Protective Effects of Metoprolol and Ascorbic Acid During the Development of Diabetic Cardiomyopathy

by

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Abstract

The existence of a heart muscle disorder specific to Diabetes Mellitus (DM) has been proposed, termed, Diabetic Cardiomyopathy (DCM). DCM is defined as the presence of an early asymptomatic diastolic dysfunction that eventually progresses to overt systolic dysfunction in the absence of ischemic or valvular heart disease. Metabolic impairment and increased oxidative stress have been highlighted as causes. The β-blocker metoprolol is known to improve function in diabetic rat hearts, possibly through amelioration of the sequelae associated with oxidative stress, without lowering oxidative stress. It is unclear if lowering oxidative stress in concert with metoprolol treatment would improve function further. Ascorbic Acid (AA) is a potent antioxidant and has been shown to improve function in the diabetic rat heart.

Hypothesis:

We propose that metabolic changes that occur during diabetes elevate oxidative stress, leading to protein damage, signaling changes, cell death and other sequelea; the eventual sum of these changes is an impairment of function. Treatment of either the sequelae of oxidative stress or oxidative stress directly will be beneficial but treatment of both will improve function further.

To accomplish our study we induced DM in male Wistar rats using 60 mg/kg streptozotocin and treated them with metoprolol at 15 mg/kg/day via osmotic

pump and/or AA at 1000 mg/kg/day via drinking water. In order to study the effect of treatment on the development of dysfunction we studied a time point before and after development of dysfunction (5 and 7 weeks, respectively). Blood was collected to assess the severity of diabetes and echocardiography performed to assess *in vivo* heart function. At termination, *ex vivo* heart function and substrate use were measured by working heart perfusion. Tissue was collected for measurements of metabolite levels and oxidative protein damage.

Function significantly worsened in association with metabolism and oxidative damage. Both drugs improved function, while only AA reduced oxidative damage. Combined treatment led to improvement in function more pronounced then single treatment. Our β-blocker and antioxidant treatment strategy focuses on oxidative stress, and not on diabetes specifically, thus it may prove useful in other disease where oxidative stress contributes to pathology.

Preface

Ethics approval for this study was attained from the Animal Care Committee at the University of British Columbia. Study 1 was listed under the certificate titled: Modulation of cardiac metabolism by metoprolol in the diabetic heart (#A06-0420). Study 2 was listed under the certificate titled: Malonyl Co-A-independent regulation of carnitine palmitoyltransferase-1 by B adrenoceptor signaling in the heart (#A07-0730).

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AA	Ascorbic Acid
AGE(s)	Advanced Glycation End Products
Akt	Protein Kinase B
AMP	Adenosine Monophosphate
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
CDM	Centre for Disease Modeling
CHO	Chinese Hamster Ovary Cells
COMET	Carvedilol or Metoprolol European Trial
CVD	Cardiovascular Disease
DAG	Diacylglycerol
DCM	Diabetic Cardiomyopathy
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
DNP	Dinitrophenol
EDTA	Ethylenediaminetetraacetic Acid
EDV	End Diastolic Volume
EF	Ejection Fraction
ESV	End Systolic Volume
FS	Fractional Shortening
GlcNAc	*N*-Acetylglucosamine
GLUT4	Glucost Transporter 4
HbA1c	Glycated Hemoglobin
HBP	Hexosamine Biosynthetic Pathway
HEPES	4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid
LPL	Lipoprotein Lipase
LVEDD	Left Ventricular End Diastolic Diameter
LVEDV	Left Ventricular End Diastolic Volume
LVESD	Left Ventricular End Systolic Diameter
LVESV	Left Ventricular End Systolic Volume
MOPS	3-(N-Morpholino)-Propanesulfonic Acid
NADH(NAD^+)	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCX	Sodium Calcium Exchanger
PKA	Protein Kinase A
PKC	Protein Kinase C
PKC-β_2	Protein Kinase C β_2
PLB	Phospholamban
PMCA	Plasma Membrane Ca^{2+} ATPase

RAAS	Renin-Angiotensin-Aldosterone-System
Rac1	Rho Family, Small GTP Binding Protein
RAGE	AGE Receptor
ROS	Reactive Oxygen Species
RPP	Rate Pressure Product
RyR	Ryanodine Receptor
SEM	Standard Error of the Mean
SERCA	Sarco/Endoplasmic Reticulum Ca2+-ATPase
STZ	Streptozotocin
T1DM	Type 2 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TUNEL	Terminal Deoxynucleotidyl Transferase (dUTP) Nick End Labeling
TWEEN	Polyoxyethylenesorbitan
UDP-GlcNAc	Uridine Diphosphate *N*-Acetylglucosamine
v/v	Volume by Volume
VLDL	Very Low Density Lipoprotein

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1. Introduction

1.1. Diabetes Mellitus

Diabetes mellitus (diabetes) is a condition of chronically elevated blood glucose levels. It is clinically defined as an 8-hour fasting plasma glucose greater then 7.0 mM, a non-fasting plasma glucose of greater then 11.1 mM or a 2-hour plasma glucose of greater then 11.1 mM on an oral glucose tolerance test [1]. The current world wide prevalence is estimated at 285 million people, and the number is fast rising, with some estimating an increase in confirmed sufferers to 438 million by the year 2030[2]. Most sufferers today are found in the developed world, however, the greatest increases in prevalence are expected to be in developing countries in Asia and Africa[3].

Diabetes is a disease of damaged insulin signaling. Insulin is a peptide hormone produced by β-cells located in the islets of Langerhans in the pancreas, and it is used to regulate blood glucose content. Diabetes develops either through failure of β-cells to secrete enough insulin, or through failure of cells to detect insulin. Regardless of the specific early cause, β-cell death, loss of insulin sensitivity and hyperglycemia will develop in most cases[4-6] . However, diabetes affects more then just glucose homeostasis, protein and lipid metabolism are also severely disturbed[7].

Type 1 diabetes is caused by the destruction of insulin secreting β-cells. The presence of antibodies targeting insulin or other antigens associated with islet cells indicate that the effector mechanism behind β-cell death is often autoimmunity, however, the underlying cause is a combination of genetic or epigenetic susceptibility and environmental stimuli [8].

Type 2 diabetes is caused by a reduction in sensitivity to insulin, termed: insulin resistance. Development of insulin resistance is due to a combination of; a genetic predisposition, often indicated by family history or race/ethnicity; and an unhealthy lifestyle, usually low physical activity, excess caloric intake and obesity[9].

Of the two major types of diabetes mellitus, Type 1 diabetes sufferers comprise about 5-10% of the total diabetic population and Type 2 diabetics comprise almost the entire remainder [2] . A third common form of diabetes, known as gestational diabetes, affects about 3.7% of non-aboriginal and 8-18% of aboriginal women during pregnancy. The presence of gestational diabetes increases the likelihood that the mother will later develop Type 2 diabetes [10].

1.2. Complications during Diabetes

Diabetes mellitus is a disease that causes severe homeostatic disturbances in the blood, thus deleterious effects can be found in many different body tissues. For instance, the effects of diabetes on the microvasculature and peripheral nervous system are: suspected to be the leading cause of new cases of blindness in adults between 20-74 years of age; are the leading cause of kidney failure, accounting for 44% of all new cases in 2008; and are the leading cause of non-traumatic lower limb

amputation[11]. Diabetes also increases the risk for macrovascular disease, including development of atherosclerosis and a 150-400% increase in the likelihood of stroke[12, 13] . In addition, diabetes has a powerful association with cardiovascular disease (CVD). CVD is the primary cause of death in both Type 1 and Type 2 diabetic patients and is the single largest component of health care expenses associated with diabetes[12].

1.3. Diabetic Cardiomyopathy

In 1972 Rubler *et al.* first proposed the presence of a diabetes specific cardiomyopathy in four patients who developed cardiac hypertrophy, interstitial fibrosis and eventually heart failure without any discernable cause[14]. In 1974 Kannel *et al.* demonstrated, in the landmark Framingham Heart Study, that diabetes is an independent risk factor for the development of heart failure. Furthermore, they showed that diabetic men displayed a greater then two-fold, and women, a five-fold increase in risk[15]. Today there is much evidence describing the increased cardiovascular disease risk to diabetic patients, however, the existence of a cardiomyopathy which develops independent of hypertension, valvular and congenital heart disease or coronary artery disease is still somewhat controversial in the eyes of some clinicians[16]. In addition, clinical societies have often not recognized it as a stand-alone entity, only Type 1 diabetes was mentioned as a cause of secondary cardiomyopathy in the 2006 American Heart Association Classification of Cardiomyopathies, and a similar assertion was made in the 2008 European Society of Cardiology scientific statement[17, 18] . *Sharma et al.* point out that diabetic

cardiomyopathy (DCM) often leads to heart failure when it is combined with ischemic heart disease or hypertension, thus the presence of DCM may not be obvious and other complications may be highlighted [19]. Similarly, Maisch argues that the problem may lay with the definition of DCM. He points out that in a clinical setting DCM will be classified using functional measurements, thus DCM may be classified as a non-ischemic or ischemic cardiomyopathy or, 'heart failure with normal ejection fraction', or 'heart failure with reduced ejection fraction' [20]. Maisch goes on to suggest a common definition for DCM - "distinct entity characterized by the presence of abnormal myocardial performance or structure in the absence of epicardial coronary artery disease, hypertension, and significant valvular disease", which is inline with accepted definition or cardiomyopathy in general[20]. A commonly reported characteristic of DCM, in both young Type 1 and 60% of well controlled normotensive Type 2 diabetics, is the development of diastolic dysfunction, defined as "early diastolic filling, prolongation of isovolumetric relaxation, and increased atrial filling" [21-23] . Thus, others extend the definition of DCM to include an early phase indicated by an asymptomatic diastolic dysfunction, which later progresses to overt systolic dysfunction and eventual heart failure[24, 25] .

Several diabetic animal models have been used to study the progression of DCM. Akita mice are a genetic model of Type 1 diabetes, they possess a single base pair mutation which causes misfolding of proinsulin, endoplasmic reticulum stress and β-cell death leading to hyperglycemia[26]. The Akita mouse can also display diastolic dysfunction in absence of systolic dysfunction, similar to the early phase of DCM in humans [27]. The ob/ob mouse model of diabetes shows a deficiency in the

hormone leptin, as a result animals become obese and eventually develop Type 2 diabetes[28]. Diastolic dysfunction has also been reported in these animals[29]. The streptozotocin (STZ) induced model of Type 1 diabetes has been used in over 7600 scientific publications, making it the most commonly used model of diabetes, and the second most commonly used animal model in research. In this model, diabetes is induced using STZ, a glucose moiety produced by the bacterium *Streptomyces griseus*, that is toxic to insulin producing β-cells in the pancreas[30]. The STZ model displays DCM, with an initial asymptomatic diastolic dysfunction followed by development of an overt systolic dysfunction. Importantly, this model does not develop hypertension, or atherosclerosis, showing that DCM can occur in the absence of any other cardiovascular complication[31-35] .

1.4. Causes of Diabetic Cardiomyopathy

A variety of molecular and structural causes have been implicated in the development of DCM. They range from abnormalities in calcium handling, structural changes within the heart, development of neuropathy, hormonal abnormalities and finally metabolic changes and oxidative stress[36]. When considering the diversity of causes of DCM, it is important to remember that all the discussed causes converge on two effector mechanisms, either reduced ventricular compliance or reduced ventricular contractility[19].

1.4.1. Calcium Handling Abnormalities

Disturbances in calcium handling are a hallmark of DCM. Calcium is essential for coupling of excitation and contraction, thus, precise control of calcium levels is essential for normal function. There are numerous studies in animal models that show reductions in expression and activity in all calcium transporters that are involved in excitation and contraction coupling. These include, sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA), Na^{+}/Ca^{2+} exchanger (NCX), ryanodine receptor (RyR), and plasma membrane Ca^{2+}-ATPase (PMCA) [36]. Some of the observed changes in activity are due to Protein kinase C, which is activated during diabetes and heart failure, and is known to phosphorylate a number of enzymes that are involved in cellular calcium handling [37-39] .

The effect diabetes has on the SERCA homologue SERCA-2a and its inhibitor phospholamban (PLB), seem to be particularly important. Protein and mRNA levels of both proteins are reduced during diabetes, and depression in SERCA activity is known to lead to calcium overload in the cytosol and impaired contraction[40, 41] . Furthermore, overexpression of SERCA in diabetic rodent models can lead to normalization of function[42].

1.4.2. Cell Death and Fibrosis

Sharma *et al*. have demonstrated development of a pro-apoptotic signaling state in the diabetic heart[34]. In addition, myocyte apoptosis and necrosis in the diabetic heart are often reported in literature[43]. Cell death has an important impact on the heart due to its limited regenerative capacity. According to Cai *et al*. cell

death will lead to "loss of contractile units, conduction disturbances, compensatory hypertrophy of myocardial cells and fibrosis" [43-45] . The molecular basis behind increased cell death centers around metabolic disturbances and the resultant oxidative stress, as well as inflammation[43].

Although the presence of increased apoptosis in the diabetic heart is difficult to doubt, the reported rates might not be as high as initially thought. Many studies use Terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) or detection of activated caspase-3 to assess apoptosis[46, 47] . However, TUNEL is not completely specific for apoptosis, as it will generate a positive signal in cells that are undergoing DNA repair [48]. Also, levels of activated caspase-3 are not always an accurate indicator of apoptosis. Sharma *et al*. have demonstrated that cleaved caspase-3 can be sequestered and deactivated by caveolins[34].

Development of myocardial fibrosis, is another key characteristic observed during DCM and is known to be at least partially due to replacement fibrosis following necrotic or apoptotic cell death[36]. There is also a relationship between fibrosis and diastolic dysfunction, as demonstrated in both Type 2 diabetic rodents and in human Type 1 diabetics with asymptomatic diastolic dysfunction[49, 50] .

Activation of Protein Kinase C β_2 (PKC-β_2) and the Renin Angiotensin-Aldosterone System (RAAS) are both involved in development of fibrosis during diabetes. PKC-β_2 is a mediator of fibrosis and its activity is known to increase during hyperglycemia. Furthermore its expression is increased in rodent models of STZ induced diabetes[51]. Overexpression of PKC-β_2 in animal models causes cardiac hypertrophy, fibrosis and left ventricular dysfunction[52]. The RAAS is also activated

during diabetes, and is thought to contribute to myocyte necrosis and development of fibrosis in the heart[36].

1.4.3. Metabolism and Oxidative Stress

1.4.3.1. Fuel Usage

In the normal heart 60-80% of ATP is derived from oxidation of fatty acids, and 20-40% from oxidation of carbohydrates, during diabetes fuel usage is severely disturbed, with a near complete loss of carbohydrate oxidation and an acceleration in fatty acid oxidation[53-55] . Changes in substrate uptake and reciprocal regulation of fatty acid and glucose oxidation are blamed for the metabolic disturbances in the diabetic heart.

GLUT4 is the primary inducible glucose transporter in the adult heart. GLUT4 is normally sequestered into intracellular vesicles, and upon stimulation by insulin or contraction it is translocated to the sarcolemma[56]. During diabetes there is a reduction in glucose transport into cardiac myocytes, this is primarily due to a reduction in total expression of GLUT4 as well as reduction in translocation of the remaining protein[53].

Lipids are supplied to tissue from lipoprotein such as chylomicrons and Very Low Density Lipoprotein (VLDL) that contain esterified fatty acids collected from the gut and the liver, respectively; or from free non-esterified fatty acids that are bound to albumin. Fatty acids from chylomicrons and VLDL, are greater in molar concentration by approximately 10-fold compared to albumin bound fatty acids[57]. Albumin bound fatty acids can be taken up by mycotyes directly (via transporters

on the myocyte surface), while lipoprotein bound fatty acid must first be released by the enzyme lipoprotein lipase (LPL) found on the vascular luminal surface of endothelial cells. During diabetes, levels of lipoproteins and albumin bound fatty acid increase, as does LPL activity at it's functional site in the vasculature, thus, fatty acid uptake into cardiomyocytes is increased[58-60] .

Fatty acid oxidation and carbohydrate oxidation are reciprocally regulated, thus, rates of glucose metabolism are further inhibited by accelerated rates of fatty acid oxidation, this effect is referred to as the Randle cycle[60]. The Randle cycle is mediated through changes in ratios of NADH/NAD^+, acetyl-CoA/free CoA and citrate levels. Accelerated fatty acid oxidation increases NADH/NAD^+ ratio, and acetyl-CoA/free CoA ratios, both of these actions inhibit the pyruvate dehydrogenase complex, reducing flux through the tricarboxylic acid cycle and preventing full oxidation of glucose[60]. Furthermore, citrate levels are also increased by fatty acid oxidation, citrate inhibits phosphofructokinase-1, a key rate controlling step in glycolysis[61].

