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Exploration of glycemic fluctuations and oxidative stress in normal and insulin resistant rats

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EXPLORATION OF GLYCEMIC FLUCTUATIONS AND OXIDATIVE STRESS IN NORMAL AND INSULIN RESISTANT RATS

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1 Preface

This Project was carried out in cooperation between the Institute of Veterinary Disease Biology, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark and Novo Nordisk A/S, Diabetes Pharmacology, Måløv, Denmark in the period 2009-2012. The work regards the investigation of fluctuating hyperglycemia and increased oxidative stress in normal and insulin resistant rats and is based on a review of the literature and experimental studies described in following manuscripts.

Paper I:
Rakipovski,G, Raun,K, Lykkesfeldt,J: Fluctuating hyperglycaemia increases oxidative stress response in lean rats compared to sustained hyperglycaemia despite lower glycaemic exposure. *Diab Vasc Dis Res* 8:295-298, 2011

Paper II:
Günaj Rakipovski, Jens Lykkesfeldt, Thomas Galbo and Kirsten Raun: Fluctuating but not sustained hyperglycaemia preferentially increases vascular oxidative stress and GLUT1 expression in rats on high fat diet. Submitted to Diabetologia (2012).

Paper III:
Günaj Rakipovski, Kirsten Raun and Jens Lykkesfeldt: Insulin resistance exacerbates vascular oxidative stress response during fluctuating hyperglycaemia. In preparation (2012).

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3 Summary

Diabetes is a serious chronic disease affecting more than 300 million people globally. Diabetes leading to the detrimental development of diabetic late stage complications is one of the main causes for a substantial lowering of life quality and early mortality. Several large scale prospect and epidemiological studies have investigated the role of hyperglycemia and the development of late stage complication. Indeed controlling blood glucose levels by lowering glycated haemoglobin (Hba1c) levels may to some extent significantly lower the risk of developing diabetes related complication however still a great number diabetes patients will develop these detrimental complications. Recently fluctuating hyperglycemia which is not necessarily reflected by the Hba1c level has been hypothesized to be an important risk factor in the development of late stage complications. Reports both in humans and rodents have shown that fluctuating hyperglycaemia induce higher levels of oxidative stress leading to cellular damage particular in the cardiovascular system thus potentially resulting in diabetes related atherosclerosis and cardiovascular disease (CVD). However, so far the exact sequences of biochemical events are still under debate. Thus in this project we set to investigate the effect of fluctuating hyperglycemia versus sustained hyperglycemia in both healthy (LEAN) and insulin resistant, diet induced obese (DIO) animal models and its role on markers of oxidative stress, expression of key enzymes and glucose metabolism in liver and aorta. We hypothesized that the liver, being one of the main regulators of glucose homeostasis, may exert important regulation of glucose metabolism during different hyperglycaemic situations and thus indirectly have an impact the severity of oxidative stress in aorta. Initially we aimed to set up an infusion protocol in catheterized animals giving well-defined and consistent fluctuating and sustained hyperglycemic profiles. The infusion protocol was a fully automated system handling both the infusion and blood sampling from the animals. In the setup animals were freely moving with *ad libitum* access to food and water thus the setup was considered as a major advantage with regards to minimizing the stress in the animals. Our major findings were that fluctuating hyperglycemia both in LEAN and in DIO rats increased systemic oxidative stress similar to chronic hyperglycemia despite a much lower overall glycemic exposure. However at tissue level we did not observe any changes in the LEAN model. Insulin resistance has shown to be an important factor in the development of diabetes related complications. Thus in the DIO model as compared to the LEAN model we found significant higher levels of $GP91^{PHOX}$ and GLUT1 protein abundance in aorta. We as well found that the DIO model had a significant lower antioxidant capacity, measured as super oxide dismutase (SOD) activity, in the aorta. Additionally when subjecting the DIO animals to fluctuating hyperglycemia the response on $GP91^{PHOX}$ and GLUT1 protein abundance in aorta were further increased and resulted in significantly higher response as compared to the group of animals receiving the exact same

amount of glucose. Moreover, fluctuating hyperglycemia as well increased malondialdehyde (MDA) and oxidized low density lipoproteins (oxLDL) in aorta. In the liver we observed increased oxidative stress but only in the insulin resistant rats subjected to chronic hyperglycemia. Interestingly the liver seemed to counteract these detrimental consequences during chronic hyperglycemia by down regulating net glucose uptake thus exerting a protective mechanism against further oxidative stress. Collectively, our data indicate that both fluctuating and overt sustained hyperglycemia may induce a higher degree of oxidative damage. Interestingly, the oxidative damage is highly compartmentalized where the cardiovascular system is primarily targeted by the fluctuating hyperglycemic profile whereas the liver is more disposed to oxidative damage by the chronic hyperglycemic profile. Insulin resistance has shown to be an important factor in the development of macrovascular complications. In our studies, the DIO model was more prone to increased oxidative stress suggesting that insulin resistance exacerbates the oxidative stress resulting from fluctuating hyperglycemia.

4 Dansk resume

Diabetes er en alvorlig kronisk sygdom som globalt set påvirker mere 300 millioner mennesker. Diabetiske sen-komplikationer som udvikles ved længere tids diabetes er den primære årsag for en signifikant forringelse af livs kvalitet og præmatur dødlighed. Adskillige prospektive og epidemiologiske studier har undersøgt sammenhænget mellem hyperglykæmi og udviklingen af diabetiske sen-komplikationer. Udfaldet af disse studier har vist at en forbedret kontrol af blod glukose niveauerne målt ved glykosyleret hæmoglobin (Hba1c) vil medføre, til en vis grad, en signifikant reduktion i risikoen for at udvikle diabetiske sen-komplikationer og dog stadig en stor del af disse diabetiske patienter vil alligevel udvikle disse alvorlige diabetiske sen-komplikation trods en forbedret hyperglykæmi. På det seneste har man foreslået at fluktuerende hyperglykæmi som nødvendigvis ikke afspejles af Hba1c niveauerne kan være en afgørende risiko factor for udviklingen af diabetiske sen-komplikationer. Flere og flere publikationer baseret både på menneske og gnaverforsøg har vist at fluktuerende hyperglykæmi inducerer højere niveauer af oxidativt stress som resulterer i cellulær skade især i det kardiovaskulære system og potentielt set kan være årsag til udviklingen af diabetes relatere arteriosklerose og hjerte-kar sygdomme. Kendskab til den eksakte biokemiske konsekvens er endnu ukendt og stadig under stor debat. Derfor - det primære formål med dette projekt var at undersøge sammenhænget mellem fluktuerende versus konstant hyperglykæmi i både raske (LEAN) og insulin resistente, diet induceret fede (DIO) rotter på markør for oxidativt stress. Desuden var formålet også at undersøge hvorledes forskellige glykæmiske profile ville påvirke regulereingen af vigtige enzymer i metabolismen for glukose både i lever og aorta. Vores hypotese var baseret på at leveren, som en af de vigtigste regulatorer for glukose balancen, under forskellige hyperglykæmiske forhold vil udøve vigtig regulering af glukose metabolismen således at graden af oxidativt stress i aorta kan påvirkes. Indledningsvis var formålet at opsætte en infusion protokol i dyr med permanente katetre med vel defineret og konsistente fluktuerende og kroniske hyperglykæmiske profiler. Både infusion af glukose og blodprøvetagninger var fuld automatiseret. Ydermere i op sættet havde dyrene frit bevægelighed og *ad libitum* adgang til foder og vand. Dette blev anset for at være en fordel med henblik på at reducere stress in dyrene. De vigtigste fund var at fluktuerende hyperglykæmi både i LEAN og i DIO rotter øgede systemisk oxidativt stress sammenlignelig med gruppen som havde kronisk hyperglykæmi trods en betydelig lavere total glukose belastning dog observerede vi ingen ændringer i væv på oxidativt stress niveauer i LEAN modellen. Insulin resistens har en betydelig effekt på udviklingen af diabetes relateret sen-komplikationer. I forhold til LEAN modellen havde DIO modellen signifikant højere niveauer af $GP91^{PHOX}$ og GLUT1 protein mængder in aorta. Derudover observerede vi at DIO modellen også havde en signifikant lavere anti-oxidativt kapacitet ved lavere super oxid dismutase (SOD)

aktivitet, i aorta. Ydermere, ved fluktuerende hyperglykæmi i DIO modellen observerede vi at $GP91^{PHOX}$ og GLUT1 protein mængden i aorta var yderligere forhøjet. Desuden observerede vi at malondialdehyd (MDA) og oxideret lav densitets lipoprotein (oxLDL) var signifikant forhøjet i aorta ved fluktuerende hyperglykæmi. I leveren observerede vi også forhøjet niveauer af oxidativt stress men kun i DIO modellen ved kronisk hyperglykæmi. Bemærkelsesværdigt, leveren modvirkede disse skadelige effekter ved kronisk hyperglykæmi ved at nedregulere netto glukose optag i leveren og derved udøve en beskyttende mekanisme mod yderligere oxidativt stress. Samlet set, data indikerer at både fluktuerende og kronisk hyperglykæmi inducerer højere niveauer af oxidativt stress. Niveauerne af oxidativt stress virkede til at være organ specifikt hvor det kardio vaskulære system virker til at være mere sårbar over for fluktuerende hyperglykæmi hvorimod leveren var mere udsat for oxidativt stress ved kronisk hyperglykæmi. Insulin resistens er en vigtig faktor for udviklingen af diabetiske sen-komplikationer og DIO modellen under hyperglykæmiske forhold havde en ydermere forværret oxidativt stress.

5 List of abbreviations

ACC:	Acetyl-CoA Carboxylase
ADA	American Diabetes Association
AKT1:	Serine/threonine Protein Kinase 1
ApoE:	Apo-lipoprotein E
ASC:	Ascorbic acid
ATP	Adenosine-5'-triphosphate
BHT:	Tetramethoxypropane Butylated Hydroxytoluene
CAT	Catalase
cGMP:	Cyclic Guanosine Monophosphate
CHG:	Constant High Glucose
CLG:	Constant Low Glucose
CVD:	Cardiovascular Disease
DCCT:	Diabetes Complications and Control Trial
DHA:	Dehydroascorbic acid
DIO:	Diet Induced Obesity
DNA:	Deoxyribonucleic Acid
$FADH_2$:	Flavin Adenine Dinucleotide
FAS:	Fatty Acid Synthase
FFA:	Free Fatty Acid
FLU:	Fluctuating Hyperglycemia
G-6-P:	Glucose-6-Phosphate
G-6-Pase:	Glucose-6-Phosphotase
GAPD:	Glyceraldehyde Phosphate Dehydrogenase
GK:	Glucokinase
GKRP	Glucokinase Regulatory Protein
GLY:	Glycogen
GLUT:	Glucose Transporter
GSH	Glutathione
GPx	Glutathione Peroxidase
H2O2:	Hydrogen Peroxide
H_2SO_4	Dihydrosulphuric acid
Hba1c:	Glycated Haemoglobin
ICAM:	Intercellular Adhesion Molecule 1
IGT:	Impaired Glucose Tolerance
IL-6:	Interleukin-6
IRS:	Insulin receptor Substrate
LEAN:	Lean rats on regular chow
NADH:	Nicotinamide Adenine Dinucleotide Dehydrogenase
NADPHox:	Nicotinamide Adenine Dinucleotide Phosphate Oxidase
NGT	Normal Glucose Tolerance
NO:	Nitric Oxide
NOS:	Nitric Oxide Synthase
OGTT:	Oral Glucose Tolerance Test
oxLDL:	Oxidized Low Density Lipoproteins
PA	Phosphotungstic Acid
PEPCK:	Phosphoenolpyruvate Carboxykinase
ROS:	Reactive Oxygen Species
SOD	Superoxide Dismutase
STOP-NIDDM:	Study to Prevent Non-Insulin-Dependent Diabetes Mellitus
Screbp1c:	Sterol-Regulated Element Binding Protein 1c
STZ:	Streptozotocin
T1D:	Type 1 Diabetes
T2D:	Type 2 Diabetes
TBA	Dithiobarbituric Acid
TG:	Triglycerides
TNF-α:	Tumor Necrosis Factor-α
UKPDS:	United Kingdom Prospect Diabetes Study Diabetes Diabetes
USA:	United States of America
VCAM:	Vascular Cell Adhesion Molecule 1
VEC:	Vascular Endothelial Cells
VEH:	Vehicle
WHO:	World Health Organization

6 Aim

Recently it has been suggested that postprandial or fluctuating hyperglycemia may be an independent risk factor for the development of diabetes related complications. Increased oxidative stress during fluctuating hyperglycemia has been hypothesized as an important component in the development diabetes related late complications. However the exact biochemical event undergoing during fluctuating hyperglycemia and the suggested induction of oxidative stress is still under debate. Therefore the overall purpose of this project was to investigate the role of fluctuating hyperglycemia on the development of oxidative stress in rats. Initially we aimed to set up an infusion protocol in non-restrained catheterized animals giving well-defined and consistent fluctuating and sustained hyperglycemic profiles. Diabetes is a multifactorial disease including dyslipidemia, obesity, insulin resistance and inflammation collectively known as the metabolic syndrome. All these components have shown to be major contributors to oxidative stress and consequently to the development of diabetes related late complication thus, in order to investigate the effect of glucose itself or the combination of hyperglycemia and the metabolic syndrome on markers of oxidative stress we chose to apply our infusion protocol in both lean healthy and in obese, insulin resistant rats. Finally we investigated the regulation of key enzymes responsible for glucose metabolism in liver and aorta. We hypothesized that the liver, being the main regulator of glucose homeostasis, may exert important regulatory mechanisms of the glucose metabolism during different hyperglycaemic situations.

7 Introduction

Diabetes in general

Diabetes is a serious chronic health disease with increasing prevalence globally. Recent estimates by the World Health Organisation (WHO) shows that approximately 300 million adults have diabetes and in year 2030 the number of patients diagnosed with diabetes will reach approximately 500 million globally (1). Diabetes is a metabolic disease that involves the effect or endogenous production of the vital hormone insulin (2). Insulin the main mediator of glucose uptake into cells and tissues is the most important regulator of glucose levels in the body (2). There are two major types of diabetes: Type 1 diabetes (T1D) which accounts for approximately 10% of all cases and type 2 diabetes (T2D) which accounts for approximately 90% of diabetes cases (2). T1D, formerly known as juvenile diabetes or insulin dependent diabetes mellitus commonly occurs in childhood. T1D is considered as an autoimmune disease where the insulin producing β-cells of pancreas are destroyed by the immune system. Eventually T1D patients will have complete insulin deficiency and treatment with an exogenous supplementation of insulin is essential for survival (2;3).
T2D, formerly known as non-insulin dependent or adult-onset diabetes, is characterized by a resistance to the action of insulin, a relative deficiency of insulin production, or both(2).

Insulin resistance

The vast majority of patients with T2D are obese and recent epidemiological studies have shown that insulin resistance due to obesity is the main risk factor for the development of T2D (4). Insulin resistance which is described as a defective action of insulin to exert proper metabolism of carbohydrates, but also lipids and proteins in primarily in liver, skeletal muscle and adipose tissue will lead to glucose intolerance(5). In adipocytes, insulin promotes energy storage by increasing glucose and free fatty acid (FFA) uptake, and stimulating lipogenesis while inhibiting lipolysis (6). In skeletal muscle, insulin enhances glucose and amino acid uptake and stimulates glycogen (GLY) and protein synthesis (7). In both adipocytes and skeletal muscle insulin activates the translocation of the glucose transporter 4 (GLUT4) enabling glucose uptake (6;7). In liver insulin activates the transcriptional regulator sterol-regulated element binding protein 1c (Screbp1c) which promotes the regulation of several enzymes in the regulation of liver glucose metabolism resulting in net glucose uptake and de novo synthesis of GLY and triglycerides (TG). Screbp1c's increases the expression of glucokinase (GK) pyruvate kinase, lipoprotein lipase, fatty acid synthase (FAS) and acetyl-CoA carboxylase (ACC) and decreases gluconeogenic enzyme expression such as fructose 1,6-

bisphosphatase, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphotase (G6Pase) (reviewed in (8)). Liver GK, the rate limiting enzyme for net liver glucose uptake synthesizing the phosphorylation of glucose to glucose-6-phosphate (G-6-P), has been shown to be an important regulator of whole body glucose homeostasis (9). Liver GK with a Km =10-12mM has a low affinity to glucose(10) and during basal glucose levels (4-6 mM) the major part of the GK is bound to its GK regulatory protein (GKRP) in the nucleus. However when subjected to high glucose concentrations (>10mM) GK is released from the GKRP and translocated to the cytoplasm and becomes active (11).

In liver, adipocytes and skeletal muscle insulin exerts its effect by binding to its receptor. This in turn enables the insulin receptor to phosphorylate several different isoforms of the insulin receptor substrate (IRS) on tyrosine residues. This phosphorylation serves as a docking site for downstream effectors such as the serine/threonine protein kinase 1 (Akt1). This phosphorylation step of IRS by insulin receptor is considered to be a critical step in insulin signalling and numerous studies suggests that the major cause of insulin resistance is via phosphorylation of insulin receptor and/or IRS on certain serine/threonine residues instead of tyrosine residues. In particular, phosphorylation of the IRS molecules on specific serine/threonine residues, for instance serine 307, by certain kinases results in decreased ability of insulin receptor to phosphorylate tyrosine residues of IRS and eventually result in degradation of the IRS, thereby impairing insulin signalling (reviewed in (12)). Multiple hormones and metabolites have been shown to be implicated in this process. For instance, tumour necrosis factor-alpha (TNF-α)(13) and FFA(14;15), the levels of which are increased in obesity(16;17), have been shown to impair insulin signalling via serine/threonine phosphorylation of insulin receptor and/or IRS. Moreover studies in rodents have shown that long term high fat feeding will induce whole body insulin resistance with a concomitant reduction in liver GK (18) and muscle/adipose GLUT4 (19;20) expression and activity resulting in impaired glucose tolerance (IGT).

Glycated Haemoglobin

Regardless type of diabetes the main hallmark of diabetes is elevated glucose levels or hyperglycemia in the circulation. According to recent guidelines by the United states National Diabetes Data Group, diabetes is defined as having fasting glucose levels above 7 mM or 2-hour plasma glucose levels after an 75 g oral glucose tolerance test (OGTT) above 11.1 mM (21). Glycated haemoglobin (Hba1c) is a weighted measure of the average blood glucose level over the past 90 days in humans and 30 days in rodents thus reflecting the degree of glucose exposure over a certain period of time and is often used to measure how well diabetes is being managed (22;23). Patients with diabetes, especially type 1, routinely test their blood glucose levels, and according to Nathan et al, there is a direct, consistent, and linear relationship

between mean glucose and Hba1c (24). Therefore Hba1c is a good indicator of how well glucose levels are being managed over time and has become the "Golden Standard" for both managing and diagnosing diabetes.

Diabetes late complications

Diabetes affects the life quality of many individuals with pre-mature morbidity consequently leading to early mortality and these detrimental consequences of diabetes is strongly associated with the development of late stage diabetes complications (25;26). Diabetic complications are often divided into microvascular and macrovascular complications. Microvascular complications defined as the destruction of small blood vessels will lead to the development of diabetic eye disease in form of retinopathy, kidney failure in form of nephropathy, peripheral vascular disease, such as amputation of foot and leg and gastro-paresis in form of neuropathy (27;28). Macrovascular complications are defined as the detrimental effects on the larger vascular system such as the arteries and heart. These may include the development of hypertension, endothelial dysfunction, atherosclerosis and cardiovascular disease (CVD) (29;30).
Diabetes is the leading cause for newly diagnosed kidney failure and blindness and more than 60 % of non-traumating leg amputations are among diabetes patients. Furthermore deaths due to heart disease and strokes are more prevalent among diabetic patients than the general population and accounts for approximately 65% of all pre-mature mortality among diabetes patients (31). Due to the very high incident of diabetes and the numerous diseases related to diabetes it is a very costly disease. Diabetes is a significant financial burden to many national economies. Recent reports shows that in the United States of America (USA) diabetes costs approximately 132 billion US dollars and accounting for the vast majority of the budget used for healthcare (32). The magnitude of both health economic costs but also disease related detrimental consequences of diabetes a better understanding of diabetes related late complications are highly warranted.

Microvascular complications

Diabetic retinopathy affecting approximately 40% of all diabetes patients is the leading cause of new cases of legal blindness among adults aged 20 to 74 years in USA (33). The development of diabetic retinopathy is highly dependent on the severity and duration of hyperglycemia. There are several proposed pathological mechanisms by which diabetes may lead to development of retinopathy. Increased substrate availability in form of glucose may lead to excess free radical production in the retina resulting in loss of pericytes and thickening of basement membranes (reviewed in (34)).

According to recent reports by the American Diabetes Association (ADA) diabetic nephropathy is the leading cause of renal failure in USA affecting approximately 50% of all diabetic patients (35). The pathological changes to the kidney include increased glomerular basement membrane thickness, micro-aneurysm formation, mesangial nodule formation and poducyte loss all leading to renal dysfunction. The underlying mechanism of injury may also involve some or all of the similar mechanisms as in diabetic retinopathy (reviewed in (36)).
Diabetic neuropathy is recognized by the ADA as "the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes". As with other microvascular complications, risk of developing diabetic neuropathy is proportional to both the magnitude and duration of hyperglycemia. The precise nature of injury to the peripheral nerves from hyperglycemia is not known but is likely related to increased free radical production. Peripheral neuropathy in diabetes may manifest in several different forms, including sensory, focal/multifocal, and autonomic neuropathies. More than 80% of amputations occur after foot ulceration or injury, which can result from diabetic neuropathy. Neurological dysfunction may also occur in most organ systems and can manifest by gastro-paresis, constipation, anhidrosis, bladder dysfunction, erectile dysfunction, exercise intolerance, resting tachycardia, silent ischemia, and even sudden cardiac death (reviewed in (37;38)).

Macrovascular complications

The risk of developing macrovascular complications such as CVD among diabetes patients is considerable higher as compared to healthy individuals and it accounts for the vast majority of deaths among patients with diabetes(39). The central pathological mechanism in macrovascular disease is the process of atherosclerosis, which leads to narrowing of arterial walls throughout the body. The pathogenesis of atherosclerosis in patients with diabetes is considered multifactorial affected by hypertension, dyslipidemia, hyperglycemia and chronic inflammation damaging the arterial wall and inducing endothelial dysfunction. In response to vessel wall injury and inflammation monocytes then infiltrate the arterial wall and differentiate into macrophages, which accumulate oxidized lipids, such as oxidized low density lipoproteins (oxLDL), to form foam cells. Subsequently, foam cells stimulate macrophage proliferation and attraction of T-lymphocytes. T-lymphocytes, in turn, induce smooth muscle proliferation in the arterial walls and collagen accumulation. The net result of the process is the formation of a lipid-rich atherosclerotic lesion with a fibrous cap. Rupture of this lesions leads to acute vascular infarctions (reviewed in (40)). Endothelial dysfunction has been demonstrated as one of the initial events in the process of diabetes related atherosclerosis. Reduced production or bioavailability of nitric oxide (NO), a vaso-relaxing molecule, may lead to "stiffening" of vessels and endothelial dysfunction affecting the blood pressure(41). Recently several research groups

have demonstrated that oxLDL particles(42) and the reduction of NO bioavailability(43) are both products of increased free radical production and the unifying theory by Brownlee and college's(27) describing that hyperglycemia increases free radical production, such as super oxides from the mitochondria may suggest that increased oxidative stress as the casual link between diabetes and the development of diabetes related complications (Described in more details in section Oxidative stress and endothelial dysfunction).

Oxidative stress in diabetes

Oxidative stress as the main pathological consequence

During the past decade much focus has been put on the role of oxidative stress in many diseases. There is a growing recognition that oxidative stress may be an important event contributing to the pathogenesis of complications associated with diabetes (44). Individuals both with IGT and diagnosed diabetes and experimentally induced diabetic animal models have shown hyperglycemia induced oxidative stress, probably resulting from an attenuated endogenous anti-oxidant system and/or increased promotion of free radical generation (44-46). Elevated levels of free radicals may cause cell membrane damage due to peroxidation of lipid membranes, protein glycation, and depletion of antioxidant defence enzyme systems all of which can lead to cell and tissue damage and thus contribute to the development and progression of diabetes related late stage complications (47).

Oxidative stress has been defined as a persistent imbalance between the production of reactive oxygen species (ROS) and the natural occurring antioxidant defence mechanism(47). ROS are small charged molecules that include free radicals such as superoxide ($O_2\bullet-$), hydroxyl (•OH), peroxyl ($\bullet RO_2$), hydroperoxyl ($HRO_2\ \bullet-$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO-$)(48). ROS molecules are highly reactive that may cause tissue damage by altering the structure of lipids, proteins and deoxyribonucleic acid (DNA) (48). Increased oxidative stress has been linked with the development of several diseases such as cancer(49) and Parkinson(50). Based on the indications that production of ROS is elevated in diabetic patients oxidative stress has been suggested to play a causal role in the development of diabetes related complications(51). However, in addition to inducing macromolecular damage, ROS can also function as signalling molecules to activate various cellular stress-sensitive pathways (52;53).

Sources of oxidative stress in diabetes

The mitochondria have been described as the main source of ROS production in diabetes(48). Increased flux through the glycolysis and the Krebs-cycle will lead to elevated levels of

nicotinamide adenine dinucleotide dehydrogenase (NADH) and flavin adenine dinucleotide ($FADH_2$). When entering the oxidative phosphorylation, the final step of Adenosine-5'-triphosphate (ATP) production in the mitochondria, the increased electron flow in the mitochondria will increase the membrane potential. In turn this will inhibit electron transport at complex III, increasing the half-life of the free-radical intermediates of coenzyme Q (ubiquinone). As a consequence oxygen molecules are reduced to superoxide (Illustration 1).

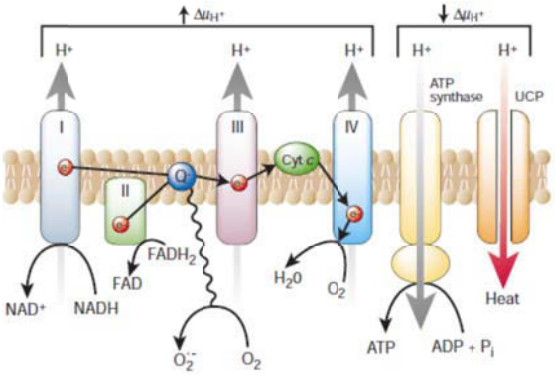


Illustration 1. Figure adapted from (27).Production of super oxides by the electron transport chain in the mitochondria. Increased hyperglycemia derived electron donors (NADH and $FADH_2$) generates a high mitochondrial membrane potential by pumping protons across the mitochondrial inner membrane. This inhibits electron transport at complex III, increasing the half-life of coenzyme Q (ubiquinone). As a consequence oxygen molecules are reduced to superoxide.

The increased superoxide production in the mitochondria may down regulate glyceraldehyde phosphate dehydrogenase (GAPDH), a rate limiting enzyme in the glycolysis thus resulting in increased glycolytic flux through the polyol, hexosamine, Protein Kinase C (PKC) or advanced glycated end (AGE) product pathway. All these four glycolytic pathways have been shown to be involved in the development of diabetes related late complications(27). Lately the PKC pathway has gained a lot attention. The nicotinamide adenine dinucleotide phosphate oxidase (NADPHox) enzyme complex another important source of ROS production particular in vessels is activated upon increased levels of PKC (54;55). NADPHox is an enzyme complex of six subunits (Illustration 2). There are two trans-membrane proteins, $P22^{PHOX}$ and $GP91^{PHOX}$, which constitute the catalytic component. The other subunits $P47^{PHOX}$, $P67^{PHOX}$, $P40^{PHOX}$ and a GTPase, such as Rac, are localized in the cytoplasm during the inactivated state. Physical separation of these subunits provides important regulation of activity and free radical production by preventing any basal free radical production by NADPHox(56).
Increased protein levels of $P22^{PHOX}$, $P47^{PHOX}$ and $P67^{PHOX}$ have been observed in human vessels from patients with T2D (57)while $P22^{PHOX}$ and $GP91^{PHOX}$ expression, both at mRNA and protein level were shown to be up regulated in vessels from diabetic rats (58;59). The specific mechanisms underlying enhanced NADPHox activity in T2D are yet to be fully elucidated but

recent studies have indicated that hyperglycemia stimulated increase in PKC levels may increase the phosphorylation of the cellular subunit $p47^{PHOX}$ and $p67^{PHOX}$ stimulating its membranous translocation and thus activates the NADPHox complex (60). Additionally, others have shown that activating PKC in cell cultures either by high glucose levels or by a specific PKC activator increases the Rac activity thus increasing the NADPHox activity(61). Furthermore, recent reports have shown increased NADPHox during obesity resulting in elevated systemic oxidative stress inducing dysregulation of important adipokines, such as leptin and adiponection thus resulting in the development of the insulin resistance and later on the progression of T2D(62).

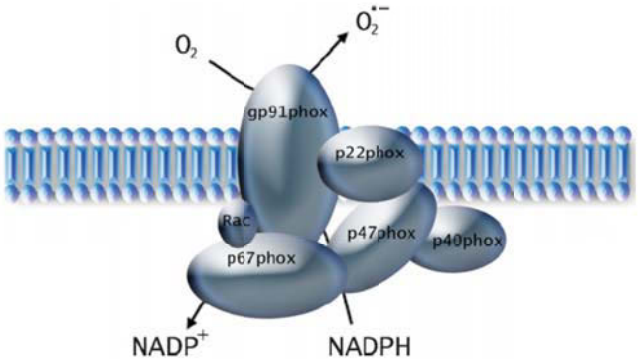


Illustration 2 NADPH oxidase enzyme complex. Illustration adapted from (63).

