratory Sciences, Department of Physiology and Pharmacology, West Virginia University School of Medicine, Morgantown, West Virginia, USA: 2007-Present. Defended on May 17th, Expected Graduation Summer 2012.

M.S in Pharmaceutical Biotechnology, Dr. M.G.R Medical University, Chennai, India: 2002-2004.

B.S in Pharmacy, Vishwabharati College of Pharmaceutical Sciences, Jawaharlal Nehru Technological University, Hyderabad, India: 1997-2001.

SKILLS

Proficient in mammalian cell culture, sub-cellular fractionations and organelle isolations.

Designed an innovative laboratory protocol for measuring red blood cell flux and velocity that facilitated easy visualization of rodent peripheral microcirculation using intra-vital video microscopy.

Optimization of multiple protocols new to lab, problem-solving skills, adept at data analysis and highly developed interpretation skills.

Techniques: *In vivo* Digital Microscopy, Animal models of Diabetes, Rodent Surgical Techniques, Mammalian and Microbial Cell Culture, Immunoprecipitation, Flow Cytometry, Real time RT-PCR, ELISA, Mitochondrial Complex Activity and Respiration Assays, Immuno-blotting, Native gel and 2D-gel electrophoresis.

AWARDS and HONORS

Ad hoc reviewer, Membership committee, Society for experimental biology and medicine: 2009-present.

Julie Betschart symposium second place winner, School of Medicine, West Virginia University, Morgantown, 2009.

Graduate school student travel award, School of Medicine, West Virginia University, Morgantown, 2009.

Caroline tum Suden/Frances A. Hellebrandt Professional Opportunity Award, American Physiological Society. EB'2009, New Orleans, LA.

Young Investigator Award, Society for Experimental Biology and Medicine. EB'2009, New Orleans, LA.

Zweifach Student Travel Award, The Microcirculatory Society. EB'2009, New Orleans, LA, 2009.

Professional Skills Travel Fellowship, American Physiological Society. Lake Buena Vista, FL, 2009.

Selected Presentation, E.J. Van Liere Memorial Convocation, WVU School of Medicine, Morgantown, WV, 2008.

PUBLICATIONS

1. C-peptide Reduces Mitochondrial Superoxide Generation by Restoring Complex I Activity in High Glucose-Exposed Renal Microvascular Endothelial cells. **Vejandla, H**, Hollander, JM, Kothur, A, and Brock, RW. *ISRN Endocrinol.* 2012; 162802 Epub 2012 Apr 10.
2. Brock, RW, Shelton, KR, Kothur, A, MacMillan-Crow, LA & **Vejandla, H**. C-peptide Ameliorates Microvascular Dysfunction during Type I Diabetes by Restoring Endothelial NADPH. *Diabetes/Metabolism Research & Reviews* (DMRR-07-RES-209: IN REVISION).
3. **Vejandla, H**, Kothur A and Brock, RW. C-peptide Confers Anti-oxidative Protection in Renal Cortical Endothelial Cells during Type I diabetes by preventing the Inhibition of Glucose-6-Phosphate Dehydrogenase Activity (In preparation).

ABSTRACTS

Vejandla H, Kothur A, Dabkowski E.R, Hollander J. M, Frisbee J.C and Brock R.W. C-peptide confers protection in renal cortical endothelial cells during Type I diabetes by preventing the phosphorylation of glucose-6-phosphate dehydrogenase. *EB'2009 - Abstract #4865*

Vejandla H, Frisbee J.C, Dabkowski E.R, Hollander J.M, Kothur A and Brock R.W. Hyperglycemia-induced mitochondrial dysfunction and oxidant generation in mouse renal microvascular endothelial cells is reversed by C-peptide. *EB'2009 - Abstract #4918*

Vejandla H, E. R. Dabkowski, J. M. Hollander and R.W. Brock, "C-peptide Reverses Hyperglycemia-induced Mitochondrial Dysfunction and Oxidants in Murine Renal Microvascular Endothelial cells" - Jackson Cardiovascular-Renal Meeting, Center for Excellence In Cardiovascular-Renal Research, The University of Mississippi Medical Center, Jackson, Mississippi. 2008

Vejandla H and Robert W Brock, "C-Peptide- Waiting To Join The Battle Against Diabetic Renal Dysfunction"- Oral presentation, E.J. Van Lier Memorial Convocation, West Virginia University School of Medicine, Morgantown, West Virginia. 2008

Vejandla H and Philip Palade, "Hydrogen Peroxide and 4-Hydroxynonenal Downregulate L-Type Calcium Channel Expression in Rat Aortic Vascular Smooth Muscle Cells"- Poster presentation, Student Research Day, University of Arkansas for Medical Sciences, Department of Pharmacology and Toxicology, Little Rock, Arkansas. 2007

Vejandla H and Satish Kumar D, "Study of Hypoglycemic activity of an Ayurvedic Polyherbal Formulation in Alloxan Induced Diabetic Rat Model"- Poster and oral presentation at Indian pharmaceutical congress, Chennai, India. 2003

Vejandla H and Sudhakar G, "Anti-inflammatory and Analgesic Activity of a Proprietary Herbal Drug in Rat" – Oral presentation at Indian pharmaceutical congress, Hyderabad, India. 2000

Vejandla H, "Synthesis and Biological Evaluation of Condensed/ Substituted Isoxazole Derivatives"- Oral presentation at Vishwabharati College of Pharmaceutical Sciences, Guntur, India. 1999

PROFESSIONAL AFFILIATIONS

American Society for Pharmacology and Experimental Therapeutics, 2007- present

Microcirculatory Society, 2007- present

American Heart Association, 2007- present

American Physiological Society, 2007- present

Licensed Pharmacist- Andhra Pradesh Pharmacy Council, India, 2001- present

Indian Pharmacy Council, 2000- present

Technical Skills

Cell culture techniques: Isolation of primary cultures of hepatocytes using Seglen's perfusion technique, culturing of rat kidney proximal tubule, murine renal endothelial cell, A7R5 and PC-12 cell lines, isolation and culturing of mouse liver and kidney endothelial cells using immunomagnetic separation and fluorescent cell imaging.

Animal techniques: Establishing murine type 1 diabetic model using streptozotocin and alloxan, implantation of subcutaneous osmotic pumps in mice and live imaging of microcirculation in mouse using intra-vital video microscopy.

Molecular and Biochemical techniques: Real time RT-PCR, Radioimmuno assay, ELISA, Mitochondrial complex activity and respiration assays, Immunoblotting, Blue native gel electrophoresis, Native gel electrophoresis and 2D-gel electrophoresis.