METABOLIC AND PHYSIOLOGICAL STUDIES IN A RAT MODEL OF TYPE 2 DIABETES

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METABOLIC AND PHYSIOLOGICAL STUDIES IN A RAT MODEL OF TYPE 2 DIABETES

by

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Abstract

Mild hyperhomocysteinemia is a risk factor for many diseases, including cardiovascular disease. We determined the effects of insulin resistance and of Type 2 diabetes on homocysteine and amino acid metabolism using Zucker diabetic fatty rats (ZDF/Gmi-fa/fa and ZDF/Gmi +/?). Plasma total homocysteine was reduced in ZDF fa/fa rats by 24% in the pre-diabetic insulin-resistant stage while in the frank diabetic stage there was a 59% reduction. Hepatic activities of several enzymes that remove homocysteine: cystathionine β-synthase, cystathionine γ-lyase and betaine:homocysteine methyltransferase were increased as well as methionine adenosyltransferase. Cystathionine β-synthase and betaine:homocysteine methyltransferase mRNA levels and the hepatic level of S-adenosylmethionine were also increased in the ZDF fa/fa rats. Studies with primary hepatocytes showed that homocysteine export and the transsulfuration flux in cells from ZDF fa/fa rats were particularly sensitive to the presence of betaine, which reduced these fluxes. Interestingly, the liver betaine concentration was found to be significantly lower in the ZDF fa/fa rats at both 5 and 11 weeks. We conclude that the decreased insulin action and the elevation of the counter-regulatory hormones observed in diabetes may be responsible for the more efficient homocysteine removal seen in the ZDF fa/fa rats.

Amino acids were measured in plasma, liver and skeletal muscle and the ratios of plasma: liver and plasma: skeletal muscle, were calculated (Chapter 4). At the insulin-resistant stage, the plasma concentrations of the gluconeogenic amino acids aspartate, serine, glutamine, glycine and histidine were decreased in the ZDF fa/fa rats while taurine, α-aminoadipic acid, methionine, phenylalanine, tryptophan and the three

branched-chain amino acids were significantly increased. At the diabetic stage, a larger number of gluconeogenic amino acids showed decreased plasma concentrations. The three branched chain amino acids again showed elevated plasma concentrations. In the liver and the skeletal muscles, many of the gluconeogenic amino acids showed lowered concentrations at both stages while the levels of one or all of the branched-chain amino acids were elevated. These changes in amino acid concentration are similar to changes seen in Type 1 diabetes. However, it is evident that insulin resistance alone is capable of bringing about many of the changes in amino acid metabolism observed in Type 2 diabetes.

The next part of the study focused on the effects of the insulin-sensitizing drug, Rosiglitazone, on homocysteine metabolism (Chapter 5). Rosiglitazone is an agonist of the peroxisome proliferator activated receptor γ and is used as an anti-diabetic agent to treat Type 2 diabetes. Male ZDF fa/fa and ZDF fa/+ rats, aged 6 weeks were each divided into 2 groups: Rosiglitazone-treated (RSG) and untreated, and were killed at 12 weeks of age. Rosiglitazone treatment was able to maintain a normal plasma glucose level in the ZDF fa/fa (RSG) rats. At 12 weeks of age, ZDF fa/fa (untreated) rats developed type 2 diabetes as indicated by a 3-fold increase in plasma glucose, while plasma insulin level was similar to ZDF fa/+ rats. The significant reduction observed in plasma homocysteine in the ZDF fa/fa (untreated) rats was returned towards normal by Rosiglitazone treatment. The elevated activity of the transsulfuration enzyme, cystathionine γ-lyase, in the ZDF fa/fa (untreated) rats was corrected by Rosiglitazone treatment while cystathionine β-synthase was unaffected. The elevated activity of betaine:homocysteine methyltransferase observed in the ZDF fa/fa (untreated) rats was further increased by Rosiglitazone

treatment. That the increased activity of betaine:homocysteine methyltransferase in the ZDF fa/fa (RSG) rats may be a function of increased lipid output from the liver in the face of the lipid redistribution seen with Rosiglitazone is supported by the changes that were observed in phospholipid metabolism.