Antioxidants

During states of oxidative stress the function and the role of oxidative processes in biological and macromolecular systems can be protected by antioxidants(47). Cellular defences against oxidative stress involve dietary and endogenous antioxidants to scavenge and quench ROS in the cell(47). Vitamins C and E are diet-derived antioxidants that detoxify free radicals directly. Vitamin E, a lipophilic antioxidant reacts directly with peroxyl and superoxide radicals and protects membranes from lipid peroxidation(47). The most compelling evidence for an effect of vitamin E in diabetes is on protection against lipid peroxidation, whereas effects on protein and DNA oxidation are less pronounced(64). Vitamin C also known as ascorbic acid (ASC) is probably the most important vascular antioxidant and is essential both as a potent radical scavenger and as a redox modulator of vascular functions (65). Working as an electron donor, ASC is oxidized via a radical intermediate to dehydroascorbic acid (DHA) (66), which is subsequently recycled back to its reduced form inside e.g. erythrocytes and endothelial cells (67). ASC levels in plasma and tissues have been reported to be lowered in diabetic patients and animals and to correlate negatively with early markers of endothelial dysfunction and DNA damage (68-70). Furthermore studies have as well shown that administration of ASC may

counteract hyperglycemic induced endothelial dysfunction thus suggesting that ASC may play a pivotal role in the protection against oxidative stress (71-73).
Endogenous cellular enzymatic and non-enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), or glutathione (GSH) are the most powerful antioxidants in the organism and have been shown to play an important role in diabetes (74). Various isoforms of SOD are found both in the mitochondria, cytoplasm and the nucleus of the cell (75). SOD converts super oxides produced in the body into hydrogen peroxide (H_2O_2) and because of its mitochondrial localization, SOD is considered as the first line of defense against oxidative stress (75). In diabetes the SOD activity and expression in various cells and tissues has been shown to be attenuated thus resulting in increased levels of ROS (76;77). Moreover in patients with coronary artery disease SOD expression was found to be significantly reduced (78). The mechanism for the lowering of SOD activity and expression in diabetes is not fully known however recent studies suggests that due to hyperglycemia the SOD enzyme may be more susceptible to glycation which may result in down regulation of enzyme activity thus resulting in elevated ROS levels (79).
GPx is a selenium-dependent enzyme using the reduced form of GSH as substrate. GPx with the specific H^+ donation by GSH catalyzes the reduction of hydrogen peroxide and lipid-hydroperoxides to water and oxygen (47). In diabetic animals both in liver (80) and aorta (60) and in human vascular endothelial cells (VEC)(81) exposed to high glucose concentrations an abnormal glutathione redox cycle was observed, resulting in an impairment of reduced GSH-dependent hydrogen peroxide degradation.
CAT is located in peroxisomes and catalyses the decomposition of H_2O_2 to water and oxygen(47). Observations regarding CAT activity in diabetes have shown to be complex and in some cases unpredictable. In various tissues from diabetic animals, CAT has shown to be either decreased or increased or in some cases no changes(47).
However, even though some reports have shown either increased activity or no changes in the antioxidant system it is in general acknowledged that the antioxidant capacity in diabetes, both dietary and endogenous, is in overall down regulated either in activity or content leading to a disturbance in the balance between oxidants and pro-oxidant resulting in excess bioavailability of ROS and the induction of oxidative stress.

Biomarkers for oxidative stress

An important prerequisite to evaluate oxidative stress are robust, validated, and reproducible biomarkers of oxidative stress *in vivo* during metabolic disease. Generally, there is an agreement that oxidative stress is highly compartmentalized and therefore the one ultimate biomarker of oxidative stress is unlikely to be identified. Consequently, a range of markers of oxidative stress and damage is necessary to adequately describe the redox status. Biomarkers

of oxidative stress can be classified into several groups: reactive oxygen species, by- or end-products of oxidative damage, antioxidant enzymes and redox status of endogenous antioxidants (82). As highly sensitive markers of oxidative damage in mammalian systems, biomarkers may provide information on three progressive levels of disease outcome: (i) as measurable endpoints of oxidative damage to proteins, amino acids, oxidised lipids, oxidised DNA bases, (ii) as functional markers of blood flow and platelet aggregation and (iii) as endpoints related to a specific disease (83). In diabetes several biomarkers of oxidative stress in different biological samples have been investigated. Of the many biological targets of oxidative stress, lipids are the most involved class of biomolecules. Lipid oxidation gives rise to a number of secondary products. These products are mainly aldehydes, with the ability to further worsen the oxidative damage(47).

Malondialdehyde

Malondialdehyde (MDA) is the most studied product of polyunsaturated fatty acid peroxidation(84). Several studies have shown increased MDA both in plasma and urine and as well in various tissues (47;85). Moreover increased MDA have been found in the atherosclerotic plaques promoted by diabetes(86). The level of MDA is often determined as its natural reactant with thiobarbituric acid producing fluorescent thiobarbituric acid reacting substances (TBARS). This reaction, although simple and reproducible, is unfortunately rather non-specific because thiobarbituric acid reacts with many other carbonyl-containing compounds (87). The use of high performance liquid chromatography (HPLC) coupled with pre-column thiobarbituric acid derivitization can; however, identify the specific malondialdehyde-thiobarbituric acid complex, providing a more specific assay for MDA in biological fluids (87).

8-Isoprostanes

8-Isoprostanes (8-IsoP) are regarded as a specific marker of oxidative stress and are produced by peroxidation of arachidonic acid, a highly unsaturated fatty acid(47). 8-IsoPs are stable, robust molecules detectable in all human tissues and biological fluids. Based on available data, quantification of these compounds in either plasma or urine is representative of their endogenous production and thus gives a highly precise and accurate index of *in vivo* oxidant stress. 8-IsoPs are originally quantified by the use of HPLC methods (88) however lately specific ELISA methods have been developed (89). In diabetes several studies have shown increased levels of 8-IsoP level primarily in urine(90;91) but also in plasma (92) and these alterations have shown to correlate with reduced endothelial function(93;94).

Insulin resistance and oxidative stress

Recently, The Framingham Heart study implying a community based cohort showed that oxidative stress measured as urinary content of 8-IsoP correlated positively with diabetes but also with body mass index (BMI) a measure for obesity(95). Likewise a large number of reports both in humans and animals have suggested that oxidative stress is increased during obesity (62;96). Both high fat feed and genetically obese insulin resistant animal models have shown increased ROS production in liver (97;98), muscle and adipose tissue (62;99;100). Furthermore, cell based studies have reported that increased levels of ROS may disturb the translocation of GLUT4 to the cell membrane thus attenuating the insulin mediated glucose uptake in skeletal muscle and adipose tissue(101;102). In the liver the study by Takamura et al in T2D subjects showed that ROS levels in liver positively correlated with increased expression of gluconeogenic enzymes resulting in increased liver glucose production (103). This hypothesis that ROS may affect the glucose utilization in liver, muscle and adipose tissue is further supported by numerous cell based studies showing that ROS molecules may affect the insulin signalling cascade by augmenting the degradation of the IRS1 thus inducing insulin resistance(104;105). In fact this down regulation of glucose uptake in specifically insulin responsive tissue has been suggested as a protective mechanism against further oxidative stress (106).

Oxidative stress and endothelial dysfunction

The vascular endothelium, composed of trillion endothelial cells is the largest organ in the human organism and is defined as the inner surface of veins and arteries (107). The vascular endothelium is an important organ having a pivotal role in maintaining cardiovascular homeostasis under physiological conditions by releasing vasoactive substances regulating the vascular tone. The endothelium releases vasoactive substances in response to mechanical stimuli such as pressure or shear stress and hormonal stimuli in form of insulin. NO formed by the Nitric Oxide Synthase (NOS) is a potent signalling molecule and vasodilator, responsible for mediating many functions of the endothelium, including control of vascular tone, inhibition of platelet aggregation and leukocyte adhesion, and suppression of vascular smooth muscle cell (VSMC) proliferation. On its release from endothelial cells, NO diffuses to the lumen of the vessel where it exerts its effects on platelet and blood element functions, and to adjacent VSMC where it induces vasodilation and inhibits vascular remodelling and smooth muscle cell proliferation via activation of soluble guanylyl cyclase, present in the cytosol. Activation of guanylyl cyclase causes an increase in intracellular cyclic guanosine monophosphate (cGMP), which in turn activates protein kinase G (PKG), leading to reduced intracellular calcium and vasodilation(Reviewed in (108)). A reduced bioavailability of NO is one of the initial events in

the development of endothelial dysfunction (109) and reduced NO levels has been observed in obese (110) and insulin resistant T2D patients (111-113). Increased ROS production have shown to accelerate NO oxidation, yielding the highly reactive peroxynitrit (ONOO-) as a by-product, which in turn is capable of inducing oxidation of macromolecules such as lipids, proteins and DNA (47). NADPHox has been described as being the main source for ROS production in the vascular endothelium and positively correlated with endothelial dysfunction and clinical risk factors for CVD in humans (114-116). In addition inhibition of the enzyme is seen to improve endothelial function in both rat and human blood vessels (117).
In diabetes VEC's have been described to be more prone to oxidative damage compared to other cell types. VECs are referred to as "glucoseblind", due to their inability to down regulate the rate of glucose transport when exposed to high glucose levels for longer periods (118). These observations have led investigators to suggest that VEC are unique in their inability to modify glucose transport when exposed to extracellular high glucose concentrations, and thant an unregulated influx of high glucose levels may cause overproduction of ROS, which may consequently impair VEC functions (119;120). Glucose in VEC is taken up by facilitated diffusion mediated trough GLUT1 by an insulin independent manner(121). Studies both in human and bovine VEC's have shown a lack of down regulation of GLUT1 when exposed to chronic high levels of glucose (118). Excess glucose may activate PKC by several mechanisms, which consequently increases NADPHox activity (27;114;122). In addition increased oxidative stress has been shown to increase the adhesion of monocytes into the vessel wall by increased expression of Intercellular Adhesion Molecule 1 (ICAM1), Vascular Adhesion Molecule (VCAM1) and E-selectin which is thought to be an initial step in the formation of atherosclerotic plaques (123).

Postprandial hyperglycemia and Oxidative stress

Definition of postprandial hyperglycemia

The classic definition of postprandial glucose refers to blood glucose concentrations after ingestion of a meal. Many factors are defining the glucose profile after a meal including timing, quantity, composition of the meal in terms of carbohydrate content and the hormonal response in particular insulin and glucagon secretion and action. To fully absorb a meal it takes approximately 4-6 hours thus it still remains open when is the proper time point to measure blood glucose and use as a marker for postprandial hyperglycemia. In healthy subjects the prandial glucose profile is strictly controlled and will only in very rear cases exceed 7.8 mM while in diabetic subjects the postprandial phase is often characterized as a rapid and large change in blood glucose levels(124). Although, according to the ADA postprandial

hyperglycemia is defined as having blood 2-hour blood glucose after a meal or a 75 g OGTT challenge above 7,8 mM (125).

Postprandial hyperglycemia and diabetic late complications

As earlier described the development of diabetes related complications are highly dependent on the level of hyperglycemia. Although, using Hba1c as a marker for assessing the risk of developing diabetes related late complications has raised some concerns(125;126). Hba1c has not a biunivocal (one-to-one) correlation with glycemic profiles (127;128), meaning that subjects with the same mean glycemic value and Hba1c level could have different glycemic profiles. In example, as shown in Illustration 3, diabetic subjects (A and B) having similar mean and fasting plasma glucose levels may have a different glycemic profile. Thus sudden fluctuations in glucose levels may not necessarily be accounted for by Hba1c.

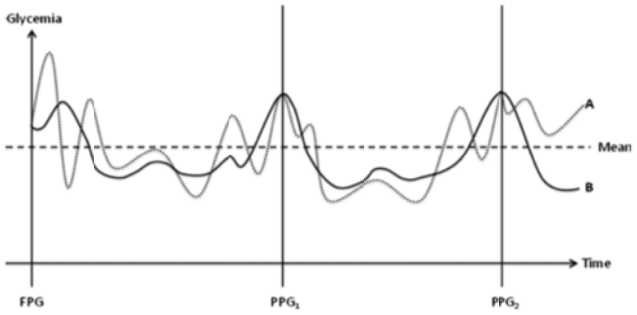


Illustration 3. Glycemic profile of two diabetic subjects, A and B. Both subjects have similar fasting glucose and Hba1c levels but different glycemic profiles.

The United Kingdom Prospect Diabetes Study (UKPDS) was initiated at 1977 enrolling more than 3500 newly diagnosed patients T2D. This study was design in order to investigate whether glycaemic control in T2D would prevent the development of diabetes related late complications. The major outcome of this study was that by 1 % reduction of Hba1c may significantly reduce the incidents of all kinds of diabetes related late complications. However when the glycemic lowering effects were distinguished between micro- and macrovascular complications only a significant lowering effect of microvascular complications were evident and not on macrovascular complications (129;130). These findings were further supported by another prospect study the Diabetes Complications and Control Trial (DCCT) on T1D patients showing that T1D patients with similar Hba1c levels had different risk profiles of developing retinopathy in particular. The investigators hypothesized that repeated incidents of post prandial hyperglycemia, which is necessarily not accounted for by Hba1c, may represent an independent risk factor for the development of diabetes related late complications (Reviewed in (131;132)). In support of these findings several epidemiological studies have been

published, showing indications that postprandial hyperglycemia both in diabetic but also in healthy individuals may be an important risk factor for the development of diabetes related macrovascular complications. In these studies, as outlined in Table 1, it was shown that 1 or 2-hour glucose levels after a 75g OGTT or a test meal challenge may be a better predictor of CVD and mortality in diabetes patients than Hba1c.

Table 1 Epidemiological studies showing an association between postprandial hyperglycemia with risk of CVD and mortality. RR: relative risk; HR: Hazard risk. Table adapted from (133)with modification.

STUDY	Year of publication	Study setup	Conclusion
Chicago Heart Study(134)	1997	12220 NGT/IGT/T2D white and black American men	CVD mortality had a RR:1.18 in patients with 2-h post-challenge glucose > 8.9 mM versus normo-glycemic patients.
Rancho Bernado study(135)	1998	1858 T2D Caucasian adults of European ancestors in California.	HR for CVD (2.6) and mortality (2.9) in patients with 2-h post-challenge glucose > 11.1 mM versus normo-glycemic patients.
Hoorn Study(136)	1999	2363 IGT/T2D men and women	CVD mortality had a RR:3.31 in patients with 2-h post-challenge glucose > 11.1 mM versus normo-glycemic patients.
Honolulu Heart Program(137)	1999	8006 Japanese-American men with IGT/T2D	CVD mortality had a RR:3.49 in patients with 2-h post-challenge glucose > 12.5 mM versus normo-glycemic patients.
Mauritius/Fiji/Nauru study(138)	1999	9179 NGT/IGT/T2D men and women from maurtius	CVD mortality had a HR:2.3 (men), 2.6 (women) in patients with 2-h post-challenge glucose > 11.1 mM versus normo-glycemic patients.
DECODE(139)	2001	22514 IGT men and women from Europe	HR for all-cause mortality = 1.73 for 2-h post-challenge glucose >11.2 mM; HR for CVD mortality = 1.40; HR for CHD mortality = 1.56; HR for stroke mortality = 1.29
Framingham Offspring study(140)	2002	3370 NGT/IGT American men and women	RR for CVD in patients with 2-h post-challenge glucose >11.1 mmol/L = 1.42
Cardiovascular health study(141)	2002	4014 NGT/IGT/T2D American men and women	HR for CVD = 1.29 for 2-h post-challenge glucose > 8.5 mM.
DECODA(142)	2004	6817 IGT men and women from Asia	RR all-cause mortality for 2h post-challenge glucose >11.1 mM = 2.80; RR of CVD mortality for 2h post challenge glucose >11.1 mM = 3.42.
Whitehall Study (143)	2006	17869 NGT/IGT male from United Kingdom	HR in patients with 2-h post-challenge glucose >11.1 mM for CVD mortality = 3.2 and stroke mortality = 1.16 vs.normoglycemic control subjects

In the STOP-NIDDM, one of the first intervention trials investigating the role of post prandial hyperglycemia, subjects with IGT were treated with an α-amylo glycosidase inhibitor, delaying the uptake of glucose from the gut thus targeting post prandial hyperglycemia. In this study a reduction in post prandial hyperglycemia showed a great impact both in the risk of progression to T2D (36% reduction) and also in the development of hypertension (34% reduction) and cardiovascular events (49% reduction)(144-147). Moreover in a study by Esposito *et al* in T2D patients, lowering the incidents of post prandial hyperglycemia by an insulin releasing agent

had a great impact on carotid intima-media thickness, a clinical marker for atherosclerosis, and systemic vascular inflammation (148). Based on the above described epidemiological and intervention trials it was hypothesized that having poorly controlled glycemia such as repeated incidents of post prandial or fluctuating hyperglycemia will increase the risk of developing diabetes related macrovascular complications(132;149;150).
Importantly none of the above described studies were designed to investigate the role of oxidative stress in the development of diabetic late complication. However, lately much effort has been put to investigate whether fluctuating hyperglycemia may increase oxidative stress and thereby promote the development diabetes related late complications however, so far the exact sequences of biochemical events have not been fully elucidated.

Human mechanistic in vivo studies

Initial studies by Ceriello and colleges reported that sudden increase in glycemic levels both in healthy and in diabetic subjects resulted in a significant lowering of systemic antioxidant levels indicating attenuated production of ROS (151-153). The study by Monnier *et al* (90) in patients with T2D showed that with higher degree of fluctuating hyperglycemia urinary marker for oxidative damage were increased thus suggesting that fluctuating hyperglycemia may increase oxidative stress and that this may represent an Hba1c independent risk factor for the development of particularly diabetes related macrovascular complications. Recently similar findings were reported both in T2D patients and in IGT obese subjects suggesting that postprandial hyperglycemia is an independent predictor of cardiovascular events even in non-diabetic individuals (154-156). A more mechanistic approach was done by Ceriello et al. Here both healthy and type 2 diabetic subjects were challenged with intra venouse infusion of either sustained or pulsatile glucose. The group of patients receiving the pulsatile infusion showed increased oxidative stress by increased plasma 3-nitrotyrosin levels and endothelial dysfunction measured by flow mediated dilation. These detrimental effects by fluctuating hyperglycemia were normalized when co-infusing ASC counteracting the increased ROS production (71;72). As described in section Antioxidants, ASC levels in blood is often used as a marker for total anti-oxidant capacity. In connection Choi et al reported that the level of DNA damage was inversely correlated with ASC plasma concentrations (70). In a recent study in T2D patients ASC supplementation reaching supra physiological plasma concentrations decreased postprandial plasma MDA levels and improved plasma lipid profiles, suggesting that ASC supplementation may be beneficial in reducing post prandial induced oxidative stress(157). In support of these findings a recent study by Meugnier et al in healthy volunteer's subjected to an acute glucose infusion showed a global down regulation of gene expression involved in the endogenous anti-oxidant system both in skeletal muscle and in

adipose tissue (158). Thus it has been suggested that a reduction in antioxidant capacity as one of the causes for increased oxidative stress during fluctuating hyperglycemia(132).

Animal models

Several different murine animal models have been used for the study of fluctuating hyperglycemia and oxidative stress. Unlike in humans mice and rats do not develop spontaneous diabetes thus either gene manipulated or chemically induced diabetic models are often preferred (159).

Recently much attention has been put into the novel non-obese T2D rat model, Goto-Kakizaki. The Goto-Kakizaki rat is developed from selective breeding of the Wistar rat for glucose intolerance. The Goto-Kakizaki rat displays a reduced β-cell function resulting in a marked glucose intolerance and frank diabetes. The model does not display obesity, dyslipidaemia or insulin resistance (160;161). The Goto-Kakizaki rat has proved to be an important model for studying the effect of fluctuating hyperglycemia and the risk for developing diabetes related macrovascular complications independent of obesity, dyslipidemia or insulin resistance. Increase in number of monocytes adhering to endothelial cells in thoracic aorta has been reported during an acute glycaemic pulse. Like-wise chronic fluctuating hyperglycemia induced by scheduled feeding in Goto-Kakizaki rats also induced a greater degree of monocyte adhesion and increased VCAM1 expression and this effect was significantly higher than in *ad libitum* fed sustained hyperglycaemic rats, despite much lower Hba1c levels(162;163). Similar as in the human situation (144-147) treating against fluctuating hyperglycemia with insulin or acabarose (α-amyloglucosidase inhibitor) without affecting the Hba1c had significant effect on the degree of monocyte adhesion and intima thickening of the vessel wall (164).

Chemically induction of diabetes by either streptozotocin (STZ) or alloxan is far the most used model. Both STZ and alloxan are highly toxic agents to the insulin producing β-cells resulting in β-cell apoptosis and diminished insulin levels(165). Numerous studies have shown increased oxidative stress in STZ induced diabetic models (76;166). However a more mechanistic approach was evident in the study by Horvath et al. Here STZ induced diabetic rats with fluctuating glucose levels obtained by multiple low dose fast acting insulin injections had considerable higher activity levels of the nuclear enzyme poly(ADPribose) polymerase (PARP) in thoracic aorta than in non-treated overt sustained hyperglycemic rats (167). PARP plays a pivotal role in numerous physiological mechanisms such as DNA repair, regulation of genomic stability and gene expression and increased oxidative stress, was suggested as the casual effect. In addition in the same study ex-vivo analysis on thoracic aorta also showed that the fluctuating glucose group had a weaker response to acetylcholine-induced endothelium dependent vascular relaxation. Fluctuating hyperglycemia has also been investigated in non-diabetic models. Recently challenging apo-lipoprotein E (APOE) deficient mice which a more

prone to develop atherosclerotic plaques with daily oral glucose boluses accelerated the formation of aortic lesions (168). Interestingly in none of the above described studies showed effects on oxidative stress parameters in neither urine nor plasma. Obesity and insulin resistance has been described as significant contributors to the induction of oxidative stress and the fact the above described models are neither obese nor insulin resistant may have resulted in these findings.

In vitro studies

An often used approach to investigate the effect of fluctuating hyperglycemia and oxidative stress is cell based assays. Culturing cells are generally considered as un-physiological, and caution is needed in extrapolating results to the in vivo situation, even though many studies have been conducted showing a clear connection between fluctuating hyperglycemia and increased oxidative stress.

A set of cell based studies by Quagliaro et al demonstrated that subjecting human VEC's to intermittent high glucose levels induced higher degree of apoptosis (169-171). A later study showed that the increased rate of apoptosis by intermittent high glucose was related to increased oxidative stress through PKC activation of the NADPHox enzyme (172). In a similar study by Piconi et al it was shown that enriching the medium with SOD these detrimental effects were diminished and markers of oxidative stress were lowered thus again conforming that fluctuating hyperglycemia may induce increased oxidative stress (123). Also in another study by Piconi et al the effects of constantly high and intermittently high glucose on oxidative stress and adhesion molecule (ICAM1, VCAM1 and E-selectin), as well as on interleukin (IL)-6 expression in human VEC's were investigated. It was found that intermittently high glucose was more effective in triggering the generation of nitrotyrosine and inducing the expression of adhesion molecules and IL-6 than stable high glucose (173). Other cell types than aortic endothelial cells have also shown to be more vulnerable to exposure of intermittent high glucose. In INS-1 pancreatic β-cells intermittent hyperglycemia induced a higher degree of apoptosis and decreased the insulin secretory capacity more than chronic hyperglycemia (174;175).

All together as shown in Illustration 4, presence of excess ROS and the induction of oxidative stress in diabetes and diabetes related metabolic disease is clearly a multifactorial metabolic consequence. As reviewed above hyperglycemia is a crucial factor for the excess production of ROS either through mitochondria or the NADPHox system. However, the exposure of glycemia can be described by the function of two components. Either the chronic sustained or the fluctuating hyperglycaemic state. The chronic state is integrated in the level of Hba1c and as earlier described is well acknowledged that the lowering of Hba1c will as well lower the risk of developing microvascular complications. However several direct and indirect evidences support

the concept that fluctuating hyperglycaemia may have a significant impact on the level of oxidative stress. This detrimental effect by fluctuating hyperglycemia has been observed both in diabetic but importantly also in healthy subjects. Fluctuating hyperglycemia has shown to affect the antioxidant capacity and inflammatory responses resulting in excess ROS formation and oxidative stress. The suggested biochemical processes that may be involved are increased NADPHox activity affecting the expression of ICAM and VCAM and resulting in increased oxLDL accumulation in vessels. Moreover several other investigations as well points out that during the pre-diabetic state, recognized by obesity and insulin resistance may also be an important player in this context. Obesity may also increase the inflammatory responses similar to that seen during fluctuating hyperglycemia. In addition increased TNF-α and other inflammatory markers may result in excess ROS and the development of insulin resistance. In turn - insulin resistance affecting both glucose and lipid metabolism primarily in liver, skeletal muscle and adipose tissue resulting in a disturbed substrate (glucose and FFA) disposal may result in excess substrate availability in endothelial cells. Particularly insulin independent glucose uptake may be increased through GLUT1 and thus result in increased NADPHox and vascular oxidative stress.

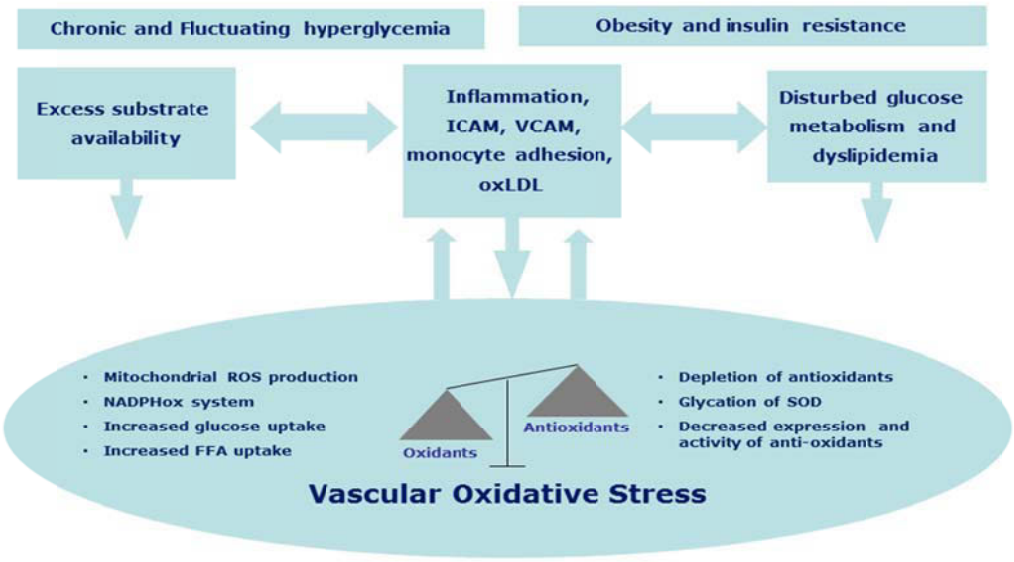


Illustration 4 An overwiev of the metabolic concquences during hyperglycemia and obesity casuing increased vascular oxidative stress.

8 Material and Methods

Animals

All animal experimentation was approved by the Danish Animal Experimentation Inspectorate under the Ministry of Justice and carried out by trained and licensed personnel.

Lean model: Sprague-Dawley male rats (Taconic, Denmark), were used in the study. Upon arrival to the animal unit, the animals were allowed an acclimatization period of at least one week. During the entire study period animals had free access to standard chow diet (Type 1324, Altromin GmBH, Germany) and water (tap water added 1% citric acid). Animals were housed four in each cage in temperature (22 ± 2 °C) and humidity (50± 20 %) controlled rooms. The circadian rhythm was 12h light: 12h dark (Lights on at 06:00 AM).

DIO model: Sprague-Dawley male rats (Taconic, Denmark), were used in the study. Upon arrival to the animal unit, the animals were put on a high fat diet (D12492, Research Diets Inc., USA) for at least 20 weeks in order to develop obesity, insulin resistance and dyslipidemia. During the entire study period, animals had free access to food and water (tap water added 1% citric acid). Animals were housed in pairs in temperature (22 ± 2 °C) and humidity (50± 20 %) controlled rooms. The circadian rhythm was 12h light: 12h dark (Lights on at 06:00 AM).

Surgical procedures

Similar surgical procedures were performed in both animal models. Prior to surgery animals were treated with Anorfin® (0.03 mg/kg; 2ml/kg; GEA A/S, Denmark), a fast acting analgesic and subsequently kept on anaesthesia with Isofluran® (Baxter Pharmaceuticals Inc., USA) and 30%: O_2 / 70%: N_2O (Illustration 5).

Illustration 5. Anesthetized rat placed on a heated laboratory table with suction. The animal was shaved and cleansed with chloride-hexidin at the insertion site.

Silicon catheters (Tygon Microbore Tubing, S-50-HL, Cole Parmer, UK.), kept overnight in 70% ethanol were filled with 100IU/ml heparin (Leo Pharma Nordic, Denmark) in 0,9%NaCl and surgically placed approximately 5 cm in artery carotid (app. 160mm in length = app. 16µl mrk. red) and approximately 2-3 cm in right jugulary vein (app. 130mm in length = app. 13µl, mrk. blue). The jugular vein was used for infusion and the carotid artery for blood sampling (Illustration 6).

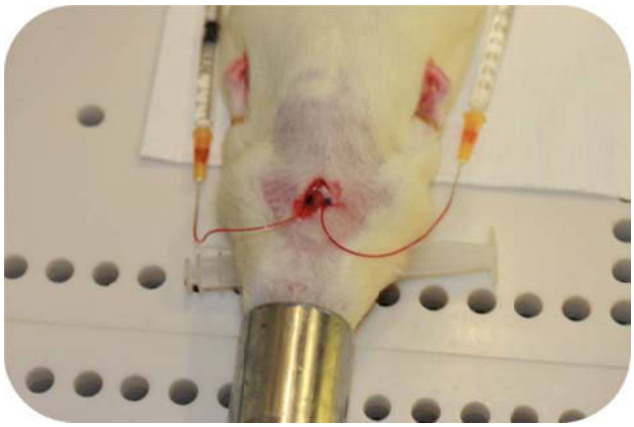


Illustration 6. Surgically inserted permanent catheters in the carotid artery and jugulary vein.

Subsequently a mid-scapular incision (app. 2 cm) on the back, adjacent to the insertion site was made. The subcutaneous tissue was spread by a hemostat creating a pocket. Subsequently the catheters were lead subcutaneously around the neck and through a replacement button (Instech Laboratory Inc., USA) and the replacement button was inserted into the subcutaneous cavity. Finally the catheters were filled with 500IU/ml heparin (LEO Pharma Nordic, Denmark) in HAEMACCEL® and the wound around the button was closed with non-absorbable suture (USP 3/0, Johnson & Johnson, USA). After surgery animals were single

housed and allowed 7-8 days of post-surgery recovery before experimentation. During the recovery period animals were treated with Rimadyl® (5 mg/kg; 2ml/kg; Pfizer, USA) and Baytril® (10 mg/kg; 2ml/kg; Bayer AG, Germany). Body weight and food intake was followed daily. Animal's post-surgery not reaching the same body weight as in the pre-surgical state was not used in the studies.