1.4.3.2. Lipid Accumulation

Increased lipid uptake and oxidation by the diabetic heart has several deleterious consequences. Accelerated fatty acid uptake decreases the diabetic heart's oxygen efficiency, the *ex vivo* oxygen requirements for Type 1 and Type 2 diabetic rodent hearts are increased by 57% and 85%, respectively. A similar effect is observed when fatty acid levels that the heart is exposed to are increased, the

same group reported a 15% increase in control hearts when they were perfused with a high versus low concentration of fat[62].

Another consequence of increased lipid uptake and oxidation is the development of lipotoxicity, or cellular dysfunction and death associated with lipid accumulation[63]. A substantial amount of evidence has emerged linking excess lipid supply and accumulation in myocytes with cardiomyopathy, both in humans with impaired metabolic regulation and in animal models of diabetes and other metabolic disorders[63-65] . Certain fatty acids have been shown to induce apoptosis in several different cell types, including cardiomyocytes[66]. Often referred to as 'palmitate induced apoptosis', the effect seems to be most pronounced with long chain saturated fatty acids, such as palmitate (C16:0) and stearate (C18:0), shorter chain lengths and unsaturated fatty acids do not seem to cause lipotoxicity[67, 68] . The key effector mechanism in palmitate-induced apoptosis is believed to be production of ceramide and increased oxidiatve stress. Ceramides are a lipid signaling molecule that are involved in propagating pro-apoptotic signaling and are synthesized from saturated fatty acids like palmitate[69]. Furthermore, cell permeable ceramide analogues generate the same effect as palmitate and inihibiton of ceramide synthesis often prevents apoptosis in the presence of palmitate[70]. However, ceramide production is not essential for palmitate induced apoptosis in all cell types, as isolated cardiomyocytes from chick embryos, Chinese Hamster Ovaries (CHO) and H4IIE liver cells undergo apoptosis via a ceramide-independent pathway[71-73] . On the other hand, oxidative stress appears to be an essential mediator of palmitate-induced cell death, as generation of reactive oxygen and

nitrogen species increases during lipid accumulation in a variety of cell types and apoptosis can be prevented using agents that scavenge reactive species[73-76] .

1.4.3.3. Hyperglycemia

Similar to excess lipid accumulation, excess glucose load during diabetes can cause significant cellular damage and eventually lead to myocyte death, hyperglycemia's toxic effects are referred to as, glucotoxicity[77]. The clinical relevance of hyperglycemia is well recognized. For every 1% increase in levels of glycated hemoglobin, (HbA1c, glucose permanently modifies a portion of total hemoglobin upon long term exposure, greater glucose concentrations yield modification of more hemoglobin) there is an 8% increase in risk of heart failure[78]. Furthermore, elevated plasma glucose, even without diabetes, is a predictor for the development of cardiovascular disease[79]. Although there are many factors and processes involved in the pathologic effects of glucotoxicity, the primary effector mechanisms revolve around protein glycation, formation of reactive oxygen species and glucose flux through alternate pathways[25, 77] .

Glycation, or 'non-enzymatic glycosylation', is a posttranslational modification where a carbohydrate group is added to a protein, lipid or nucleic acid molecule. Glycation can affect the activity of proteins, such as p53; a transcription factor known to regulate cell death[80]. Increased glycosylation of p53 in isolated myocytes exposed to hyperglycemia has been demonstrated to increase its activity, leading eventually to activation of the local RAAS, production of angiotensin II and increase in ratio of pro-/anti-apoptotic proteins[77]. Glycation is also responsible for

the production of Advanced Glycation End products (AGEs). AGEs are proteins, lipids or nucleic acids that have been modified as a result of increases in oxidative stress and excess carbohydrates[81]. AGE formation is deleterious not only because proteins are structurally modified, but also because they can activate pro-inflammatory pathways when they bind their receptors, RAGE. Furthermore, formation of the AGE-RAGE complex can activate the NADPH oxidase complex, leading to further reactive oxygen species (ROS) production[82, 83] .

NADHP oxidase mediated ROS production is also dependent on several other signaling molecules and proteins such as, diacylglycerol (DAG), angiotensin II and Rac1[84, 85] . During hyperglycemia *de novo* DAG synthesis has been demonstrated to increase. DAG is a second messenger signaling molecule that is a physiological activator of Protein Kinase C (PKC), activated PKC is known to stimulate NADPH oxidase activity[82, 84] . Angiotensin II is an effector molecule produced by the RAAS. During diabetes the RAAS is locally activated, the resultant angiotensin II stimulates NADPH oxidase activity[36, 85] . Rac is a small guanosine triphosphate binding protein that is a member of the NADPH oxidase complex and is essential for its formation, Rac1 is the major isoform in the cardiomyocyte[86, 87] . Rac1 was recently demonstrated to be essential for the development of hyperglycemia induced apoptosis in cardiomyocytes, and this role was demonstrated to be mediated through NADPH oxidase[88].

Glucotoxicity can also result from diversion of glucose from the oxidative pathway into alternate metabolic pathways[19, 53] . During diabetes, glucose uptake is significantly reduced but not completely abolished. However, due to inhibition of

glucose oxidation and glycolysis caused by fatty acid oxidation, the small portion of glucose that enters the cell can be shunted towards the polyol or hexosamine biosynthetic pathways [89].

The polyol pathway consists of an NADPH requiring reduction of glucose to sorbitol via aldose reductase and an oxidation of sorbitol to fructose via sorbitol dehydrogenase[90, 91] . During diabetes, excess glucose enters this pathway and stimulates aldose reductase but not sorbitol dehydrogenase, thus excess flux through the polyol pathway leads to reduction of the NADPH/$NADP^+$ ratio. NADPH is required to produce glutathione, a major endogenous antioxidant, thus polyol flux can weaken cellular antioxidant defenses[90].

Under physiologic conditions the Hexosamine Biosynthetic Pathway's (HBP) role is to act as a fuel sensor, and to help partition fuels to the appropriate storage sites within the body[92]. Carbohydrates enter this pathway as fructose-6-phosphate, immediately before the rate controlling phosphofructokinase-1-mediated step in glycolysis. The final product of the HBP is UDP-*N*-acetylglucosamine (UDP-GlcNAc), this then serves as a substrate for *O*-GlcNAc transferase, which then attaches the GlcNAc moiety to specific sites on target proteins[93]. The HBP is implicated in a number of cellular processes, including intracellular signaling, modification of protein degradation and modulating protein-protein interaction[93, 94] . During diabetes, flux through the HBP is increased, possibly due to inhibition of PFK-1 via the Randle cycle and accelerated fatty acid oxidation[19]. Recently, Rajamani *et al*. demonstrated increased GlcNAc tagging of the pro-apoptotic Bad protein in myocyte

during hyperglycemia, furthermore, GlcNAc tagging prevented interaction of Bad and the apoptosis inhibitor, BCl-2[95].

1.5. Selected Treatment Strategies

1.5.1. β-Blockers

β-adrenergic receptors are a class of G-protein-linked receptors (β-receptors) that accept catecholamines, such as epinephrine and norepinephrine, and signal as part of the sympathetic nervous system. All three subtypes of β-receptors are found in the heart, β_1, β_2, and β_3, however, β_1 is the most abundant and has the most powerful effect on contractile function[96-98] . Outcomes of β receptor signaling are dependent on the specific heterotrimeric G proteins they couple to; β_1 and β_2 can couple to stimulatory G_s proteins while β_2 and β_3 can couple to the inhibitory G_i protein. Signaling through G_s leads to activation of adenylyl cyclase triggering increases in cAMP levels and activation of Protein Kinase A (PKA), while signaling through G_i inhibits adenylyl cyclase. PKA phosphorylates several different sarcolemmal proteins including L-type Ca^{2+} channels and phospholamban, these actions enhance calcium influx into the myocyte and calcium uptake into the sarcoplasmic reticulum. Thus the net effect of G_s signaling is enhanced contraction[99, 100] .

β-adrenergic receptor antagonists (β-blockers), are a class of drugs that bind and block the action of one particular, or several, β-receptors, thus, they block sympathetic signaling and have acute negative inotropic and chronotropic effects[101].

Due to their suppression of contracton, β-blockers were originally considered dangerous for heart failure sufferers[102-104] . However, it is now known that with chronic treatment they improve cardiac function, and reduce morbidity and mortality in heart failure patients. Consequently, their use is currently strongly supported by clinical guidelines and by consensus[105-107] .

β-blockers are not created equal in terms of receptor specificities and chemical properties. Currently only three β-blockers, bisprolol, metoprolol and carvedilol, have been approved for patients undergoing heart failure. Metoprolol and bisprolol are β_1 selective inverse agonist, meaning that they bind and block the β_1 receptor (and β_2 at high doses) but also reduce receptor signaling below basal levels[108]. Carvedilol is a nonselective β blocker which also displays antagonism for the α_1 receptor. Carvedilol is also known to have clinically relevant antioxidant and vasodilating properties[109].

1.5.1.1. β-Blockers and Diabetic Cardiomyopathy

The mechanism of β-blockers therapeutic effect during heart failure is thought to center around their ability to mitigate excessive adrenergic drive, helping normalize impaired calcium handling[110]. Increased adrenergic signaling has also been demonstrated during DCM, as has the associated reduction in β_1 receptor sensitivity and expression, and impaired calcium handling[36, 111, 112] . Thus, long term β-blocker therapy may help to ameliorate some of the dysfunction observed during DCM. In a series of studies by Sharma *et al*. the effect of metoprolol on STZ

diabetic rats was examined[19, 33, 34, 112, 113]. Metoprolol in this model was demonstrated to ameliorate functional impairments in isolated perfused working hearts, improving reduced hydraulic power, rate pressure product and cardiac output[33]. Independent to its functional effects, metoprolol also partially ameliorated metabolic disturbances during diabetes, with Sharma *et al*. reporting reductions in fatty acid oxidation and secondary increases in glucose oxidation[33, 34]. Finally, metoprolol also appeared to switch the diabetic heart away from an activity pattern promoting activation of the pro-apoptotic Bad and inhibition of anti-apoptotic BCl-2, to inhibition of Bad and activation of BCl-2, while not reducing oxidative DNA damage. Thus metoprolol improved function and reduced the sequela of diabetes and oxidative stress without reducing oxidative stress[34].

However, the concept of treating diabetics with β-blockers is somewhat controversial. Much of the controversy centers around the belief that β-blockers will reduce awareness of symptoms of hypoglycemia, however, there is direct scientific evidence from studies in human diabetics and normals refuting this claim[114-116] . Another perceived negative consequence of β-blockade, and metoprolol in particular, is that it may promote development of new cases of diabetes. This belief stems largely from retrospective analysis of data from the Carvedilol Or Metoprolol European Trial (COMET), where it was found that metoprolol tartrate was associated with an increase in new onset diabetes of 10.1% compared to 8.7% for carvedilol[117]. However, there has been much criticism of these findings, as the actual comparison being made is to carvedilol treated patients, thus there is no proof that metoprolol actually triggers new onset diabetes.

Furthermore, an unreccomended low dose of the non-clinically proven metoprolol tartrate (50 mg, twice daily; quick release formulation) was used instead of the more effective metoprolol succinate (slow release formulation) [118]. Comments from cardiologist Dr. John McMurray summarize these results best, "a proven dose of carvedilol is clearly superior to a non-recommended, low dose of a short acting formulation of metoprolol…"[119].

1.5.2. Ascorbic Acid

There are many sources of increased oxidative stress during diabetes, including altered fuel usage, accumulation of lipids and hyperglycemia. This increased oxidative stress, as discussed above, is thought to contribute to the contractile dysfunction observed during DM. The cardioprotective effects of a number of antioxidant molecules have been evaluated, including, β-carotene, vitamin E and Ascorbic Acid (AA). To date, there have been many encouraging findings in epidemiological studies. High β-carotene intake has been shown to reduce cardiovascular risk in the Nurses Health Study, reduce cardiovascular mortality and myocardial infarction in the elderly in the Massachusetts Health Care Panel Study, and reduce cardiovascular risk in the Health Professionals Follow Up Study[120-122] . Vitamin E has been associated with reduced cardiovascular death and risk in the Nurses Health and Health Professional Follow Up Study[120-122]. AA is among one of the most common antioxidants available and it has proven effective in reducing cardiovascular risk in both the National Health and Nutrition Examination Survey and the Eastern Finland Study[120, 123, 124].

Basic scientific studies have also provided evidence for a therapeutic role for antioxidants. In a study by Qin *et al*. it was demonstrated that a combination treatment of AA and vitamin E was able to reduce oxidative stress, increase BCl-2 expression and lower Caspase 3 activity in rabbit hearts post myocardial infarction [125]. Furthermore, the ability of the β-blocker carvedilol to reduce infarct size is matched by SB 211475, a metabolite of carvedilol without any adrenergic receptor blocking ability, but possessing its antioxidant strength[126]. Finally, Dai *et al*. were able to show that oral AA intake could lead to partial amelioration of myocardial dysfunction, including filling rates, in a dose dependent manner, in the STZ diabetic rat. Furthermore, they demonstrated that AA was able to lower elevated plasma triglycerides, cholesterol and free fatty acid levels, again in a dose dependent manner[127].

In sharp contrast to the findings in support of antioxidant therapy in epidemiological and basic science studies lies the largely negative data from clinical trials[128, 129]. Generally speaking, clinical trials have not been able to demonstrate a clear therapeutic relationship between antioxidants and cardiovascular disease, however, several methodological aspects are outlined by Ye *et al.* and Steinhubl *et al*. that may explain the discrepancy. First, clinical trials often select antioxidants based on ease of availability and deliverability. For example, in clinical studies a synthetic vitamin E is often used, whereas natural vitamin E consists of 8 different forms with differing properties. Next, the duration of study for a clinical trial is often shorter then an observational study, running only 5 years or so, whereas observational studies could span decades. Finally, the study population selected

during clinical trials is often older and has preexisting disease, therefore treatment periods are much shorter, for example: 2 years of antioxidant therapy after 40 years of oxidative stress. In comparison, in animal studies treatment often begins before overt disease is present and prospective studies often begin when subjects are younger and healthier. Although clinical trials provide greater control of variables then epidemiological studies, due to their shortcomings, their findings in regard to antioxidant therapy are at best inconclusive [128, 129] .

1.6. Hypothesis and Study Objectives

Primary Hypothesis (see Scheme 1):

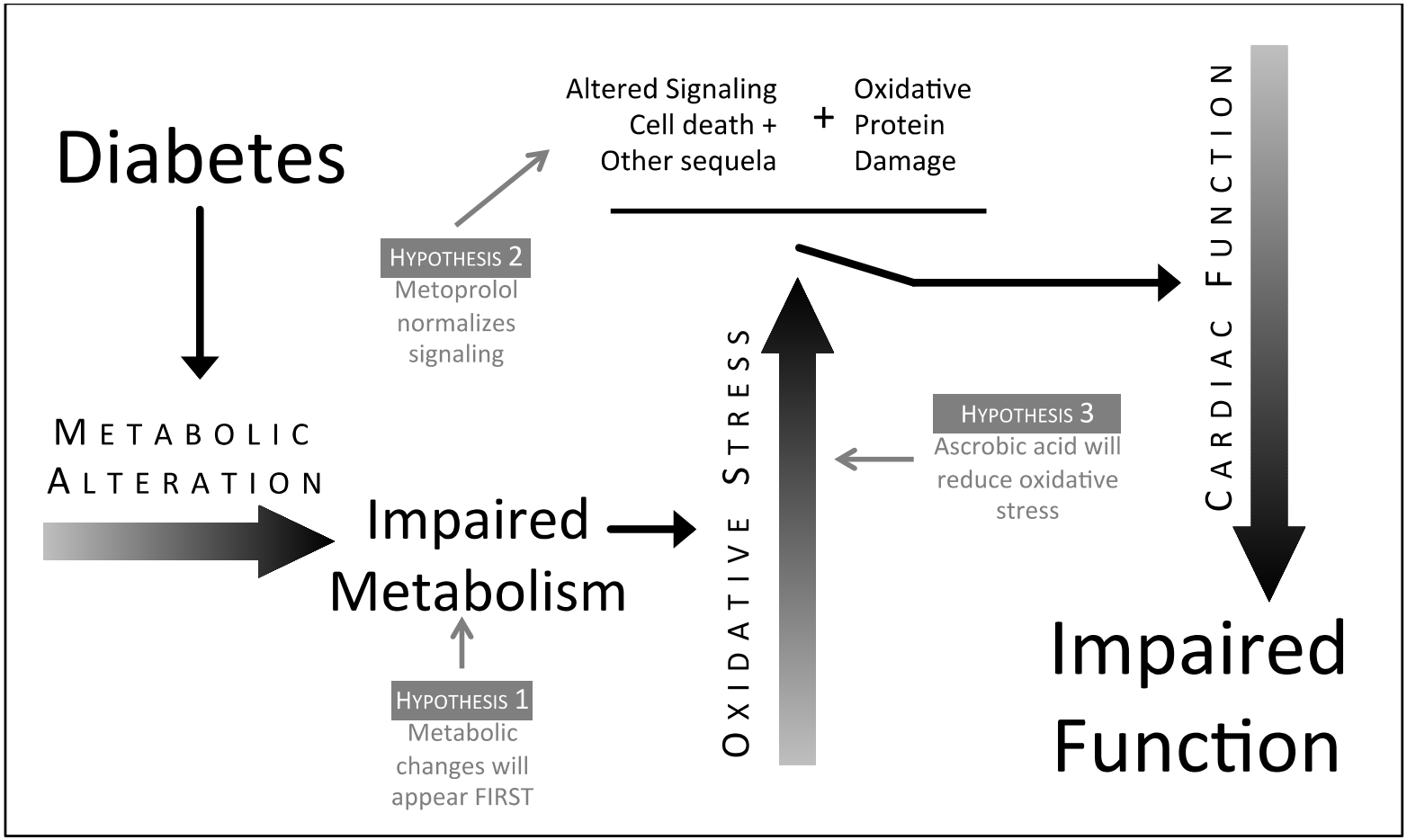
We propose that metabolic changes that occur during diabetes elevate oxidative stress, leading to protein damage, signaling changes, cell death and other sequelea; the eventual sum of these changes is an impairment of function. Treatment of either the sequelae of oxidative stress or oxidative stress directly will be beneficial but treatment of both will improve function further.