Type 1 and Type 2 diabetes as well as insulin resistance prior to the development of Type 2 diabetes have been reported to cause endothelial dysfunction. Hyperglycemia has been suggested to play a major role in this development of endothelial dysfunction. Hyperhomocysteinemia has also been identified as a causative agent of endothelial dysfunction. Our earlier studies (Chapter 3 and 5) showed plasma homocysteine to be lower in both insulin resistance and during Type 2 diabetes in ZDF fa/fa rats. We, therefore, designed a study to determine the effects of increased glucose and decreased homocysteine on the function of mesenteric arteries and the effects of normalization of these two parameters with the treatment of Rosiglitazone. 6 week old ZDF fa/+ and ZDF fa/fa rats were randomly assigned to RSG treated and untreated groups as in Chapter 3, and were killed at 6, 12 and 18 weeks of age.

The endothelium dependent vasorelaxation in response to acetylcholine, the proteinase-activated receptor-2 (PAR2) agonist 2-furoyl-LIGRLO-amide (2fli) and arachidonic acid were preserved in the ZDF fa/fa mesenteric arteries despite the hyperinsulinemia at 6 weeks and the hyperglycemia at 12 and 18 weeks. Despite the normalization of plasma glucose and near normalization of plasma homocysteine with the treatment of Rosiglitazone the endothelium dependent vasorelaxation in response to the above agonists did not change, reflecting the lack of any endothelial defects in the mesenteric arteries of these rats.

The inability of the nitric oxide synthase inhibitor N_{ω}-Nitro-L-arginine methyl ester hydrochloride (L-NAME) to completely abolish vasorelaxation in response to both acetylcholine and 2fli demonstrates the important role played by endothelium-derived-hyperpolarizing factor in the relaxation of small caliber blood vessels in response to these 2 agonists. Studies conducted with inhibitors of cyclo-oxygenase 1/2 (COX1/2) and calcium activated potassium channels (K_{Ca}) to identify the possible nitric oxide synthase independent mechanisms of vasorelaxation indicated both COX and Kca channels to be involved. Kca channels however, were shown to play a greater role than COX 1/2 as previously reported.

We also observed an increased sensitivity towards the PAR2 agonist 2fli by the ZDF fa/fa rat vessels. This may be consistent with the increase in pro-inflammatory substances in the ZDF rat model, as PAR2 expression has been shown to increase during stress to the blood vessels and in the presence of various cytokines and pro-inflammatory substances.

Our data also did not indicate a reduction in vascular smooth muscle cell responsiveness to nitric oxide in the ZDF fa/fa rats compared to ZDF fa/+ rats. Both groups of rats however, displayed a reduced relaxation response to sodium Nitroprusside compared to previously reported values. Treatment with Rosiglitazone was able to successfully restore this responsiveness in both groups of rats. A similar increase in responsiveness was also seen in the ZDF fa/+ and ZDF fa/fa untreated rat vessels as well as ZDF fa/fa (RSG) vessels incubated with L-NAME.

The normal endothelium dependent vasorelaxation displayed by the ZDF fa/fa rats despite the hyperglycemia indicates that hyperglycemia alone may not be able to cause

endothelial dysfunction. Since plasma homocysteine levels in these rats were decreased, it leaves us with the interesting possibility that homocysteine may act as a balance point for development of endothelial dysfunction in this model of Type 2 diabetes.

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List of Abbreviations

2fli: 2-furoyl-LIGRLO-amide

AA: Arachidonic acid

ACh: Acetylcholine

ADMA: Asymmetric dimethylarginine

AGAT: L-arginine:glycine amidinotransferase

ANTS: 8-aminonaphthalene-1,3,6-trisulfonic acid

ATP: Adenosine triphosphate

BCAA: Branched chain amino acid

BCKDC: Branched chain α-ketoacid dehydrogenase complex

BH_4: Tetrahydrobiopterin

BHMT: Betaine:homocysteine methyltransferase

BMI: Body mass index

BSA: Bovine serum albumin

cAMP: Cyclic adenosine mono phosphate

CBS: Cystathionine β-synthase

CE: Cholesteryl ester

CETP: Cholesteryl ester transfer protein

CGL: Cystathionine γ-lyase

CN: control

CoA: Coenzyme A

COMT: Catechol-O-methyltransferase

COX: Cyclooxygenase

CT: CTP: phosphocholine cytidyltransferase

CVD: Cardiovascular disease

DDAH: Dimethylarginine dimethylaminohydrolase

DTT: Dithiothreitol

ECF: Extracellular fluid volume

EDHF: Endothelium-derived hyperpolarizing factor

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene-Glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraaceticacid

ELISA: Enzyme- Linked ImmnoSorbent Assay

eNOS: Endothelial Nitric oxide synthase

FFA: Free fatty acids

GAA: Guanidinoacetic acid

GAMT: Guanidinoacetate methyltransferase

GFR: Glomeruler filtration rate

GLUT: Glucose transporter

GMP: Guonosine mono phosphate

GNMT: Glycine *N*-methyltransferase

GPC: Glycerophosphorylcholine

Hcy: Homocysteine

HDL: High density lipoprotein

HHcy: Hyperhomocysteinemia

HPLC: High performance liquid chromatography

ICAA: Intracellular concentration of amino acids

ICAM-1: Intercellular adhesion molecule 1

ICF: Intra cellular fluid volume

IMCL: Intra myo-cellular lipid

iNOS: Inducible nitric oxide synthase

IRS-1: Insulin receptor substrate-1

Kca: Calcium activated potassium channels

LCACoA: Long chain fatty acyl CoA

LDL: Low density lipoprotein

L-DOPA: L-3,4-dihydroxyphenylalanine

LNAA: Large neutral amino acid

L-NAME: N_{ω}-Nitro-L-arginine methyl ester hydrochloride

LOX: Lipoxygenase

LOX-1: Lectin-like oxidized LDL receptor 1

MAT: Methionine adenosyltransferase

MCP 1: Macrophage chemoattractant peptide 1

MI: Myocardial infarction

MMP: Matrix mettalloproteinases

MRS: Magnetic resonance spectroscopy

MS: Methionine synthase

MTHFR: Methylenetetrahydrofolate reductase

NAD^+: Nicotinamide adenine dinucleotide (oxidized)

NADH: Nicotinamide adenine dinucleotide (reduced)

NADPH: Nicotinamide adenine dinucleotide phosphate (reduced)