Study protocols

MR scanning

The day before experimentation body composition was evaluated in live, conscious animals by quantitative nuclear magnetic resonance spectroscopy (EchoMRI 3-in-1 Animal Tissue Composition Analyzer; Echo Medical Systems, Houston, TX). A system test was performed routinely at the beginning of each measurement day, and the equipment was calibrated by scanning a calibration holder containing a known amount of fat to test the validity of measurement. Subsequently the animal was carefully put into a container, placed in the machine and scanned. The measurement was done within 120 sec and the animal was placed back into its cage. The fat content was calculated as the percentage of total body weight.

Glucose infusion

On the day of experimentation all animals had their food removed at 6.00 AM and transferred to clean cages. All animals had their catheters checked for liability. The carotid artery was connected to an Accusampler® (DiLab AB, Sweden) for automated blood sampling whereas the jugular vein catheter were connected to the infusion pump (World Precision Instruments Inc., USA). Experimentation started after 6 hours of fast around 12 pm. The animals were given intravenous infusion of glucose (50% glucose solution; Fresenius Kabi AG, Germany) or saline for 72 (LEAN) or 96 (DIO) hours as outlined in Table 2. Briefly, Animals were divided into four groups (n=7-8) and given either a continuous high (CHG) or low (CLG) or pulsatile (FLU) infusion of glucose for 72 (Lean) or 96 (DIO) hours. The FLU and CLG groups received equal amount of glucose in total through the study period although with different infusion profiles whereas the CHG received three times the amount of glucose in total. The controls received a constant infusion of saline (VEH) equal to the CLG group.

Table 2 Infusion rates (ml/kg/h). The table describes one cycle of vehicle or glucose infusion and the amount of glucose infused per 24 hours. The cycle was repeated continuously during the complete period of 72 hours for the lean animals or 96 hours for the DIO animals.

		0 – 2 min	2 – 32 min	32 – 152 min	Glucose (g/kg/24 hours)
VEH	LEAN	1.2	1.2	1.2	0
	DIO	1.0	1.0	1.0	0
CHG	LEAN	4.0	4.0	4.0	48.0
	DIO	3.0	3.0	3.0	36.0
CLG	LEAN	1.2	1.2	1.2	14.4
	DIO	1.0	1.0	1.0	12.0
FLU	LEAN	30.0	4.5	0	14.6
	DIO	30.0	3.5	0	12.4

The infusions were controlled by a software developed in house (InsulinExtrazaganza, version 1.01). The software was developed in Labview (National Instruments™, USA). As shown in Illustration 7, in the software time (min) for infusion, infusion rates (ml/min/kg), weight (kg) and syringe inner diameter (mm) were filled in. Subsequently the infusion protocol was initiated by clicking on start.

Illustration 7. A screen shot of the InsulinExtravaganza software for infusion. The illustration shows the pulsatile glucose infusion profile with nine glucose pulses in 24 hours.

All blood samples were drawn by the Accusampler®, in K_3-EDTA coated tubes under cooling and immediately centrifuged (4°C; 5000rpm; 3 min). As shown in Illustration 8 blood samples (50ul) for glucose and insulin were drawn at predefined time points. The time points were chosen in order to cover the entire circadian rhythm during the various glucose profiles. Briefly, on each experimental day two periods of fluctuating glucose were covered. The first spike of the day was measured daily and then the every second period each day. On each period blood glucose levels were measured at time points -10, 15, 42 and 60 min. relative to initiation of each pulsatile glucose infusion.

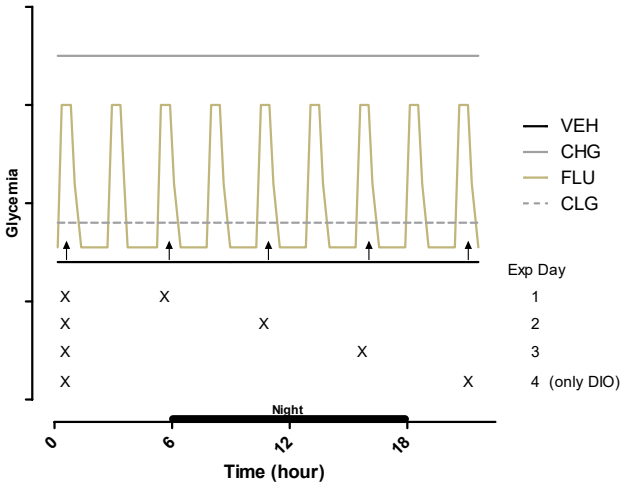


Illustration 8. The illustration shows the expected glucose profiles during 24 hours. In order to cover the complete circadian rhythm blood sampling were performed at predefined time points.

Blood samples (200ul in total) for MDA, ASC, 8-IsoP and FFA were drawn at time points 0, 24, 48 and 72 and 96 (only DIO) hours. Plasma was immediately separated (4°C; 5000rpm; 3 min) and the plasma was divided into several aliquots. For MDA and 8-IsoP plasma aliquots were immediately frozen in dry ice and kept in -80°C until analysis. For ASC the plasma was acidified with an equal volume of 10% (w/v) freshly made meta-phosphoric acid containing 2 mmol/L of disodium-EDTA. The precipitate was removed by centrifugation (4°C; 5000rpm; 3 min) and the supernatant was stored in -80 °C until analysis. For FFA an aliquote of plasma was transferred into sodium fluoride (NaF; 5mg/ml) coated tubes and stored in -80 °C until analysis. In order to account for the haemodilution due to the infusion the haematocrit value were as well determined at the same time points and used to normalize the level of plasma MDA, ASC, 8-IsoP and FFA content.

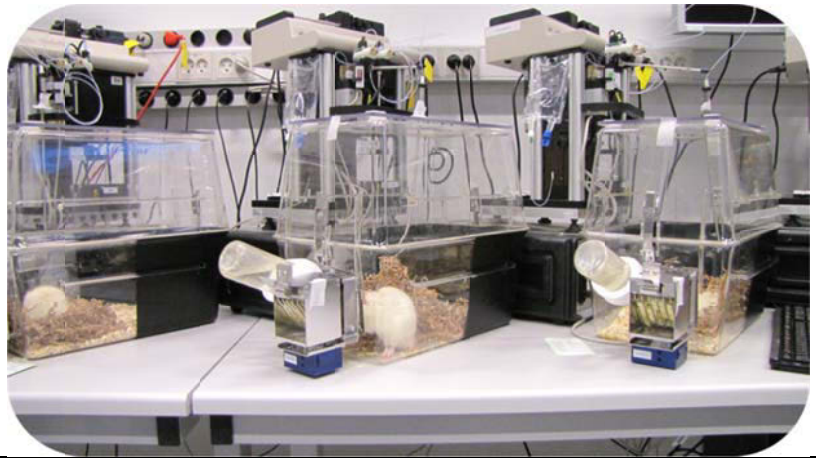


Illustration 9. An illustration of the Accusampler setup. Animals were able to move freely and have free access to food and water during the complete experimentation period.

After 72 or 96 (only DIO) hours of glucose or vehicle infusion animals were disconnected from the Accusampler® and infusion pumps. The animals were anesthetized with Isofluran® and cut opened at the abdominal area and following tissues will be excised and preserved in liquid nitrogen: Liver, left lateral lobe; Thoracic aorta; Gastrocnemius muscle; Kidney and Mesenteric white adipose tissue. Consequently animals will be euthanized by an injection of pentobarbital (100ul; 200mg/ml) in the heart.

Biochemical analyses.

Chemicals

All chemicals were of the highest quality available. Tris-[2-carboxyethyl] phosphine hydrochloride, phosphotungstic acid (PA), tetramethoxypropane, Butylated hydroxytoluene (BHT), dithiobarbituric acid (TBA) were obtained from Fluka (Milwaukee, IL, USA). meta-Phosphoric acid, disodium EDTA, glacial acetic acid, n-Butanol, dihydrosulphuric acid (H_2SO_4) were obtained from Merck (Darmstadt, Germany).

Glucose

For glucose measurement, plasma samples (10 µl) were diluted in 500 µl of EBIO buffer solution (Eppendorf AG., Hamburg, Germany) and kept on ice until analysis by immobilized glucose oxidase methodology (EBIO Plus Autoanalyzer; Eppendorf AG., Germany).

Insulin

For insulin, plasma samples (10 µl) were transferred to cooled micronic racks and stored at -80°C until analysis by rat insulin sensitive enzyme-linked immunosorbent assay (ELISA) as

described by the assay kit manufacturer (Cat no.:90010, Chrystal Chemistry, USA). Briefly, plasma insulin was determined as single measurements. 5 µl of plasma was incubated with 95 µl sample dilution (supplied by the assay kit manufacturer) for 2h at 4°C. After washing plates were incubated with 100µl rat insulin enzyme conjugate for 30 min at ambient temperature. Next – plates were washed and incubated with 100µl substrate for 11 min. Reaction was stopped and the plate was read at (450 – 630) nm on a SpectraMax ELISA reader (Molecular Devices Inc., CA, USA).

Ascorbic acid

For total ascorbic acid (total ascorbate acid = reduced ASC + DHA measured after reduction with tris-[2-carboxyethyl] phosphine hydrochloride) and DHA analysis. The analytical procedure using HPLC with coulometric detection has been described in detail elsewhere (66;176).

Malondialdehyde

Lipid oxidation was assessed by measuring MDA in plasma, liver or aorta as its genuine TBA adduct by HPLC with fluorescence detection as described previously (177). Briefly, 10 µL of plasma or tissue homogenate (1:9 in dPBS) was placed in a microcentrifuge tube; 500 µL of 42 mmol/L H_2SO_4 was then added and mixed gently, after which 125 µL of 100 g/L PA was added and vortex-mixed. After 5 min at room temperature, the mixture was centrifuged (3 min at 16 000*g*). MDA is associated with the lipoprotein, and consequently was contained in the pellet. The supernatant was discarded, and the precipitate was re-suspended in 300 µL of 42 mmol/L H_2SO_4 and 45 µL of 100 g/L PA, after which the sample was centrifuged (3 min at 16 000*g*). The supernatant was again discarded, and the pellet was re-suspended in 350 µL of H_2O, 50 µL of 0.7 mmol/L BHT in 200 µL/L 96% ethanol, and 100 µL of TBA reagent consisting of 6.7 g/L TBA in H_2O diluted 1:1 with glacial acetic acid. The mixture was heated immediately for 60 min at 95 °C and cooled on ice; the $MDA(TBA)_2$ adduct was then extracted with 500 µL of *n*-butanol. The layers were separated by centrifugation (3 min at 16 000*g*). Calibration curves were constructed using tetramethoxypropane (0.1–5.0 mM for plasma; 2.5–50 mM tissue homogenates). The $MDA(TBA)_2$ was determined on an automated HPLC gradient system by fluorescence detection (excitation, 515 nm; emission, 553 nm) as described in details in (177).

8-Isoprostanes

Plasma 8-Isoprostanes were measured as described by the assay kit manufacturer (Cat no.:516351, Cayman Chemicals, USA). Briefly 40 µl of plasma were purified from proteins and phospholipids by Waters Ostro 96-wells plate 25 mg (Cat. No.: 186005518, Waters Inc., USA). Subsequently all samples were air dried (Heto-Vac, Biostadt, Denmark) and re-suspended with

120 µl EIA buffer supplied by the assay kit manufacturer. Samples were then analyzed in duplicate's 50µl on each 96 well pre-coated strips also supplied by the assay manufacturer. Plates were read at (405 - 420) nm on a SpectraMax ELISA reader (Molecular Devices Inc., CA, USA).

Liver triglycerides and glycogen

Liver TG and GLY content were determined by homogenizing weighed liver tissue with a reagent consisting of a sodium acetate buffer mixed with Triton X-100 for 15 seconds by use of homogenizer, Polytron PT 3000 (PT-DA 3007/2 generator, IKA-Werke Germany). Immediately after homogenization, the sample was placed in a bath of boiled water for two minutes, and thereafter kept on slush ice for fast cooling. After cooling, the homogenate was centrifuged and the homogenate was analyzed for TG on the Hitachi 912 analyser, using a commercially available enzymatic triglyceride assay (Cat. No.: 11488872; Roche diagnostics; Switzerland). For glycogen analysis, 25 µl amyloglucosidase (Sigma-Aldrich) was added to the homogenate and it was placed at 20°C overnight before analysis, using a commercially available enzymatic glucose assay (Cat. No.: 11447521; Roche diagnostics; Switzerland) on a Hitachi 912 analyzer (Hitachi, Japan).

Super oxide dismutase activity

SOD in liver and aorta was assessed as described by manufacturer (Cat. No.: 19160, Sigma Aldrich). Briefly, Liver or aorta homogenates (1:9 dPBS, pH=7.45) were centrifuged and the supernatant was used for quantification of SOD activity. The SOD levels were determined in triplicates and expressed in U/mg protein. Plates were read at 450 nm on a SpectraMax ELISA reader (Molecular Devices Inc., CA, USA).

Oxidized LDL

Oxidized LDL in aorta homogenates were assayed by ELISA on pre-coated 96-well strip plates as described by the assay manufacturer (Cat. No.: E90527Ra, Uscn Life Science Inc, China). Briefly, supernatants from aorta lysates, prepared as described above were diluted x300-1200 in dPBS, ph=7.45 and quantified in duplicates and expressed as ng/mg protein. Plates were read at (450) nm on a SpectraMax ELISA reader (Molecular Devices Inc., CA, USA).

Western blotting

Tissue protein lysates were assayed with antibodies against GS (Cell Signalling Technology, USA), GLUT1 and $GP91^{PHOX}$ (Abcam, USA) GK and Srebpc1 (2A4) (Santa Cruz, USA). Protein levels were normalized to β-Actin (Abcam, USA). Secondary antibodies were horseradish peroxidise-coupled and ECL reagent (BioVision, USA) was used for detection. Quantification

was performed using ImageGuage 4.0 (Fujifilm, Japan). Briefly, 50 mg of liver or aorta were homogenized in 1 ml protein lysate buffer containing (Cell Extraction Buffer, Invitrogen, Life Technologies Ltd, UK; Protease inhibitor, P2714, Sigma Aldrich & AEBSF, Calbiochem) by and TissueLysser II (Qiagen, USA). Protein content of the supernatant was determined in order to ensure that the gel was loaded with equal amount of protein form each sample. Prior to load of gel (Criterion 4-12% Bis-Tris gel, BioRad, USA) each sample was added a reducing agent (NuPage®, NP0009, Invitrogen, Life Technologies Ltd, UK) and sample loading buffer (NuPage®, 10482055, Invitrogen, Life Technologies Ltd, UK). The protein bands were transferred to nitrocellulose membrane (IBlot™ Gel transfer, Invitrogen, Life Technologies Ltd, UK). After blocking of the membrane (#37543, Blocking buffer, Thermo Fisher Scientific, USA) the blots were incubated with antibodies as outlined in
Table 3

Table 3. List of antibodies used in western blot analyses.

Primary antibody				**Secondary antibody**		
Against	Dilution		vendor	Against	Dilution	Vendor
	Liver	Aorta				
Glucokinase	1:1000	N/A	Cat. No.: sc-11416, Santa Cruz Biotechnology Inc., USA	Rabbit	1:3000	Cat. No.: 31460, Thermo Fisher Scientific, USA
Glycogen synthase	1:500	N/A	Cat. No.: 3893, Cell Signalling Technology Inc., USA	Rabbit	1:3000	Cat. No.: 31460, Thermo Fisher Scientific, USA
Screbp1c	1:500	N/A	Cat. No.: sc-8984, Santa Cruz Biotechnology Inc., USA	Rabbit	1:3000	Cat. No.: 31460, Thermo Fisher Scientific, USA
GLUT1	N/A	1:100	Cat. No.: Ab-652, Abcam, USA	Rabbit	1:3000	Cat. No.: 31460, Thermo Fisher Scientific, USA
$GP91^{PHOX}$	1:500	1:500	Cat. No.: 611415, BD Transduction Laboratories™, USA	Mouse	1:3000	Cat. No.: 31470, Thermo Fisher Scientific, USA
B-actin	1:5000	1:5000	Cat. No.: Ab6276, Abcam, USA	Goat	1:3000	Cat. No.: 31430, Thermo Fisher Scientific, USA

Horseradish peroxidise-coupled and ECL reagent (BioVision, USA) was used for detection. Quantification was performed using ImageGuage 4.0 (Fujifilm, Japan).

RT-PCR

RNA was isolated from tissue using the RNeasy Mini Kit (Qiagen, USA). cDNA was synthesized using the iScript kit (Bio-Rad, USA). Primer-probesets were from TaqMan Gene Expression Assays (Applied Biosystems, USA) (except Srebp1c) and PCR reactions were performed using a TaqMan Master mix (Applied Biosystems, USA) and a MX3000P system (Agilent, USA). Srebp1c

custom primer-probe sets were purchased from Applied Biosystems with the sequences: Forward: CGCTACCGTTCCTCTATCAATGAC; Reverse: AGTTTCTGGTTGCTGTGCTGTAAG; Probe: GTGGTGGGCACTGAGGC. Ct values were normalized to those of Ppib.

Statistics

Statistical analyses of the results were performed by using SAS JMP software (version 8.1 for Windows, SAS institute, Cary, NC). Plasma MDA, ASC, %DHA, 8-IsoP and FFA were analysed using ANOVA with time 0 hours as covariant and then a fit model with Group, Time and Group x Time interaction as model based variables. Liver and aorta metabolic parameters were analysed using two-way ANOVA having Group, Diet and Group x Diet interaction as model based variables. In cases of statistical significance, Tukey's post hoc test was applied. A p-level less than 0.05 was considered statistically significant.

9 Results

Animal data

Table 4

	Lean (n=30)	DIO (n=30)
Age (Weeks)	20-23	27-31
Weight (gram)	400-430	700-800
Fat content (%)	18	31

Animals on a normal diet (LEAN) had significantly lower body weight and body fat content as compared to DIO animals.

Plasma Glucose

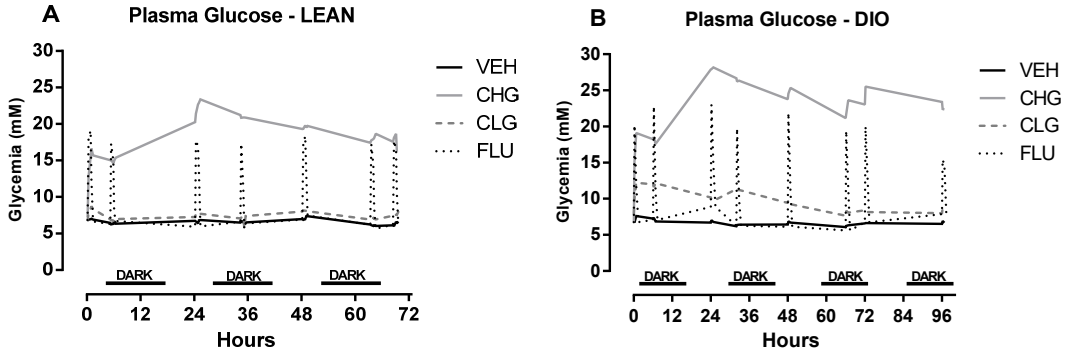


Figure 1. Plasma Glucose profiles in lean (A) and DIO (B) rats. Data are shown as means, (n=7-8).

At baseline both the LEAN and the DIO rats had similar and normal glycemic level (5-6 mM). In the CHG groups, hyperglycaemia (PG > 22 mM) were immediately manifested and maintained throughout the study. In the DIO rats, the CHG group reached slightly higher levels of chronic hyperglycemia (27 mM) as compared to the LEAN-CHG. Like-wise in the CLG groups, receiving one third the amount of glucose as the CHG groups, the DIO rats showed a rapid increase of glucose levels to app. 12mM but levelled out during the experimentation and was almost back to normal at the end of the study whereas in the LEAN situation the CLG group showed very slightly elevated levels of glycemia (7-8 mM). In the FLU groups, receiving the same amount of glucose as the CLG groups but by pulsating infusion, we managed to simulate repeated incidents of hyperglycemic spikes. Again in the DIO rats the level of

hyperglycemia reached during these spikes seemed to be slightly higher as compared to their LEAN counterpart. The plasma glucose levels were consistently elevated to about 20-22 mM in the DIO rats and 18-20 mM in the LEAN rats at peak level and returned to basal levels within 30 min after infusion of glucose was paused.

Plasma Insulin

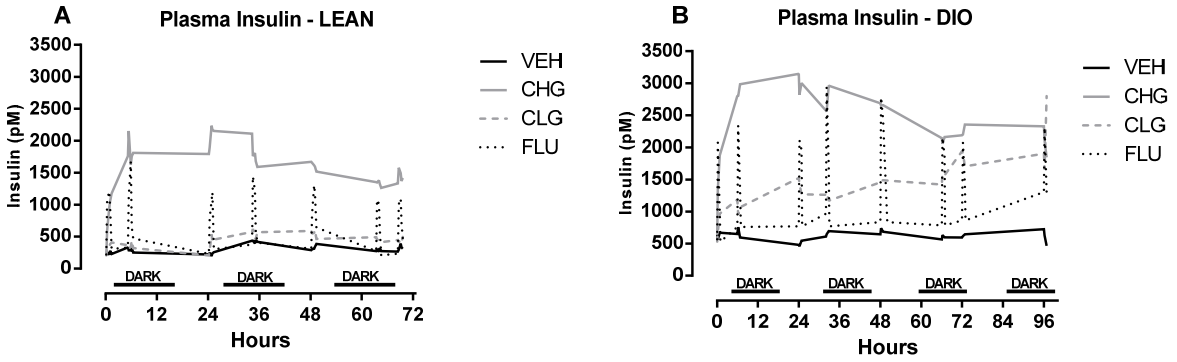


Figure 2. Plasma Insulin profiles in lean (A) and DIO (B) rats. Data are shown as means, (n=7-8).

As expected, at baseline the plasma insulin levels were significantly higher in the DIO rats as compared to the LEAN rats (LEAN: 215 ± 10 pM vs. DIO: 596 ± 32 pM; $p<0.0001$). The DIO rats also reached markedly higher levels of hyperinsulinemia both during chronic low (CLG) and high (CHG) glucose infusion as compared to its LEAN counterparts. Notably, in the DIO rats the CLG rats showed a gradually increase in plasma insulin levels probably counteracting the increased glucose levels as seen in Figure 1B. Again, during pulsatile glucose infusion the DIO rats reached higher levels of plasma insulin at peak level than the LEAN rats (LEAN: 1200-1500 pM; DIO: 2000-2500 pM). Altogether, in general besides having significant higher levels of basal plasma insulin levels the DIO rats as well responded with higher levels of plasma insulin during glucose infusion as compared to the LEAN groups indicating insulin resistance

Plasma FFA

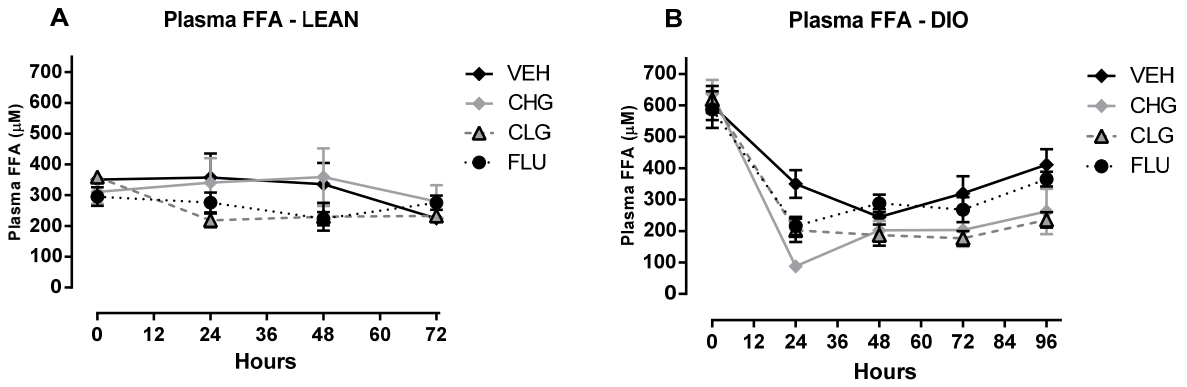


Figure 3. Plasma FFA profiles in lean and DIO rats. Data are shown as means ± SEM (n=7-8).

Plasma FFA levels were monitored daily. At semi-fasted state (6 hours) the DIO rats had significantly higher levels of plasma FFA as compared to the LEAN rats (LEAN: 327 ± 15 µM vs. DIO: 612 ± 23 µM; p<0.0001). In the DIO rats the ANOVA revealed a significant change during time (p=0.0008) and among groups (p=0.0007) but no significance in time*group interaction (p=0,056). In addition, the post hoc test showed a significant reduction in plasma FFA during chronic infusion of glucose (CHG & CLG: p<0.05 vs. VEH; both cases) but not during fluctuating hyperglycemia. In the LEAN groups plasma FFA levels were not affected during glucose infusion as compared to the control groups (p=ns vs. VEH).

Plasma MDA

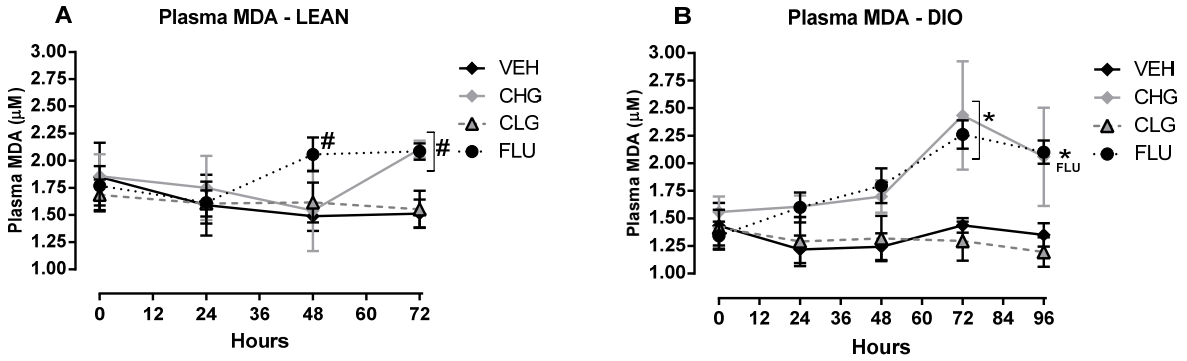


Figure 4. Plasma MDA profiles in lean (A) and DIO (B) rats. Data are means ± SEM, n=7-8. #p<0.05 vs. VEH-LEAN, *p<0.05 vs. VEH-DIO.

Plasma MDA was measured as a biomarker of oxidative damage to lipids. At baseline (t=0 hour) plasma MDA did not differ among groups. In both LEAN and DIO rats ANOVA revealed a significant interaction between time and groups (p<0.0001; both cases). In the lean situation, Tukey's post hoc test showed a significant increase in plasma MDA levels in the FLU group at 48 and 72 hours (48 h: VEH: 1.45 ± 0.15 vs. FLU 2.1± 0.16; 72 h: VEH: 1.5 ± 0.13 vs. FLU:

1.9 ± 0.1; p<0.05), and in the CHG group at 72 hours (VEH: 1.5 ± 0.1 vs. CHG: 2.1 ± 0.1; p<0.05). In the DIO situation we observed similar changes in plasma MDA as seen in the LEAN rats. Plasma MDA was significantly increased in the FLU group at 72 and 96 hours were (72 h: VEH: 1.44 ± 0.06 vs. FLU 2.26± 0.13; 96 h: VEH: 1.35 ± 0.1 vs. FLU: 2.1 ± 0.1; p<0.05 all cases), and in the CHG group at 72 hours (VEH: 1.44 ± 0.1 vs. CHG: 2.45 ± 0.43; p<0.05). In both models we did not observe any changes in plasma MDA for the VEH and CLG group. These findings indicate that the level of systemic oxidative stress is independent of the glycemic exposure but rather the dependent on the glycemic profile.

Plasma 8-IsoP

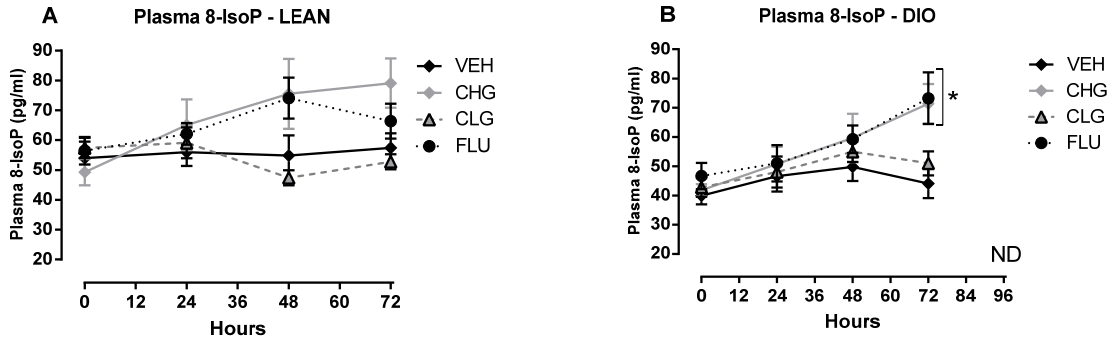


Figure 5. Plasma 8-IsoP profiles in lean (A) and DIO (B) rats. Data are shown as means ± SEM (n=7-8). *p<0.05 vs. VEH-DIO. ND=no data

In order to support the finding above we as well analysed the level of plasma 8-IsoP during the time course. In the LEAN model the ANOVA showed a significant difference among group (p=0.0136). The post hoc test showed a significant difference for the CHG group (p=0.0127 vs. VEH) but not in the FLU (p=0.063 vs. VEH). However when comparing the FLU group with the CLG group, which received the same amount of glucose in total, we observed a significant higher level of 8-IsoP (p=0.023 vs. CLG). In the DIO model the ANOVA revealed a significant interaction between group and time (p=0.03). The post hoc test showed a significant difference in FLU and CHG at the 72 hour time point (p<0.05 vs. VEH; both cases).