We will address our hypothesis in two ways. First, we will observe the development of metabolic impairment, oxidative damage and DCM by studying a time point before (referred to as Study 1) and a time point after (Study 2) development of overt cardiac dysfunction. Second, we will use two drugs that are known to ameliorate functional impairment, metoprolol and ascorbic acid, to study

how they affect oxidative damage and function from Study 1 to Study 2. Thus our hypothesis can be broken down into three sub-hypotheses:

1. *Disturbances in metabolism will appear before the development of overt dysfunction, while changes in oxidative protein damage will appear most prominent at the point of dysfunction.*
2. *Both metoprolol and ascorbic acid will improve cardiac function, however, only ascorbic acid will reduce oxidative stress.*
3. *Combined metoprolol and ascorbic acid treatment will improve function further then single treatment.*

We feel that our approach will provide us with new insights into DCM. By assessing changes in metabolism, oxidative damage and function from Study 1 to Study 2, we will be able to clarify the sequence of events involved in the development of cardiac dysfunction. Furthermore, by assessing the effect of our drug treatments, we will be able to report whether reduction of oxidative stress will supplement the beneficial effects observed with metoprolol. Finally, our study should provide insight into the mechanism of action of metoprolol and ascorbic acid.



Scheme 1. Outline of overall hypothesis (black text) and sub-hypotheses (grey).

2. Methods

2.1. Animals/ Treatment Groups

Animals were cared for in accordance with the guidelines of the Canadian Council on Animal Care. The Animal Care Committee at the University of British Columbia, Office of Research Services, approved the protocol for animal care. All animal experiments were conducted at the Genetically Engineered Models Facility located at the James Hogg Research Centre. Animals were divided into two study groups, titled Study 1 and Study 2. For both studies, weight matched (200-220 g) Male Wistar rats were used. Animals from Study 1 were purchased from Charles River (St. Constant, Quebec), while Study 2 animals were obtained from the Centre for Disease Modeling (CDM, University of British Columbia, Vancouver, British Colombia). The strain of animal purchased from both sources was Wistar, and animals were all male with the same approximate age at the time of study. Furthermore, the CDM sourced animals used in Study 2 display similar cardiac functional and metabolic characteristics during diabetes similar to rats from Charles River and elsewhere[33, 130, 131] . Unless noted, all animals were allowed *ad libitum* access to standard rat chow and water.

For Study 1, animals were given one week after arrival to acclimatize and were then randomly divided into either diabetic or control groups. Diabetes was induced by a single intravenous injection of streptozotocin (STZ) at 60 mg/kg body

weight (60 mg/ml STZ in a sterile saline, 0.9% NaCl w/v solution, delivered at 1 µl/g body weight) into the caudal vein. Control animals were injected with sterile saline only. Blood was collected at one-week post STZ and at termination; all animals were fasted for 5 hours before collection to allow stabilization of plasma insulin levels. Plasma glucose and insulin were measured at one-week post STZ to ensure induction of diabetes (this time point will hereafter be known as 'induction of diabetes'). Similar measurements were also made at termination. One-week post induction of diabetes (Week 2 in Scheme 2), the control and diabetic animals (C, D) were divided into metoprolol treated (CM, DM), ascorbic acid treated (CA, DA), and metoprolol with ascorbic acid treated (CMA, DMA). Metoprolol was administered via subcutaneous Alzet 2ML4 osmotic pumps, from the Durect Corporation (Cupertino, California), at a dose of 15 mg/kg/day, respectively. Ascorbic acid was delivered at a dose of 1000 mg/kg/day in the drinking water. β -blocker and ascorbic acid treatment lasted for a total of four weeks. Animals were terminated, and their hearts collected and perfused at five week post-induction of diabetes, a period during which metabolic abnormalities have occurred, but overt cardiac dysfunction is reportedly not yet evident [130]. A final blood collection from the chest cavity was made after excision of the heart; urine was also collected from the bladder (Scheme 2).

Study 2 was identical, with the following exceptions: Alzet 2006 osmotic pumps were used, treatment lasted six weeks and termination occurred seven weeks post-induction of diabetes, a period during which both metabolic abnormalities and cardiac dysfunction are reportedly evident [130] (Scheme 2).

2.2. Echocardiography

Heart function was measured *in vivo* by echocardiography using the VEVO 770 High Resolution Imaging System with a RMV 716 probe, all from Visual Sonics (Toronto, Ontario). All animals were anesthetized with 2 % isoflurane. Left ventricular end diastolic and end systolic diameters (LVEDD and LVESD, respectively) were measured. Calculations were automatically generated by the manufacturer's software using the following formulas: Fractional Shortening (FS, %) = [(LVEDD – LVESD)/LVEDD] ×100%, Left Ventricular End Diastolic Volume (LVEDV, µl) = [7.0/(2.4 + LVEDD)] x $(LVEDD)^3$ x 1000, Left Ventricular End Systolic Volume (LVESV, µl) = same as previous except using LVESD in place of LVEDD, ejection fraction (EF, %) = [(LVEDV – LVESV)/LVEDV] ×100%. All values represent an average of a minimum of three measurements from each animal. Values generated for each animal were then combined to produce a value for its treatment group.

2.3. Function and Fuel Usage

2.3.1. Perfusion Conditions

Cardiac function and metabolism were measured as previously described [132-134]. At termination, animals were anesthetized by 4% isofluorane anesthesia and the hearts were isolated and perfused as working heart preparations using a modified Krebs-Henseleit solution (perfusion buffer), supplemented with substrates at physiologically relevant concentrations. Perfusion buffer consisted of 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 5.5 mM glucose, 0.5

mM lactate, 20 μU/ml insulin, and 0.6 mM palmitate bound to 3% BSA [132]. Hearts were perfused in working heart mode for 30 minutes, during this time metabolic and functional measurements were made every 6 minutes. After completion of perfusion, hearts were freeze clamped, weighed, and stored at -80°C for further analysis.

2.3.2. Function

In order to measure heart rate and peak systolic pressure, a pressure transducer, from Viggo-Spectramed (Oxnard, California), was inserted into the afterload line. Cardiac output and aortic flow were measured using external flow probes attached to the preload and aortic outflow lines; probes were purchased from Transonic Systems Inc. (Ithaca, New York). From these measurements, cardiac output, coronary flow (cardiac output – aortic outflow), rate pressure product (heart rate x peak systolic pressure) and hydraulic work (cardiac output x peak systolic pressure) were calculated[135].

2.3.3. Fuel Usage

In all animals from Study 1, and selected animals from Study 2, the rates of glucose oxidation and palmitate oxidation were quantified by measuring $^{14}CO_2$ and $^{3}H_2O$ produced by oxidation of [^{14}C]glucose and [^{3}H]palmitate, respectively. In the remainder of the hearts from Study 2, glycolysis and palmitate oxidation rates were quantified by measuring $^{3}H_2O$, and $^{14}CO_2$, produced by oxidation of [^{3}H]glucose and

[^{14}C] palmitate, respectively. All radioisotope labeled glucose and palmitate were purchased from Perkin-Elmer (Woodbridge, Ontario).

In order to accurately measure $^{3}H_2O$ released into the perfusion buffer it must first be separated from the [^{3}H]glucose or [^{3}H]palmitate present. To separate $^{3}H_2O$ we loaded 200 µl of collected perfusion buffer samples into a cap-less 500 µl centrifuge tube and placed this inside of a larger 7 ml scintillation vial containing 500 µl of water. After sealing the scintillation vial, we allowed the $^{3}H_2O$ to evaporate from the smaller tube at 60°C for 24 hours. Samples were then moved to a refrigerator and incubated at 4°C to allow evaporated $^{3}H_2O$ to re-condense into the larger outer tube. After 24 hours, the cap-less tubes were removed and the radioactivity of recovered water within the 7 ml scintillation tube was measured using a Beckman LS6500 Liquid Scintillation Counter (Mississauga, Ontario). All samples were run in duplicate and control tubes loaded with known amounts of $^{3}H_2O$ were also run in order to measure efficiency of $^{3}H_2O$ collection. $^{3}H_2O$ was purchased from Perkin-Elmer (Woodbridge, Ontario).

In order to accurately measure oxidation of ^{14}C containing substrates, we must account for both the $^{14}CO_2$ released into the atmosphere and the $^{14}CO_2$ converted to $H^{14}CO_3^-$ that was released into the perfusion buffer. This procedure has been previously described[133] . Briefly, $^{14}CO_2$ was captured by bubbling gas produced inside of the sealed working heart perfusion rig through a solution of the strong base, 10-X hyamine hydroxide (1 M methylbensethonium hydroxide in methanol), hyamine samples were then collected and stored. $H^{14}CO_3^-$ was extracted from the perfusion buffer by converting it to $^{14}CO_2$ using 9N H_2SO_4, it was then

captured onto filter paper soaked with 10-X hyamine hydroxide. The radioactivity of the 10-X hyamine samples and hyamine soaked filter paper was measured using a Beckman LS6500 Liquid Scintillation Counter (Mississauga, Ontario).

2.4. Metabolism

Plasma glucose concentrations were measured using the Beckman Glucose Analyzer II. Plasma insulin was measured using the radioimmunoassay kit from Linco supplied by Cedarlane (Burlington, Ontario). Plasma cholesterol, and triglycerides were determined by colorimetric assay kits available from Sigma (St. Louis, Missouri). Whole blood ketone levels were measured using the CardioChek analyzer from Polymer Technology Systems (Indianapolis, Indiana).

2.4.1. Tissue Triglyceride Assay

Myocardial triglyceride content was measured in 30-40 mg of tissue, as previously described [136]. Briefly, tissue was powdered in liquid nitrogen cooled mortar and pestle, then transferred to glass test tubes with 3 ml of a 2:1 chloroform:methanol (v/v). After a 1 hour room temperature incubation mounted on a shaking platform, 0.6 ml of 0.05% H_2SO_4 was added and tubes were left overnight at 4°C to separate liquid phases. The lower liquid phase was collected, 1 ml of 1% Triton-X100 in chloroform (v/v) was added and the samples were dried under N_2 gas at 45°C. Samples were then reconstituted in 500 µl PBS and assayed using a colorimetric triglyceride kit purchased from Caymen Chemical supplied by

Cedarlane (Burlington, Ontario).

2.4.2. *Tissue Glycogen*

Myocardial glycogen content was measured in 95-110 mg of powdered tissue as previously described [137] . Briefly, tissue was boiled in 0.3 ml of 30% KOH for 1 hour in pre-weighed Corex® test tubes topped with glass marbles, tubes were manufactured by Corning and supplied by VWR International (Edmonton, Alberta). Samples were allowed to cool, then 0.2 ml of Na_2SO_4 and 2 ml of absolute ethanol were added to each tube. Tubes were left at -20°C over night for ethanol precipitation of released glycogen. The following day, samples were spun at 3500 × g, the supernatant which contained free glucose, was discarded. The pellet was washed in 66% ethanol and then boiled in 1 ml of 2N H_2SO_4 for 3 hours to hydrolyze glycogen to glucose. After cooling, 0.5 ml of a 1M MOPS buffer was added and samples were individually brought to a pH of 6.8-7 using NaOH. The Corex® tubes were weighed to determine the end sample dilution volume. Finally, samples were assayed using a colorimetric glucose assay kit purchased from Caymen Chemical supplied by Cedarlane (Burlington, Ontario).

2.5. Oxidative Stress Assessment, Oxyblot

Increased oxidative stress has been shown to cause the introduction of carbonyl groups into protein, therefore oxidative stress was assessed by measuring changes in the number of carbonyl groups present in whole heart homogenates. For

homogenization, 30-40 mg of powdered heart tissue was weighed into liquid nitrogen cooled tubes and 0.5 ml of cold total protein extraction buffer plus β-mercaptoethanol, (total protein extraction buffer: 20 mM HEPES, 1 mM ethylenediamine tetraacetic acid (EDTA), 250 mM sucrose, 100 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 100 mM sodium fluoride, 5 µl/ml protease inhibitor cocktail from Sigma-Aldrich (St. Louis, Missouri) and 2% (v/v) β-mercaptoethanol) was added. Tissue was then homogenized using an Ultra-Turrax TR-10 tissue homogenizer, from Rose Scientific Ltd. (Edmonton, Alberta), for 2 x 5 second bursts. Tissue homogenates were then spun at 500 g in order to separate soluble proteins from membrane components and nuclei, the supernatant was then collected for further analysis.

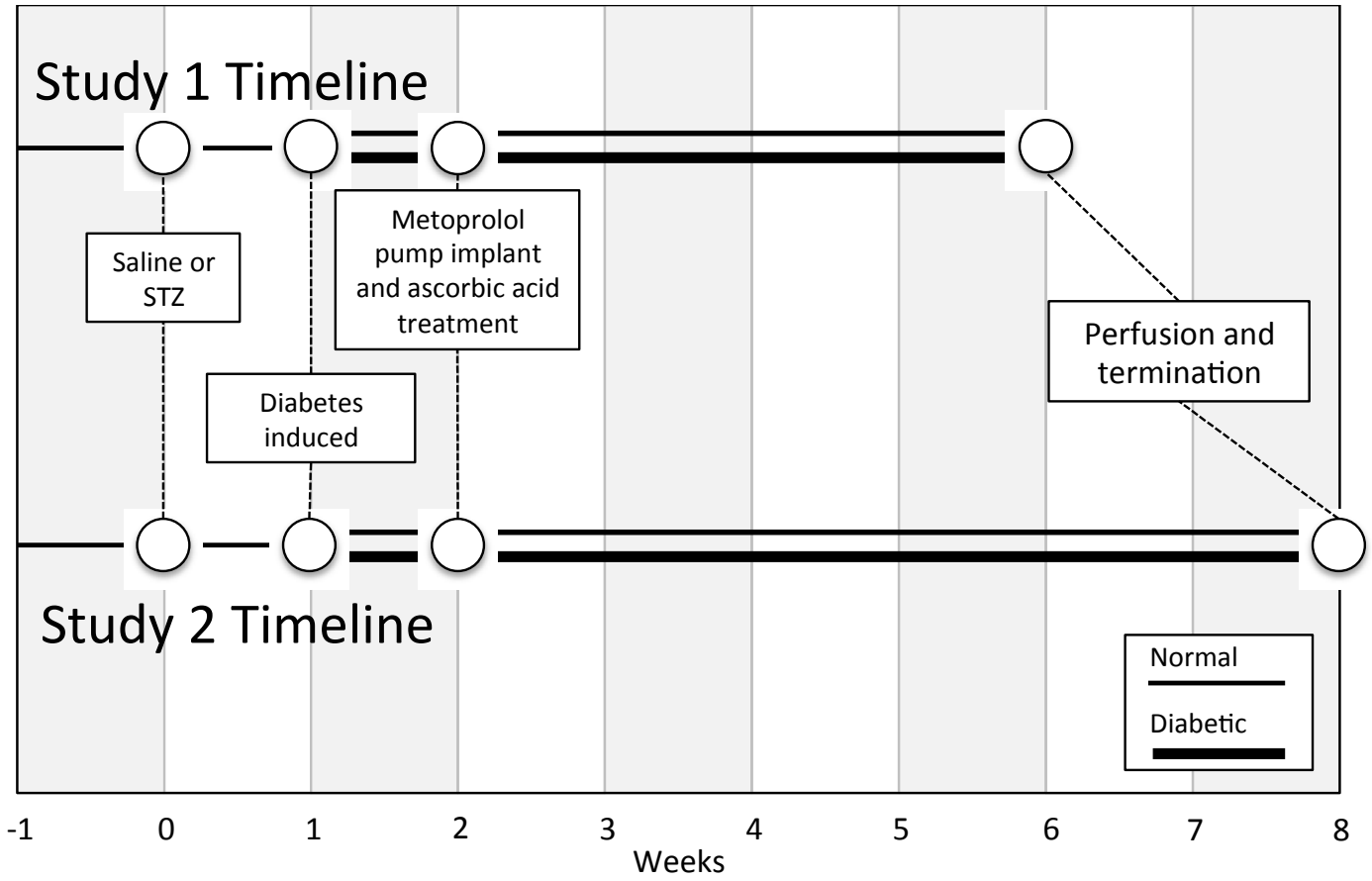
Homogenates were analyzed using the OxyBlot kit from Millipore supplied by Cedarlane (Burlington, Ontario). This kit was used to label carbonyl groups with a 2,4-dinitrophenylhydrazone (DNP) tag. After tagging samples were blotted directly onto nitrocellulose membranes.