NF κB: Nucler factor kappa B

NMR: Nuclear magnetic resonance

NO: Nitric oxide

NOD: Non obese diabetic

NOS: Nitric oxide synthase

OR: Odds ratio

PAI-1: Plasminogen activator inhibitor-1

PAR 2: Proteinase activated receptor-2

PC: Phosphatidylcholine

PCho: Phosphorylcholine

PDME: Phosphatidyl-dimethyl-ethanolamine

PE: Phosphatidylethanolamine

PEMT: phosphatidylethanolamine *N*-methyltransferase

PEPCK: Phosphoenolpyruvate carboxy kinase

PI3 Kinase: Phosphatidylinositol 3 kinase

PKC: Protein kinase C

PL: Phospholipid

PLP: Pyridoxal 5' phosphate

PMS: Phenazine methosulfate

PPAR: Peroxisome proliferator activated receptor

RBP-4: Retinol binding protein-4

ROS: Reactive oxygen species

RSG: Rosiglitazone

RT PCR: Reverse transcriptase polymerase chain reaction

SAH: S-adenosylhomocysteine

SAHH: S-adenosylhomocysteine hydrolase

SAM: S-adenosylmethionine

SHMT: Serine hydroxymethyltransferase

SNP: Sodium Nitroprusside

SRB 1: Scavenger receptor B1

SSC: Sulfosalicylic acid

SSPG: Steady state plasma glucose

TCA: Trichloroacetic acid

TG: Triacylglycerol

tHcy: Total homocysteine

TM: Thrombomodulin

TNF-α: Tumor necrosis factor-α

TRAM-34: 1((2-chlorophenyl) dipenylmethyl]-1H-pyrazole

TZD: Thiazolidinedione

VCAM 1: Vascular adhesion molecule 1

VLDL: Very low density lipoprotein

ZDF: Zucker diabetic fatty

Chapter 1

Introduction

1.1 History and Epidemiology

The original hypothesis linking hyperhomocysteinemia (HHcy) with atherothrombotic vascular disease was proposed after the observation of severe arteriosclerotic lesions in two children with elevated plasma homocysteine (Hcy) concentrations and homocysteinuria (McCully, 1969). This led to the proposal that even less severe increases in plasma Hcy may pose a risk. Since then, a plethora of prospective, retrospective and cross-sectional studies have appeared that link moderate HHcy to atherosclerotic disease. These studies show that patients with coronary, cerebrovascular or peripheral arterial occlusive disease have mean plasma total homocysteine (tHcy) levels greater than control subjects. Kang *et al* (1992) reported mean plasma tHcy to be significantly higher both in male and female patients with coronary artery disease when compared to controls with angiographically normal coronary arteries. Since cross-sectional studies deal with patients who already exhibit disease characteristics, they do not answer the question whether HHcy gives rise to the coronary artery disease or *vice versa* (Stampfer and Malinow, 1995). Two large prospective studies address this question. The Physicians' Health Study, which studied male physicians in the United States, showed that a plasma Hcy concentration only 1.7 μmole/liter or 12 % greater than the upper limit of normal was associated with a 3.4-fold increase in the risk of myocardial infarction (MI) (Stampfer *et al*, 1992). The Tromso study, which investigated over 21,000 subjects between the ages of 12-61, showed a relative risk for coronary heart disease of 1.32 for an increase in serum Hcy of 4 μmol/liter after adjusting for possible confounders (Arnesen *et al*, 1995). Meta-analyses confirm Hcy's status as an independent risk factor although they disagree on its strength. A meta-analysis of 27 studies relating Hcy to

coronary, cerebrovascular and peripheral arterial vascular disease showed a very strong relationship between these diseases and tHcy (Boushey *et al*, 1995). It was concluded that a 5 μmol/liter increment in tHcy was comparable to a 0.5 mmol/liter increment in cholesterol in increasing the incidence of coronary artery disease. A more recent meta-analysis that examined 30 prospective and retrospective studies confirmed that increased plasma Hcy is an independent predictor of ischemic heart disease but of only moderate strength (Hcy studies collaboration, 2002). The same group (Klerk *et al*, 2002) has reported a meta-analysis of the MTHFR 677TT polymorphism, which tends to increase plasma Hcy, particularly in the context of low folate. They found that individuals with the MTHFR 677TT genotype had a significantly higher risk of cardiovascular disease (CVD) especially when found in conjunction with a low folate status. The uncertainty about the degree of risk associated with elevated plasma Hcy may be attributed to a number of factors, but Stamm and Reynolds (1999) have emphasized one important factor: tHcy in plasma may not be the most appropriate marker of CVD risk. In particular, risk may be more closely linked to either cellular Hcy or to a particular component of plasma Hcy.

There are certain situations in which the risk attributable to Hcy may be elevated. Certainly, there is evidence that HHcy is a stronger risk factor in patients with Type 2 diabetes (Hoogeveen *et al*, 2000) and in patients with existing coronary disease. In the latter group, Nygard *et al* (1997 A) found a direct relationship between plasma Hcy levels and overall mortality; after a follow up of years from the confirmation of coronary artery disease, Kaplan-Meier estimates of mortality were 3.8% for patients with tHcy levels less than 9 μmol/liter, 8.6% for patients with Hcy level between 9 and 14.9 μmol/liter, and 24.7% for those with levels 15 μmol/liter or higher.

There is considerable evidence that the cardiovascular risk posed by Hcy is graded; there does not appear to be a threshold effect. A cross sectional study of elderly subjects from the Framingham Heart Study clearly demonstrated a graded increase in the level of extra-cranial stenosis. Similar observations were made in several other studies with regard to different cardiovascular disease states (Arnesen *et al*, 1995, Genest *et al*, 1990).

1.2 Plasma Forms of Hcy

Although the term Hcy is used generically, plasma contains several different forms of this amino acid. Plasma tHcy is made up of free and protein-bound Hcy. The free Hcy consists of homocysteine, homocystine and cysteine-homocysteine mixed disulphides (Kang *et al*, 1992) and a protein-bound fraction is linked to proteins by disulphide linkage, principally to cysteine 34 of albumin (Figure 1.1). Typically, in humans, the protein-bound fraction makes up the bulk of plasma tHcy accounting for >70%, homocystine and cysteine-homocysteine mixed disulphides make up 5-15% each, while only trace amounts ($\leq$ 1%) are found as free reduced homocysteine (Jacobsen, 2001). These different forms of Hcy along with reduced, free-oxidized and protein bound forms of cysteine and cysteinylglycine comprise a dynamic system (Ueland *et al*, 1996). A change in any one of these species leads to alterations in the thiol redox status (Ueland *et al*, 1996). Despite the existence of these different forms, typically the parameter measured is "total plasma Hcy", since assays for the separate forms are not yet satisfactory for routine clinical measurement. HHcy is categorized as being moderate,

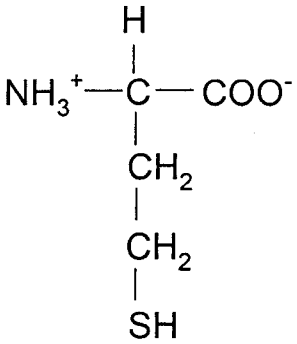
intermediate or severe with basal tHcy values of between 15 and 30, between 31 and 100 and greater than 100 μmol/liter respectively (Kang *et al*, 1992).