Plasma Ascorbate

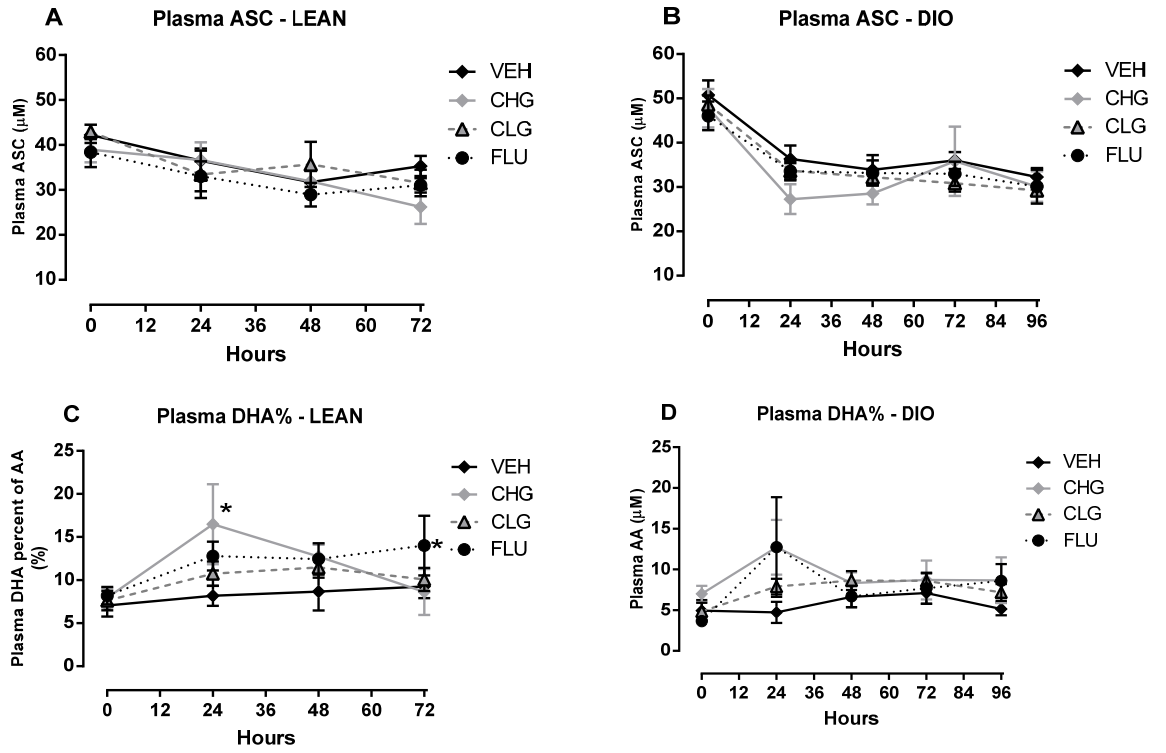
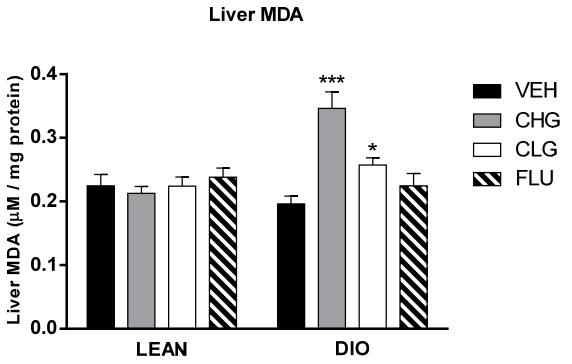


Figure 6. Plasma ASC profiles in lean (A) and DIO (B) and plasma %DHA in lean (C) and DIO (D). Data are shown as means ± SEM (n=7-8). *p<0.05 vs. VEH-DIO.

Plasma ASC has been described as one of the most important antioxidants in the organism. Besides its very efficient reducing capabilities it's also responsible for regeneration of other anti-oxidants such vitamin E. We measured plasma ASC both the reduced and oxidized form and used it as an indicator for the redox balance in plasma. In both models the ANOVA revealed a significant decrease in plasma ASC levels during time ($p<0.0001$, both cases) but not among groups. In the LEAN model plasma ASC levels decreased gradually during the time course to levels around 30-35 µM at the end of the study. In the DIO model the plasma ascorbate was decreased to the same level of around 30-35 µM already apparent after 24 hours of experimentation and subsequently the level of plasma ASC plateaued for the rest of the period. Interestingly in the LEAN situation the ASC oxidation ratio measured as %DHA of total ASC showed a treatment dependable significant difference ($p<0.05$). The post hoc test as well revealed a significant difference for the CHG group at the 24 hour time point (VEH: 8.1 ± 1.1 vs. CHG: 16.1± 4.5; $p<0.05$) and for the FLU group at 72 hour time point (VEH: 9.2 ± 1.3 vs. FLU: 15.7 ± 3.1; $p<0.05$). We did not observe any significant changes in the DIO model.

Liver MDA



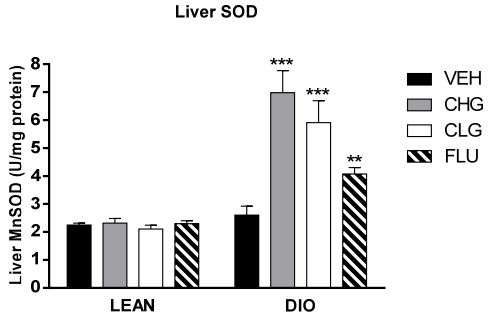
Results from two-way ANOVA for Diet and Group

Source of Variation	**P value**
Interaction	< 0,0001
Group	0,0014
Diet	0,0093

Figure 7. Liver MDA. Data are means ± SEM, n=7-8. *p<0.05, ***p<0.0001 vs. VEH-DIO.

The level of liver MDA was not different among the two models as judged by the MDA levels of the two controls (VEH-LEAN vs. VEH-DIO; p=ns.). As shown in the table above the two-way ANOVA showed a significant interaction between diet and groups (p<0.0001). The post hoc test verified that in DIO rats liver MDA levels were significantly increased in the CHG (p<0.0001 vs. VEH) and CLG (p<0.05 vs. VEH) as compared to controls. We observed no differences among the groups in LEAN rats (p=ns; all cases). These data may indicate that insulin resistant rats are more susceptible to increased ROS production but only when challenged with chronic glucose infusion.

Liver SOD



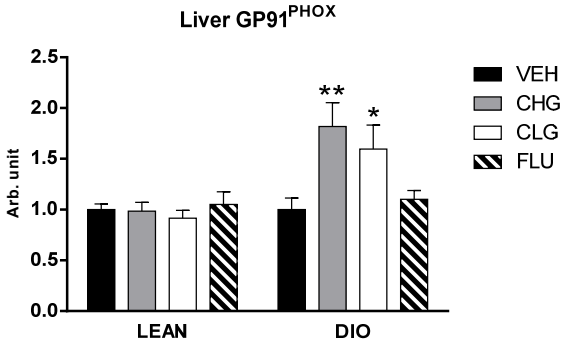
Results from two-way ANOVA for Diet and Group

Source of Variation	**P value**
Group*Diet	< 0,0001
Group	< 0,0001
Diet	< 0,0001

Figure 8. Liver SOD. Data are means ± SEM, n=7-8. **p<0.001, ***p<0.0001 vs. VEH-DIO.

We observed a clear interaction between group and diet (p<0.0001). Animals chronically infused with glucose had a more pronounced impact on the SOD levels showing a 2.7 and 2.3 fold higher levels of SOD activity for the CHG group (p<0.0001 vs. VEH) and CLG group (p<0.0001 vs. VEH) respectively. The FLU group had also higher levels of SOD activity (p<0.001 vs. VEH) but to a lesser degree as seen in the CHG and CLG group. Yet again – we observed no difference between the two controls (p=ns).

Liver GP91^{PHOX}



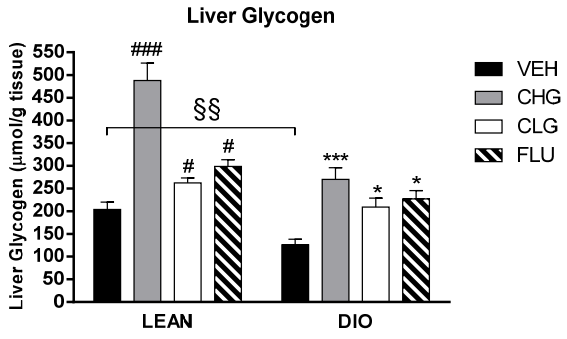
Results from two-way ANOVA for Group and Diet

Source of Variation	P value
Group*Diet	0,0033
Group	0,0668
Diet	0,0010

Figure 9. Liver GP91^{PHOX} protein expression. The level of GP91^{PHOX} protein expression was quantified as the relative protein abundance to β-actin. Data are shown as relative expression to VEH-LEAN. Data are means ± SEM, n=7-8. *p<0.05, **p<0.001 vs. VEH-DIO.

The two-way ANOVA showed an interaction between group and diet (p=0.0033). We observed no change among the groups in the LEAN model. However in the DIO model CHG (p<0.001) and CLG (p<0.05) had significant higher GP91^{PHOX} protein levels as compared to controls.

Liver Glycogen



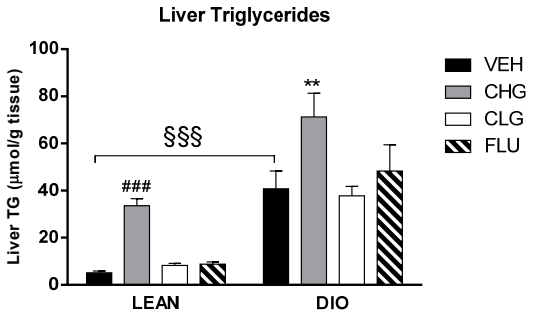
Results from two-way ANOVA for Group and Diet

Source of Variation	P value
Group*Diet	0,0080
Group	< 0,0001
Diet	< 0,0001

Figure 10. Liver GLY. Data are means ± SEM, n=7-8. §§p<0.001, ###p<0.0001, #p<0.05 vs. VEH-LEAN; *p<0.05, ***p<0.0001 vs. VEH-DIO.

The two-way ANOVA revealed a significant interaction between group and diet (p=0.008). As shown in figure 10, all intervention groups had a significant increase in liver GLY levels where the CHG, due to being exposed to three times higher amounts of glucose, showing the largest difference. Of notice, even though the two models were exposed to the same amount of glucose especially the CHG in the DIO model did not show the same rise in liver GLY as seen for in the LEAN animals. Indeed insulin resistance can affect the livers capability of de novo GLY synthesis and as expected insulin resistant rats had significant lower levels of liver GLY (LEAN: 203 ± 17 vs. DIO: 125 ± 12; p<0.001).

Liver Triglycerides



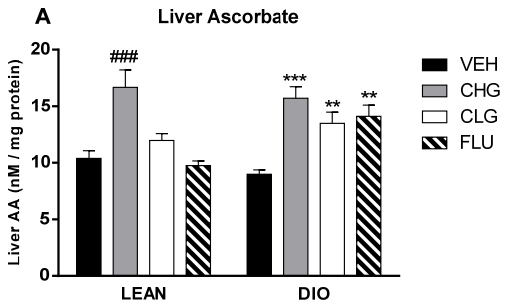
Results from two-way ANOVA of Group and Diet

Source of Variation	P value
Interaction	0,8018
Group	< 0,0001
Diet	< 0,0001

Figure 11. Liver TG. Data are means ± SEM, n=7-8. §§§p<0.0001, ###p<0.0001 vs. VEH-LEAN; **p<0.001 vs. VEH-DIO.

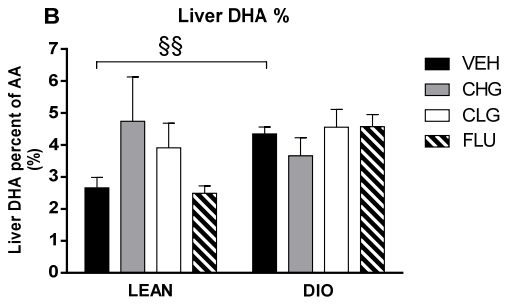
As expected long term high fat feeding significantly increases liver TG accumulation (p<0.0001). As judged by the two-way ANOVA there was no interaction between group and diet. The post hoc test revealed that subjecting both LEAN and DIO rats to high overt chronic hyperglycemia as for the CHG groups will significantly increase the accumulation of liver TG as compared to its respective control groups (LEAN: p<0.0001, DIO: p<0.001).

Liver Ascorbate



Results (Fig. 12A) from two-way ANOVA of Group and Diet

Source of Variation	P value
Group*Diet	0,0085
Group	< 0,0001
Diet	0,1918



Results(Fig. 12B) from two-way ANOVA of Group and Diet

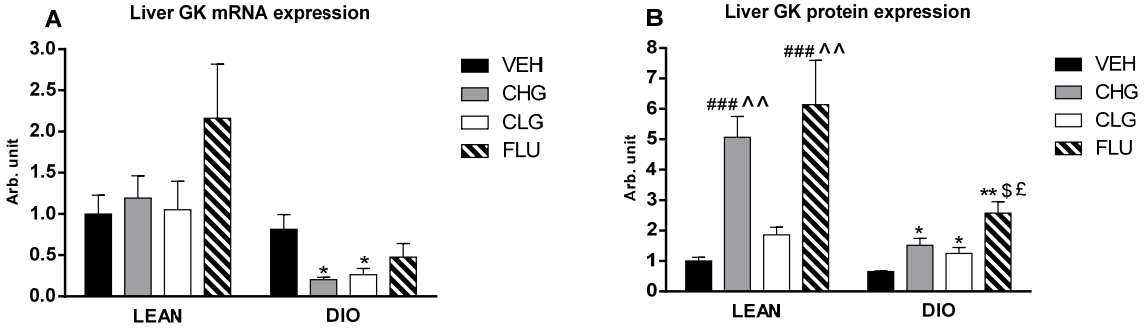
Source of Variation	P value
Interaction	0,1900
Group	0,6062
Diet	0,1167

Figure 12. Liver ASC (A) and %DHA (B). Data are means ± SEM, n=7-8. §§p<0.001, ###p<0.0001 vs. VEH-LEAN; **p<0.001, ***p<0.0001 vs. VEH-DIO.

Unlike in humans – rats are capable of synthesizing de novo ascorbic acid where the main substrate is glucose. The two-way ANOVA showed a significant interaction between group and diet. In both models subjecting rats to overt sustained hyperglycemia increased liver ascorbate

accumulation with up to 50%. Interestingly the FLU and CLG group had only increased liver ascorbate in the DIO model and not in the LEAN situation. Data as well showed no significant differences between the LEAN and DIO controls (p=ns). The %DHA showed as well no statistical significance among groups. Of notice, evaluating the difference in %DHA between the LEAN and DIO controls we found a significance increase in the DIO model (p<0.001) thus indicating that long term high fat feeding may disturb the redox in liver.

Liver Glucokinase



Results (Fig. 13A) from two-way ANOVA of Group and Diet

Source of Variation	P value
Group*Diet	0,286
Group	0,2942
Diet	0,0017

Results (Fig. 13B) from two-way ANOVA of Group and Diet

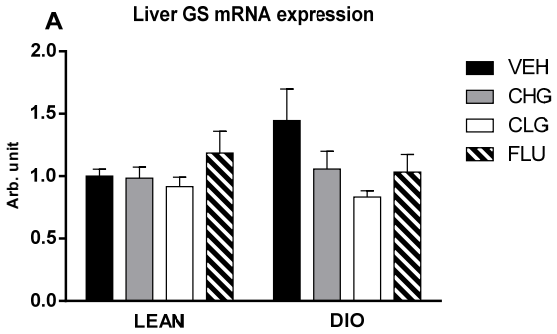
Source of Variation	P value
Group*Diet	0,101
Group	0,0004
Diet	0,0018

Figure 13. Liver GK mRNA(A) and protein(B) expression. The level of GK protein expression was quantified as the relative protein abundance to β-actin. Data are shown as relative expression to VEH-LEAN. Data are means ± SEM, n=7-8. ###p<0.0001 vs. VEH-LEAN, ^^p<0.001 vs. CLG-LEAN, *p<0.05, **p<0.001 vs. VEH-DIO, $p<0.05 vs. CHG-DIO, £p<0.05 vs. CLG-DIO.

GK the rate limiting enzyme for glucose uptake in liver, was analysed by RT-PCR (Figure 13A) and western blotting (Figure 13B). The GK protein abundance was significantly affected among groups (p=0.0004) and diet (p=0.0018). In the LEAN model we observed an app. 5-fold increase in GK protein levels for the CHG and FLU group (p<0.0001 vs. VEH-DIO; both cases) but not no changes in the CLG group (p=ns). In the DIO model the GK protein levels were significantly increased in all intervention groups. The CHG and CLG group showed approximately a 2 fold increase in GK protein abundance as compared to the controls (p<0.05; both cases). Interestingly, the FLU group had an even higher effect on the GK protein level, displaying a 4 fold increase compared to the controls (p<0.0001), which was also significantly higher than the CHG and CLG groups (p<0.05; both cases). Of notice even though we observed a significant increase in GK protein abundance in the DIO model this increase was significantly less than observed in the LEAN model.

In the GK mRNA level (Fig. 3D) we observed a diet dependent significance (p=0.0017). The post hoc test revealed a reduction in the CHG and CLG groups (p<0.05 vs. VEH; all cases) but not in the FLU group.

Liver Glycogen synthase



Results (Fig. 14A) from two-way ANOVA of Group and Diet

Source of Variation	P value
Group Die	0,1460
Group	0,0767
Diet	0,4582

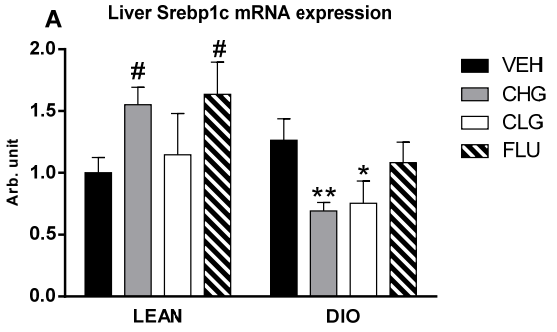
Results (Fig. 14B) from two-way ANOVA of Group and Diet

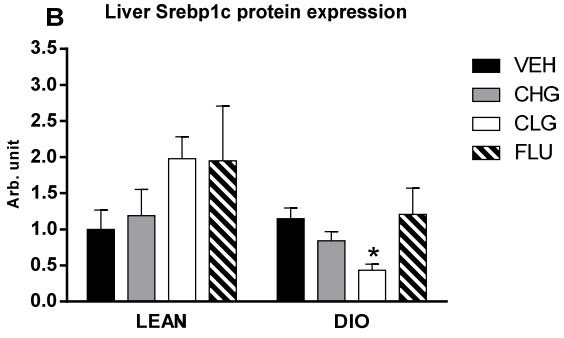
Source of Variation	P value
Group*Time	0,2914
Group	0,9376
Time	0,0697

Figure 14. Liver GS mRNA(A) and protein(B) expression. The level of GS protein expression was quantified as the relative protein abundance to β-actin. Data are shown as relative expression to VEH-LEAN. Data are means ± SEM, n=7-8.

As outline in tables above we observed no significant effect on GS mRNA or protein expression levels.

Liver Screbp1c





Results (Fig. 15A) from two-way ANOVA of Group and Diet

Source of Variation	P value
Group*Time	0,2134
Group	0,2998
Diet	0,0304

Results (Fig. 15B) from two-way ANOVA of Group and Diet

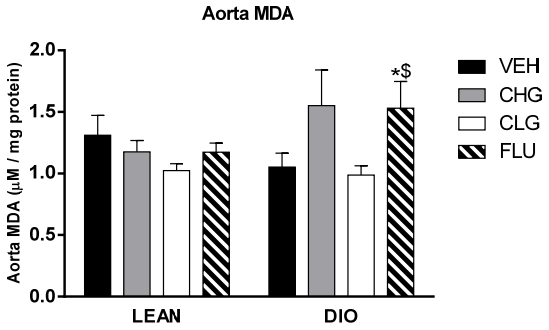
Source of Variation	P value
Group*Time	0,2052
Group	0,4896
Diet	0,0391

Figure 15. Liver Screbp1c mRNA(A) and protein(B) expression. The level of Screbp1c protein expression was quantified as the relative protein abundance to β-actin. Data are shown as relative expression to VEH-LEAN. Data are means ± SEM, n=7-8. #p<0.05 vs. VEH-LEAN, *p<0.05, **p<0.001 vs. VEH-DIO.

According to the two-way ANOVA diet had a significant effect on the level of Screbp1c mRNA levels (p=0.0304). In the LEAN situation we observed a significant increase in the CHG (p<0.05) and FLU (p<0.05) as compared to the VEH-LEAN. In contrary in the DIO model the

CHG (p<0.001 vs. VEH-DIO) and CLG (p<0.05 vs. VEH-DIO) showed a significant lowering of Screbp1c mRNA levels. The protein abundance was also significantly affected by diet (p=0.0391). In the LEAN model we did not observe any significant changes among group and in the DIO model only the CLG group showed a significant reduction in Screbp1c protein abundance (p<0.05 vs. VEH-DIO).

Aorta MDA



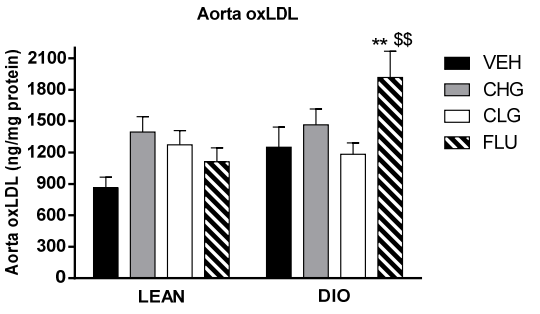
Results from two-way ANOVA for Group and Diet

Source of Variation	P value
Group*Diet	0,0678
Group	0,0239
Diet	0,2582

Figure 16. Aorta MDA. Data are means ± SEM, n=7-8. *p<0.05 vs. VEH-DIO, $p<0.05 vs. CLG-DIO.

We observed a significant difference in aorta MDA among groups (p=0.0239). The post hoc test showed no effect on aorta MDA in LEAN animals. However, in DIO rats we observed a significant increase FLU group as compared to VEH and CLG animals (p<0.05; both cases).

Aorta oxLDL



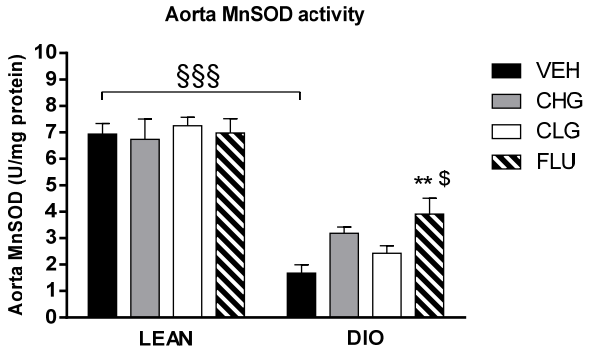
Results from two-way ANOVA for Group and Diet

Source of Variation	P value
Group*Diet	0,0387
Group	0,0280
Diet	0,0169

Figure 17. Aorta oxLDL. Data are means ± SEM, n=7-8. **p<0.001 vs. VEH-DIO, $$p<0.001 vs. CLG-DIO.

The two-way ANOVA showed a significant interaction between groups and diet (p=0.0387). OxLDL has been shown to be increased in high fat feed rats but we did not observe any significant difference between the two control groups (p=ns). In DIO rats we observed a significant increase in the FLU as compared to the VEH and CLG groups (p<0.001; both cases).

Aorta SOD



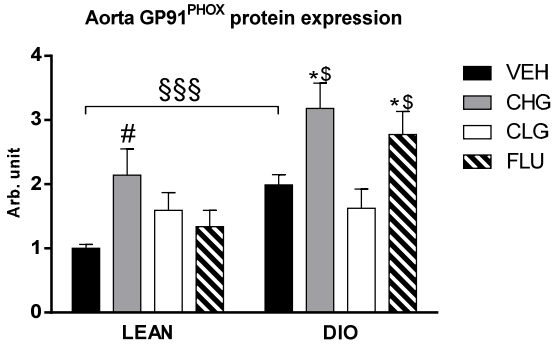
Results from two-way ANOVA for Group and Diet

Source of Variation	P value
Group*Diet	0,0664
Group	0,1204
Diet	< 0,0001

Figure 18. Aorta SOD activity. Data are means ± SEM, n=7-8. **p<0.001 vs. VEH-DIO, $p<0.05 vs. CLG-DIO.

As evident from Figure 18 animals subjected to long term high fat feeding had significantly lower SOD activity as compared to LEAN rats (VEH-LEAN vs. VEH-DIO, p<0.0001). In LEAN rats the different glucose infusion paradigms had no effect on SOD activity. However in DIO rats fluctuating hyperglycemia increased significantly SOD activity as compared to VEH (p<0.001) and CLG (p<0.05). These data may indicate that during insulin resistance the endogenous anti-oxidant system in aorta may be markedly reduced and predisposed to oxidative stress.

Aorta GP91^{PHOX}



Results from two-way ANOVA for Group and Diet

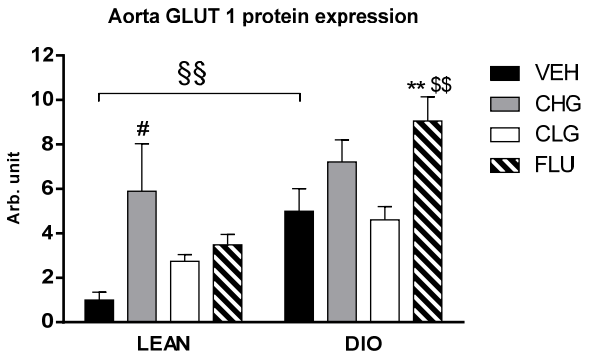
Source of Variation	P value
Group*Diet	0,0941
Goup	0,0024
Diet	0,0002

Figure 19. Aorta GP91^{PHOX} protein expression assessed by western blotting. The level of GP91^{PHOX} protein expression was quantified as the relative protein abundance to β-actin. Data are shown as relative expression to VEH-LEAN. Data are means ± SEM, n=7-8. #p<0.05 vs. VEH-LEAN, *p<0.05 vs. VEH-DIO, $p<0.05 vs. CLG-DIO.

The two-way ANOVA revealed no significant interaction between group and diet (p=0.0941) but significance at group (p=0.0024) and diet (p=0.0002). In LEAN rats only the CHG group showed a significant increase in GP91^{PHOX} protein expression (p<0.05). In DIO rats, yet again they seemed to be more susceptible for changes as compared to the LEAN rats. Initially, the

protein abundance of GP91^{PHOX} was significantly higher in the DIO controls as compared to the LEAN controls ($p<0.0001$). Moreover, in DIO rats both the CHG and the FLU group showed significant higher level of GP91^{PHOX} protein expression of ($p<0.05$ vs. VEH & CLG; all cases)

Aorta GLUT1



Results from two-way ANOVA for Group and Diet

Source of Variation	P value
Group*Diet	0,1325
Group	0,0009
Diet	< 0,0001

Figure 20. Aorta GLUT1 protein expression assessed by western blotting. The level of GLUT1 protein expression was quantified as the relative protein abundance to β-actin. Data are shown as relative expression to VEH-LEAN. Data are means ± SEM, n=7-8. #p<0.05 vs. VEH-LEAN, **p<0.001 vs. VEH-DIO, $$p<0.001 vs. CLG-DIO.

The two-way ANOVA revealed a significant difference both among groups ($p=0.0009$) and diet $p<0.0001$). Interestingly we found that the GLUT1 protein expression was significantly increased in DIO controls as compared to LEAN controls ($p<0.0001$). In LEAN rats we observed a 6-fold increase in the CHG group but no changes in the other groups. In the DIO model we observed a significant increase in the FLU group as compared to VEH and CLG ($p<0.001$; both cases).

Overview of results

Table 5. Summary of results. The arrows indicate the effect of different hyperglycemic profiles in LEAN and DIO rats relative to their respective controls. Arrows in DIO-VEH indicates the effects relative to LEAN-VEH. Upwards arrows indicate up-regulation or increase. Downwards arrows indicate down regulation or decrease. - : No changes, 1xarrow: low, 2xarrow: moderate; 3xarrow: high.

		LEAN				DIO				
		VEH	**CHG**	**CLG**	**FLU**	**VEH**	**CHG**	**CLG**	**FLU**	**Remarks**
Metabolic parameters										
Plasma Insulin		-	↑↑	-	↓↑	↑	↑↑↑	↑	↓↑	-Chronic hyperglycemia down regulates net hepatic glucose uptake as a consequences of increased ROS production. -Fluctuating hyperglycemia increases GLUT1 protein expression in aorta
Plasma FFA		-	-	-	-	↑↑↑	↓			
Liver GLY		-	↑↑↑	↑↑	↑↑	↓	↑↑	↑	↑	
Liver TG		-	↑↑	-	-	↑	↑↑↑	-	-	
Liver GK expression		-	↑↑↑	-	↑↑↑	-	↓↓↓	↓↓↓	-	
Liver Screbp1c expression		-	↑↑↑	-	↑↑↑	-	↓↓	-	↓↓	
Liver GS expression		-	-	-	-	-	-	-	-	
Aorta GLUT1		-	↑	-	-	↑	↑↑	-	↑↑↑	
Oxidative stress										
MDA	Plasma	-	↑	-	↑	-	↑	-	↑	Fluctuating hyperglycemia increases systemic oxidative stress relatively higher than sustained hyperglycemia Only chronic hyperglycemia affects the liver oxidative stress levels. Fluctuating hyperglycemia increases oxidative stress and oxLDL accumulation
	Liver	-	-	-	-	-	↑↑↑	↑	-	
	Aorta	-	-	-	-	-	↑	-	↑↑	
Plasma 8-IsoP		-	↑	-	↑	-	↑	-	↑	
DHA%	Plasma	-	-	-	↑	-	-	-	-	
	Liver	-	-	-	-	↑	-	-	-	
$GP91^{PHOX}$	Liver	-	-	-	-	-	↑↑	↑	-	
	Aorta	-	↑↑	-	-	↑↑	↑↑↑	-	↑↑↑	
Aorta oxLDL		-	-	-	-	-	-	-	↑	
Antioxidants										
ASC	Plasma	-	-	-	-	-	-	-	-	Insulin resistance decreases aorta SOD levels.
	Liver	-	↑↑↑	-	-	-	↑↑↑	↑	↑	
SOD	Liver	-	-	-	-	-	↑↑↑	↑↑	↑	
	Aorta	-	-	-	-	↓↓↓	-	-	↑	

10 Discussion

Substantial efforts have been made to clarify if repeated incidents of short term hyperglycemia are an Hba1c independent risk factor for endothelial dysfunction and the development of macro and as well micro-vascular complications in diabetes (90;163;167). Recent reports both in humans and rodents have shown that intermittent hyperglycaemia induce higher levels of oxidative stress leading to cellular damage in particular in the cardiovascular system thus potentially resulting in diabetes related atherosclerosis and CVD (71). A more mechanistic approach have been achieved in cell based studies on both human and bovine VECs showing that intermittent glucose levels compared to chronic high glucose levels induces a higher degree of oxidative stress by increased activity levels of the NADPHox resulting in increased cell apoptosis(169;170;173;178). However, so far the exact sequences of biochemical events undergoing during fluctuating hyperglycemia are still under debate. Thus in this project we set to investigate the role fluctuating hyperglycemia versus sustained hyperglycemia in both LEAN and DIO animal models and its role on markers of oxidative stress, expression of key enzymes and glucose metabolism in liver and aorta. We hypothesized that the liver, being one of the main regulators of glucose homeostasis, may exert important regulation of glucose metabolism during different hyperglycaemic situations and thus indirectly impact the severity of oxidative stress in aorta.