After the membranes had fully absorbed samples, they were blocked for 1 hour with blocking buffer (2.5% bovine serum albumin in tris-buffered saline plus 0.1% polyoxyethylenesorbitan, (TWEEN)) and were incubated at room temperature for two hours with primary anti-DNP antibody that was supplied with the kit. Membranes were then washed with tris-buffered saline plus TWEEN (2 rinses and 3 x 5 minute washes), incubated for 1 hour at room temperature with a supplied horse radish peroxidase tagged secondary antibody followed by another set of washes. Detection of blotted proteins was accomplished using the Super Signal

West Femto Maximum Sensitivity substrate, from Pierce Biotechnology supplied by Fisher Scientific (Ottawa, Ontario). Images were taken using the ChemigeniusQ Image Analyzer, purchased from Geneflow (Alexandria, Virginia), densitometry analysis was conducted using the software program ImageJ, from the National Institutes of Health (Bethesda, Maryland).

2.6. Statistics

Values are expressed as means ± Standard Error of the Mean (SEM). When the means of more then two groups was compared, the One-Way Analysis of Variance technique was used, with Bonferroni post-hoc analysis. When the means of two groups were compared a Student's t-test was performed. For all analysis a pre-adjustment α level of 0.05 was chosen. All analysis was performed using Prism 5 software from GraphPad Software Inc. (La Jolla, California). The Statistical Consulting and Research Laboratory reviewed and approved the statistical analysis performed for this study[138].



Scheme 2. Outline of treatments for Study 1 and Study 2. For both studies, animals were allowed to acclimatize for 1 week, after which they were separated into control and diabetic groups. Diabetic animals were injected with streptozotocin (STZ, 60 mg/kg body weight), and control animals with saline. Diabetes was confirmed by measuring blood glucose levels 1 week post injection. 1 week post diabetes confirmation, osmotic pumps containing saline or metoprolol (15 mg/kg/day) were implanted. At the same time ascorbic acid treatment (1000 mg/kg/day, in drinking water) was initiated in selected animals. For Study 1, perfusion and termination were performed 5 weeks post diabetes confirmation, for Study 2, this occurred 7 weeks post diabetes confirmation.

3. Results

3.1. General Characteristics and Plasma Parameters

3.1.1. Study 1

General physical characteristics, nutrient intake and a list of plasma parameter values during Study 1 can be found in Table 1 and Table 2. Body weight was significantly reduced in diabetic animals and was unaffected by any drug treatments. Heart weight was unchanged in diabetic animals, or by any treatment. Diabetic animals were also observed to consume 2-fold more food and 4-fold more water than the untreated control animals (Table 1).

Decreased plasma insulin and elevated glucose levels were observed in diabetic rats, indicating a perturbation of glucose homeostasis, as expected. Treatment of diabetic rats with metoprolol or ascorbic acid had no effect on these parameters. Plasma triglyceride levels were also significantly disturbed during diabetes. Metoprolol, but not ascorbic acid, treatment partially ameliorated changes in triglyceride content in diabetic animals. Plasma cholesterol levels were unaffected by diabetes or treatment. Plasma ketone levels appeared to double in all diabetic groups, however, a full statistical analysis of plasma ketone levels was not possible because of the lack of a sufficient number of animals in the control untreated group (Table 2).

3.1.2. Study 2

General physical characteristics, nutrient intake and a list of plasma parameter values during Study 2 can be found in Table 1 and Table 2. All diabetic animals experienced significantly reduced body and heart weights compared to untreated control animals. All diabetic animals consumed a half-fold more food and four-fold more water than untreated controls, both observed changes were significant (Table 1).

Glucose homeostasis was severely disturbed in all diabetic groups; insulin levels were significantly reduced and an associated rise in plasma glucose was observed. Treatment had no effect. Plasma triglyceride and ketone levels were significantly increased during diabetes. Treatment with metoprolol had no effects in control or diabetic animals, however, ascorbic acid caused reductions in plasma triglyceride content in both control and diabetic animals that did not achieve statistical significance. Plasma ketone levels were not altered by treatment. Plasma cholesterol levels were significantly increased in diabetic animals compared to controls. Metoprolol alone had no effect on plasma cholesterol levels, however, ascorbic acid with or without metoprolol, lowered cholesterol levels to a point where they were no longer significantly different from the untreated control (Table 2).

3.2. *In vivo* Cardiac Function of Diabetic Rats

3.2.1. Study 1

Cardiac function was assessed *in vivo* by echocardiography; volume and function measurements observed during Study 1 can be found in Table 3 and Figure 1A. Heart rate, *in vivo,* was significantly reduced in all diabetic hearts compared to controls. Metoprolol had a significant negative chronotropic effect in control heart, and when combined with diabetes, produced a significant and nearly additive reduction in metoprolol treated diabetic heart rates. Ascorbic acid did not have an effect on heart rates. Ejection Fraction (EF), Fractional Shortening (FS), End Systolic and Diastolic Volume (ESV and EDV) were not significantly altered in diabetic animals compared to controls. Treatment had little effect on EF, FS and ESV, except for the combined metoprolol and ascorbic acid treatment, which significantly reduced EF and FS, and significantly raised ESV in diabetic hearts. EDV was significantly increased in all metoprolol treated groups compared to controls. Stroke volume was not significantly modified by diabetes; metoprolol delivered alone did significantly raise stroke volume in both control and diabetic hearts, however (Table 3). Cardiac output was not significantly affected by diabetes or any treatment (Figure 1).

3.2.2. Study 2

Volume and function measurements observed during Study 2 can be found in Table 3 and Figure 1B. Heart rate was significantly reduced in all diabetic animals

and in metoprolol treated control animals. Diabetic animals treated with metoprolol experienced a further reduction in heart rate, however this effect was not statistically significant. Ascorbic acid had no effect on heart rates. EF and FS were significantly reduced in all diabetic animals compared to untreated controls, except for EF in diabetics treated with ascorbic acid alone. ESV and EDV were both increased by diabetes, but neither change was significant. Treatment with metoprolol caused a significant increase in ESV in control animals, and in ESV and EDV in diabetic animals. Stroke volume was unaffected by diabetes or treatment (Table 3). Cardiac output was lower in all diabetic animals; however, this change was only significant in animals treated with metoprolol alone (Figure 1).

3.3. Ex vivo Cardiac Function of Diabetic Rats

3.3.1. Study 1

In order to assess changes in cardiac performance independent of whole body effects of both diabetes and treatment, function was also measured *ex vivo* during working heart perfusion. Functional parameters observed during Study 1 can be found in Table 4 and Figure 2A and 2B. Heart rate measured *ex vivo* demonstrated that diabetes had a significant negative chronotropic effect. Metoprolol treatment demonstrated a significant positive chronotropic effect when it was given alone in control hearts, and raised diabetic heart rates in treated groups to a point where they were no longer significantly different from controls. Ascorbic acid had no significant effect except that it appeared to somewhat blunt

metoprolol's increase of heart rate, as demonstrated in the control dual treated group. There were no significant changes in peak systolic pressure, rate pressure product, and hydraulic work caused by diabetes or treatment (Table 4). Coronary flow and cardiac output were not significantly affected by diabetes (Figure 2A,B). Treatment with ascorbic acid did significantly increase coronary flow rates in diabetic hearts when administered in combination with metoprolol, and in control hearts with or without metoprolol (Figure 2A).

3.3.2. Study 2

Functional parameters observed during Study 2 can be found in Table 4 and Figure 2C and 2D. Diabetic hearts experienced a significant reduction in heart rates compared to the untreated control. Metoprolol, when administered alone, significantly raised rates above the untreated diabetic; these rates were also no longer significantly different from controls. Treatment of diabetic animals with ascorbic acid alone had no effect on heart rates, and when given with metoprolol, seemed to remove metoprolol's normalizing effect. There were no significant treatment effects on control heart rates. Peak systolic pressure was unchanged by diabetes or treatment. Rate Pressure Product (RPP) was significantly reduced by diabetes. Treatment with metoprolol alone significantly increased RPP compared to untreated diabetics and ascorbic acid alone partially ameliorated RPP, raising it to a level that was not significantly different from control. However, both these correcting effects disappeared when metoprolol and ascorbic acid treatments were given in the same animal. Cardiac work and coronary flow were significantly

reduced during diabetes. Treatment of diabetics with metoprolol alone had no effect, however, ascorbic acid with or without metoprolol, caused increases in both parameters substantial enough that they were no longer different from control (Table 4 and Figure 2C). Cardiac output was significantly reduced during diabetes. Metoprolol alone had no significant effect. Cardiac output of ascorbic acid-treated diabetic animals was not statistically different from those of control animals indicating a partial amelioration of diabetes-induced reductions in cardiac output. When both drugs were combined cardiac output was significantly increased compared to the untreated diabetic group (Figure 2D).

3.4. Substrate Oxidation and Metabolite Content

3.4.1. Study 1

Oxidation rates of exogenous, radiolabeled, palmitate and glucose measured *ex vivo* during working heart perfusion are shown in Figures 3A and 3B. Palmitate oxidation rates were significantly accelerated by 20% in diabetic hearts compared to controls. All treatments significantly lowered palmitate oxidation rates in diabetic hearts and rates in all diabetic treated groups were not significantly different from control (Figure 3A). Oxidation of exogenous glucose was reduced to nearly undetectable levels in diabetic hearts. Although treatment with metoprolol or ascorbic acid individually did not alter glucose oxidation rates, combined treatment did cause a noticeable, but not significant, increase. Treatment had no effect on exogenous glucose oxidation in control hearts (Figure 3B).

Tissue glycogen content was measured in hearts flash frozen after working heart perfusion, data for Study 1 is charted in Figure 4A. Glycogen content was higher in all diabetic hearts, however, changes were most pronounced and only significant in the dual metoprolol and ascorbic acid treated group. Treatment had no effect on control hearts (Figure 4A).

Tissue triglycerides were extracted and measured in hearts flash frozen after working heart perfusion, data for Study 1 is charted in Figure 4B. Cardiac triglyceride content was not significantly modified by diabetes or any treatment (Figure 4B).

3.4.2. Study 2

Oxidation rates of exogenous, radiolabeled, palmitate and glucose for Study 2 are displayed in Figure 3C and 3D. Palmitate oxidation rates were significantly increased by 70% in diabetic compared to controls animals. Treatment with metoprolol caused a non-significant reduction in diabetic hearts only while ascorbic acid had no effect on control or diabetic animals. Dual treatment significantly reduced rates of palmitate oxidation in diabetic animals (Figure 3C). Glucose oxidation rates were significantly lowered to almost undetectable levels in diabetic hearts. Treatment with metoprolol or ascorbic acid had no significant effects on glucose oxidation in control or diabetic hearts (Figure 3D).

Tissue glycogen content for Study 2 is displayed in Figure 4C. Tissue glycogen content was significantly increased in diabetic hearts compared to untreated controls. Metoprolol treatment appeared to raise glycogen levels in

diabetic hearts over the untreated diabetic group, however changes were not significant. Ascorbic acid administered alone or with metoprolol had no effect on glycogen levels in diabetic hearts. Control hearts were unaffected by treatment (Figure 4C).

Tissue triglyceride content for hearts from Study 2 is displayed in Figure 4D. Tissue triglyceride levels appeared higher in the untreated diabetics compared to controls, however changes were not significant. Treatment did not have any significant effects (Figure 4D).

3.5. Oxidative Protein Damage

3.5.1. Study 1

Oxidative protein damage as measured by OxyBlot analysis for hearts from Study 1 can be found in Figures 5 and 6. There was a 60% rise in oxidative protein damage in diabetic hearts during Study 1, however this change was not significant (Figure 5).

The effect of treatment on oxidative protein damage for hearts from Study 1 can be found in Figure 6A and 6B. Separate charts are displayed for control and diabetic hearts in order to illustrate that the analyses for these two groups were completed on separate immunoblots. Comparisons can, therefore, only be made within the control treatments or diabetic treatments, but not across control and diabetic treatments. In control hearts, metoprolol treatment caused increases in oxidative protein damage, while ascorbic acid did not appear to have any effect. In

diabetic hearts only metoprolol alone caused increased damage while both ascorbic acid treated hearts caused reductions. However, none of these treatment effects were significant (Figure 6A,B).

3.5.2. Study 2

During Study 2 there was a significant, 100% rise in oxidative protein damage in diabetic hearts compared to control hearts (Figure 5).

The effect of treatment on oxidative protein damage for hearts from Study 2 can be found in Figure 7A and 7B. In control hearts metoprolol treatment did not appear to have an effect, however, ascorbic acid with or without metoprolol significantly lowered oxidative protein damage (Figure 7A). In diabetic hearts, an identical pattern was observed, however changes were not significant (Figure 7B).

Table 1 - General characteristics

	Control untreated **C**	STZ untreated **D**	Control metoprolol **CM**	STZ metoprolol **DM**	Control ascorbic acid **CA**	STZ ascorbic acid **DA**	Control metoprolol + ascorbic acid **CMA**	STZ metoprolol + ascorbic acid **DMA**
Study 1								
N	10	8	10	10	10	10	10	11
Body Weight (g)	476±15	385±9[a]	488±11	377±18 (9)[a]	475±12	380±20 (8)[a]	467±11	356±9[a]
Heart Weight (g)	1.75±0.04	1.67±0.07	1.91±0.07	1.68±0.06 (9)	1.77±0.05	1.62±0.05 (8)	1.76±0.05	1.57±0.03
Food (g/day)	33±3	54±1[a]	32±1	47±2	30±1	58±7[a]	30±1	52±5 (10)[a]
Water (ml/day)	49±1	223±10[a]	47±1	181±15[a]	35±2	194±12[a]	34±2	183±17 (10)[a]
Study 2								
N	10	12	10	12	4	8	8	6
Body Weight (g)	447±11	259±21[a]	480±18	283±16[a]	412±16	286±12[a]	423±9	277±37[a]
Heart Weight (g)	1.93±0.08	1.47±0.07[a]	1.94±0.06	1.43±0.05[a]	1.77±0.12	1.61±0.06[a]	1.78±0.09	1.37±0.10[a]
Food (g/day)	31±1	42±2 (14)[a]	34±3	41±2[a]	27±0	46±1[a]	27±1	37±3
Water (ml/day)	42±1	160±8 (14)[a]	41±2	149±10[a]	26±0	161±9[a]	30±1	137±8[a]

Table 1 - Values are means ± SEM (N number, if different from above, is noted in brackets). Diabetes was induced (D, DM, DA, DMA) with a 60 mg/kg streptozotocin (STZ) injection into the caudal vein, control animals received equivalent volumes of saline. Two weeks post STZ, metoprolol treatment (15 mg/ kg/ day; CM, CMA, DM, DMA), and ascorbic acid treatment (1000 mg/kg/day; CA, CMA, DA, DMA) were started. Study 1 and Study 2 animals were terminated 6 and 8 weeks after STZ injection, respectively. All values were measured at or near termination, food and water measurements are averages over the last two weeks before termination. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=$p<0.05$ vs. untreated control (C). b=$p<0.05$ vs. untreated diabetic (D).