"Total plasma Hcy" does not encompass all forms of Hcy in plasma. Hcy thiolactone (Figure 1.1) can be formed in all human cell types; in general, its concentration is markedly increased under conditions of elevated plasma Hcy. It is a cyclic thioester formed as a result of an error-correcting process in aminoacyl-tRNA synthetase (Jakubowski and Fersht, 1981). Protein homocysteinylation, by means of the formation of an amide bond between the carbonyl group of Hcy thiolactone and the ε-amino group of lysyl side chains in cellular and extracellular proteins also occurs and can change the function of the protein so modified (Jakubowski, 1999). Such homocysteinylation of proteins, such as low density lipoproteins, has been proposed as the basis for some of the pathological consequences of HHcy. The N-linked Hcy, which can account for up to 23% of plasma Hcy, is not included in the calculation of plasma tHcy (Jakubowski, 2002). Hcy thiolactone is hydrolyzed to Hcy by Hcy thiolactonase, an enzyme associated with high density lipoprotein (Jakubowski, 2000).

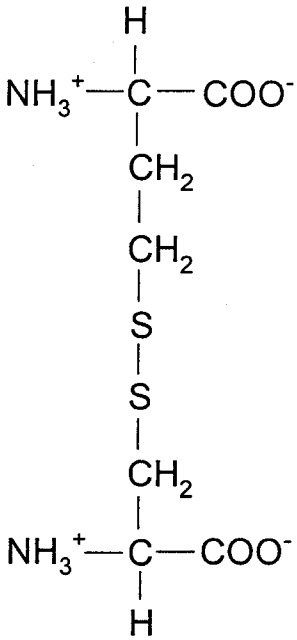
1.3 Metabolism

The liver has been shown to play a significant role in the regulation of plasma Hcy levels (Stead *et al*, 2000) because of its full complement of enzymes involved in methionine and Hcy metabolism. Hcy is an intermediate in the pathway of methionine metabolism. The only dietary-essential sulfur-containing amino acid in mammals, methionine contributes to a variety of fundamental biological processes, including protein

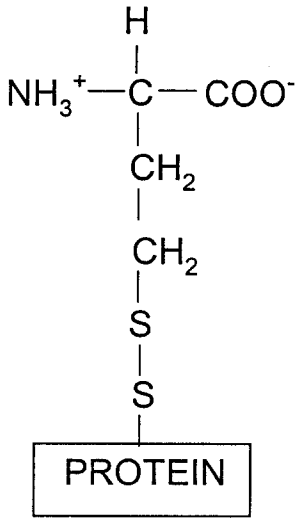
Figure 1.1. Plasma forms of Hcy



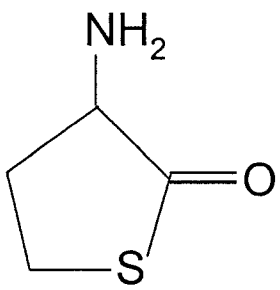
Homocysteine



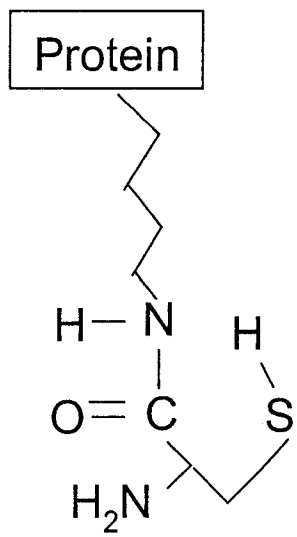
Homocysteine-Cysteine
(Mixed Disulphide)



Protein-bound
homocysteine (S-linked)