Initially we aimed to set up an infusion protocol in catheterized animals giving well-defined and consistent fluctuating and sustained hyperglycemic profiles. The infusion protocol was a fully-automated system handling both the infusion and blood sampling from the animals. In the setup animals were freely moving with *ad libitum* access to food and water thus the setup was considered as a major advantage with regards to minimizing the stress in the animals. In fact stress is known as a significant contributor to disturbed insulin and glucose levels (179) thus having the animals in unstressed environment we reduced the impact of this cofounder. The infusion protocols also gave us the opportunity to study more precisely whether it is the amount of glucose or the profile that affects the level of oxidative stress. As given in Table 2 in both models the FLU and CLG received the exact same amount of glucose but with different infusion rates whereas the CHG group received three times the amount of glucose. As shown in Figure 1 we managed to have both a fluctuating and an overt sustained hyperglycemic profile that was consistent throughout the study periods. In the DIO model the hyperglycemic profiles tended to be slightly higher probably due to their insulin resistance.

In our studies (Paper I, II & III) we found increased plasma MDA and 8-IsoP levels both during sustained overt hyperglycaemia (PG > 20mM) and in animals with fluctuating plasma glucose profiles. Indeed, overt ROS production has been shown to correlate with glycaemic exposure (27;171), but importantly, the observed effects on plasma MDA and 8-IsoP in our studies

seemed to be independent of the overall glycemic exposure since the FLU group received only one third of the amount glucose given to the CHG groups suggesting that elevated levels of systemic oxidative stress is more likely dependent on the glycemic profile rather than the total amount of glucose exposed to. Other mechanisms that are independent of the total glycaemic exposure may be involved in the generation of oxidative stress. A reduction or a delayed adaptability of the systemic antioxidant system to elevated ROS production may as well contribute to increased oxidative stress and favour the development of diabetes complications (158;180;181). To address these questions whether the systemic antioxidant capacity was affected during fluctuating glucose, we used ASC as a biomarker for the systemic antioxidant status. ASC is an important water soluble antioxidant placed low in the antioxidant hierarchy and thus acts both as a radical scavenger in vivo as well as in regenerating other antioxidants (66). Studies have reported decreased levels of plasma ASC in diabetic patients and animal models (68;69) and also a direct correlation between the DNA damage, plasma ASC levels and glycaemic control in T2D patients (70). In our studies, plasma ASC was significantly reduced in all groups during time the course but no difference we observed across groups. Interestingly in the LEAN model the level of plasma ASC was gradually decreasing during the time course reaching levels of around 35 μM at the end of the study whereas in the DIO model the decrease of plasma ASC, also to levels around 35μM, were apparent already after 24 hours and plateaued for the remainder of the study period. This earlier response in in the DIO model could be mediated by the insulin resistance which has shown to affect the ROS production (62;96). Albeit, rats—in contrast to humans—are capable of de novo ASC synthesis in the liver (182) and a possibly decrease of plasma ASC may be compensated for by increased liver ASC production and output. Indeed in our study we found increased levels of liver ASC in particular in the overt sustained hyperglycemic groups (CHG). Thus mobilisation of liver glucose which is the main substrate for ASC synthesis may account for the lack of difference in ASC profiles observed. Nonetheless we did find that the oxidation ratio of ASC, measured as %DHA, was significantly affected during fluctuating hyperglycemia but only in the LEAN model. In agreement with its use as a biomarker of redox imbalance, an increased oxidation ratio suggests that the capacity to recycle DHA to ASC is overwhelmed by the rate of ASC oxidation (69;183;184). However, by the fact that rats are capable of synthesizing ASC may imply that the mere plasma antioxidant status measured as ASC is a poor biomarker of oxidative stress in these rat models.

In the first study (Paper I) we used lean rats with no signs of insulin resistance or dyslipidaemia. In that study we did not observe any oxidative changes at tissue level. The moderate, albeit significant, changes in plasma MDA and oxidation ratio as compared to the effects seen in diabetic models (47;68;85;185), may reflect that lean animals are metabolically more robust and to a large extent capable of handling hyperglycemic episodes

without experiencing severe induction of oxidative stress and endothelial dysfunction. In fact, normal rats have been shown to metabolically adapt to chronic infusion of glucose by increasing their insulin sensitivity to some extent (186). Also - the development of diabetes related endothelial dysfunction is clearly composed of several metabolic disorders and besides hyperglycaemia, insulin resistance and dyslipidaemia have been shown to play important roles in endothelial function (187;188). Thus in the next study (Paper II), we added the challenge of a high fat diet to either sustained or intermittent hyperglycaemia in rats with insulin resistance, dyslipidaemia or fatty liver for a more "realistic" model of the effects of hyperglycaemia on oxidative stress seen in T2D.

Liver is an important organ for maintaining whole body glucose homeostasis. Increased liver oxidative stress has been demonstrated both during chronic glucose infusion (189) and in diet induced insulin resistant animal models (98;190). In the DIO model, we observed a marked increase in both liver MDA and SOD but only in the groups receiving a constant infusion of glucose and not in the fluctuating hyperglycemic group. The membrane bound NADPHox subunit, $GP91^{PHOX}$, has been described as being the primary regulator of NADPHox activity and in studies where hepatocytes were subjected to chronic high glucose, expression of $GP91^{PHOX}$ was significantly increased along with increased NADPHox activity (191). The significant increase in liver $GP91^{PHOX}$ protein expression in the DIO model for the sustained hyperglycaemic groups may indicate increased ROS production by the NADPHox and consequently, the induction of oxidative stress. These effects on oxidative stress markers were not present during fluctuating hyperglycaemia. This may suggest that the liver is more prone to increased ROS production and oxidative stress when subjected to sustained hyperglycaemia but only in the state of insulin resistance. The increased levels of oxidative stress in livers of insulin resistant rats may be a result of increased liver substrate uptake. Elevated insulin levels during insulin resistance and the increased substrate availability during long term high fat feeding may result in increased substrate uptake (192). To answer these questions we investigated the effect on key enzymes in the liver responsible for glucose uptake and utilization during these different glycemic challenges. As expected, the GK protein levels were markedly increased during glucose infusion. Interestingly, the increase in the LEAN model was markedly higher as seen for the DIO model. Insulin resistance has shown to reduce the level of liver GK and thus disturb whole body glucose homeostasis (193). However at mRNA level, the DIO rats subjected to chronic hyperglycemia had a significant lower expression of liver GK. The down regulation of GK expression in the groups receiving the chronic glucose infusion may indicate a lowering of net glucose uptake by the liver. Although it should be emphasized that this only represents a "snap shot" of the time course and multiple adaptive processes may have been involved during the days of glucose infusion. In fact studies have shown that the liver may exert biphasic controlling actions of glucose homeostasis in the hyperglycaemic

state. At initial stages of hyperglycaemia, the liver facilitates glucose uptake while at later stages, the liver develop insulin resistance and facilitates glucose output (186;194). The level of GK expression is primarily controlled by insulin and studies have shown that long term high fat feeding will induce liver insulin resistance with a concomitant reduction in GK expression and activity (193). ROS molecules has been shown in several studies to be an important signal molecule and direct effects of increased ROS production in liver, muscle and adipose tissue has been demonstrated to consequently induce insulin resistance and act as a protective mechanism against further oxidative stress (195;196). During chronic glucose infusion, liver TG and GLY were markedly increased. Hypothetically, the accumulation of TG and GLY may exert a protective mechanism against excess substrate availability. Studies have revealed that over expressing di-acyl glycerol acyl transferase (DGAT), the rate limiting enzyme for TG synthesis in liver, will protect the liver against FFA induced oxidative stress (197). In contrast, pharmacological inhibition of the DGAT during high fat feeding will indeed improve liver steatosis due to decreased TG levels but paradoxically worsen liver damage probably through increased FFA availability inducing inflammation in from TNF-α and ROS production(198). The activity of liver DGAT is inversely regulated with the accumulation of TG (199) thus in the CHG animals a limited capacity for further TG and GLY accumulation may have caused a "spill" over of substrate in form of glucose and FFA thus activating the NADPHox. Assuming that this interpretation is correct, the liver plays an important role in protecting organs with insulin independent glucose uptake from excess glucose challenge only limited by livers capacity to synthesize and accumulate GLY and TG. Thus in our set up the liver probably counteracted the potential detrimental response by down regulated liver glucose uptake and probably exerted a protection against further oxidative stress. Paradoxically, the protective down regulation of glucose uptake by insulin responsive tissues may leave VEC's as important targets of hyperglycaemia mediated oxidative insult (120).

Cultured aortic endothelial cells subjected to intermittent hyperglycaemia has been shown to produce higher levels of ROS as compared to cells subjected to sustained hyperglycaemia (169;178). In Paper II we showed in the DIO model that fluctuating but not sustained hyperglycaemic status increased MDA and oxLDL in aorta. Additionally we observed significant higher levels of $GP91^{PHOX}$ and GLUT1 protein abundance in aorta. Likewise, presumably as an adaptive preventive mechanism against increased oxidative stress, the SOD activity was markedly increased as well. Interestingly in Paper III we found that the overall SOD activity levels in the DIO model compared to the LEAN were markedly lower. Attenuated antioxidant status in aorta has been reported both in insulin resistant and in diabetic rats (200). This down regulation of the endogenous antioxidant capacity in the insulin resistant state may have caused an imbalance between the production and the scavenging of ROS thus resulting in oxidative stress. Moreover influx of glucose in endothelial cells is facilitated by GLUT1 in an

insulin independent manner (121) and excess glucose may activate PKC by several mechanisms, which consequently increases oxidative stress by activating NADPHox (27;114;122). In connection we found a marked increase in GLUT1 and GP91^{PHOX} protein expression both during fluctuating and sustained hyperglycaemia. In diabetics, increased oxLDL accumulation in vessels has been shown to promote foam cell formation in vascular smooth muscle thus leading to plaque formation and the development of atherosclerosis (42;201). Additionally, oxLDL has itself been shown to produce oxidative stress in endothelial cells via activation of a NADPHox (202). The level of oxLDL is strongly correlated to hyperglycemia and studies in T2D patients have shown that after a meal oxLDL is increased (153;203).These findings may indicate that in the state of insulin resistance during fluctuating hyperglycaemia aorta may be subjected to excess glucose uptake affecting the activity of NADPHox thus leading to increased oxidative stress and oxLDL accumulation.

In perspective, a role for insulin in these studies must as well be considered. In a study by Ellger et al. where normal rats where chronically infused with either insulin or glucose or both, it was found that endothelial function was improved by insulin through its action of reducing hyperglycaemia (192). In this regard, hyperinsulinemic conditions both in normal and T2D subjects and in rodents as well have been shown to increase hepatic de novo GLY and TG synthesis (204;205). In line with these data, we observed a marked increase in liver GLY and TG in the CHG group. Insulin may thus exerts its effects by increasing the expression and activity of GLUT's and lipogenic enzymes in the liver and thereby increase liver glucose uptake (205). This action by insulin, facilitating net liver glucose uptake as seen for the CHG group, may hypothetically also exert an indirect protection of the vascular endothelium during excess glucose exposure. In the FLU group this effect of insulin may be absent due to the short term exposure to hyperinsulinemia. Thus in our studies insulin may to some extent be a confounder and additional studies evaluating markers of oxidative stress and glucose disposal in insulin sensitive versus non-insulin sensitive tissues under hyperinsulinemic clamp conditions may be an approach in order to investigate these mechanistic consequences. In this context an approach to avoid the effects of insulin may be to diminish endogenous insulin production by treating animals with STZ. As previously described introducing diabetes by STZ is often the choice of model when studying diabetic late complications (76;166). However as emphasized in Paper III insulin resistance is an important factor in the development of diabetes related late complication. Inducing insulin resistance by high fat feeding and then combining it with STZ may be an approach to study the effect of hyperglycemia in combination with insulin resistance without the confounding effects by insulin. This kind of set up as well allows to study the more long terms effects of different hyperglycemic effects on levels of oxidative stress.

Another approach is to use atherosclerotic prone ApoE mice which develop stable plaque lesions very aggressively(206). Moreover studies have shown that manipulating these mice

either by high fat feeding (207) or by STZ induction of diabetes (208) will accelerate the plaque formation compared to non-diabetic lean ApoE mice. This model will as well allow studying the long term effects of hyperglycemia. Additionally by implementing the use of tracer techniques makes it possible to study the level of glucose uptake by aorta more accurately, which has been shown to have an important effect in the plaque formation (209). Collectively, our data indicates that both fluctuating and overt sustained hyperglycemia may induce a higher degree of oxidative damage. Interestingly the oxidative damage is highly compartmentalized where the cardiovascular system is primarily targeted by the fluctuating hyperglycemic profile whereas the liver is more disposed to oxidative damage by the chronic hyperglycemic profile. Additionally these detrimental oxidative changes seemed to be more prone in the state of insulin resistance. In conclusion, our data provides substantial in vivo support for the hypothesis that fluctuating hyperglycaemia induces relatively higher levels of oxidative stress in the cardiovascular system compared to sustained hyperglycaemia. This is in agreement with the clinical evidence that fluctuating hyperglycaemia is an independent risk factor for diabetes related endothelial dysfunction, atherosclerosis and cardiovascular disease.

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12 Paper I, II, III

Paper I

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Fluctuating hyperglycaemia increases oxidative stress response in lean rats compared to sustained hyperglycaemia despite lower glycaemic exposure

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Abstract

Objective: To compare the effect of fluctuating glucose with sustained hyperglycaemia on systemic oxidative stress during 72 h of glucose infusion.

Methods: Catheterised male Sprague–Dawley rats were given either a continuous high (CHG), low (CLG) or pulsatile (FLU) infusion of glucose or saline (VEH) for 72 h. Plasma ascorbate oxidation ratio (AOR) and malondialdehyde (MDA) were used as biomarkers of oxidative stress and damage.

Results: The FLU group showed significant increases in both plasma AOR and MDA at 48 and 72 h ($p < 0.05$ all cases), whereas the CHG group, despite being infused with three times the amount of glucose, only showed increased MDA levels at 72 h time point ($p < 0.05$).

Conclusion: Our data suggests that fluctuating glucose levels lead to oxidative stress similar to that of sustained hyperglycaemia despite a much lower total glycaemic exposure. Thus, our data supports the notion that fluctuating glucose may be relatively more deleterious than sustained hyperglycaemia.

Keywords

endothelial dysfunction, fluctuating glucose, malondialdehyde, oxidative stress

Introduction

Fluctuating hyperglycaemia has been suggested to be an independent risk factor for the development of macrovascular complications in diabetes. Increased oxidative stress resulting from an overproduction of reactive oxygen species (ROS) has been suggested as the underlying mechanism.[1,2] Recently, reports have shown poor regulation of several glycolytic genes and transport mechanism during fluctuating compared to sustained hyperglycaemia,[3,4] and this lack of adaptability may cause oxidative stress. However, the mechanisms by which glucose fluctuations may impair macrovascular function remain unclear and call for better defined animal models. This study examined the effect of fluctuating versus sustained high or low levels of glucose on markers of systemic oxidative stress and hepatic glucose metabolism in catheterised rats.

Materials and Methods

Thirty male Sprague–Dawley rats (400–430 g; Taconic, Ejby, Denmark), were housed under controlled conditions (temperature, 22 ± 2°C; humidity, 50 ± 20%; light, 12 h) with water and chow (Altromin GmBH, Lage, Germany) *ad libitum*. Animals were catheterised (S-50-HL; Tygon, Saint Gobain, UK) in the jugular vein for infusion and in the carotid artery for blood sampling and treated with Baytril (Bayer AG, Leverkusen, Germany) and Rimadyl (Pfizer A/S, Ballerup, Denmark) for 3 days post-surgery. The animals were randomised into four groups ($n = 7–8$) and were connected to an Accusampler (DiLab, Lund, Sweden) for automated blood sampling and glucose infusion (50% glucose; Fresenius Kabi AG, Bad Homburg, Germany) and received either a continuously

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high (CHG; 4.0 ml/kg/h), continuously low (CLG; 1.2 ml/kg/h) or pulsating (FLU; 0–2 min, 30 ml/kg/h; 2–30 min, 4.0 ml/kg/h, i.e. nine daily infusions separated by 2.5 h) glucose infusion for 72 h. Controls received a constant saline infusion (VEH, 1.2 ml/kg/h). Blood was collected in K_3-EDTA coated tubes and centrifuged (4°C, 5000 rpm, 3 min). Plasma malondialdehyde (MDA) and ascorbate oxidation ratio (AOR, i.e. the % of dehydroascorbic acid of total ascorbate) were measured daily as biomarkers of oxidative stress. Plasma protein was used to correct for haemodilution. Plasma glucose and insulin were analysed throughout the study covering the entire circadian rhythm. After 72 h, animals were anaesthetised with isofluran and liver was excised and preserved in liquid nitrogen. Animals were euthanised by intra-cardiac injection of pentobarbital (100 µl; 200 mg/ml). Plasma glucose was analysed by glucose oxidase methodology (EBIO Plus; Eppendorf AG, Hamburg, Germany). Plasma insulin was analysed by ELISA (Chrystal Chemistry, Downers Grove, IL, USA). Plasma MDA and AOR were analysed as described previously.[5,6] Liver triglycerides and glycogen were determined in homogenised tissue (sodium acetate 0.15 mol/l and 0.75% Triton X-100, pH = 4.9). Homogenate was boiled (2 min), cooled (0°C) and liver TG determined on the supernatant (Cat#11488872; Roche, Basel, Switzerland). For glycogen, liver homogenate was incubated (20°C/24 h) with amyloglycosidase before analysing total glucose.

Statistical analyses were carried out by SAS JMP software (version 8.1; SAS institute, Cary, NC, USA). Data were analysed by ANOVA with random effect using group and time as factors. In the case of significance, an appropriate *post hoc* test was performed.

Results

Baseline measurements were not different between groups. Hyperglycaemia (18 mmol/l) and hyperinsulinaemia (1500 pmol/l) were immediately manifested in the CHG group and maintained throughout the study. The FLU group showed regular oscillation of glucose (16–18 mmol/l) and insulin (1300 pmol/l) at peak level and returned to normal levels between pulses. The CLG group displayed slight increases in both glucose (8 mmol/l) and insulin (300 pmol/l) throughout the study.

Both plasma AOR and MDA were significantly affected by the intervention (Table 1). FLU had 50% higher plasma MDA over the entire time course compared to controls ($p < 0.05$) and 40% higher than CLG ($p < 0.05$). FLU showed a significant increases at time points 48 and 72 h

Table 1. Biochemical measurements of oxidative stress (malondialdehyde and ascorbate oxidation ratio) in plasma and liver triglycerides/glycogen in rats subjected to various glucose infusion regimens

Measurement	Time (h)			
	0	24	48	72
Plasma malondialdehyde				
VEH	58 ± 6	39 ± 7	49 ± 7[a]	44 ± 7[a]
CHG	60 ± 5	55 ± 6	44 ± 6[a]	85 ± 7[b]
CLG	58 ± 3	52 ± 5	46 ± 5[a]	43 ± 5[a]
FLU*	58 ± 2	54 ± 5	71 ± 6[b]	74 ± 6[b]
Plasma ascorbate oxidation ratio				
VEH	7.0 ± 1.2	8.5 ± 2.3[a]	8.3 ± 3.1	9.7 ± 2.6[a]
CHG	7.4 ± 1.1	16.0 ± 2.1[b]	12.4 ± 2.1	8.4 ± 2.3[a]
CLG	7.6 ± 1.1	11.1 ± 2.1[a]	10.8 ± 2.0	10.8 ± 2.2[a]
FLU**	8.3 ± 1.0	12.2 ± 1.8[a]	11.9 ± 1.8	15.4 ± 1.8[b]
Liver biochemistry	Triglycerides		Glycogen	
VEH	5.1 ± 0.8		187 ± 22	
CHG	33.6 ± 3.0***		488 ± 39***	
CLG	8.2 ± 0.9*		263 ± 11*	
FLU	8.8 ± 0.9*		299 ± 15*	

Plasma data were analysed by two-way ANOVA with random effect using time and group as factors. In the case of significance, *post hoc* tests were carried out using Tukey's test correcting for multiple comparisons. Different characters indicate significantly different groups by time point ($p < 0.05$). Liver data were analysed by one-way ANOVA using group as the factor. In the case of significance, intervention groups were compared to VEH by Dunnet's *post hoc* test. A p value <0.05 was considered statistically significant.

*FLU significantly different from both VEH and CLG by ANOVA ($p < 0.05$); **FLU significantly different from VEH by ANOVA ($p < 0.05$).

For further details, see materials and methods section. Data are shown as mean ± SEM (n = 7–8).

VEH: vehicle, CHG: constant high glucose, CLG: constant low glucose, FLU: fluctuating glucose.

Results for plasma malondialdehyde are given as nmol/mg protein.

Results for plasma ascorbate oxidation ratio are given as % oxidised of total ascorbate.

($p < 0.05$ *vs*. VEH, both cases) whereas CHG was only affected at 72 h ($p < 0.05$ *vs*. VEH). FLU also showed 50% increased plasma AOR *vs*. VEH ($p < 0.05$).

In liver (Table 1), CHG showed 2.5-fold increased GLY and 6-fold increased TG ($p < 0.0001$ *vs*. VEH; both cases), whereas FLU and CLG showed only moderate changes in GLY and TG ($p < 0.05$ *vs*. VEH; both cases).

Discussion

Oxidative damage to lipids is considered an important factor in the progression of macrovascular complications in diabetes.[7] Increased plasma MDA was found in both CHG and FLU. Notably, this effect was earlier and more pronounced in FLU than in CHG. Also, FLU had a higher AOR showing that the capacity to recycle ascorbate is overwhelmed by its oxidation rate, indicating increased oxidative stress. Oxidative stress has been shown to correlate with glycaemic exposure,[8] but we found this relationship to be more complex since FLU received only one third of the glucose amount given to CHG animals. Thus, oxidative stress apparently also depends on the glycaemic profile. In agreement, human and animal studies have previously shown fluctuating glucose to impair vascular function presumably due to increased oxidative stress.[1,2]

Other mechanisms independent of glycaemic exposure may cause increased oxidative stress. Cultured endothelial cells subjected to sustained hyperglycaemia down-regulated glucose transport protein 1 (GLUT 1) expression whereas short-term hyperglycaemia had no effect on GLUT 1 expression or activity.[4] This attenuated metabolic adaptability during repetitive hyperglycaemia may, hypothetically, leave the endothelium exposed to excess glucose influx resulting in oxidative stress. Moreover, a potential role of insulin in the lack of adaptability must be considered as well. A recent study, where rats were chronically infused with insulin, glucose or both found that insulin improved endothelial function through its hyperglycaemia reducing action.[9] Insulin increases expression and activity of GLUTs and lipogenic enzymes particularly in liver and thereby hepatic glucose uptake.[10] This action may exert an indirect protection of the vascular endothelium during sustained high-glucose exposure. In FLU animals, insulin protection may be absent due to the short-term hyperinsulinaemia observed there. However, additional studies evaluating oxidative stress and glucose disposition in insulin sensitive versus non-sensitive tissues during hyperinsulinaemia are needed to support this hypothesis.

Conclusion

Our study shows that compared to sustained hyperglycaemia, fluctuating glucose induces earlier signs of lipid oxidation and redox imbalance despite a lower total glycaemic exposure. This supports the hypothesis that poorly controlled hyperglycaemia in diabetic patients, regardless of mean glucose levels, may be an independent risk factor for the development of diabetic complications.

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Conflict of interest

The authors declare there are no conflicts of interest.

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Paper II

Fluctuating but not sustained hyperglycaemia preferentially increases vascular oxidative stress and GLUT1 expression in rats on high fat diet

Running title: Fluctuating glucose and oxidative stress

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Abstract

Aim; Fluctuating hyperglycaemia resulting in elevated oxidative stress has been suggested as an important risk factor in development of macrovascular complications in diabetes. Here, we investigated the effects of sustained vs. fluctuating hyperglycaemia in insulin resistant rats on markers of oxidative stress, enzyme expression and glucose metabolism in liver and aorta. We hypothesized that the liver's ability to regulate the glucose homeostasis under varying states of hyperglycaemia may indirectly affect oxidative stress status in aorta.

Material and Methods; Animals were infused with continuously high (CHG), low (CLG) or pulsating (FLU) glucose or saline (VEH) for 96 h and oxidative stress status and key regulators of glucose metabolism in liver and aorta were investigated.

Results; Fluctuating hyperglycaemia showed a similar response in plasma lipid oxidation as constant hyperglycaemia. Likewise—in aorta—FLU and CHG displayed increased expression of GLUT1, NADPH oxidase and super oxide dismutase, while only the FLU group showed increased accumulation of oxidative stress and oxLDL in aorta.

Conclusion; Fluctuating hyperglycaemia induced relatively higher levels of oxidative stress systemically and in aorta in particular than sustained hyperglycaemia and thus our study provides in vivo mechanistic support to the clinical observations that fluctuating hyperglycaemia is an independent risk factor for diabetes related macrovascular complications.

Keywords; Oxidative stress, Fluctuating hyperglycemia, Macrovascular complications

Abbreviations

CHG	Constant High Glucose
CLG	Constant Low Glucose
FLU	Fluctuating Glucose
GLY	Glycogen
MDA	Malondialdehyde
NOX	NADPH oxidase
oxLDL	oxidised low density lipoprotein
PG	Plasma Glucose
PI	Plasma Insulin
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
TG	Triglycerides
VEC	Vascular endothelial cells
VEH	Vehicle

Introduction

Recent epidemiological studies as well as controlled intervention trials have shown that despite having similar average glycaemic exposure—as measured by HbA1c—the risks of developing micro and macro-vascular complications can be different in diabetic patients suggesting the need for renewed focus on other risk markers (1;2). Thus, postprandial hyperglycaemia that is typically not reflected in an altered HbA1c status has been hypothesized to be an important and independent risk factor for the incident of cardiovascular events in diabetic patients (2-4). Several in vitro and in vivo studies both in humans and animals have reported that repeated episodes of short term hyperglycaemia may induce higher levels oxidative stress than sustained hyperglycaemia and thus be more detrimental to the cardio vascular system (5-8). However, the exact nature and sequence of the biochemical and molecular events during fluctuating hyperglycaemia are still not fully understood.

Recently, it has been reported that during chronic hyperglycaemia cells may metabolically adapt to situations of excess glucose exposure and thus exert a protection against oxidative stress (9). One putative mechanism may be the development of insulin resistance and the resulting down-regulation of glucose uptake (10). In liver, glucose uptake and metabolism are strictly regulated by several enzymes such as the glucokinase (GK), glycogen synthase (GS) and the Sterol Regulatory Element-Binding Proteins (Srebp1c)(11). The expression of liver GK, the rate limiting enzyme for glucose uptake, is primarily controlled by insulin and down regulation of liver GK has been shown both in insulin resistant and diabetic models (12;13). Paradoxically, the protective down regulation of glucose uptake by insulin responsive tissues may leave vascular endothelial cells (VEC) as important targets of hyperglycaemia mediated oxidative insults (14). Excess glucose influx in VEC by facilitated diffusion through the GLUT1 (15) may activate protein kinase C (PKC) and consequently augment the activity of the NADPH oxidase enzyme complex (NOX) resulting in increased reactive oxygen species

(ROS) and oxidative stress (16-18). This in turn may increase the accumulation of oxidised low density lipoprotein (oxLDL) in vessels and consequently lead to plaque formation and atherosclerosis (19). ROS molecules may be neutralized by the superoxide dismutase (SOD) but studies have shown that this defence mechanism may be depressed during diabetes thus leading to a further oxidative damage (20). In the present study, we assessed the effects of sustained vs. fluctuating hyperglycaemia in high fat fed rats accompanied with insulin resistance on markers of oxidative stress, expression of key enzymes and glucose metabolism in liver and aorta. We hypothesized that the liver, being one of the main regulators of glucose homeostasis, may exert important regulation of glucose metabolism during different hyperglycaemic situations and thus indirectly impact the severity of oxidative stress in aorta.

Materials and Methods

Animals and surgery. The study was approved by the Danish Animal Experimentation Inspectorate under the Ministry of Justice and carried out by trained and licensed personnel. A total of 30 Sprague-Dawley male rats (Taconic, Denmark), were used in the study. Animals were high fat feed (D12492, Research Diets, USA) for at least 20 weeks in order to develop obesity, insulin resistance and dyslipidemia (Table 1). During the entire study period, animals had free access to food and water (tap water added 1% citric acid). Animals were housed in pairs in temperature (22 ± 2 °C) and humidity (50± 20 %) controlled rooms. The circadian rhythm was 12h light: 12h dark (Lights on at 06:00 AM). Prior to surgery, animals were treated with Anorfin® (GEA A/S, Denmark), a fast acting analgesic and subsequently kept on anaesthesia with Isofluran® (Baxter Pharmaceuticals, USA) and 30%: O_2 / 70%: N_2O. Silicon catheters (Tygon Microbore Tubing, S-50-HL, Cole Parmer, UK.) were surgically placed into the right jugular vein for infusion and in the left carotid artery for blood sampling. The catheters was externalized subcutaneously, to the mid-scapular region, filled with 500IU/ml heparin (LEO Pharma Nordic, Denmark) in HAEMACCEL® (Intervet/Schering-plough, UK) and sealed into Dacron buttons (Instech Laboratory Inc, USA). After surgery animals were single housed and allowed 7-8 days of post-surgery recovery before infusions. During the recovery period animals were treated with Baytril® (Bayer AG, Germany) and Rimadyl® (Pfizer Inc, USA) for three days.