Table 2 – Plasma parameters

	Control untreated **C**	STZ untreated **D**	Control metoprolol **CM**	STZ metoprolol **DM**	Control ascorbic acid **CA**	STZ ascorbic acid **DA**	Control metoprolol + ascorbic acid **CMA**	STZ metoprolol + ascorbic acid **DMA**
Study 1								
N	10	8	10	10	10	10	10	11
Insulin (ng/ml)	1.20±0.21	0.28±0.05[a]	0.97±0.14	0.32±0.07 (8)[a]	1.17±0.19	0.28±0.03[a]	1.31±0.29	0.26±0.04[a]
Glucose (mM)	7.3±0.3 (6)	23.1±1.2 (5)[a]	7.3±0.2 (6)	22.6±1.3 (5)[a]	7.8±0.4	22.2±0.8 (8)[a]	7.1±0.3	20.4±1.7[a]
Triglyceride (mM)	1.35±0.16	2.63±0.31[a]	1.70±0.16	1.77±0.30	1.46±0.14	2.47±0.39[a]	1.18±0.17	2.04±0.33
Cholesterol (mM)	2.18±0.11	2.35±0.14	2.00±0.09	2.33±0.24	1.88±0.09	2.49±0.20	1.98±0.07	2.85±0.34
Ketones (mM)	0.43±0.05 (2)	1.26±0.40 (4)	0.65±0.07 (6)	0.98±0.16 (5)	0.65±0.15 (8)	1.34±0.27 (8)	0.59±0.03 (8)	1.34±0.23 (8)
Study 2								
N	10	12	10	12	4	8	8	6
Insulin (ng/ml)	1.28±0.13	0.34±0.09[a]	1.85±0.35	0.39±0.14[a]	1.26±0.15	0.30±0.12[a]	1.35±0.16	0.31±0.16[a]
Glucose (mM)	9.1±0.4	22.2±1.6[a]	9.3±0.5	23.0±1.6[a]	11.0±0.6	22.9±1.4[a]	9.6±0.5	22.6±2.7[a]
Triglyceride (mM)	1.31±0.09	3.49±0.26[a]	1.42±0.14	3.34±0.32[a]	1.29±0.20	3.17±0.40[a]	1.16±0.12	2.73±0.42[a]
Cholesterol (mM)	1.92±0.08	3.72±0.41[a]	1.73±0.08	3.22±0.33[a]	1.70±0.03	3.01±0.27	1.83±0.08	2.81±0.24
Ketones (mM)	0.54±0.04 (8)	2.37±0.30[a]	0.56±0.04	2.11±0.30[a]	0.69±0.09	1.93±0.43[a]	0.63±0.04	2.42±0.44[a]

Table 2 - Values are means ± SEM (N number, if different from above, is noted in brackets). Diabetes was induced (D, DM, DA, DMA) with a 60 mg/kg streptozotocin (STZ) injection into the caudal vein, control animals received equivalent volumes of saline. Two weeks post STZ, metoprolol treatment (15 mg/ kg/ day; CM, CMA, DM, DMA), and ascorbic acid treatment (1000 mg/kg/day; CA, CMA, DA, DMA) were started. Study 1 and Study 2 animals were terminated 6 and 8 weeks after STZ injection, respectively. All values were measured at or near termination, food and water measurements are averages over the last two weeks before termination. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=$p<0.05$ vs. untreated control (C). b=$p<0.05$ vs. untreated diabetic (D).

Table 3 - Functional parameters as measured by echocardiography

	Control untreated **C**	STZ untreated **D**	Control metoprolol **CM**	STZ metoprolol **DM**	Control ascorbic acid **CA**	STZ ascorbic acid **DA**	Control metoprolol + ascorbic acid **CMA**	STZ metoprolol + ascorbic acid **DMA**
Study 1								
N	10	9	10	9	10	8	10	11
Heart Rate (BPM)	387±9	323±9[a]	331±8[a]	281±9[a b]	380±10	301±12[a]	317±8[a]	272±8[a b]
Ejection Fraction (%)	73.4±2.0	69.1±3.0	73.0±1.4	70.8±2.1	73.4±1.4	66.2±1.6	69.8±1.6	65.0±1.6[a]
Fractional Shortening (%)	44.3±1.8	40.9±2.5	44.0±1.2	42.2±1.8	44.2±1.3	38.3±1.3	41.1±1.3	37.4±1.2[a]
End Systolic Volume µl	84±12	120±15	106±8	123±14	90±7	128±10	110±11	141±11[a]
End Diastolic Volume µl	305±23	377±21	391±15[a]	411±25[a]	338±11	374±15	361±24[a]	398±16[a]
Stroke Volume µl	220±13	257±14	284±10[a]	288±14[a]	248±8	246±6	251±16	257±7
Study 2								
N	10	13	10	12	4	8	8	6
Heart Rate (BPM)	359±12	287±12[a]	293±8[a]	261±8[a]	338±19	293±14[a]	295±8[a]	258±22[a]
Ejection Fraction (%)	73.0±1.6	66.8±0.5[a]	68.7±1.0	64.7±1.1[a]	66.9±1.9	66.3±1.2	70.6±2.3	64.7±2.1[a]
Fractional Shortening (%)	43.7±1.4	38.4±0.4[a]	40.2±0.8	37.0±0.9[a]	38.6±1.5	38.1±1.0[a]	41.9±1.9	36.9±1.7[a]
End Systolic Volume µl	81±8	112±6	125±12[a]	129±8[a]	110±3	112±6	107±12	116±13
End Diastolic Volume µl	300±19	335±16	395±31	362±15[a]	334±13	332±12	358±23	325±28
Stroke Volume µl	219±15	224±10	270±19	233±9	224±14	220±8	251±16	209±17

Table 3 - Values are means ± SEM (N number, if different from above, is noted in brackets). Animals were anesthetised with 2% isoflurane during the measurement period. STZ was delivered at 60 mg/kg by IV injection, metoprolol at 15 mg/kg/day by osmotic pump and ascorbic acid at 1000 mg/kg/day in drinking water. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=$p<0.05$ vs. untreated control (C). b=$p<0.05$ vs. untreated diabetic (D).

Table 4 - Functional parameters as measured during working heart perfusion

	Control untreated **C**	STZ untreated **D**	Control metoprolol **CM**	STZ metoprolol **DM**	Control ascorbic acid **CA**	STZ ascorbic acid **DA**	Control metoprolol + ascorbic acid **CMA**	STZ metoprolol + ascorbic acid **DMA**
Study 1								
N	10	7	10	9	10	8	10	11
Heart Rate (BPM)	251±6	227±5[a]	275±6[a]	245±7	253±4	226±5[a]	264±4	245±3
Peak Systolic Pressure (mm Hg)	108±2	112±2	104±3	109±4	110±1	115±2	109±1	110±1
Rate Pressure Product (BPM x mm Hg/ 1000)	27.0±0.8	25.3±0.4	28.5±0.8	26.5±0.7	28.0±0.5	25.9±0.5	28.7±0.5	27.0±0.5
Cardiac Work (ml x mm Hg/min x 1000)	84.9±2.6	84.3±2.4	81.5±4.1	80.3±5.4	94.9±1.9	95.1±4.4	95.8±1.8	89.8±2.0
Study 2								
N	10	12	10	12	4	8	8	6
Heart Rate (BPM)	234±4	202±2[a]	239±3	223±5[b]	240±4	209±7[a]	260±12	210±6[a]
Peak Systolic Pressure (mm Hg)	119±2	111±4	122±3	113±3	120±2	118±2	112±5	116±2
Rate Pressure Product (BPM x mm Hg/ 1000)	27.8±0.8	22.4±0.8[a]	29.2±0.6	25.2±0.7[b]	28.8±0.6	24.6±1.0	28.8±0.5	24.3±1.0[a]
Cardiac Work (ml x mm Hg/min x 1000)	104.8±2.7	76.8±5.2[a]	106.7±5.1	84.5±3.7[a]	100.7±3.7	87.7±2.9	94.9±5.0	92.6±3.4

Table 4 - Values are averages of measurements taken over the perfusion period ± SEM. Hearts were perfused in working heart mode for 30 minutes with perfusion buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 5.5 mM glucose, 0.5 mM lactate, 20 µU/ml insulin, and 0.6 mM palmitate bound to 3% BSA. All animals received the same perfusion buffer, and no treatments were delivered during perfusion. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=$p<0.05$ vs. untreated control (C). b=$p<0.05$ vs. untreated diabetic (D).

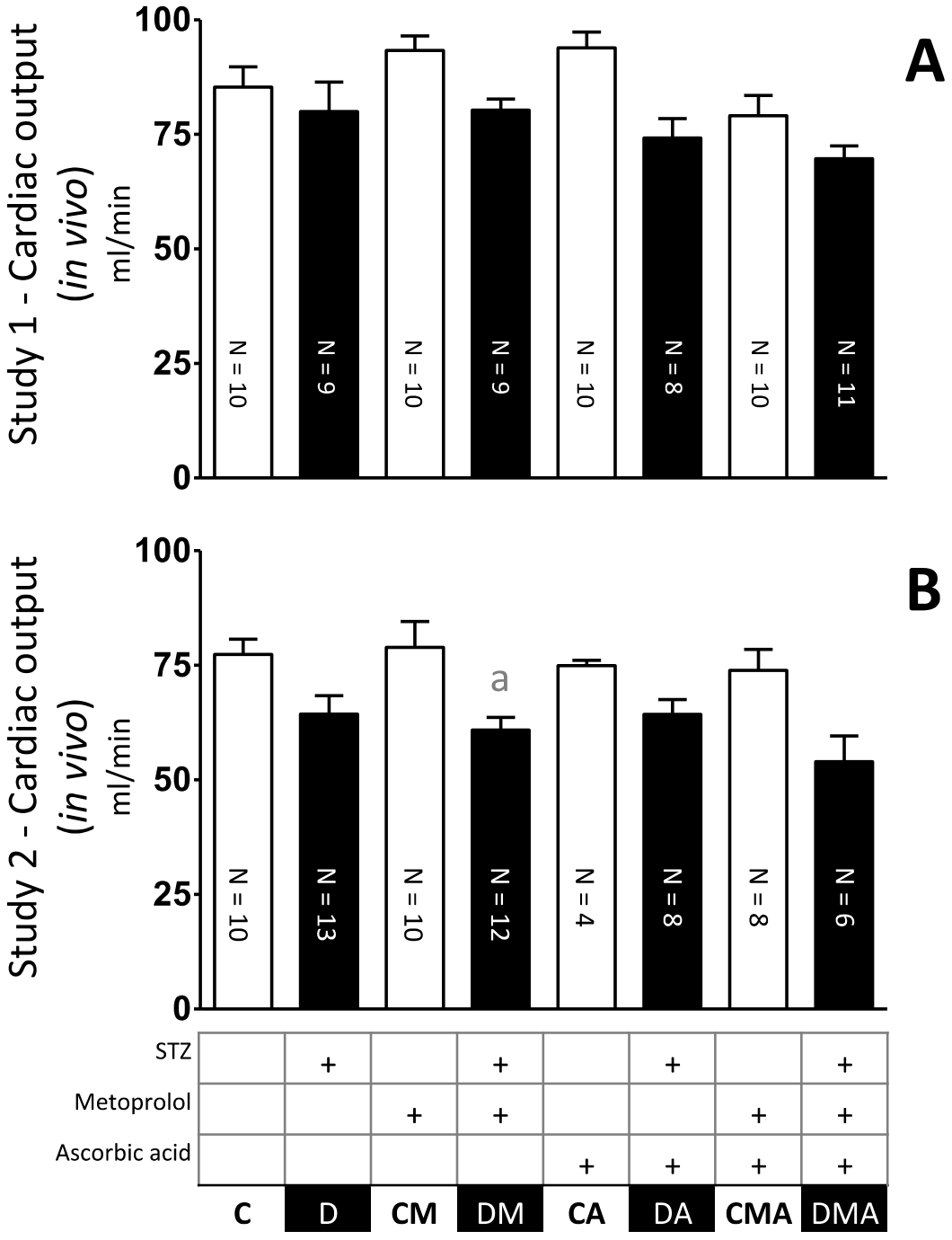


Figure 1 – *In vivo* cardiac output of control and diabetic rats treated with metoprolol and ascorbic acid during Study 1 (**A**) and Study 2 (**B**) . Values are means ± SEM. Measurements were made using the VEVO 770 High Resolution Imaging System with a RMV 716 probe. Animals were anesthetized with 2% isoflurane during measurement period. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=p<0.05 vs. untreated control (C). b=p<0.05 vs. untreated diabetic (D).

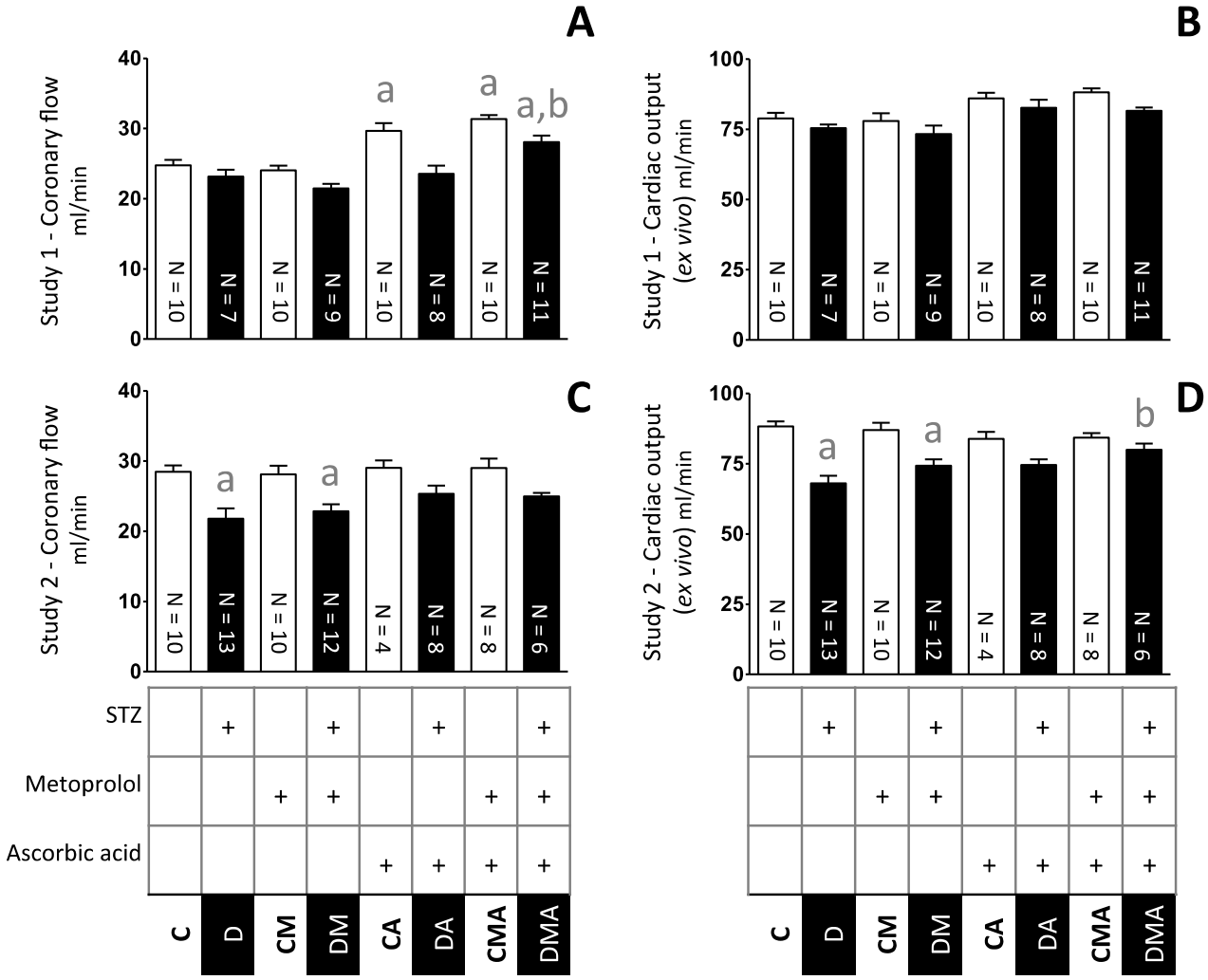


Figure 2 – Comparison of coronary flow (**A**) and cardiac output (**B**) in isolated perfused hearts during Study 1 and Study 2 (**C** & **D,** respectively). Values are averages of measurements taken over perfusion period ± SEM. Coronary flow was measured by flow probes. Hearts were perfused in working heart mode for 30 minutes with perfusion buffer containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 5.5 mM glucose, 0.5 mM lactate, 20 µU/ml insulin, and 0.6 mM palmitate bound to 3% BSA. All animals received the same perfusion buffer, and no treatments were delivered during perfusion. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=$p<0.05$ vs. untreated control (C). b=$p<0.05$ vs. untreated diabetic (D).