Homocysteine thiolactone



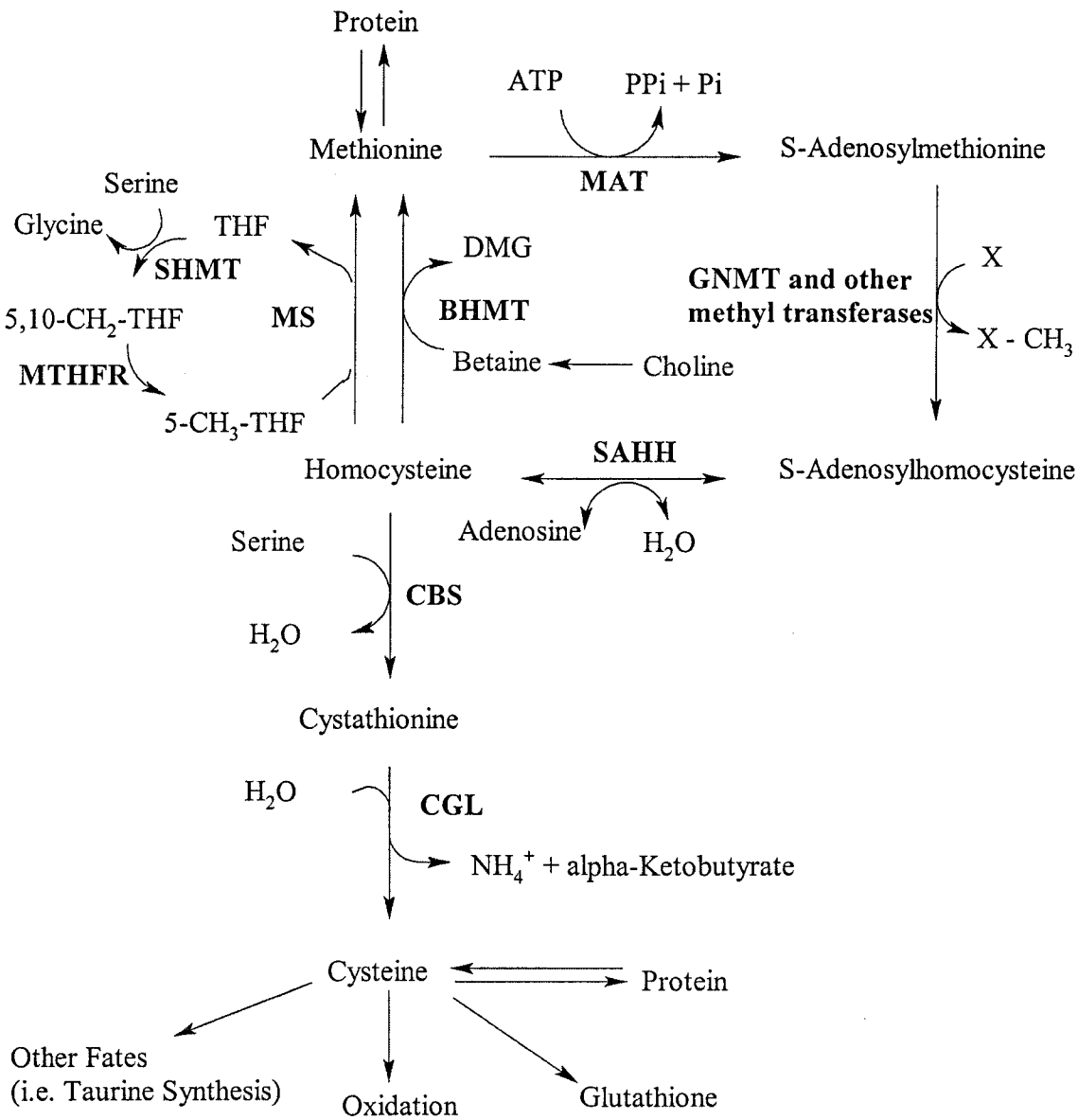
Protein-bound homocysteine
(N-linked)

synthesis, methylation reactions as S-adenosylmethionine (SAM), formation of the polyamines spermine and spermidine and the synthesis of cysteine. Hcy is a key substrate in 3 essential biological processes: (1) recycling of intracellular folates; (2) catabolism of choline and betaine; and (3) formation of the non-essential amino acid cysteine and the anti-oxidant glutathione through the transsulfuration pathway (Finkelstein, 1998).