Infusion protocol. Animals were randomized into a total of four weight matched groups (n = 7-8). Catheters were flushed with heparin solution (100 U/ml in saline; LEO Pharma Nordic, Denmark) to ensure proper function. Carotid artery catheters were connected to an Accusampler® (DiLab, Sweden) for automated blood sampling whereas the jugular vein catheters were connected to the infusion pump (World Precision Instruments Inc. USA). The

animals were given intravenous infusion of glucose (50% glucose solution; Fresenius Kabi AG, Germany) or saline for 96 hours as outlined in table 2.

Blood sampling. All blood samples were drawn by the accusampler system, in K_3-EDTA coated tubes and immediately centrifuged (4°C; 5000rpm; 3 min). Blood samples (50µl) for glucose and insulin were drawn at predefined time points in order to cover the entire circadian rhythm and the various glucose profiles. Blood samples (100µl) for MDA were drawn at time points 0, 24, 48, 72 and 96 hours.

Study termination. After 96 hours of infusion, animals were disconnected from the accusampler system and infusion pumps and anesthetized with isofluran. The abdominal cavity was opened and liver and thoracic aorta was quickly excised, rinsed, and preserved in liquid nitrogen. Subsequently and while still anaesthetized, animals were euthanized by an injection of pentobarbital (100µl; 200mg/ml) into the heart.

Biochemical analyses. For glucose measurement, plasma samples (10 µl) were diluted in 500 µl of EBIO buffer solution (Eppendorf, Hamburg, Germany) and analysed by immobilized glucose oxidase methodology (EBIO Plus autoanalyzer; Eppendorf, Germany). Plasma insulin were analysed by rat insulin sensitive ELISA (Chrystal Chemistry, USA). Lipid oxidation was assessed by measuring malondialdehyde (MDA) in plasma, liver and aorta as described previously (21). Liver triglycerides (TG) and glycogen (GLY) content were determined by homogenizing weighed liver tissue with a reagent consisting of a sodium acetate buffer mixed with Triton X-100 for 15 seconds by use of homogenizer, Polytron PT 3000 (PT-DA 3007/2 generator, IKA-Werke Germany). Immediately after homogenization, the sample was placed in a bath of boiled water for two minutes, and thereafter kept on slush

ice for fast cooling. After cooling, the homogenate was centrifuged and the homogenate was analysed for TG on a Hitachi 912 analyser, using a commercially available enzymatic triglyceride assay (Cat. No. 11488872; Roche diagnostics; Switzerland). For glycogen analysis, 25 µl amyloglucosidase (Sigma-Aldrich) was added to the homogenate and it was placed at 20°C overnight before analysis, using a commercially available enzymatic glucose assay (Cat. No. 11447521; Roche diagnostics; Switzerland) on a Hitachi 912 analyzer. SOD in liver and aorta was assessed as described by manufacturer (Sigma Aldrich Cat. No. 19160). Oxidized LDL in aorta was assessed as described manufacturer (Uscn Life Science Inc; China, Cat. No. E90527Ra).

Western Blotting. Tissue protein lysates were assayed with antibodies against Glycogen Synthase (Cell Signalling Technology, USA), GLUT1 and gp-91^{PHOX} (Abcam, USA) GK and Srebpc1 (2A4) (Santa Cruz, USA). Protein levels were normalized to β-Actin (Abcam, USA). Secondary antibodies were horseradish peroxidise-coupled and ECL reagent (BioVision, USA) was used for detection. Quantification was performed using ImageGuage 4.0 (Fujifilm, Japan).

Real-time PCR. RNA was isolated from tissue using the RNeasy Mini Kit (Qiagen). cDNA was synthesized using the iScript kit (Bio-Rad). Primer-probesets were from TaqMan Gene Expression Assays (Applied Biosystems) (except Srebp1c) and PCR reactions were performed using a TaqMan Master mix (Applied Biosystems) and a MX3000P system (Agilent). Srebp1c custom primer-probe sets were purchased from Applied Biosystems with the sequences: Forward: CGCTACCGTTCCTCTATCAATGAC; Reverse: AGTTTCTGGTTGCTGTGCTGTAAG; Probe: GTGGTGGGCACTGAGGC.
Ct values were normalized to those of Ppib.

Statistics. Statistical analyses of the results were performed by using SAS JMP software (version 8.1 for Windows, SAS institute, Cary, NC). Plasma MDA were analysed using ANCOVA with time 0 hours as covariant and then a fit model with time, treatment and time x treatment interaction as model based variables. Liver and aorta metabolic parameters were analysed using ANOVA. In cases of statistical significance, Tukey's post hoc test was applied. A p-level less than 0.05 was considered statistically significant.

Results

Fluctuating hyperglycaemia and oxidative stress (Fig. 1). At baseline all groups had similar glycaemic level (5-6 mmol/l). In the CHG group, hyperglycaemia (PG > 22 mmol/l) were immediately manifested and maintained throughout the study. The CLG group, receiving one third the amount of glucose of the CHG group, displayed a moderate increase in glycaemia (PG ~ 12 mmol/l) at the initial phase of experimentation but levelled out during the time course. The FLU group, receiving the same amount of glucose as the CLG group but by pulsating infusion, showed the expected fluctuations in plasma glucose. The plasma glucose levels were consistently elevated to about 20-22 mmol/l at peak level and returned to basal levels within 30 min after infusion of glucose was paused. Plasma MDA was measured as a biomarker of oxidative damage to lipids (Fig. 1B). At baseline (T=0 h) plasma MDA concentrations did not differ among groups and during time course the VEH and CLG showed no changes. The ANCOVA revealed a significant difference both in time and treatment and in time x treatment interaction for the CHG and FLU groups ($p<0.05$ in all cases). Plasma MDA was significantly increased in the FLU group at 72 and 96 hours were (72 h: VEH: 1.44 ± 0.06 vs. FLU 2.26± 0.13; 96 h: VEH: 1.35 ± 0.1 vs. FLU: 2.1 ± 0.1; $p<0.05$ all cases), and in the CHG group at 72 hours (VEH: 1.35 ± 0.1 vs. CHG: 2.45 ± 0.43; $p<0.05$).

Liver Biochemistry (Fig. 2). Overt hyperglycaemia which was evident in the CHG group induced a significant higher level of liver MDA compared to all other groups (Fig. 2A; $p<0.0001$ vs. VEH; $p<0.05$ vs. CLG & FLU). Liver MDA was also significantly increased in the CLG group ($p<0.05$ vs. VEH). Similarly, we observed a significant increase in SOD activity (Fig. 2B) in the CHG ($p<0.001$ vs. VEH) and CLG ($p<0.05$ vs. VEH) groups. Protein expression of gp-91^{PHOX} (Fig. 2C), the major subunit of the NOX, was also significantly

increased for the CHG group as compared to VEH and FLU group ($p<0.05$; both cases). Although not statistically significant ($p=0.067$ vs. VEH), the gp-91^{PHOX} protein level was approximately 50% higher for the CLG as compared to the FLU and VEH group. Interestingly, neither of the analysed parameters were significantly affected in the FLU group.

Liver Expression (Fig. 3). As expected, all groups receiving glucose infusion showed a significantly increased accumulation of TG and GLY in liver (Fig. 3A & B). The CHG group showed a 2.5 fold increase in GLY and a 2 fold increase in TG levels as compared to the controls ($p<0.0001$; both cases). The FLU and CLG group showed moderate but significant changes in both GLY and TG ($p<0.05$; all cases). These results may indicate that the sustained hyperglycaemic group (CHG) had a significant higher rate of hepatic glucose uptake. GK the rate limiting enzyme for glucose uptake in liver, was analysed by western blotting (Fig. 3C). The CHG and CLG group showed approximately a 2 fold increase in GK protein level as compared to the controls ($p<0.05$; both cases). Interestingly, the FLU group had an even higher effect on the GK protein level, displaying a 4 fold increase compared to the controls ($p<0.0001$), which was also significantly higher than the CHG and CLG groups ($p<0.05$; both cases). In contrast, the GK mRNA level (Fig. 3D) was significantly reduced in the CHG and CLG groups ($p<0.05$ vs. VEH; all cases) but not in the FLU group. Protein expression of GS (Fig. 3E & F), the major regulator of glycogenolysis, was significantly reduced in the CLG group ($p<0.05$) but not in the CHG and FLU as compared to the controls. Finally, we assessed the hyperglycaemic effect on Srebp1c (Fig 3G & H), a major regulator of lipogenic enzymes upstream in the glycolytic pathway. We observed a marked reduction in both protein and mRNA levels in the CLG group ($p<0.05$ vs VEH; both cases) but only at mRNA level for the CHG group ($p<0.05$ vs. VEH). The FLU group was not significantly affected as compared to controls.

Aorta biochemistry (Fig. 4 & 5). As illustrated in figure 4A, fluctuating hyperglycaemia induced significant higher levels of aortic MDA and oxLDL as compared to controls and the CLG group ($p<0.05$; both cases). The aortic SOD levels (Fig. 4B) were significantly affected in the FLU and CHG groups ($p<0.05$ vs. VEH; both cases). In contrast to the liver, glucose uptake in aorta is not mediated by the action of insulin but primarily by facilitated diffusion through the GLUT1. In aorta, the FLU group showed a significant increase in GLUT1 and $gp91^{PHOX}$ protein expression as compared to the VEH and CLG group (Fig. 5, $p<0.05$; both cases). Furthermore, the abundance of GLUT1 and $gp91^{PHOX}$ protein was also significantly increased in the CHG group ($p<0.05$ vs. VEH; both cases). Neither of the analysed parameters in aorta was affected in the CLG group.

Discussion

Substantial efforts have been made to clarify if repeated incidents of short term hyperglycaemia are an independent risk factor for endothelial dysfunction and the development of macro vascular complications in diabetes(8;22;23) and recent reports both in humans and rodents have shown that intermittent hyperglycaemia induce higher levels of oxidative stress leading to cellular damage in particular in the cardiovascular system thus potentially resulting in diabetes related atherosclerosis and CVD (7);8;22;23). However, so far the exact sequences of biochemical events have not been elucidated.

In a previous study using lean rats with no signs of insulin resistance or dyslipidaemia, we showed that intermittent hyperglycaemia compared to sustained hyperglycaemia may induce relatively higher levels of oxidative stress systemically but not at tissue level (24). The moderate, albeit significant, changes in plasma MDA and oxidation ratio as compared to the effects seen in diabetic models (25-28), may reflect that lean animals are metabolically more robust and to a large extent capable of handling hyperglycaemic episodes without experiencing severe induction of oxidative stress and endothelial dysfunction. Indeed, the development of diabetes related endothelial dysfunction is clearly composed of several metabolic disorders and besides hyperglycaemia, insulin resistance and dyslipidaemia have been shown to play important roles in endothelial function (29;30).

In the present study, we added the challenge of a high fat diet to either sustained or intermittent hyperglycaemia in rats with insulin resistance, dyslipidaemia (Table 1) or fatty liver for a more realistic model of the effects of hyperglycaemia on oxidative stress. We hypothesized that the liver, being the main regulator of glucose homeostasis, may exert important regulatory mechanisms of the glucose metabolism during different hyperglycaemic situations. In fact, this regulation may affect the disposal and consequently the exposure of

glucose to aorta and therefore have an indirect effect on the severity of oxidative stress e.g. on the vascular system in particular.

Lipid oxidation, measured as MDA, is a widely used biomarker of cellular oxidative stress in diabetics (31), and studies both in diabetic patients and animal models have found increased lipid oxidation in both tissue and biological fluids (27;28). In agreement with this rationale, a recent study showed a positive correlation between increased MDA in plasma and vascular dysfunction during hyperglycaemia both in insulin resistant and diabetic patients (32). In this study, we also found increased plasma MDA levels both during sustained overt hyperglycaemia (PG > 20mM) and in animals with fluctuating plasma glucose profiles. These findings may reflect an earlier onset of oxidative stress in the FLU group and consequently a potentially more detrimental effect on the endothelium than the CHG group. Indeed, overt ROS production has been shown to correlate with glycaemic exposure (18;33), but importantly, the observed effect on plasma MDA in our study seems to be independent of the total glycaemic exposure since the FLU group received only one third of the amount glucose given to the CHG group. This suggests that the effect on plasma MDA is independent of the total amount of glucose to which the rats were exposed to but rather dependent on the glycaemic profile.

Increased liver oxidative stress has been demonstrated both during chronic glucose infusion (34) and in diet induced insulin resistant animal models (35;36). In line with these findings, we observed a marked increase in liver MDA and SOD in the groups receiving a constant infusion of glucose but not in the fluctuating hyperglycaemic group. The membrane bound NOX subunit, gp-91^{PHOX}, has been described as being the primary regulator of NOX and in studies where hepatocytes were subjected to chronic high glucose, expression of gp-91^{PHOX} was significantly increased along with increased NOX activity (37). The significant increase in liver gp-91^{PHOX} protein expression for the sustained hyperglycaemic groups may indicate

increased ROS production by the NOX and consequently, the induction of oxidative stress. Interestingly, this effect on oxidative stress markers was not present during fluctuating hyperglycaemia despite, being subjected to equal amounts of glucose as the moderate hyperglycaemic group (CLG). This may suggest that the liver is more prone to increased ROS production and oxidative stress when subjected to sustained hyperglycaemia but not during fluctuating hyperglycaemia. Studies have shown a direct link between higher glucose uptake rates and increased NOX. In order to answer these questions, we elucidated the effect on key enzymes in the liver responsible for glucose uptake and utilization. As expected, the GK protein levels were markedly increased during constant glucose infusion of glucose while the FLU group had an even higher protein level. However, at the mRNA level, only the chronically infused animals showed a marked reduction of liver GK while the FLU group was unaffected. The down regulation of GK expression in the groups receiving the chronic glucose infusion may indicate a lowering of net glucose uptake by the liver. However, it should be emphasized that this only represents a ~~–s~~nap shot" of the time course and multiple adaptive processes may have been involved during five days of glucose infusion. In fact studies have shown that the liver may exert biphasic controlling actions of glucose homeostasis in the hyperglycaemic state. At initial stages of hyperglycaemia, the liver facilitates glucose uptake while at later stages, the liver develop insulin resistance and facilitates glucose output (38;39). The level of GK expression is primarily controlled by insulin and studies have shown that long term high fat feeding will induce liver insulin resistance with a concomitant reduction in GK expression and activity (40). ROS molecules has been shown in several studies to be an important signal molecule and direct effects of increased ROS production in liver, muscle and adipose tissue has been demonstrated to consequently induce insulin resistance and act as a protective mechanism against further oxidative stress (9;10) . Assuming that this interpretation is correct, the liver plays an

important role in protecting organs with insulin independent glucose uptake from excess glucose challenge only limited by its capacity to synthesize GLY and TG. However, this important metabolic regulation may be lacking or disturbed during fluctuating glucose. In order to address this question, we looked at oxidative stress status in aorta.

Cultured aortic endothelial cells subjected to intermittent hyperglycaemia has been shown to produce higher levels of superoxide as compared to cells subjected to sustained hyperglycaemia (5;6). In agreement with this, we showed *in vivo* that fluctuating but not sustained hyperglycaemic status increased MDA and ox-LDL in aorta. Likewise, presumably as an adaptive preventive mechanism against increased oxidative stress, the SOD activity was markedly increased as well. Influx of glucose in endothelial cells is facilitated by GLUT 1 in an insulin independent manner (15) and excess glucose may activate PKC by several mechanisms, which consequently increases oxidative stress by activating NOX (16;18;41). In connection we found a marked increase in GLUT1 and $gp91^{PHOX}$ protein expression both during fluctuating and sustained hyperglycaemia. In diabetics, increased ox-LDL accumulation in vessels has been shown to promote foam cell formation in vascular smooth muscle thus leading plaque formation and the development of atherosclerosis (19). Additionally, oxidized LDL has itself been shown to produce oxidative stress in endothelial cells via activation of a NOX (42). These findings may indicate that during fluctuating hyperglycaemia aorta may be subjected to excess glucose uptake affecting the activity of NOX thus leading to increased oxidative stress and ox-LDL accumulation.

As outlined in figure 6, our data supports our hypothesis that during overt hyperglycaemia, the liver exerts a protective effect against glucose induced oxidative stress by increasing the rate of glucose uptake and facilitating GLY and TG synthesis. These mechanisms are saturable and a spill over of glucose metabolites in the liver leads to increased ROS production. The liver counteracts these deleterious processes by down regulating expression

of liver key enzymes important for glucose uptake and metabolism thereby protecting the liver from further oxidative stress. In situations of fluctuating hyperglycaemia, this adaptation is lacking. In the aorta, where glucose is primarily taken up by facilitated diffusion, the lack of glucose uptake by liver leave the aortic endothelial cells exposed to excess glucose resulting in increased oxidative stress in the vasculature. In conclusion, our data provides substantial in vivo support for the hypothesis that fluctuating hyperglycaemia induces relatively higher levels of oxidative stress in the cardio-vascular system compared to sustained hyperglycaemia. This is in agreement with the clinical evidence that fluctuating hyperglycaemia is an independent risk factor for diabetes related endothelial dysfunction, atherosclerosis and cardiovascular disease.

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Duality of interest

All authors declare no conflict of interest that could influence the present work

Contribution statement

The study was conceived and planned by all authors. GR and TG performed the experiments. Data analysis and interpretation was performed by all authors. GR drafted the manuscript which was revised by KR and JL.

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Tables

TABLE 1.

Animal data. Data from lean animals was converted from previously published study (24).

	DIO (n=30)	LEAN (n=30)
Body weight (g)	679 ± 14	423 ± 8
Plasma Insulin (pM)	640 ± 68	198 ± 22
Plasma FFA (μM)	637 ± 44	276 ± 45

TABLE 2.

Infusions rate (ml/kg/h). The table describes one cycle of vehicle or glucose infusion and the amount of glucose infused per 24 hours. The cycle was repeated continuously during the complete infusion period of 96 hours.

	0 – 2 min	2 – 32 min	32 – 152 min	Glucose (g/kg/24 hours)
VEH	1.0	1.0	1.0	0
CHG	3.0	3.0	3.0	36.0
CLG	1.0	1.0	1.0	12.0
FLU	30.0	3.5	0	12.4

Figures

Figure 1

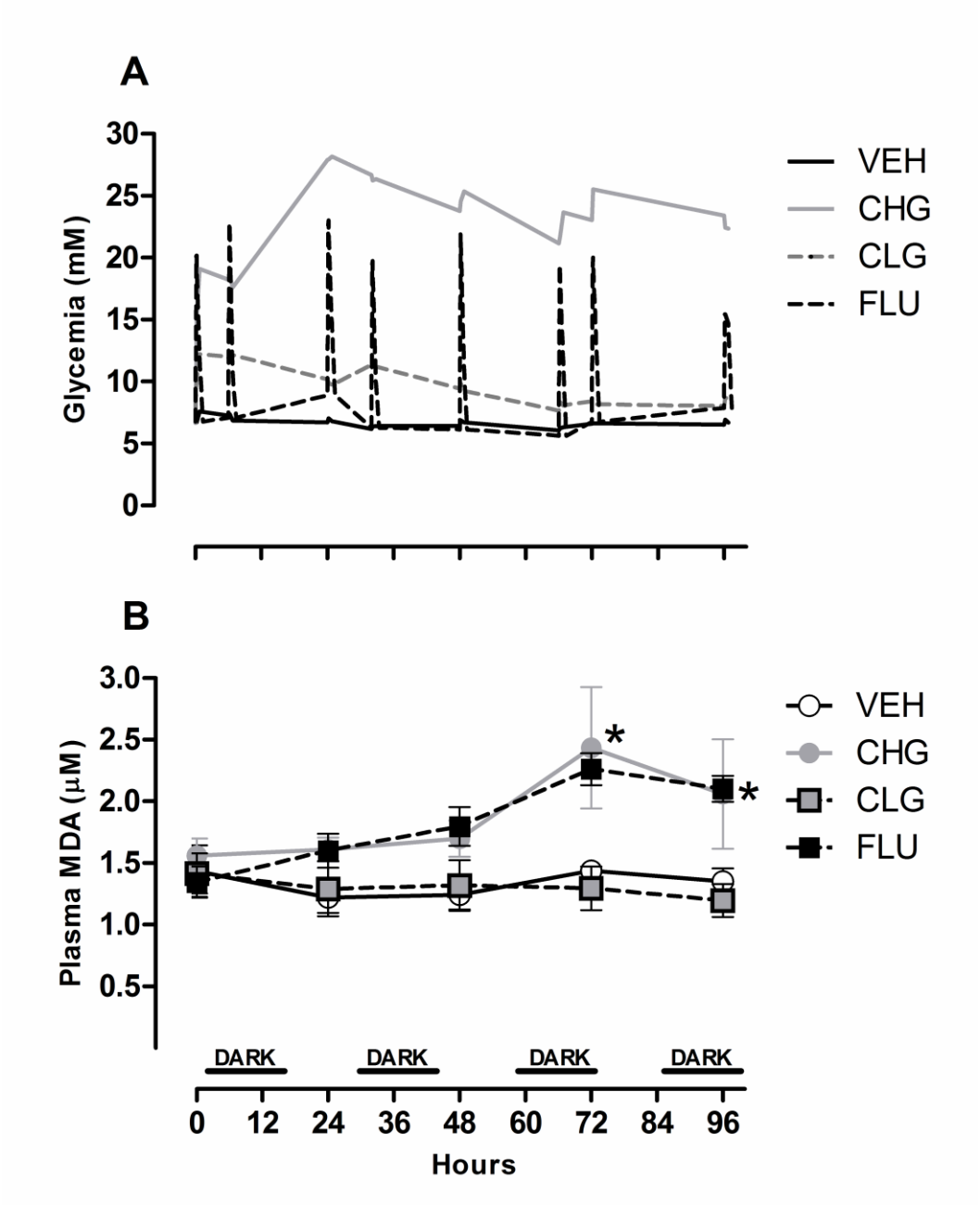

Figure 2

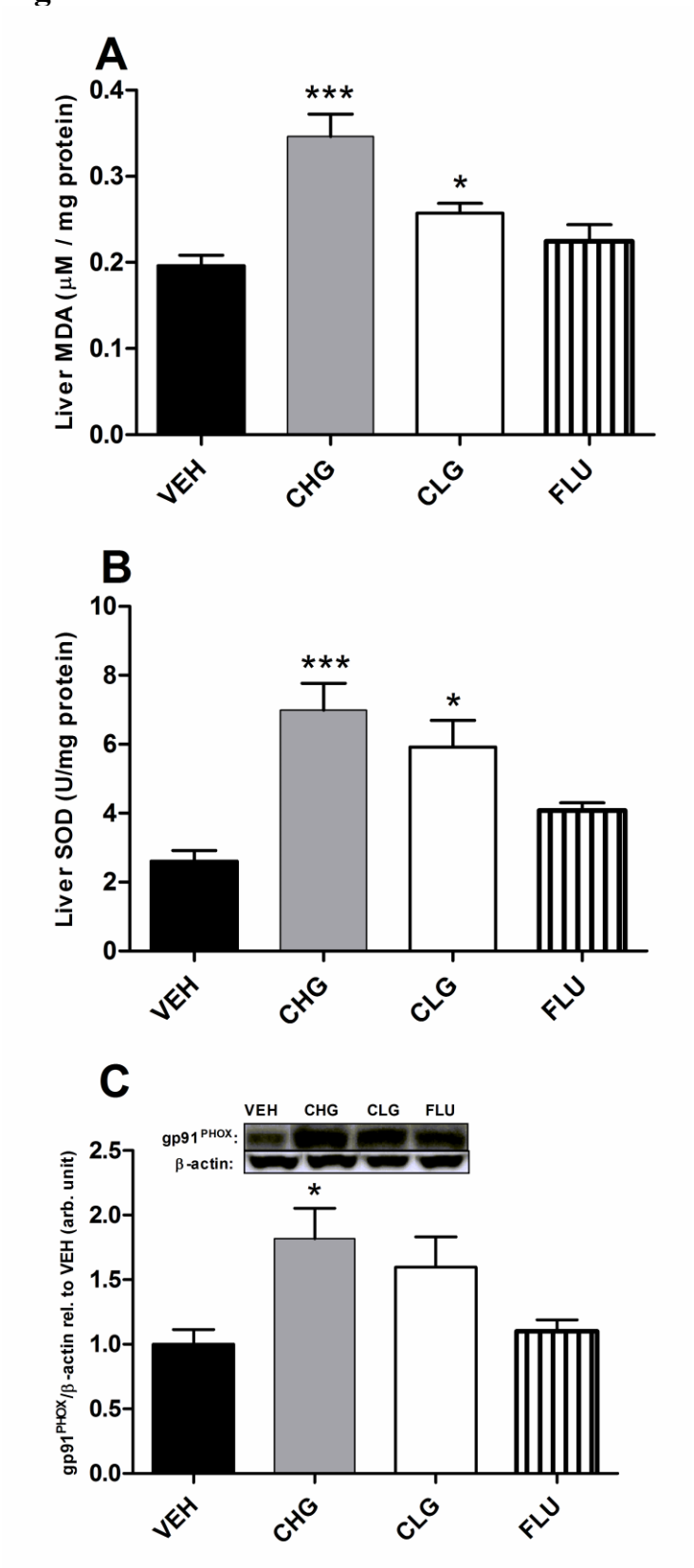
A
Liver MDA (μM / mg protein)
0.4
0.3
0.2
0.1
0.0
***
*
VEH
CHG
CLG
FLU
B
Liver SOD (U/mg protein)
10
8
6
4
2
0
***
*
VEH
CHG
CLG
FLU
C
VEH
CHG
CLG
FLU
gp91 PHOX:
β-actin:
gp91PHOX/β-actin rel. to VEH (arb. unit)
2.5
2.0
1.5
1.0
0.5
0.0
*
VEH
CHG
CLG
FLU

Figure 3

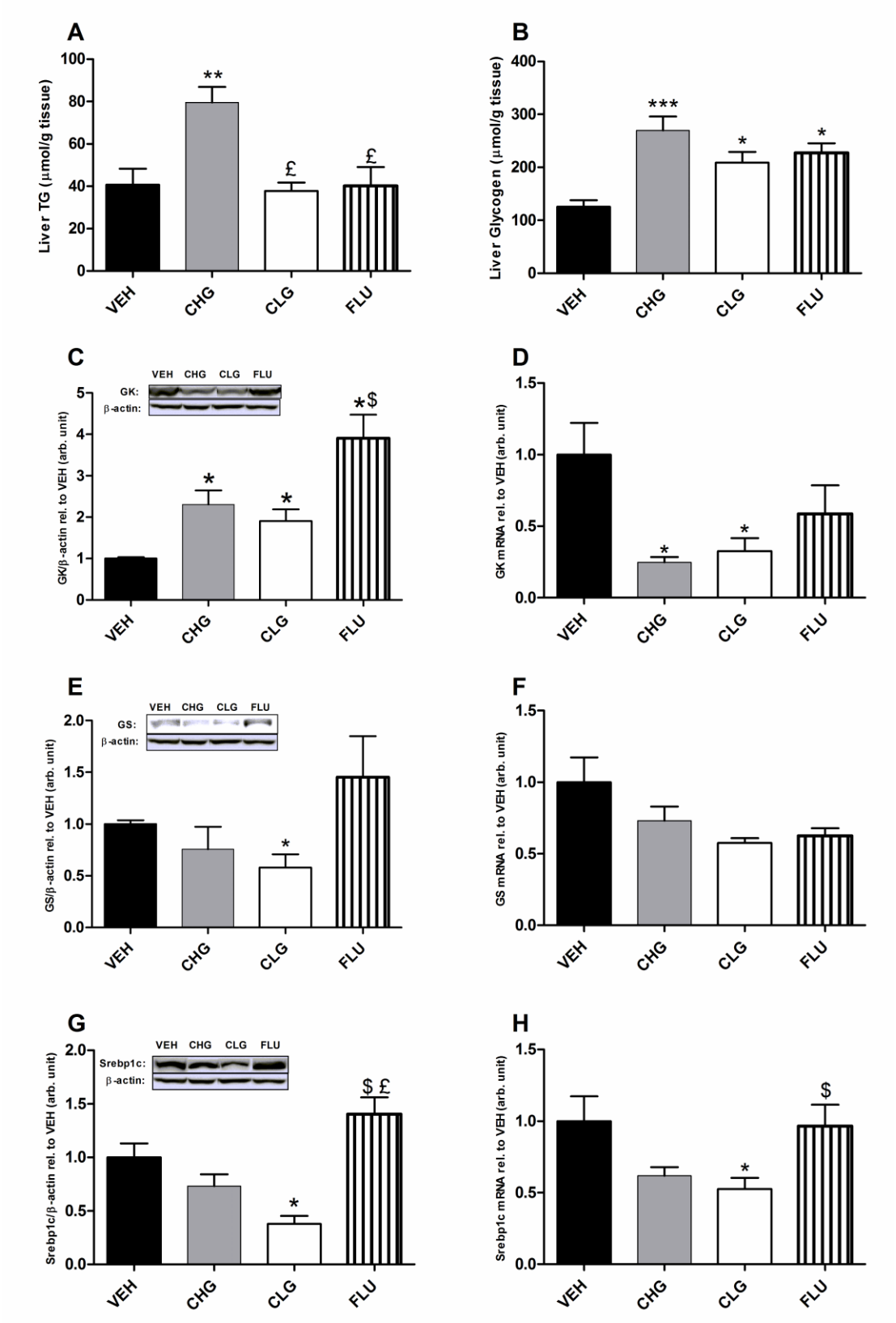
A
Liver TG (µmol/g tissue)
100
80
60
40
20
0
**
£
£
VEH
CHG
CLG
FLU
B
Liver Glycogen (µmol/g tissue)
400
300
200
100
0
***
*
*
VEH
CHG
CLG
FLU
C
VEH CHG CLG FLU
GK:
β-actin:
GK/β-actin rel. to VEH (arb. unit)
5
4
3
2
1
0
*
*
*$
VEH
CHG
CLG
FLU
D
GK mRNA rel. to VEH (arb. unit)
1.5
1.0
0.5
0.0
*
*
VEH
CHG
CLG
FLU
E
VEH CHG CLG FLU
GS:
β-actin:
GS/β-actin rel. to VEH (arb. unit)
2.0
1.5
1.0
0.5
0.0
*
VEH
CHG
CLG
FLU
F
GS mRNA rel. to VEH (arb. unit)
1.5
1.0
0.5
0.0
VEH
CHG
CLG
FLU
G
VEH CHG CLG FLU
Srebp1c:
β-actin:
Srebp1c/β-actin rel. to VEH (arb. unit)
2.0
1.5
1.0
0.5
0.0
*
$ £
VEH
CHG
CLG
FLU
H
Srebp1c mRNA rel. to VEH (arb. unit)
1.5
1.0
0.5
0.0
*
$
VEH
CHG
CLG
FLU