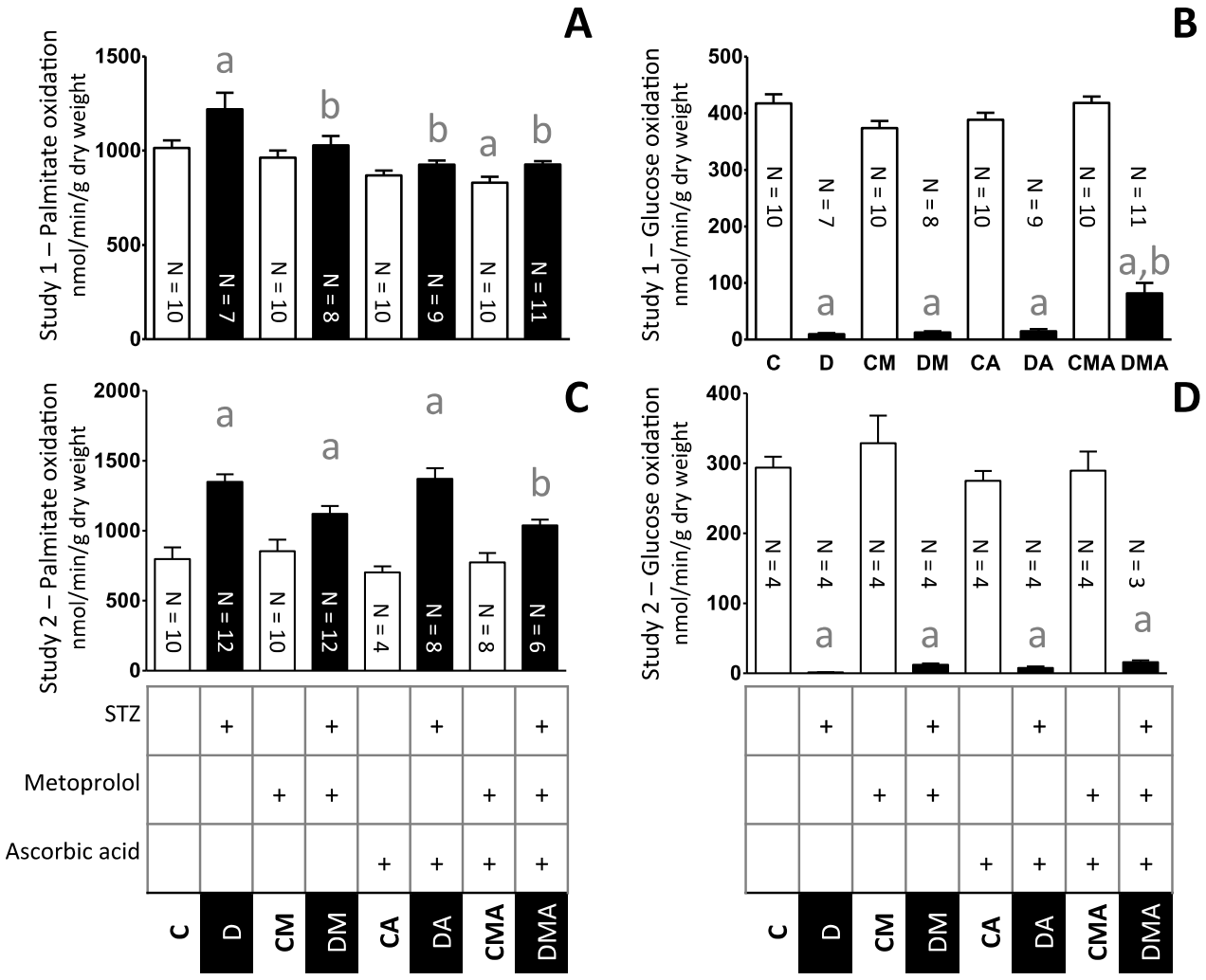


Figure 3 – Comparison of palmitate oxidation (**A**) and glucose oxidation (**B**) in isolated perfused hearts during Study 1 and Study 2 (**C** & **D,** respectively). Values are averages of measurements taken over a 30 minute time period ± SEM. Palmitate and glucose oxidation rates were studied by measuring generation of oxidized carbon-14 and $^{3}H_{2}O$ from radiolabeled substrates. Perfusion buffer consisted of 5.5 mM glucose, 0.5 mM lactate, 0.6 M palmitate bound to 3% BSA, and 20 µU/ml insulin. All animals received the same perfusion buffer, and no treatments were delivered during perfusion. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=p<0.05 vs. untreated control (C). b=p<0.05 vs. untreated diabetic (D).

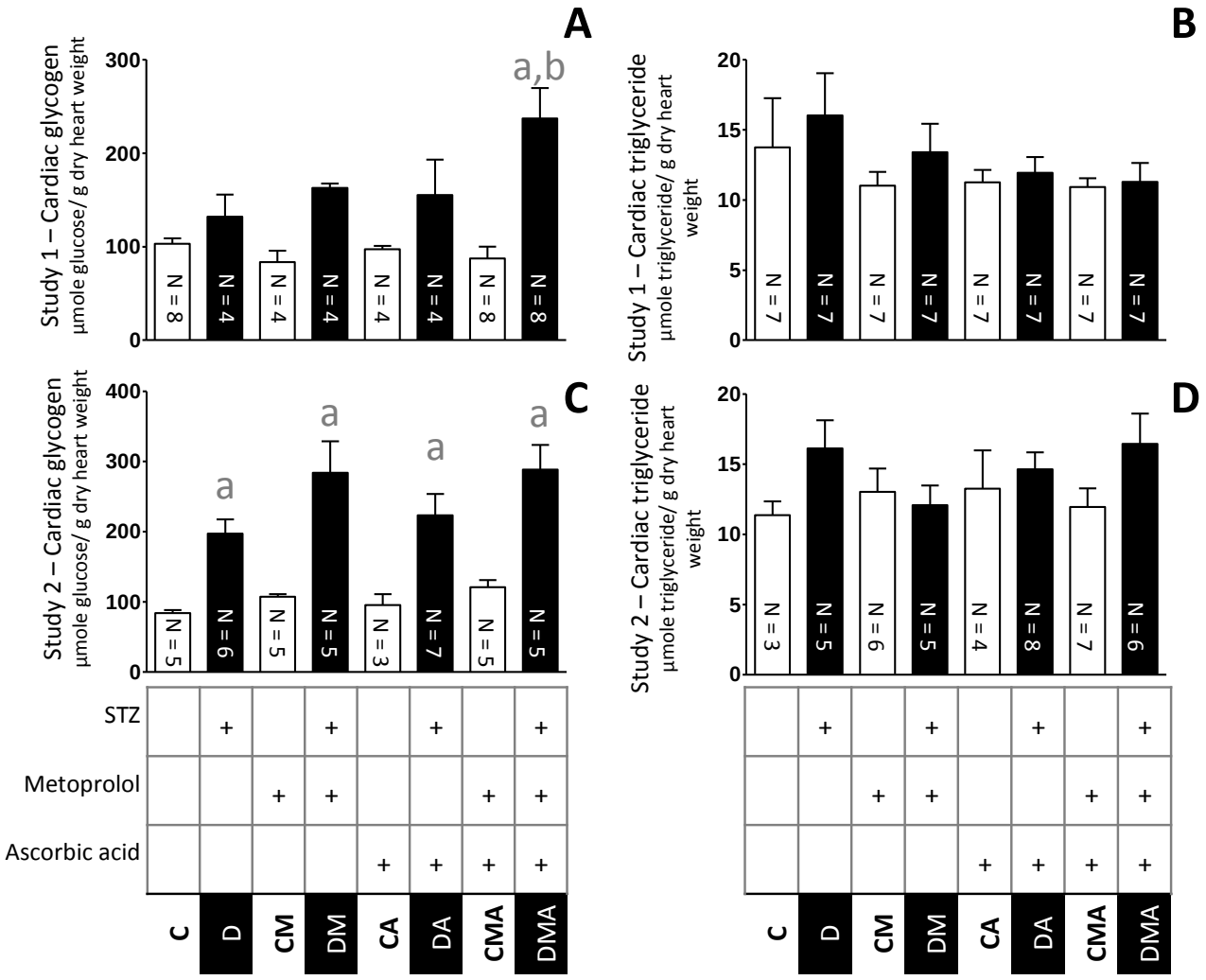
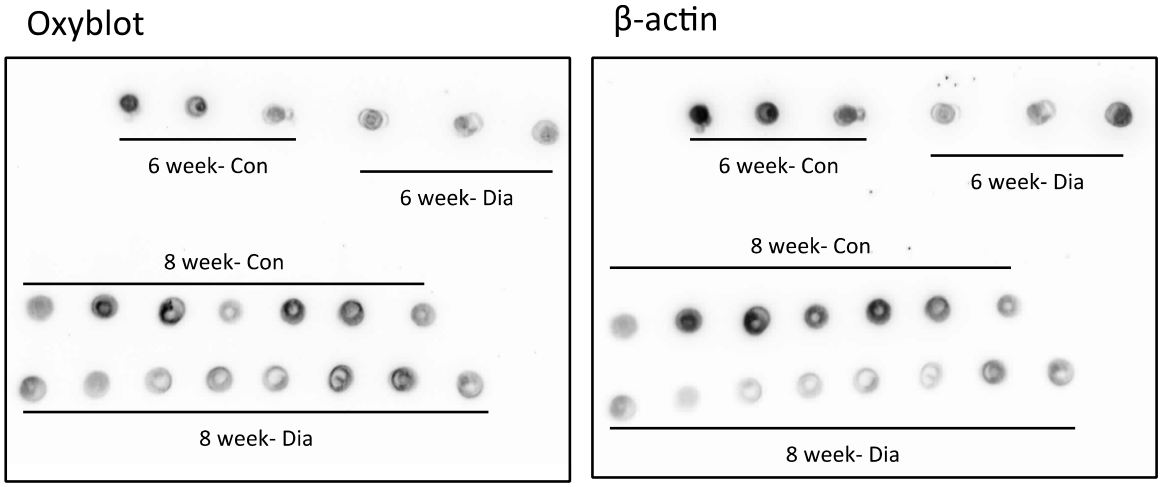


Figure 4 – Cardiac glycogen (**A**) and cardiac triglyceride (**B**) content of hearts from Study 1, and Study 2 (**C** & **D**, respectively). Value are group means, error bars represent SEM. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=p<0.05 vs. untreated control (C). b=p<0.05 vs. untreated diabetic (D).



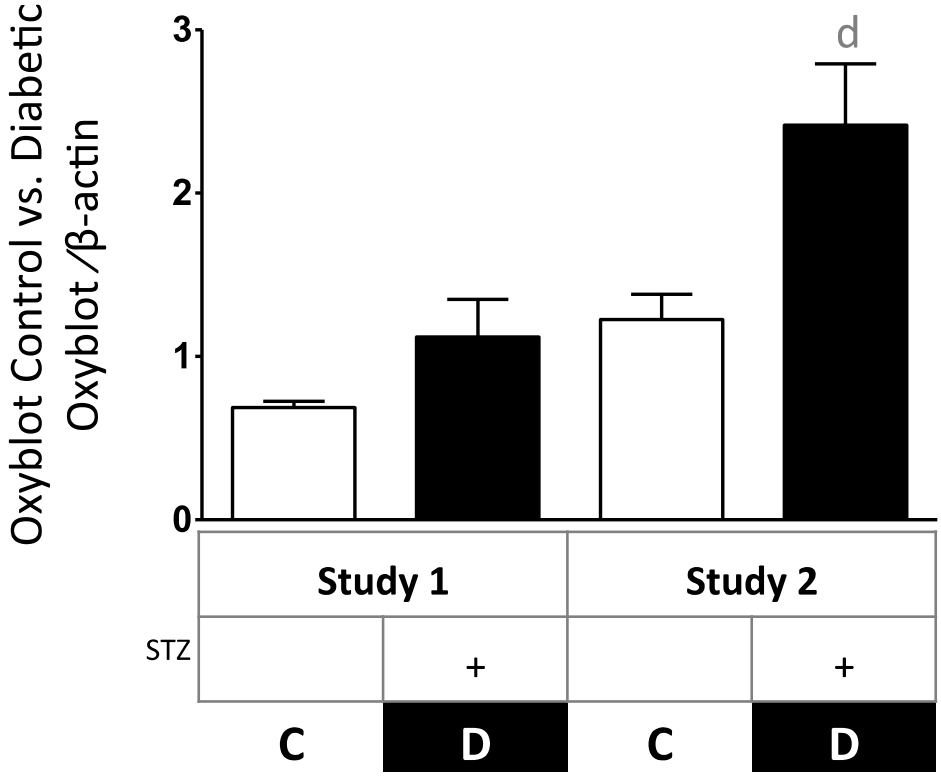


Figure 5 – Comparison of oxidative protein damage in hearts from Study 1 and Study 2. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. c=p<0.05 vs. Study 1 control (C). d=p<0.05 vs. Study 2 control (D).

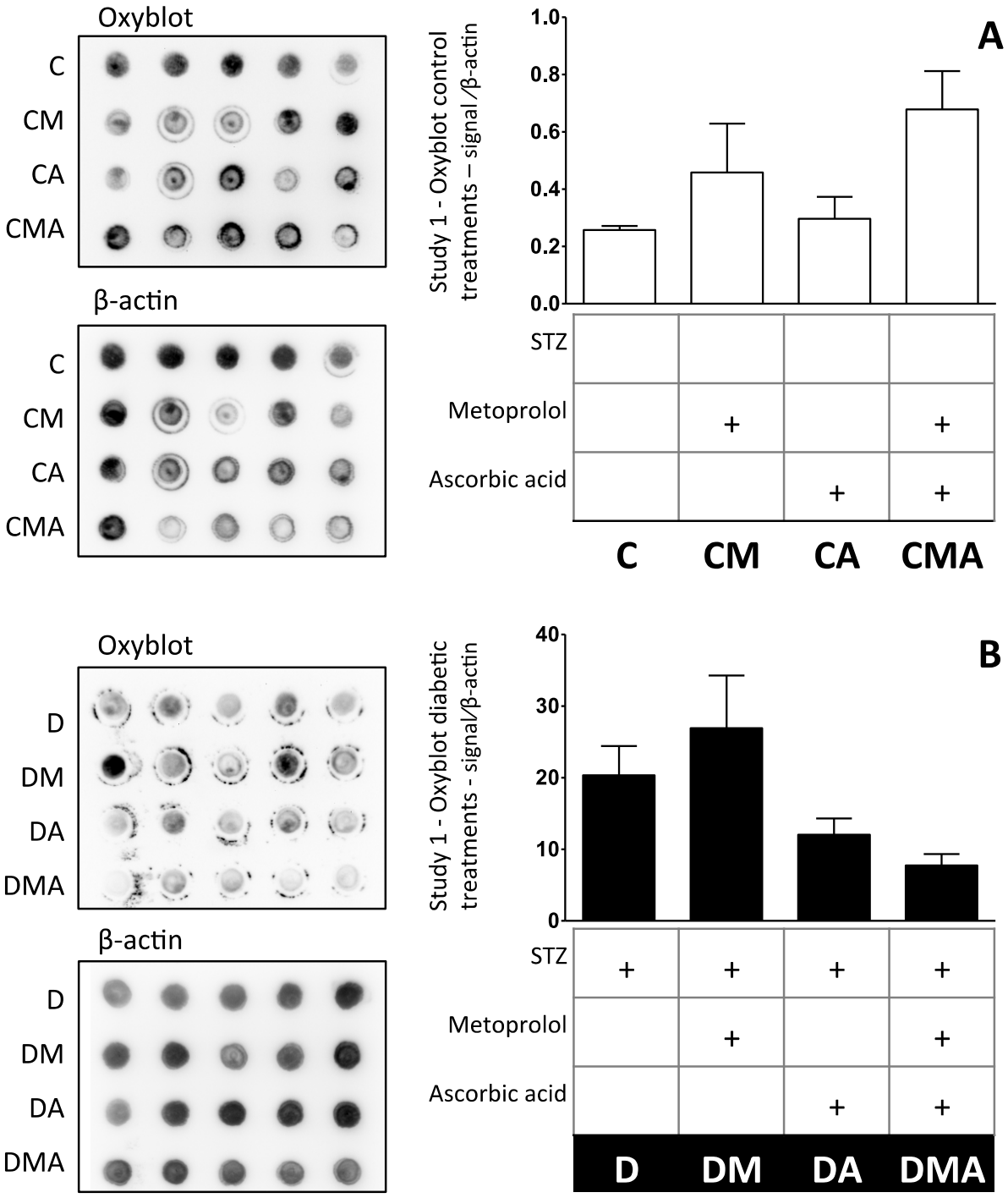


Figure 6 – Comparison of oxidative protein damage from control (**A**) and diabetic (**B**) hearts from Study 1. Y-axis scales are arbitrary units, comparisons across separate charts can not be made. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=p<0.05 vs. untreated control (C). b=p<0.05 vs. untreated diabetic (D).

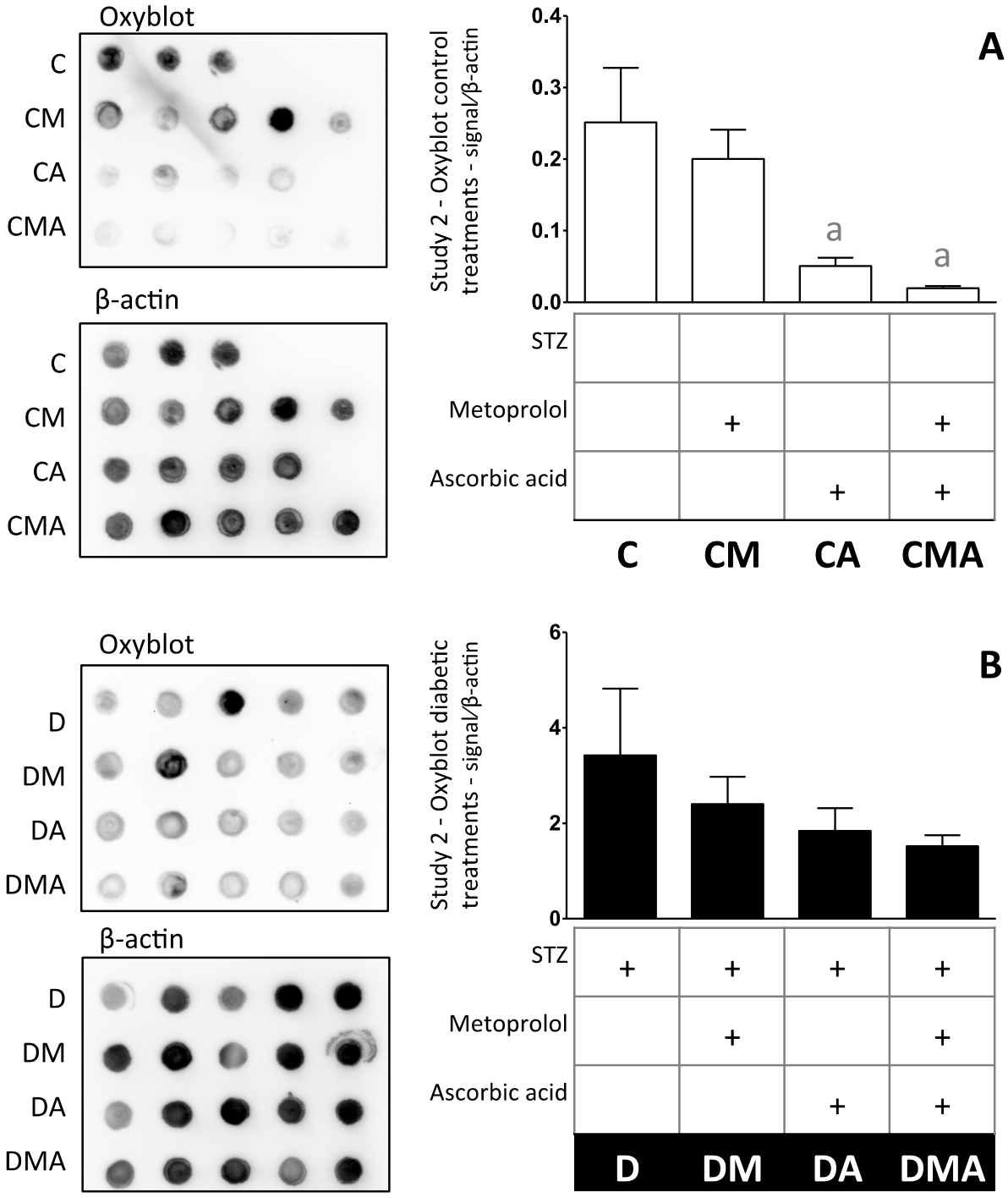


Figure 7 – Comparison of oxidative protein damage from control (**A**) and diabetic (**B**) hearts from Study 2. Y-axis scales are arbitrary units, comparisons across separate charts can not be made. Statistical analysis was by 1-Way ANOVA with Bonferroni post hoc test. a=p<0.05 vs. untreated control (C). b=p<0.05 vs. untreated diabetic (D).

4. Discussion

4.1. Overview of Study

The purpose of this study was to investigate the hypothesis (See Scheme 3 for outline of hypothesis and findings):

We propose that metabolic changes that occur during diabetes elevate oxidative stress, leading to protein damage, signaling changes, cell death and other sequelea; the eventual sum of these changes is an impairment of function. Treatment of either the sequelae of oxidative stress or oxidative stress directly will be beneficial but treatment of both will improve function further.

We approached our question in two ways. First, we chose to investigate contributions of potentially relevant factors in the development of diabetic cardiac dysfunction by comparing metabolic, functional and oxidative stress parameters measured before overt dysfunction (Study 1 – Diabetes week 5) and after the development of overt cardiac dysfunction (Study 2 – Diabetes week 7). We were able to show that cardiac function significantly worsened in concert with increases in metabolic disturbance and oxidative protein damage.

Second, we set out to determine if metoprolol's beneficial effects could be supplemented by reduction of oxidative stress by ascorbic acid. We observed that

both drugs improved cardiac function and had metabolic effects, while only ascorbic acid appeared to reduce oxidative protein damage. When metoprolol and ascorbic acid were combined the observed improvement in function was more powerful then the drugs alone.

4.2. General Physical Characteristics – Body Weight and Heart Weight are More Perturbed with Disease Progression

Diabetic animals from Study 1 displayed significant reductions in mass as compared to controls (Table 1). Weight loss in diabetic animals is usually due to loss of diaphaseal bone due to disturbances in calcium homeostasis and loss of muscle, adipose tissue and liver due to insulinopenia with tissue loss evident within 1 week following onset of diabetes[139, 140] . The observed mass gap doubled by the time Study 2 was terminated 2 weeks later. This finding is not novel, others have also shown that the mass gap increases as diabetes progresses[130, 141] .

Heart weights during Study 1 were not significantly lower in diabetics as compared to controls (Table 1). However, in Study 2, diabetic heart weights had dropped significantly by about 25%. Hoit *et al.* reported that heart weights began to trend lower at 8 weeks after induction of diabetes, a time point which is shortly after Study 2[130]. We also observed that our treatments had no effect on heart weight, however, Hanada *et al*. and Sharma *et al*. previously reported that metoprolol treatment causes reductions in heart weight (Table 1). These studies differed from ours in several ways. Firstly, metoprolol was used to reverse

isoproterenol induced cardiac hypertrophy in Hanada's study. Heart weights in treated animals were returned to normal and not below. Secondly, in Sharma's study, a much higher dose of metoprolol was used (75 mg/kg/day) and treatment was delivered by daily intraperitoneal injection. This high but transient dose likely has different effects and is not fully comparable to our lower (15 mg/kg/day) and more constant dosing.

4.3. Plasma Triglyceride and Cholesterol – Levels and Persistence of Disturbance Worsen with Disease Progression

4.3.1. Triglycerides

We observed an increase in plasma triglyceride of 94% in Study 1 diabetic rats and 160% in Study 2 (Table 2). These results are in line with observations in literature that also show rises in diabetic animals at more advanced time points [33, 127, 141, 142].

Metoprolol treatment reduced plasma triglyceride levels in diabetics in Study 1 but had no effect during Study 2, indicating that disturbances in triglyceride levels not only become more pronounced, but they also become more resistant to treatment (Table 2). Results for Study1, but not Study 2, are in keeping with similar findings in the literature. Sharma *et al*. performed their research in an identical model to ours at a time point in between Study 1 and Study 2 and found an approximate 40% rise in diabetic triglyceride content and that metoprolol normalized this rise[33]. Results are confirmed by others who have found that STZ

treated Sprague-Dawley rats at the same time point show a 70% increase in plasma triglyceride that was corrected by metoprolol[131]. However, results from Study 2 are not corroborated by results of Olbrich *et al.* who preformed a long-term study on metoprolol treated STZ rats. They found that metoprolol still had a corrective effect, but only noted a 10% increase in triglyceride levels at their 6 month time point, much lower then observed in Study 2[143]. This finding likely indicates that their animals were not as metabolically perturbed and thus their metabolic phenotype was at a point that could be rescued, similar to those in Study 1. The changing effect of metoprolol in Study 1 versus Study 2, likely reflects the increase in severity of metabolic disturbances over the short span of time.

Ascorbic acid administered alone did not produce significant reductions in plasma triglyceride content, although values trended lower then the untreated diabetics (Table 2). Dai *et al.* performed a similar study to ours and found that at a time point equivalent to Study 2, diabetic triglyceride levels were 4-fold higher than control levels and were significantly reduced using ascorbic acid, with the reduction occurring in a dose dependent manner. Importantly, the highest dose was equivalent to that used in this study[127]. It is unclear why Dai found such high triglyceride values in diabetics, their model was nearly identical to ours, differing only slightly in the STZ dose (55 vs. 60 mg/kg for us). The improved performance of ascorbic acid in their hands can likely be explained by the fact that they began treatment 3 days post STZ administration, whereas it was delayed 2 weeks in our study.

Clinically, metoprolol has been shown to increase triglyceride levels in non-diabetic hypertensive patients and in type 2 diabetic patients [144-146] . Ascorbic acid has been shown by some to reduce triglyceride levels in type 2 diabetics[147].

4.3.2. Cholesterol

Plasma cholesterol content in diabetics was significantly elevated in Study 2 but not in Study 1 (Table 2). Akula *et al*. also observed an increase in cholesterol levels in Sprague-Dawley rats as diabetes progressed. The degree of increase was also similar, with a doubling in levels observed from week 4 to week 8. Cholesteral increased in a similar manner from week 5 to week 7 in the present study (Study 1 and Study 2) [141].

Metoprolol had no effect on plasma cholesterol levels in our study, and in two similar studies in Wistar and Sprague-Dawley rats with STZ induced diabetes (Table 2) [33, 131] .

Ascorbic acid partially ameliorated increased plasma cholesterol levels during Study 2. Dai *et al*. also observed ascorbic acid's cholesterol lowering effects, with 600 and 1000 mg/kg/day lowering levels in a dose dependent manner[127]. Ascorbic acid's cholesterol lowering abilities are shared by other potent antioxidants, such as alpha lipoic acid, coenzyme Q10 and resveratrol[148-150] . It is interesting to note that each of these agents also increase plasma ascorbic acid levels. Although this finding raises the possibility of a deeper role for this vitamin, the effect may simply reflect replenishment of the body's antioxidant reserves as levels of other non-enzymatic antioxidants also increase[150].

In humans, plasma cholesterol is not modified by metoprolol in type 2 diabetic patients, but is reduced by ascorbic acid[146, 147] .

4.4. Heart Function – Relationship to Disease Progression

Heart function in diabetic rat hearts compared to controls decreased significantly from Study 1 to Study 2. Echocardiography and isolated working heart perfusion revealed virtually no changes to any diabetic functional measurements compared to controls during Study 1 (Table 3,4, Figure 1A, 2A, 2B). In contrast, diabetic hearts in Study 2 showed reductions in fractional shortening and ejection fraction as measured be echocardiography, and rate pressure product, cardiac work, coronary flow and cardiac output as measured by working heart perfusion (Table 3,4 and Figure 1B, 2C, 2D). These findings are consistent with the work of several others who have demonstrated cardiac dysfunction in STZ treated animals near the 7 week time point of Study 2 [33, 127, 130] .

Use of non-invasive and invasive methods to study cardiac function highlights the fact that disturbed *ex vivo* heart function does not always correlate with *in vivo* dysfunction. Study 2 showed reductions in cardiac output as measured by working heart perfusion, but not as measured by echocardiography (Figure 2D and 1B, respectively). This discrepancy has been previously reported. Akita mice, which display a genetic form of Type 1 diabetes, showed reduced cardiac output as measured by echocardiography at 54 weeks of age, while reductions in cardiac

power and left ventricular developed pressure measured by working heart perfusion becomes evident at 24 weeks[26]. In studies using STZ treated rats similar to that in the current study, cardiac dysfunction as measured by echocardiography, only subtly appeared as reductions in filling rate at 8 weeks and changes in chamber volume only appeared at 12 weeks[130, 141] . In contrast, studies using working heart perfusion showed differences by 6 weeks of diabetes in STZ treated rats [33, 127] .

There are several explanations for the discrepancy between *in vivo* and *ex vivo* function. First, *in vivo* cardiac output, and other related functional parameters, are largely dependent on factors affecting venous return and not by the heart itself, thus contractile problems could actually be masked by changes in mean systemic pressure, vascular compliance and blood volume[151]. Second, during echocardiography animals are anesthetized, and the hearts unchallenged, thus functional problems might not be obvious. This is supported by the fact that when isoproterenol was used to increase heart rate in diabetic rats, differences in function as measured by echocardiography, became evident as early as 5 weeks of diabetes, whereas unchallenged hearts showed no changes at 5 weeks and only subtle changes in filling rate at 8 weeks[130]. Third, there is the possibility that worsened function observed on the working heart apparatus may be a result of poor recovery after an ischemic period that exists between the removal of the heart from the chest cavity and the mounting of the heart on the perfusion apparatus. However, this is unlikely as there is considerable evidence that hearts from STZ treated animals actually recover better then control hearts after an ischemic period[152-154] , as long as the duration is short and diabetes is not too severe[155]. Finally, there are

important differences in the conditions in which the heart operates during perfusion and echocardiography. During echocardiography diabetic hearts are exposed to 3-fold higher glucose concentrations, 4-fold lower insulin, 2.5-fold more triglycerides and a more then 4-fold increase in ketone levels compared to control hearts, with accompanying neurohormonal signals attempting to ensure proper tissue perfusion (Table 2). This is important as changes in substrate availability and hormone levels are known to cause functional modifications, thus *in vivo* perfusion conditions may have partially normalized function in the diabetic heart[134, 156] . In contrast, during working heart perfusion all substrate and hormone levels are identical, highlighting intrinsic differences in the hearts themselves.

Metoprolol and ascorbic acid improved *ex vivo* but not *in vivo* function in diabetic hearts during Study 2. When metoprolol was given alone it was able to significantly improve rate pressure product; ascorbic acid alone modified rate pressure product, cardiac (hydraulic) work, coronary flow and cardiac output to a point where they were no longer significantly different from controls. Dual metoprolol and ascorbic acid treatment did the same with hydraulic work and coronary flow, but significantly improved cardiac output above the untreated diabetic group (Figure 1B, 2C, 2D and Table 4).

It should be noted that our results with metoprolol are less pronounced then those reported in the literature. Sharma *et al* observed a significant increase in cardiac output and hydraulic work in metoprolol treated diabetic rats. However, in Sharma's study function was found to be far worse in the untreated diabetic than in the present study. Where we observed a 25% reduction in cardiac output, they

showed a 60% reduction (Figure 2D). Metoprolol in their hands only improved the treated animals to a level that was very close to our untreated diabetic animals[33]. Thus it is possible that the reason they saw a greater effect with metoprolol was because cardiac function was disturbed to a greater extent.

The improvements in function we observed with ascorbic acid are similar to previously reported results. Dai *et al.* noted that ascorbic acid improved left ventricular developed pressure and left ventricular end diastolic pressure in STZ treated rats[127]. Other researchers have also shown that results observed with ascorbic acid can be extended to other antioxidants; Koksoy *et al.* noted similar improvements in pressure related parameters after treatment with sodium selanate and omega-3 fish oil supplemented with vitamin E[157].

The additive functional effects observed with dual treatment seem to imply that metoprolol and ascorbic acid did not directly act on the same site. Sharma *et al.* have proposed that metoprolol's functional improvement effects are, at least partially, mediated through normalization of signaling, sequestration of cell death mediators and reduction in myocardial fibrosis, but not reduction of oxidative stress [34, 112]. Positive effects observed with antioxidants, on the other hand, are thought to be regulated through reduction in oxidative damage to proteins[157].