Figure 4

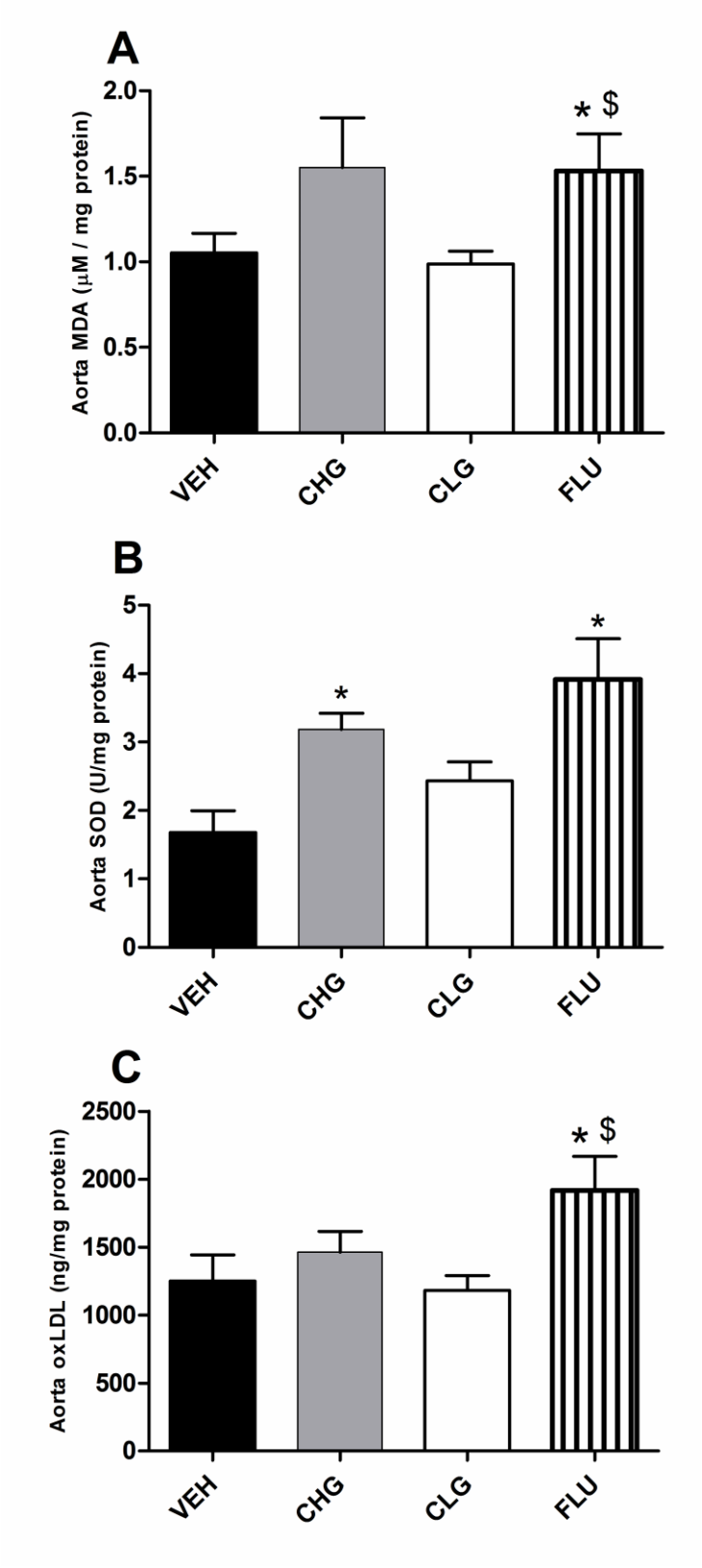
A
Aorta MDA (µM / mg protein)
2.0
1.5
1.0
0.5
0.0
* $
VEH
CHG
CLG
FLU
B
Aorta SOD (U/mg protein)
5
4
3
2
1
0
*
*
VEH
CHG
CLG
FLU
C
Aorta oxLDL (ng/mg protein)
2500
2000
1500
1000
500
0
* $
VEH
CHG
CLG
FLU

Figure 5

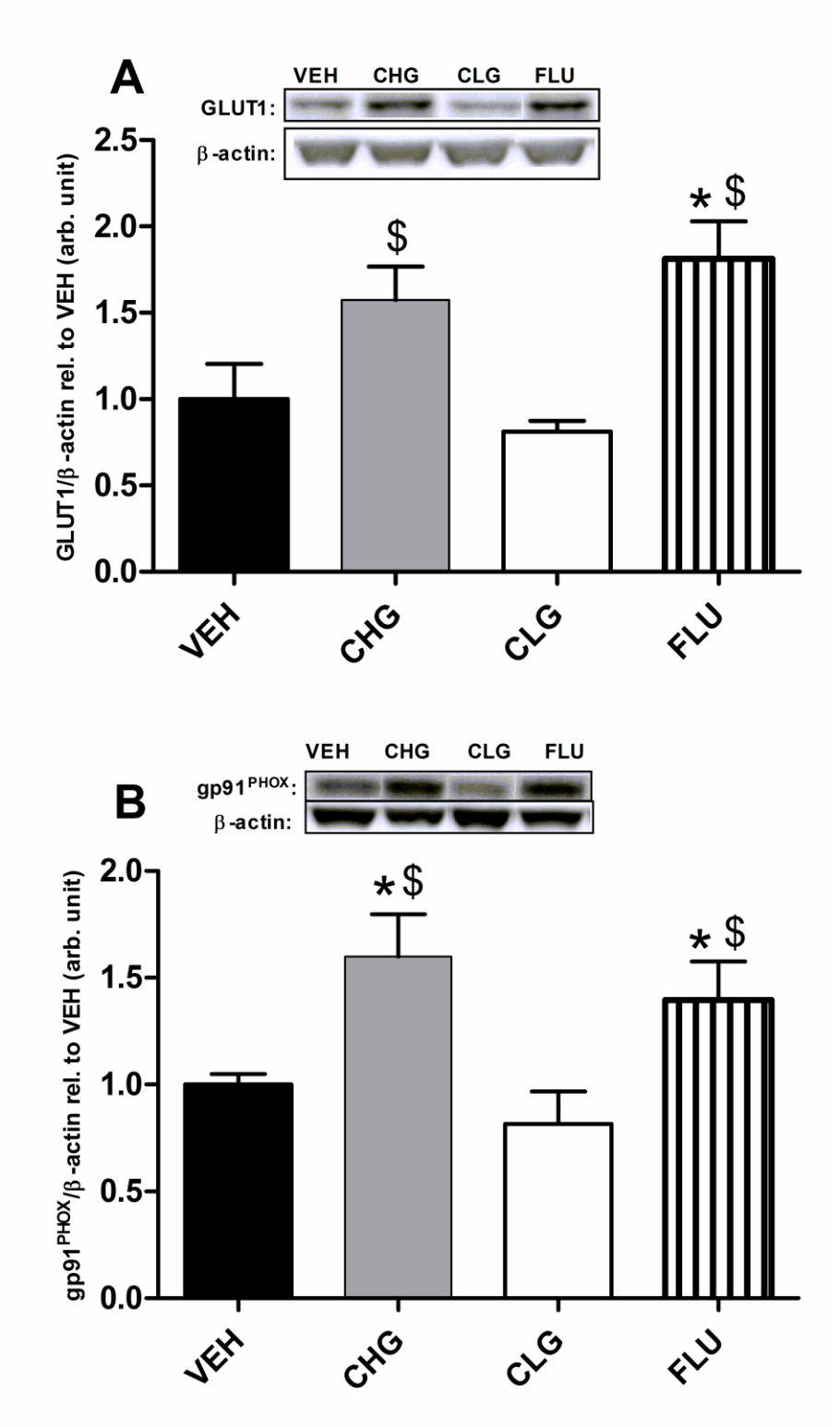
A
VEH
CHG
CLG
FLU
GLUT1:
β-actin:
GLUT1/β-actin rel. to VEH (arb. unit)
2.5
2.0
1.5
1.0
0.5
0.0
$
* $
VEH
CHG
CLG
FLU
B
VEH
CHG
CLG
FLU
gp91PHOX:
β-actin:
gp91PHOX/β-actin rel. to VEH (arb. unit)
2.0
1.5
1.0
0.5
0.0
* $
* $
VEH
CHG
CLG
FLU

Figure 6

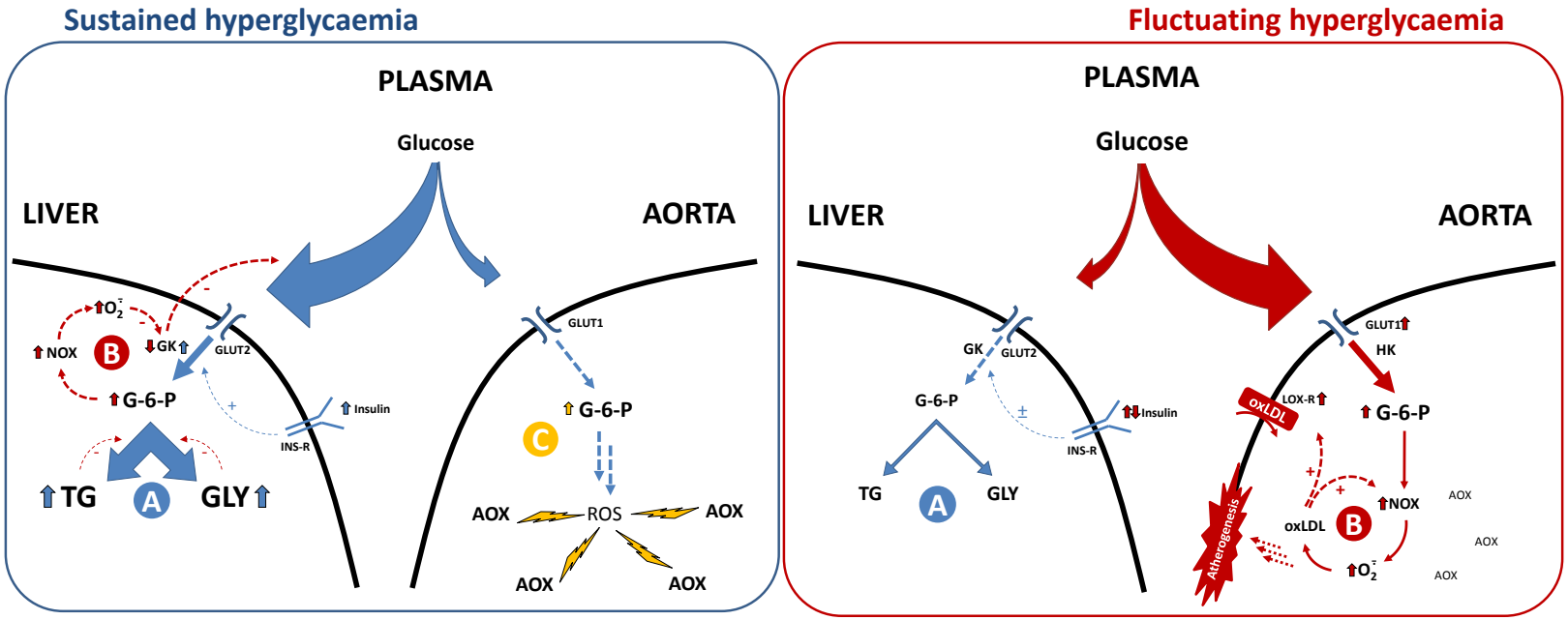
Sustained hyperglycaemia
PLASMA
Glucose
LIVER
AORTA
NOX
B
GK
GLUT2
G-6-P
Insulin
INS-R
TG
A
GLY
GLUT1
G-6-P
C
AOX
ROS
AOX
AOX
AOX
Fluctuating hyperglycaemia
PLASMA
Glucose
LIVER
AORTA
GK
GLUT2
G-6-P
Insulin
INS-R
TG
A
GLY
GLUT1
HK
LOX-R
oxLDL
G-6-P
NOX
oxLDL
B
Atherogenesis
AOX
AOX
AOX

Figure Legends

Figure 1. Fluctuating hyperglycaemia increases systemic oxidative stress status independent of glycaemic exposure. Plasma glucose (A) was followed throughout the complete study period. The FLU group were subjected to nine glucose pulses daily and the glucose levels were determined at selected time points covering the complete circadian rhythm. Plasma MDA (B) was monitored daily at the exact same time point. Data are means ± SEM, n=7-8. *$p < 0.05$ vs. VEH.

Figure 2. Sustained but not fluctuating hyperglycaemia increases oxidative stress in liver. Liver MDA (A), SOD activity (B) and protein expression of $gp91^{PHOX}$ (C) were determined in liver homogenates. Representive immunoblots of $gp91^{PHOX}$ and β-actin are shown above the bars. Data are means ± SEM, n=7-8. *p<0.05, ***p<0.0001 vs. VEH.

Figure 3. Chronic sustained hyperglycaemia down regulates key glycolytic enzymes in liver. After 96 hours of different glucose infusion paradigms liver was analysed for glycogen (A) and triglyceride (B) content and the expression level of GK (protein (C); mRNA (D)); GS (protein (E); mRNA (F)); Screbp1c (protein (G); mRNA (H)). Representive immunoblots of GK, GS, Screbp1c and β-actin are shown above the bars. Data are means ± SEM, n=7-8. *p < 0.05, **p < 0.001 and ***p<0.0001 vs. VEH. $p < 0.05 vs. CLG. £p < 0.05 vs. CHG.

Figure 4. Fluctuating hyperglycaemia induced increased oxidative stress status and accumulation of oxLDL in aorta. Aorta homogenates were analysed for MDA content (A), SOD activity (B) and oxLDL (C) accumulation. Data are means ± SEM, n=7-8. *p < 0.05 vs. VEH, $p < 0.05 vs. CLG.

Figure 5. Fluctuating and chronic overt hyperglycaemia increased GLUT1 and gp91^{PHOX} protein expression. Western Blot analyses of GLUT1 (A) and gp91^{PHOX} (B). Representive immunoblots of GLUT1, gp91^{PHOX} and β-actin are shown above the bars. Data are means ± SEM, n=7-8. *p < 0.05 vs. VEH, $p < 0.05 vs. CLG.

Figure 6. Working hypothesis: Fluctuating hyperglycaemia preferentially leads to increased glucose influx into aorta inducing ROS production and oxidative stress.

During *sustained hyperglycaemia* (left panel) the liver may exert bi-phasic metabolic events. At the initial state (A) sustained hyperglycaemia will lead to increased liver glucose uptake by increased expression of GK resulting in increased accumulation of GLY and TG. The later event (B) may be a ―spill over" of glycolytic metabolites leading to increased ROS production through increased NOX activity. Counteracting these detrimental effects ROS molecules may act as signalling molecules inducing a down regulation of GK expression and other key enzymes as well. Finally (C) the attenuated capacity of glucose uptake in the liver may result in excess facilitative diffusion of glucose in to the aortic endothelium leading to ROS production. In *fluctuating hyperglycaemic* (right panel), the initial (A) adaptive phase as seen during sustained hyperglycaemia by the liver may be lacking due to the short term hyperglycaemic ―spikes". (B) Because of increased GLUT1 expression, glucose is preferentially disposed into the aorta leading to increased ROS formation by NOX. Increased oxidative stress will then lead to accumulation of oxLDL which in turn may further increase the activity of NOX, escalating the ROS production to unphysiological levels. The increased oxLDL levels may lead to the formation of foam cells ultimately leading to atherosclerotic lesions.

Paper III

Insulin resistance exacerbates vascular oxidative stress response during fluctuating hyperglycaemia

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Keywords; Oxidative stress, Fluctuating hyperglycaemia, Metabolic syndrome, Macrovascular complications

Abstract

Aim. In the present study, we wanted to investigate to what extent the level of insulin resistance contributes to the level of oxidative stress during either fluctuating or sustained chronic hyperglycemia. **Methods.** Animals divided into eight groups (n=7-8) and given either a continuous high (CHG) or low (CLG) or pulsatile (FLU) infusion of glucose for 72 (Lean) or 96 (DIO) hours. The FLU and CLG groups received equal amount of glucose in total through the study period although with different infusion profiles whereas the CHG received three times the amount of glucose in total. The controls received a constant infusion of saline (VEH) equal to the CLG group. Plasma glucose was followed during the complete study period. Plasma MDA and 8-isoprostane were followed once daily. At termination point liver and aorta were isolated for analysis of oxidative stress levels and expression of key enzymes in the regulation of glucose metabolism. **Results.** In the LEAN model, although significant increase in aorta GLUT1 and $GP91^{PHOX}$ protein abundance during overt sustained hyperglycemia, no changes in aorta MDA, SOD or oxLDL were observed in neither of the groups. The DIO model had significant higher levels of GLUT1 and $GP91^{PHOX}$ protein expression and lower SOD activity level in aorta. Moreover subjecting DIO rats to fluctuating hyperglycemia aorta GLUT1 and $GP91^{PHOX}$ were further increased. Additionally, aorta malondialdehyde (MDA) and oxidized LDL (oxLDL) were significantly increased but not during sustained hyperglycemia. **Conclusion.** The level of oxidative was highly compartmentalized where the cardiovascular system was primarily targeted by the fluctuating hyperglycemic profile whereas the liver is more disposed to oxidative damage by the chronic hyperglycemic profile. The DIO model was more prone to increased oxidative stress suggesting that insulin resistance exacerbates the oxidative stress resulting from fluctuating hyperglycemia.

Abbreviations

8-IsoP	8-isoprostanes
BMI	Body mass index
CHG	Constant High Glucose
CLG	Constant Low Glucose
DGAT	Diacyl glycerol acyl transferase
DIO	Diet induced obesity
FFA	Free fatty acids
FLU	Fluctuating Glucose
GLY	Glycogen
Hba1c	Glycated haemoglobin
IRS	Insulin receptor substrate
MDA	Malondialdehyde
NADPHox	NADPH oxidase
NaF	Sodium Fluride
oxLDL	oxidised low density lipoprotein
PG	Plasma glucose
PKC	Protein kinase C
ROS	Reactive Oxygen Species
SOD	Super Oxide Dismutase
T2D	Type 2 diabetes
TG	Triglycerides
UCP-2	uncoupling protein 2
VEC	Vascular endothelial cells
VEH	Vehicle

Introduction

Recently, several epidemiological and case controlled intervention studies have indicated that poorly controlled glucose levels such as fluctuating hyperglycemia may be an glycated haemoglobin (Hba1c) independent risk factor for the development of diabetes related macrovascular complications (1). In support of this hypothesis, a range of studies both in humans (2;3), animal models (4;5) and cell based assays (6-8) have shown that fluctuating hyperglycemia results in a more pronounced increase of oxidative stress than in chronic hyperglycaemic situations thus potentially affecting the cardio vascular system by inducing endothelial dysfunction. On the other hand, the development of diabetes related endothelial dysfunction is clearly composed of several metabolic disorders and besides hyperglycaemia; obesity, insulin resistance and dyslipidaemia may also play important roles in endothelial function (9;10). Recently in the Framingham study, it was reported that in subjects with a higher body mass index, a measure for obesity, had significantly increased oxidative stress as measured by urinary biomarkers including 8-Isoprotanes (11). Likewise a large number of reports both in humans and animals have suggested that oxidative stress is increased during obesity (12;13) . Over nourished cells in form of excess glucose and free fatty acids (FFA) may induce the production of reactive oxygen species (ROS) through increased NADPH oxidase (ox) activity thus inducing a redox imbalance and oxidative stress (14). Insulin resistance is closely linked to obesity and the vast majority of type 2 diabetic (T2D) subjects are insulin resistant. Moreover, insulin resistance has been demonstrated to affect ROS production in vascular endothelial cells (VEC) through the action of NADPHox enzyme complex resulting in endothelial dysfunction increased oxidized low density lipoproteins (oxLDL) and the development of atherosclerosis (15;16).

We have previously shown in both lean healthy and in obese insulin resistant rats that fluctuating hyperglycemia increases systemic oxidative stress to similar levels as in overt

sustained hyperglycaemia (Paper I, Paper II). In the present study, we wanted to investigate to what extent the level of insulin resistance contributes to the level of oxidative stress during either fluctuating or sustained chronic hyperglycemia.

Materials and Methods

Animals

The study was approved by the Danish Animal Experimentation Inspectorate and carried out by trained and licensed personnel. A total of 60 Sprague-Dawley male rats (Taconic, Denmark) were used in the study. The animals were either fed a high fat feed (D12492, Research Diets, USA) for at least 20 weeks in order to develop obesity, insulin resistance and dyslipidemia (DIO, n=30)(Table 1) or a regular chow diet (Type 1324, Altromin GmBH, Germany)(LEAN, n=30). During the entire study period, animals had free access to food and water (tap water added 1% citric acid). Animals were housed in pairs in temperature (22 ± 2 °C) and humidity (50± 20 %) controlled rooms. The circadian rhythm was 12h light: 12h dark (Lights on at 06:00 AM). Prior to surgery, animals were treated with Anorfin (GEA A/S, Denmark), a fast acting analgesic, and subsequently kept on anaesthesia with isoflurane (Baxter Pharmaceuticals, USA) and 30%: O_2 / 70%: N_2O. Silicon catheters (Tygon Microbore Tubing, S-50-HL, Cole Parmer, UK.) were surgically placed into the right jugular vein for infusion and in the left carotid artery for blood sampling. The catheters was externalized subcutaneously to the mid-scapular region, filled with 500IU/ml heparin (LEO Pharma Nordic, Denmark) in HAEMACCEL (Intervet/Schering-plough, UK) and sealed into Dacron buttons (Instech Laboratory Inc, USA). After surgery, animals were single housed and allowed 7-8 days of post-surgery recovery before further experimentation. During the recovery period animals were treated with Baytril (Bayer AG, Germany) and Rimadyl (Pfizer Inc, USA) for three days.

Infusion protocol

On the day of experimentation, all animals had their food removed at 6.00 AM and were transferred to clean cages while their catheters were checked for reliability. The carotid artery

was connected to an Accusampler system (DiLab AB, Sweden) for automated blood sampling whereas the jugular vein catheter was connected to an infusion pump (World Precision Instruments Inc., USA). The dosing regimens started after 6 hours of fasting and consisted of infusion of glucose (50% glucose solution; Fresenius Kabi AG, Germany) or saline as outlined in table 2. Briefly, animals were randomly divided into four groups (n=7-8) receiving either continuous high (CHG), low (CLG), or pulsatile (FLU) infusions of glucose for 72 (LEAN) or 96 (DIO) hours. The FLU and CLG groups received equal amounts of glucose per 24 hours although with different infusion profiles whereas the CHG received three times the amount of glucose per 24 hours. Control animals received a constant infusion of saline (VEH) equal to the CLG group.

Blood sampling

Blood samples (200µl in total) for malondialdehyde (MDA), 8-isoprostanes (8-IsoP) and FFA were drawn at time points 0, 24, 48 and 72 and 96 (only DIO) hours. Plasma was immediately separated (4°C; 5000rpm; 3 min) and divided into relevant aliquots. For MDA and 8-IsoP, plasma aliquots were immediately frozen in dry ice and kept at -80°C until analysis. For FFA, an aliquot of plasma was transferred into sodium fluoride (NaF; 5mg/ml) coated tubes and stored at -80 °C until analysis. In order to account for the haemodilution due to the infusion, the haematocrit value was determined at all-time points and used for normalization of plasma concentrations.

Body composition

Body composition was evaluated in live, conscious animals by quantitative nuclear magnetic resonance spectroscopy (EchoMRI 3-in-1 Animal Tissue Composition Analyzer; Echo Medical Systems, Houston, TX) prior to experimentation. Briefly, a system test was performed routinely at the beginning of each measurement day, and the equipment was

calibrated by scanning a calibration holder containing a known amount of fat to test the validity of measurement. Subsequently the animal was carefully put into a container, placed in the machine and scanned. The measurement was done within 120 sec and the animal was placed back into its cage. The fat content was calculated as the percentage of total body weight.

Biochemical analyses

Lipid oxidation was assessed by measuring MDA in plasma, liver and aorta as described previously (17) and 8-IsoP in plasma as described by the assay kit manufacturer (Cat no.:516351, Cayman Chemicals, USA). Briefly, 40 µl of plasma were purified from proteins and phospholipids by Waters Ostro 96-wells plate 25 mg (Cat. No.: 186005518, Waters Inc., USA). Subsequently, samples were air dried (Heto-Vac, Biostadt, Denmark) and re-suspended with 120 µl EIA buffer. Finally, samples were analyzed in duplicate on 96 well pre-coated strips. Plates were read at (405 - 420) nm on a SpectraMax Plus 384 UV/VIS platereader (Molecular Devices Inc., CA, USA). SOD in liver and aorta was assessed as described by manufacturer (Sigma Aldrich Cat. No. 19160). In aorta, oxLDL was assessed as described by the assay kit manufacturer (Uscn Life Science Inc; China, Cat. No. E90527Ra). Tissue protein measurements were determined using the Bradford assay(18). Plasma FFA was determined in duplicates as described by the assay kit manufacturer (WAKO Chemicals, CA, USA).

Western blotting

Tissue protein lysates were assayed with antibodies against GLUT1 and $GP91^{PHOX}$, the major subunit of the NADPH oxidase system (Abcam, USA). Protein levels were normalized to β-Actin (Abcam, USA). Secondary antibodies were horseradish peroxidise-coupled and ECL

reagent (BioVision, USA) was used for detection. Quantification was performed using ImageGuage 4.0 (Fujifilm, Japan).

Statistics

Statistical analyses of the results were performed by using SAS JMP software (version 8.1 for Windows, SAS institute, Cary, NC). Plasma MDA were analysed using ANOVA with time 0 hours as covariant and then a fit model with time, treatment and time x treatment interaction as model based variables. Liver and aorta metabolic parameters were analysed using ANOVA. In cases of statistical significance, Tukey's post hoc test was applied. A p-level less than 0.05 was considered statistically significant.

Results

Plasma oxidative stress

Plasma MDA and 8-IsoP were measured as biomarkers of oxidative damage to lipids. Plasma MDA data have previously been published separately (Paper I, Paper II). Comparatively, plasma MDA did not differ among groups or model (LEAN vs. DIO) at baseline (t=0 hour). In both LEAN and DIO, ANOVA revealed a significant interaction between time and groups ($p<0.0001$; both cases). In LEAN animals, Tukey's post hoc test showed a significant increase in plasma MDA levels in the FLU group at 48 and 72 hours ($p<0.05$), and in the CHG group at 72 hours ($p<0.05$). In DIO animals, a similar pattern was observed. Plasma MDA was significantly increased in the FLU group at 72 and 96 hours were ($p<0.05$), and in the CHG group at 72 hours ($p<0.05$). In both models, no changes in plasma MDA for the VEH and CLG group were observed.

Plasma 8-IsoP showed a significant difference among LEAN groups by ANOVA during the time course ($p<0.05$). Post hoc test revealed a significant increase for CHG ($p<0.05$ vs. VEH) but not for FLU ($p=0.063$ vs. VEH). However, when comparing the FLU group with the CLG group, which received the same amount of glucose in total, a significant higher level of plasma 8-IsoP was observed ($p<0.05$). Among the DIO animals, ANOVA revealed a significant interaction between group and time ($p<0.05$). Post hoc test showed a significant difference in FLU and CHG vs. VEH at the 72 hour time point ($p<0.05$; both cases). Together, these findings suggest that the glycaemic profile independently influences the level of systemic oxidative stress.

Plasma FFA

Plasma FFA levels were monitored daily. At semi-fasted state (6 hours), DIO rats had significantly higher levels of plasma FFA as compared to the LEAN rats (LEAN: 327 ± 15 μM vs. DIO: 612 ± 23 μM; $p<0.0001$). In DIO rats, ANOVA revealed significant changes during time ($p<0.001$) and between groups ($p<0.001$). In addition, post hoc analyses showed a significant reduction in plasma FFA during chronic infusion of glucose (CHG & CLG: $p<0.05$ vs. VEH; both cases) but not during fluctuating hyperglycemia. In LEAN animals, plasma FFA levels were not affected by glucose infusion.

Liver oxidative stress

As confirmed by the two control groups (VEH-LEAN vs. VEH-DIO), the level of MDA in liver was not different between models. As shown in the table 3, two-way ANOVA displayed a significant interaction between diet and groups ($p<0.0001$). Post hoc tests verified that in DIO rats, liver MDA levels were significantly increased in the CHG and CLG ($p<0.001$ and $p<0.05$ vs. VEH, respectively). No differences between LEAN groups were observed.
Liver SOD activity displayed a similar pattern. As for MDA in DIO animals, significantly higher SOD activity was observed for CHG and CLG groups ($p<0.0001$ vs. VEH in both cases), but also the FLU group showed higher SOD activity ($p<0.001$ vs. VEH) although the increased was less pronounced than those seen in the CHG and CLG. Again, no differences between lean groups were observed. These data suggest that insulin resistant rats are more susceptible to increased ROS production but only when challenged with chronic glucose infusion.

Aorta oxidative stress

Significant differences in aorta MDA were observed between groups ($p<0.05$). In DIO rats, significant increases in liver MDA of the FLU group compared to VEH and CLG animals

were observed ($p<0.05$; both cases). No differences were observed among the LEAN groups. The level of oxLDL has been shown to be increased in insulin resistance (19) but no significant effect between control groups (VEH; LEAN vs. DIO, p=ns) was observed in the present study. In DIO rats, FLU oxLDL was significantly increased compared to VEH and CLG groups ($p<0.001$; both cases). Interestingly, animals subjected to long term high fat feeding (DIO) had significantly lower SOD activity compared to LEAN rats ($p<0.0001$). In LEAN animals, the different glucose infusion regimens had no effect on SOD activity. However in DIO rats, fluctuating hyperglycaemia significantly increased SOD activity compared to VEH and CLG groups ($p<0.001$ and $p<0.05$, respectively). The data indicate that the endogenous antioxidant system in aorta is markedly reduced and thus predisposed to oxidative stress during insulin resistance.

Protein expression levels

Protein expression of $GP91^{PHOX}$, the major subunit of the NADPH oxidase system, and GLUT1, the primary facilitative glucose transporter, were measured to account for adaptive responses to the dietary and infusion regimens. Two-way ANOVA revealed a significant interaction between group and diet ($p<0.001$) in liver $GP91^{PHOX}$. While no differences between LEAN groups were observed, CHG and CLG had significant higher $GP91^{PHOX}$ protein levels compared to controls among the DIO animals ($p<0.001$ and $p<0.05$ vs. VEH, respectively).