4.5. Heart Metabolism – Metabolic Alterations Worsen with Disease Progression

4.5.1. Palmitate Oxidation

Oxidation of exogenous palmitate is increased by 20% in diabetic hearts compared to controls during Study 1 and by 70% above controls by the end of Study 2 (Figure 3A, 3C). Thus, it appears that there was a large metabolic shift that occurs during the period between Study 1 and Study 2. Literature values for exogenous palmitate oxidation at early and late time points show increases in diabetic hearts that are between rates observed in Study 1 and Study 2. However, the degree of disturbance in palmitate oxidation does not correlate consistently with time point. Kewalramani *et al*. and Ghosh *et al.* both used Wistar rats treated with 55 mg/kg and both measured palmitate oxidation rates at 4 days. Kewalramani observed a 100%, while Ghosh found only a 40% increase[142, 158] . Workload is identical in both cases, and substrate concentrations are similar. Thus, there is no clear explanation why consistent palmitate oxidation values were not observed. One possibility is that hearts with lower measured substrate oxidation rates were actually metabolizing significant amounts of endogenous triglyceride stores and since only the exogenous palmitate is labeled with isotope, oxidation of endogenous fuels would be invisible.

Treatment with metoprolol and ascorbic acid completely normalized palmitate oxidation rates in Study 1 (Figure 3A). In Study 2, oxidation rates were more resistant to change. Although metoprolol consistently caused a 20% decrease in treated diabetic groups the change was not significant (Figure 3C). This is in

contrast to the work of Sharma *et al*. who showed that metoprolol treatment could lead to an approximate one-half reduction in rates. However, it is important to note that their goal was to model *in vivo* oxidation rates, so they did not provide diabetic hearts with insulin during perfusion, and provided controls with 5-fold the insulin provided in our study. As a result their palmitate oxidation rates in diabetic hearts were 400% higher then their controls. Perfusion conditions combined with their higher, 75 mg/kg/day dosage, delivered via intraperitoneal injection, might explain the larger effect they observed with metoprolol. Ascorbic acid produced no detectable effect on palmitate oxidation rates in our study, and no literature data was found on the effect of ascorbic acid on fatty acid oxidation during diabetes[33]. In animals receiving dual treatment there was a significant 30% reduction in rates, thus it appears that ascorbic acid some how sensitized hearts to the effects of metoprolol.

4.5.2. Glucose Oxidation

Glucose oxidation in diabetic hearts were lowered to nearly undetectable levels in both studies (Figure 3B, 3D). Changes in glucose oxidation rates are known to begin early and correspond with the induction of diabetes. Ghosh *et al.* showed that at 4 days post STZ injection, animals had 50% reduced glucose oxidation rates compared to controls and by 6 weeks showed oxidation rates similar to those observed in Study 1 and Study 2[158]. Glucose oxidation is reduced in diabetes partially because of inhibition of pyruvate dehydrogenase, a key regulator of glucose oxidation, caused by increased fatty acid oxidation rates and partially because of

reduced substrate availability due to reduced glucose transporter 4 (GLUT 4) expression[159].

During Study 1 and Study 2, treatment with metoprolol or ascorbic acid, individiually or in combination, showed no significnat beneficial effects. However, dual treatment in Study 1, and all treatments in Study 2 did show small increases in oxidation rates. Interestingly, the degree of increase correlates well with increases in cardiac glycogen levels, with the largest increases observed in groups with the most glycogen (Figure 3B, 3D and 4A, 4C). Although our study did not include a comprehensive analysis of cardiac glucose use, increased oxidation and increased glycogen levels might indicate increased glucose uptake. Sharma *et al.* have shown that metoprolol can cause a small but significant stimulation of glucose oxidation, however they did not observe changes in tissue glycogen levels[33]. Of note, other antioxidants are known to improve glucose uptake. Resveratrol, a potent antidoxidant, has been shown to improve glucose uptake, by increasing GLUT 4 translocation in Sprague-Dawley rats made diabetic with 65 mg/kg of STZ. In addition, the antioxidant alpha–tocopherol has been demonstrated to improve glucose uptake in diaphragm muscle from STZ treated rats[160]. Further studies are required to confirm whether our treatments caused real increases in glucose uptake and oxidation.

4.6. Oxidative Protein Damage in Diabetic Hearts Worsen with Disease Progression

Oxidative cardiac protein damage was increased by about 65% in diabetics from Study 1, although these changes are not significant. In Study 2, oxidative damage in diabetics had increased by over 95% compared to controls and the changes were statistically significant (Figure 5). The cause of the increased oxidative protein damage may be due to increases in levels of ceramide, which is a key mediator of lipotoxicity, oxidative stress and cell death[161]. Increased oxidative damage could also be due to increased glycotoxicity, or directed flow of glucose catabolism through the polyol pathway, formation of advanced glycation end products leading to reactive oxygen species production, and increased flux through the hexosamine biosynthetic pathway[91, 162, 163] .

During Study 2, when oxidative stress is most prominent, there is a large and significant reduction in oxidative protein damage associated with ascorbic acid treatment in control hearts, but a lesser and non-significant reduction in diabetic hearts (Figure 7). Although it appears that ascorbic acid, with or without metoprolol, has an approximately one-half lesser effect in diabetic animals (80 and 95% reductions in controls versus 45 and 55% in diabetics, respectively), it is important to note that oxidative damage in diabetics is doubled at this time point. Thus, in terms of absolute degree of reduction, ascorbic acid has a similar effect on both control and diabetic hearts. Ascorbic acid's reduction of oxidative stress could partially be due to its chemical antioxidant properties, but also could be due to its

ability to inhibit aldose reductase, the rate limiting enzyme in the reactive oxygen species generating polyol pathway[164].

In contrast to Study 1, during Study 2 all groups treated with metoprolol tended to show slightly lower oxidative protein damage then their respective counterparts (Figure 7). Although metoprolol is not known to have biologically relevant antioxidant activity, its ability to reduce cell death signaling increased during diabetes may improve cellular stability and reduce reactive species generation[34]. Further experiments examining the dose dependency of oxidative damage reductions using metoprolol and ascorbic acid would be useful to confirm our findings and proposed mechanisms.

4.7. Summary (see Scheme 3)

4.7.1. Progression of Diabetes from 5 to 7 Weeks - An Important Time Point in the Development of Cardiac Dysfunction

In the progression of diabetes, as demonstrated by differences between Study 1 and Study 2, a number of important changes occurred. First, diabetic animals experienced greater reductions in body weight and began to develop reductions in heart weight. Second, fatty acid oxidation in diabetic animals compared to controls increases from a mild 20% disturbance to a pronounced 100% increase. Furthermore, these changes actually become more persistent, as metoprolol and ascorbic acid lost their beneficial effects. Third, more pronounced functional impairments developed *ex vivo* then *in vivo*, indicating that intrinsic

changes occur in the myocardium over this critical time point and that they can be masked *in vivo*. Finally, oxidative protein damage in diabetic hearts worsens from 65% over controls to 95% over controls. Thus it appears that physical, metabolic, functional characteristics in the diabetic heart all worsen significantly over a relatively short 2-week period, a period over which oxidative protein damage also shows large changes.

4.7.2. Benefits of β-Blocker Therapy Supplemented with Antioxidants.

Although we observed two time points during our study, Study 2 is most useful in assessing the effects of dual therapy on metabolism, function and oxidative damage because it is here that overt dysfunction developed. During Study 2 metoprolol alone had no significant effects on metabolism or oxidative damage but did have some effect on function. Furthermore, values from metoprolol treated diabetic animals often trended more towards controls. Ascorbic acid was not observed to have an effect on metabolism except for increasing the effect of metoprolol on palmitate oxidation, also it did cause reductions in oxidative protein damage and improved function to a point where treated animals were not significantly different from controls. When both treatments were combined function was further (and significantly) improved as compared to diabetic untreated hearts.

4.7.3. Hypothesis and Conclusions

The following conclusions were made in regards to the three sub-hypotheses that we addressed during our study:

1. *Disturbances in metabolism will appear before the development of overt dysfunction, while changes in oxidative protein damage will appear most prominent at the point of dysfunction.*

During our study, we were able to demonstrate that metabolic disturbances do in fact appear before development of overt cardiac dysfunction, however, they do become more prominent once dysfunction has set in. Changes in oxidative protein damage appear before dysfunction, but do become much more pronounced after development of cardiac dysfunction. Thus, we were able to provide support for this hypothesis.

2. *Both metoprolol and ascorbic acid will improve cardiac function, however, only ascorbic acid will reduce oxidative stress.*

In our hands metoprolol and ascorbic acid improved functional parameters in the diabetic heart, with ascorbic acid having the more pronounced effect. Ascorbic acid, but not metoprolol, was able to lower oxidative stress (although changes were pronounced they were only significant in control hearts). We feel there is strong support for this hypothesis.

3. *Combined metoprolol and ascorbic acid treatment will improve function further then single treatment.*

There is strong support for this hypothesis. When the two drugs were combined, several functional parameters were raised to a point that they were not different from controls and, in the case of cardiac output, significantly improved above diabetics.

Our overall hypothesis was:

We propose that metabolic changes that occur during diabetes elevate oxidative stress, leading to protein damage, signaling changes, cell death and other sequelea; the eventual sum of these changes is an impairment of function. Treatment of either the sequelae of oxidative stress or oxidative stress directly will be beneficial but treatment of both will improve function further.

We were able to show that metabolism became significantly worse at a time point that was associated with impaired function, we were also able to show increased oxidative protein damage, a possible link between function and metabolism. Although it should be noted we have not conclusively shown a cause and effect relationship between the two. Finally, we were able to show that by

treating both oxidative stress and it's consequences, function was improved. Thus, we feel we have supported our hypothesis.

4.8. Importance of the Study

The results of this study are important because they demonstrate the effectiveness of a novel treatment strategy targeting both the signaling changes, cell death and other sequela (by metoprolol) and oxidative stress (by ascorbic acid) that are associated with STZ-induced diabetic cardiomyopathy. This strategy is especially helpful because it targets oxidative stress and its effects without interfering with oxidative lipid metabolism, avoiding problems associated with lipid accumulation. Furthermore, our results may provide insight into the superior therapeutic effects of β-blockers that incorporate antioxidant properties, such as carvedilol. Our β-blocker and antioxidant treatment strategy's focus is on oxidative stress, and not on diabetes specifically, thus it may prove helpful in other disease where metabolic disturbances contribute to oxidative stress, such as heart failure.

4.9. Future Directions

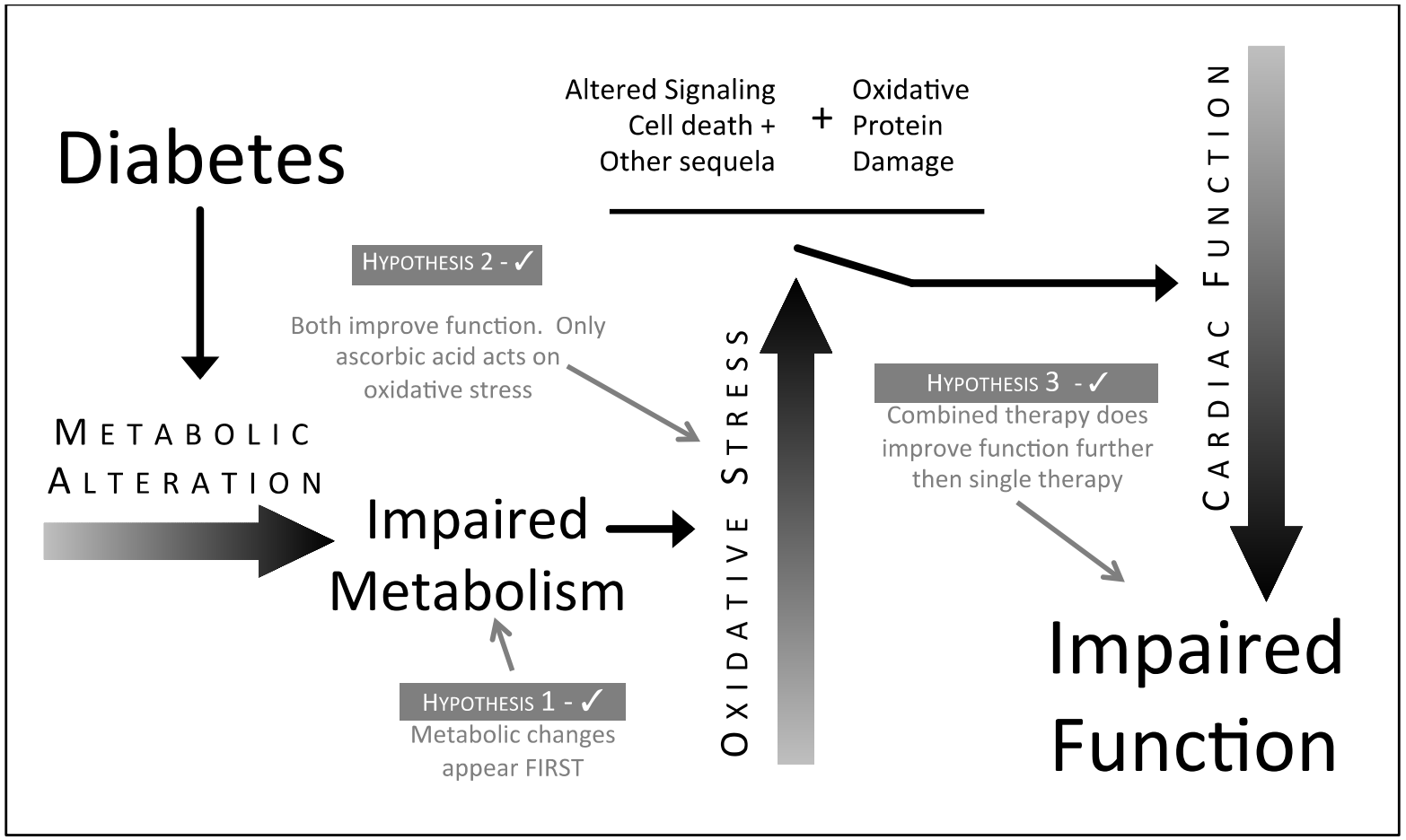
The present study sheds light onto the progression of diabetic cardiovascular disease, and especially diabetes-induced cardiac dysfunction. However, there are a number of important questions that remain. The most obvious issue is to clarify the role of metabolism in the development of cardiovascular dysfunction. To that end, one could attempt to modify metabolism further then was achieved in our study by

using higher doses of metoprolol, or using other agents that are known to inhibit long chain fatty acid oxidation, such as etomoxir, however, one would have to also lower lipid uptake in order to prevent lipid accumulation[165]. Furthermore, it would be useful to perform a full assessment of expression and activation states of key metabolic flux regulating proteins such as; hexokinase and phosphofructokinase-1, which control glycolytic flux; pyruvate dehydrogenase, which controls rate of flux through the citric acid cycle; and carnitine palmitoyltransferase-1[55]. Assessment of glucose uptake capacity in untreated and treated diabetic hearts by measurement of subcellular localization and total expression of GLUT 4 could also prove useful, particularly in explaining some of ascorbic acids effects on glucose metabolism.

Another important area to investigate would be to assess the contribution of lipotoxicity and glucotoxicity in the development of oxidative stress. This would include quantitation of ceramide levels and measuring the flux through oxidative stress causing pathways, such as the polyol pathway, and the hexosamine biosynthetic pathways. Further study of the polyol pathway in particular, by measuring expression and activity of aldose reductase, could shed further light onto the mechanism of action of ascorbic acid, as it is an inhibitor of that key enzyme[164].

Recently, metoprolol was shown to move the diabetic heart from a pro- to anti-apoptotic state. This was accomplished by shifting signaling from protein-kinase-A to protein-kinase-B signaling, and through sequesteration of activated caspase-3 by caveolins[34]. It would be useful to investigate ascorbic acid's effect on apoptotic signaling within diabetic heart as reduction of oxidative damage may result in reduced stimulus for a pro-apoptotic state.

Our experimental model of type-1 diabetes provided a clear look at cardiac dysfunction in a hyperglycemic model without complications such as hypertension. However, type-2 diabetes is the predominant form of this disease, and it is often found associated with a variety of other risk factors, including systolic hypertension [166]. Thus, it would be useful to determine if the beneficial effects we observed in our study could be maintained in a model system closer to type-2 diabetes, such as the insulin resistant and hypertensive fructose fed rat.



Scheme 3. Outline of overall hypothesis (black text) and summary of results(grey).

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