In aorta, the two-way ANOVA revealed no interaction between group and diet but significant effects of group ($p<0.001$) and diet ($p<0.001$). In LEAN animals, only the CHG group showed a significant increase in $GP91^{PHOX}$ protein expression ($p<0.05$ vs. VEH). DIO rats appeared more susceptible compared to the LEAN rats. In general, protein concentrations of $GP91^{PHOX}$ were significantly higher in the DIO controls compared to LEAN controls

(p<0.001). Moreover in DIO rats, both CHG and FLU groups showed significant higher levels of $GP91^{PHOX}$ protein expression than VEH and CLG (p<0.05; all cases).

Looking at GLUT1 protein expression, two-way ANOVA revealed a significant effects of both group (p<0.001) and diet (p<0.001). As for $GP91^{PHOX}$, GLUT1 protein expression was significantly increased in DIO controls compared to LEAN (p<0.001). In LEAN animals, a 6-fold increase in the CHG group was observed (p<0.05 vs. VEH), while in DIO animals, FLU GLUT1 was significantly increased compared to VEH and CLG (p<0.001; both cases).

Discussion

Evidence from several reports both in humans (2;3;20), animal models (4;5;21) and in cultured VEC's (7;8;22;23) has strongly indicated that fluctuating hyperglycemia may be an independent risk factor for the development of diabetes related endothelial dysfunction and macrovascular complication. Moreover, obesity with the coexistence of insulin resistance has as well been described as a detrimental metabolic consequence affecting the vascular endothelial function and increasing the risk for atherosclerosis (24-26). Increased oxidative stress resulting from either attenuated antioxidant status, increased ROS production or both has been shown to be an important pathological component in both situations thus resulting in cell dysfunction and apoptosis (27). In the present study, we wanted to investigate to what extent insulin resistance in coexistence with either fluctuating or sustained hyperglycemic profiles may affect oxidative stress status systemically, in liver and in aorta.

Lipid oxidation, measured as MDA, is a widely used biomarker of cellular oxidative stress in diabetes (28), and studies both in diabetic patients and animal models have found increased oxidative stress in both tissues and biological fluids (27;29). In agreement with this rationale, a recent study showed a positive correlation between increased MDA in plasma and vascular dysfunction during hyperglycaemia both in insulin resistant and diabetic patients (30). As previously published, both LEAN and DIO animals displayed increased plasma MDA levels both during sustained overt hyperglycaemia (PG > 20mM) and in animals with fluctuating plasma glucose profiles (Paper I, Paper II). Another well-established marker for lipid oxidation is 8-IsoP (31). Monnier *et al* showed that in T2D patients fluctuating hyperglycemia compared to overt sustained hyperglycemia may increase the level of 8-IsoP in the urine collected over 24 hours (3). In agreement with their findings and our plasma MDA data, increased levels of plasma 8-IsoP in the FLU and CHG group were found in both LEAN and

DIO animals. Increased systemic oxidative stress during obesity and insulin resistance has been shown in several reports (11). Albeit, in our study even though, the DIO rats showed clear signs of obesity and insulin resistance as judged by body weights, body fat content and HOMA index compared to LEAN, we did not observe any marked differences in systemic oxidative stress level between the two models. Indeed, blood measures of systemic oxidative stress are well-accepted as indicators of whole body redox homeostasis(27), however highly compartmentalized effects may still be observed at tissue level. The liver exerts a key role in maintaining whole body glucose and lipid homeostasis through its ability to store excess FFA in form of TG and glucose as GLY (32). During insulin resistance, lipolysis in the adiposity is not adequately inhibited resulting in increased uptake and incorporation of FFA into TG's in the liver (33). Furthermore, liver insulin resistance may also result in increased levels of gluconeogenic enzymes such as phospheonol pyruvate carboxy kinase and glucose-6 phosphatase, consequently lowering net liver glucose uptake and de novo glycogen synthesis (33). In agreement, long term high fat feeding resulted in increased liver TG and a decrease in GLY accumulation confirming the existing of liver insulin resistance (34). Both high fat feed and genetically obese insulin resistant animal models have shown increased ROS production in liver, muscle and adipose tissue (35;36). Increased levels of liver FFA and TG during high fat feeding have been shown to induce excess ROS production and cellular damage (37). In addition recent studies in hepatocytes have shown that increased FFA levels may induce higher ROS production through increased stimulation of NADPHox activity (38). However in our study, long term high fat feeding, as judged by the liver MDA and SOD levels, did not result in increased levels of oxidative stress. The duration of high fat feeding may be crucial in the induction of liver oxidative stress. A recent study by Gyeong-Min Do et al showed that oxidative stress markers and antioxidant status in liver were highly dependent on the duration of the high fat diet regime (39). The study revealed an early increase in the antioxidant system

protecting the liver from excess ROS production. Moreover, in another study applying sucrose feeding to rats resulting in excess liver lipid accumulation, it was shown that the mitochondria counteracted the increased substrate availability by increasing the expression of the uncoupling protein 2, (UCP-2) and thereby protecting the liver from excess superoxide formation (40). Interestingly, in both cases the protective mechanisms were deteriorated during the course of time inducing oxidative stress. This was closely correlated to the level of liver TG accumulated and the severity of insulin resistance developed through time. Nonetheless, when DIO rats, but not LEAN rats, were subjected to glucose infusions, both liver MDA and SOD were markedly affected in the groups receiving a constant infusion of glucose but not in the fluctuating hyperglycaemic group. Studies have demonstrated that subjecting rats to chronic infusion of glucose will increase liver oxidative stress and inflammation (41).Increased expression of the membrane bound NADPHox subunit, $GP91^{PHOX}$, being the catalytic cite for superoxide production in the NADPHox enzyme have shown in hepatocytes to correlate with increased NADPHox activity when exposed to chronic high glucose (42). This may suggest that the liver is more prone to increased ROS production and oxidative stress when subjected to sustained hyperglycaemia but only in the state of insulin resistance. During chronic glucose infusion, liver TG and GLY were markedly increased. Hypothetically, the accumulation of TG and GLY may exert a protective mechanism against excess substrate availability. Studies have revealed that over expressing di-acyl glycerol acyl transferase (DGAT), the rate limiting enzyme for TG synthesis in liver, will protect the liver against FFA induced oxidative stress (43). In contrast, pharmacological inhibition of the DGAT during high fat feeding will indeed improve liver steatosis due to decreased TG levels but paradoxically worsen liver damage probably through increased FFA availability inducing inflammation in form of TNF-α and ROS production(44). The activity of liver DGAT is inversely regulated with the accumulation of TG (45) thus in the CHG animals

a limited capacity for further TG accumulation may have caused a “spill” over of substrate in form of glucose and FFA thus activating the NADPHox. Indeed tissues and cells have shown to exert important metabolic adaptation in order to protect against excess ROS (46).

Cell based studies have shown that increased levels of ROS may disturb the translocation of GLUT4 to the cell membrane thus attenuating the insulin mediated glucose uptake in muscle and adipose tissue(47;48). The study by Takamura *et al* in T2D subjects showed that ROS levels in liver positively correlated with increased expression of gluconeogenic enzymes resulting in decreased net glucose uptake in liver (49). ROS molecules may affect the insulin signalling cascade by augmenting the degradation of the insulin receptor substrate 1 (IRS1) thus inducing impaired glucose uptake (50;51). In agreement, we observed that in the DIO model but not in the LEAN model the mRNA and protein expression of the liver GK was markedly reduced during sustained hyperglycemia (supplemental). This down regulation of glucose uptake in insulin responsive tissue has been suggested as a protective mechanism against further oxidative damage (46). Whole body glucose metabolism is composed of an “inter tissue” communication between insulin responsive and non-responsive tissues and paradoxically, the protective down regulation of glucose uptake by insulin responsive tissues may leave VEC’s as important targets of hyperglycaemia mediated oxidative insult (52). Attenuated antioxidant status in aorta has been reported both in insulin resistant and in diabetic rats(53). In support of this rationale, we found a markedly lower SOD capacity in the DIO model. Depletion of antioxidants is a well-known consequence of insulin resistance and diabetes (54). Increased substrate availability in form of glucose and particularly FFA has shown to be an important trigger of the NADPHox activity (55;56). In the study by Lambertucci et al showed that FFA in form of palmitate induced higher levels of PKC thus increasing ROS production through augmented NADPH ox activity (14). In this study, we observed significant higher levels of $GP91^{PHOX}$ and GLUT1 protein abundance in aorta.

Moreover, in contrast to liver, adding the challenge of fluctuating hyperglycemia increased MDA and ox-LDL in aorta. Likewise, presumably as an adaptive preventive mechanism against increased oxidative stress, the SOD activity was markedly increased as well. Influx of glucose in endothelial cells is facilitated by GLUT1 in an insulin independent manner (57) and excess glucose may activate PKC by several mechanisms, which consequently increases oxidative stress by activating NADPHox (58-60). In diabetics, increased ox-LDL accumulation in vessels has been shown to promote foam cell formation in vascular smooth muscle thus leading plaque formation and the development of atherosclerosis (61). Additionally, oxidized LDL has itself been shown to produce oxidative stress in endothelial cells via activation of a NADPHox (62). Possibly triggered by increased oxidative stress, insulin resistance is a complex metabolic situation where multiple pathways are involved consequently elevating the risk for endothelial dysfunction in addition adding the challenge of fluctuating hyperglycemia may further worsen this harmful situation increasing glucose uptake consequently affecting the activity of NADPHox thus leading to increased oxidative stress and ox-LDL accumulation.

Collectively, our data indicate that both fluctuating and overt sustained hyperglycemia may induce a higher degree of oxidative damage. Interestingly, the oxidative damage is highly compartmentalized where the cardio vascular system is primarily targeted by the fluctuating hyperglycemic profile whereas the liver is more disposed to oxidative damage by the chronic hyperglycemic profile. Insulin resistance has shown to be an important factor in the development of macrovascular complications. In our comparative study, the DIO model was more prone to increased oxidative stress suggesting that insulin resistance exacerbates the oxidative stress resulting from fluctuating hyperglycemia.

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Figures

Figure 1

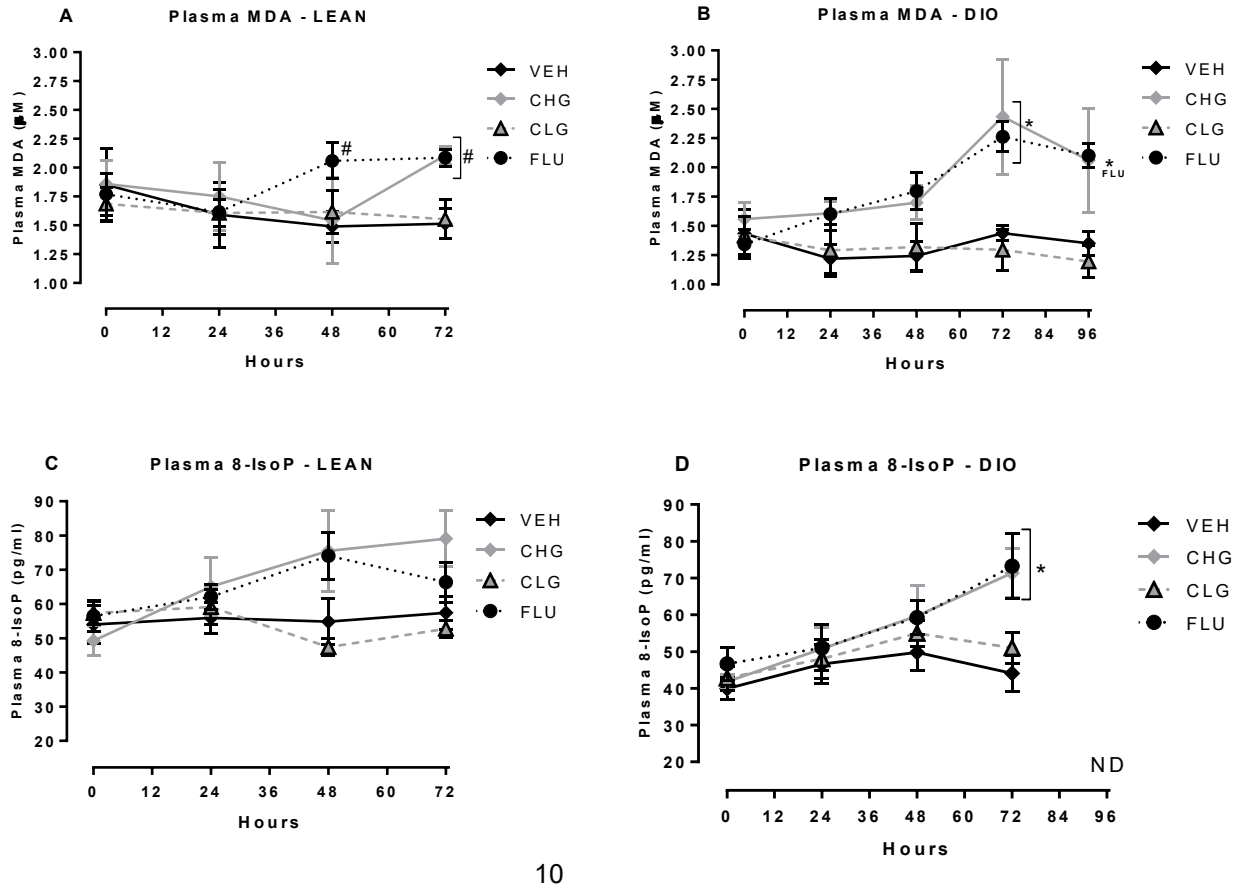
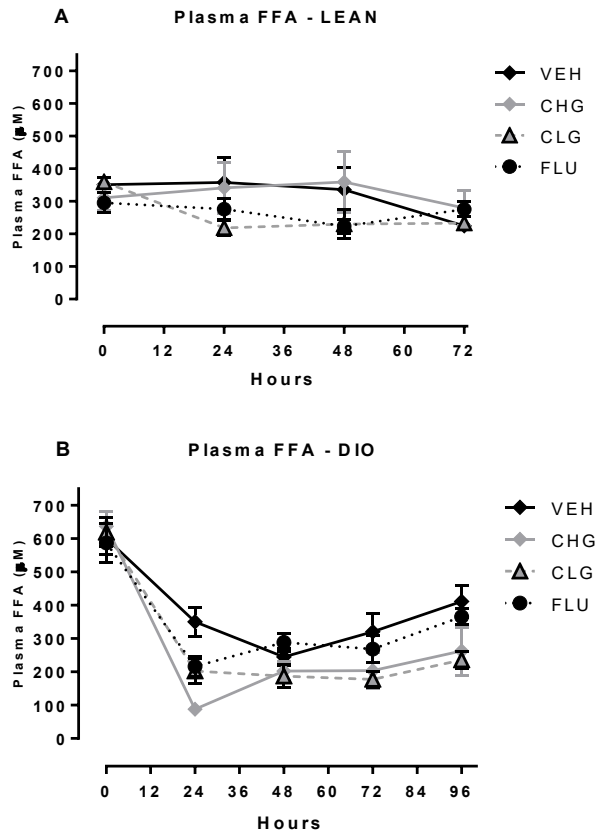


Figure 2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Hours
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CLG
FLU

Figure 3

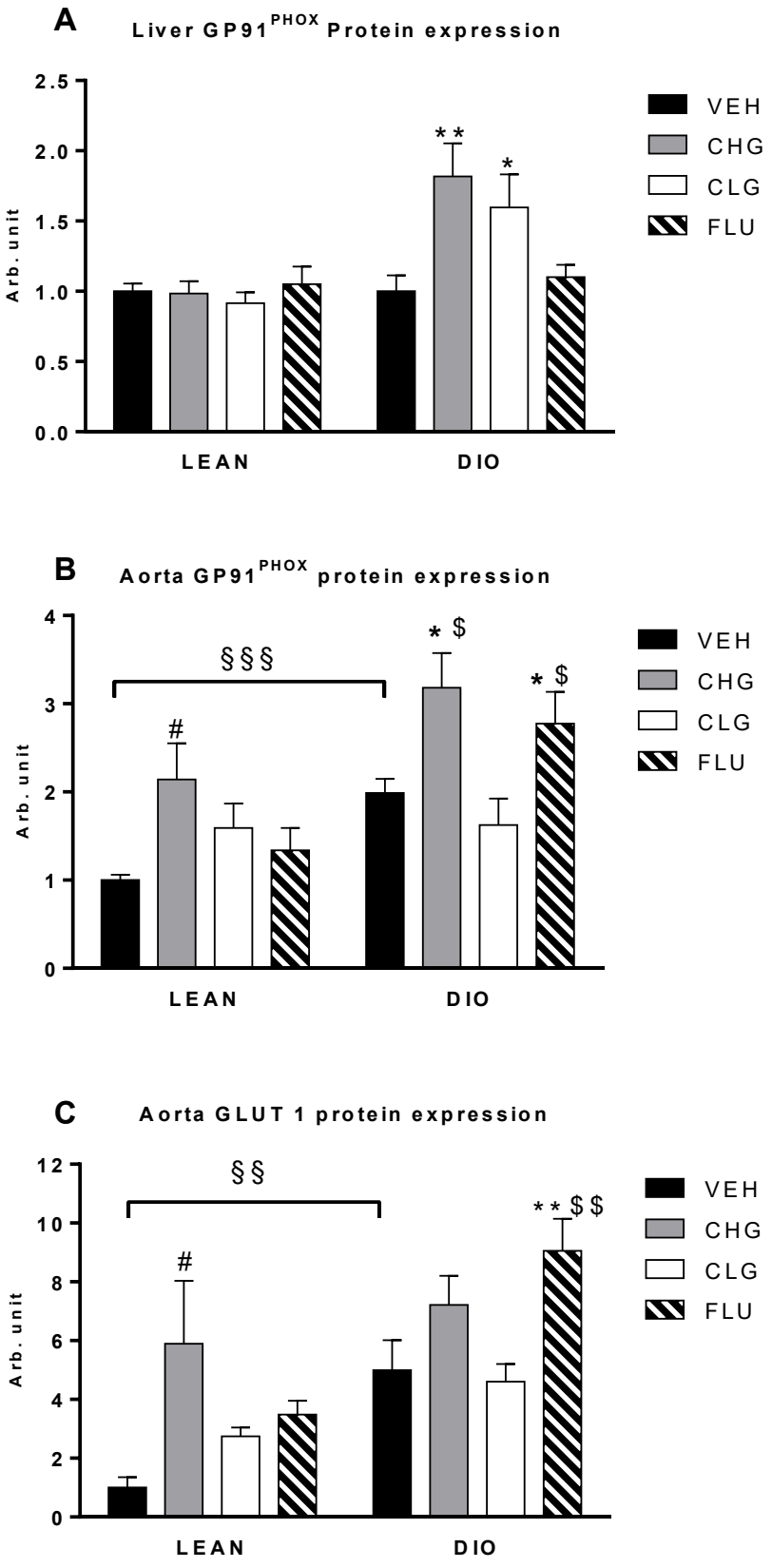
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Table 1

Table 1 Infusion rates (ml/kg/h). The table describes one cycle of vehicle or glucose infusion and the amount of glucose infused per 24 hours. The cycle was repeated continuously during the complete period of 72 hours for the lean animals or 96 hours for the DIO animals.

		0 – 2 min	2 – 32 min	32 – 152 min	Glucose (g/kg/24 hours)
VEH	LEAN	1.2	1.2	1.2	0
	DIO	1.0	1.0	1.0	0
CHG	LEAN	4.0	4.0	4.0	48.0
	DIO	3.0	3.0	3.0	36.0
CLG	LEAN	1.2	1.2	1.2	14.4
	DIO	1.0	1.0	1.0	12.0
FLU	LEAN	30.0	4.5	0	14.6
	DIO	30.0	3.5	0	12.4

Table 2

Table 2. Animal data

	LEAN	DIO
Weight (gram)	400-430	700-800
Fat content%	18	31
HOMA-IR (FPI*FPG/22.5)	66	188

Table 3

Measurements of oxidative stress markers and antioxidant capacity in liver and aorta in LEAN or DIO subjected to different glycemic profiles. Data are means ± SEM, n=7-8.

§§§p<0.0001 vs. VEH – DIO; *p<0.05, **p<0.001, ***p<0.0001 vs. VEH-DIO; $p<0.05, $$p<0.001 vs. CLG – DIO.

		LEAN				DIO				Effect of Two-way ANOVA		
		VEH	CHG	CLG	FLU	VEH	CHG	CLG	FLU	Group*Diet	Group	Diet
Liver												
	MDA (µM/mg protein)	0.23 ± 0 02	0.21 ± 0.01	0.22 ± 0.01	0.23 ± 0.01	0.20 ± 0 01	0.35 ± 0.03***	0.26 ± 0.01*	0.22 ± 0.02	<0.0001	0.0014	0.0093
	SOD (U/mg protein)	2.25 ± 0.07	2.31 ± 0.16	2.11 ± 0.14	2.29 ± 0.11	2.61 ± 0.32	6.99 ± 0.78***	5.91 ± 0.79***	4.07 ± 0.23**	<0.0001	<0.0001	<0.0001
Aorta												
	MDA (µM/mg protein)	1.31 ± 0.16	1.18 ± 0.09	1.02 ± 0.06	1.17 ± 0.07	1.05 ± 0.11	1.55 ± 0.29	0.98 ± 0.08	1.53 ± 0.22 * $	0.0678	0.0239	0.2582
	SOD (U/mg protein)	6.93 ± 0.39§§§	6.74 ± 0.76	7.25 ± 0.32	6.98 ± 0.54	1.68 ± 0.32	3.19 ± 0.24	2.43 ± 0.28	3.91 ± 0.59** $	0.0664	0.1204	<0.0001
	oxLDL (ng/mg protein)	862 ± 102	1396 ± 146	1272 ± 135	1111 ± 132	1250 ± 192	1464 ± 151	1182 ± 108	1916 ± 251** $$	0.0387	0.0280	0.0169

Figure Legends

Figure 1. Plasma MDA profiles in LEAN (A) and DIO (B) rats and plasma 8-IsoP profiles in LEAN (C) and DIO (D) rats. Data are shown as means ± SEM (n=7-8). #p<0.05 vs. VEH-LEAN; *p<0.05 vs. VEH-DIO. ND=no data.

Figure 2. Plasma FFA profiles in LEAN (A) and DIO (B) rats. Data are shown as means ± SEM (n=7-8).

Figure 3. The protein expression of $GP91^{PHOX}$ in liver (A), and aorta (B) and aorta GLUT1 (C) was quantified as the relative protein abundance to β-actin. Data are shown as relative expression to VEH-LEAN. Data are means ± SEM, n=7-8. #p<0.05 vs. VEH-LEAN; *p<0.05, **p<0.001 vs. VEH-DIO; $p<0.05, $$p<0.001 vs. CLG-DIO; §§p<0.001, §§§p<0.0001 vs. VEH-LEAN.

Supplemental

Material and Methods

Liver Triglycerides and Glycogen

Liver triglycerides (TG) and glycogen (GLY) content were determined by homogenizing weighed liver tissue with a reagent consisting of a sodium acetate buffer mixed with Triton X-100 for 15 seconds by use of homogenizer, Polytron PT 3000 (PT-DA 3007/2 generator, IKA-Werke Germany). Immediately after homogenization, the sample was placed in a bath of boiled water for two minutes, and thereafter kept on slush ice for fast cooling. After cooling, the homogenate was centrifuged and the homogenate was analyzed for TG on the Hitachi 912 analyser, using a commercially available enzymatic triglyceride assay (Cat. No.: 11488872; Roche diagnostics; Switzerland). For glycogen analysis, 25 µl amyloglucosidase (Sigma-Aldrich) was added to the homogenate and it was placed at 20°C overnight before analysis, using a commercially available enzymatic glucose assay (Cat. No.: 11447521; Roche diagnostics; Switzerland) on a Hitachi 912 analyzer (Hitachi, Japan).

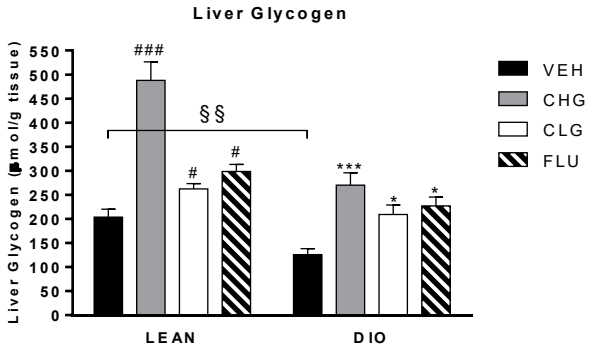
Western blotting

Tissue protein lysates were assayed with antibodies against GK (Santa Cruz, USA). Protein levels were normalized to β-Actin (Abcam, USA). Secondary antibodies were horseradish peroxidise-coupled and ECL reagent (BioVision, USA) was used for detection. Quantification was performed using ImageGuage 4.0 (Fujifilm, Japan).

RT-PCR

RNA was isolated from tissue using the RNeasy Mini Kit (Qiagen, USA). cDNA was synthesized using the iScript kit (Bio-Rad, USA). Primer-probesets were from TaqMan Gene Expression Assays (Applied Biosystems, USA) and PCR reactions were performed using a TaqMan Master mix (Applied Biosystems, USA) and a MX3000P system (Agilent, USA).

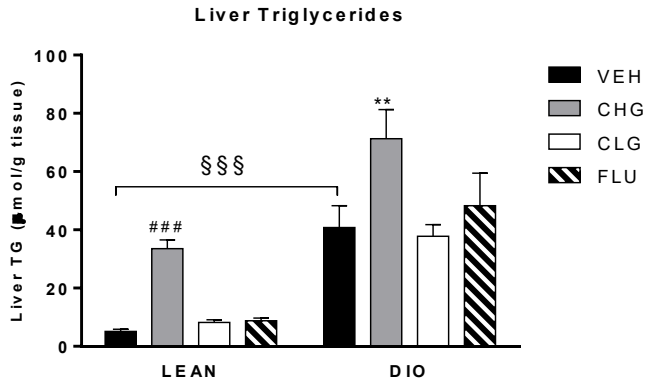
Figure 1 – Liver Glycogen



Liver GLY. Data are means ± SEM, n=7-8. §§p<0.001, ###p<0.0001, #p<0.05 vs. VEH-LEAN; *p<0.05, ***p<0.0001 vs. VEH-DIO.

The two-way ANOVA revealed a significant interaction between group and diet (p=0.008). All intervention groups had a significant increase in liver GLY levels where the CHG, due to being exposed to three times higher amounts of glucose, showing the largest difference in both models (p<0.0001). Of notice, even though the two models were exposed to the same amount of glucose especially the CHG in the DIO model did not show the same rise in liver GLY as seen for in the lean animals. Indeed insulin resistance can affect the livers capability of de novo GLY synthesis and as expected insulin resistant rats had significant lower levels of liver GLY (LEAN: 203 ± 17 vs. DIO: 125 ± 12; p<0.001).

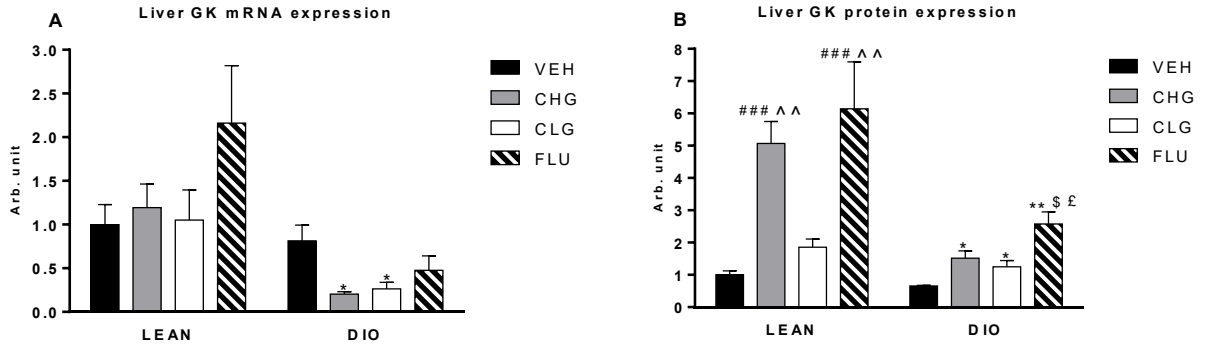
Figure 2 – Liver Triglycerides



Liver TG. Data are means ± SEM, n=7-8. §§§p<0.0001, ###p<0.0001 vs. VEH-LEAN; **p<0.001 vs. VEH-DIO.

As expected long term high fat feeding significantly increases liver TG accumulation ($p<0.0001$). As judged by the two-way ANOVA there was no interaction between group and diet. The post hoc test revealed that subjecting both lean and DIO rats to high overt chronic hyperglycemia as for the CHG groups will significantly increase the accumulation of liver TG as compared to its respective control groups (LEAN: $p<0.0001$, DIO: $p<0.001$).

Figure 3 – Liver Glucokinase



Liver GK mRNA(A) and protein(B) expression. The level of GK protein expression was quantified as the relative protein abundance to β-actin. Data are shown as relative expression to VEH-LEAN. Data are means ± SEM, n=7-8. ###p<0.0001 vs. VEH-LEAN, ^^p<0.001 vs. CLG-LEAN, *p<0.05, **p<0.001 vs. VEH-DIO, $p<0.05 vs. CHG-DIO, £p<0.05 vs. CLG-DIO.

The GK protein abundance was significantly affected among groups (p=0.0004) and diet (p=0.0018). In the lean model we observed an app. 5-fold increase in GK protein levels for the CHG and FLU group (p<0.0001 vs. VEH-DIO; both cases) but not no changes in the CLG group (p=ns). In the DIO model the GK protein levels were significantly increased in all intervention groups. The CHG and CLG group showed approximately a 2 fold increase in GK protein abundance as compared to the controls (p<0.05; both cases). Interestingly, the FLU group had an even higher effect on the GK protein level, displaying a 4 fold increase compared to the controls (p<0.0001), which was also significantly higher than the CHG and CLG groups (p<0.05; both cases). Of notice even though we observed a significant increase in GK protein abundance in the DIO model this increase was significantly less than observed in the lean model. In the GK mRNA level (Fig. 3D) we observed a diet dependent significance (p=0.0017). The post hoc test revealed a reduction in the CHG and CLG groups (p<0.05 vs. VEH; all cases) but not in the FLU group.