The pathway of methionine metabolism (Figure 1.2) consists of the ubiquitous methionine cycle and the transsulfuration pathway, which has a more limited distribution. The methionine cycle is made up of the transmethylation and the remethylation pathways. In transmethylation, methionine is converted to the high energy sulfonium compound SAM in a reaction catalyzed by methionine adenosyltransferase (MAT) (EC 2.5.1.6). ATP provides the source of the adenosyl moiety. MAT exists in three isoenzymic forms MAT I, MAT II and MAT III, which are products of two different genes *MAT 1A* and *MAT 2A* (Chou, 2000). *MAT 1A* is expressed in mature liver while *MAT 2A* is expressed in all tissues and is induced during liver growth and dedifferentiation. The liver-specific isoenzymes MAT I and MAT III are coded for by *MAT 1A.* MAT I, a tetramer, is also designated as an "intermediate Km" isozyme (Km for methionine ~ 40 μmol/liter) and is slightly inhibited by SAM. MAT III, a dimer which appears to be derived from MAT I by a post- translational modification (Cabrero and Alemany, 1988) is designated as a "high Km" isoform (Km for methionine ~ 200 μmol/liter) and is activated by its product SAM, demonstrating a strong, positive cooperative modulation at physiological methionine and SAM concentrations. MAT III allows the liver to adjust immediately to an excess influx of methionine with increased SAM formation. MAT II, found in extra-hepatic tissues and

Figure 1.2. Methionine metabolic Pathway

The abbreviations used are: MAT: Methionine adenosyltransferase, GNMT: Glycine *N*-methyltransferase, SAHH: S-adenosylhomocysteine hydrolase, BHMT: Betaine:homocysteine methyltransferase, MS: Methionine synthase, MTHFR: Methylenetetrahydrofolate reductase, CBS: Cystathionine β-synthase and CGL: Cystathionine γ-lyase, SHMT: Serine hydroxymethyltransferase.


Protein
ATP
PPi + Pi
Methionine
MAT
S-Adenosylmethionine
Serine
Glycine
THF
SHMT
DMG
BHMT
GNMT and other
methyl transferases
X
X - CH3
5,10-CH2-THF
MS
MTHFR
Betaine
Choline
5-CH3-THF
Homocysteine
SAHH
S-Adenosylhomocysteine
Adenosine
H2O
Serine
CBS
H2O
Cystathionine
H2O
CGL
NH4+ + alpha-Ketobutyrate
Cysteine
Protein
Other Fates
(i.e. Taurine Synthesis)
Oxidation
Glutathione

fetal liver and encoded by the gene *MAT 2A*, has a low Km (~8 μmol/liter) for methionine and is inhibited by S-adenosylmethionine (Sullivan and Hoffman, 1983).

SAM, once formed, appears to be retained by the cell of origin and serves as the methyl donor for virtually all known biological methylation reactions. This transfer of the methyl group of SAM to a suitable methyl acceptor is catalyzed by any one of a large number of methyltransferases, leading to the formation of such diverse cellular components as creatine, epinephrine, carnitine, phospholipids as well as methylated proteins, DNA and RNA (Clarke and Banfield, 2001). Essentially all these methyltransferases are inhibited by the common product, S-adenosylhomocysteine (SAH). However, glycine N-methyltransferase (GNMT) which converts glycine to sarcosine has unique kinetic properties. It is only weakly inhibited by SAH, has a relatively high Km for SAM and shows a sigmoidal dependence on SAM concentration (Takata *et al*, 2003). GNMT thus functions as a benign high-capacity, SAM-dependent methyltransferase that is able to convert excess methionine/SAM to sarcosine, a non toxic product. Sarcosine is then transported into mitochondria where its metabolism regenerates glycine.

The inhibition (albeit to different degrees) of essentially all of the different methyltransferases by SAH necessitates its rapid removal. S-adenosylhomocysteine hydrolase, which occurs in all cells, converts SAH to Hcy and adenosine (Finkelstein, 1998). Although the equilibrium of this reaction favors the formation of SAH it is driven forward under physiological conditions due to the rapid removal of the products, Hcy and adenosine. Adenosine can be removed by either of 2 enzymes, adenosine deaminase (EC 3.5.4.4) or adenosine kinase (EC 3.5.4.4). Hcy, on the other hand, occupies a central

position in the pathway of methionine metabolism, lying at a cross-road between the transsulfuration and the remethylation pathways. SAH can also be removed by intracellular binding to specific, saturable protein sites and by export from the cell (Finkelstein, 1998). Once exported, the kidney appears to be active in the removal of SAH from the extracellular fluid (Finkelstein, 1998). Hcy can also bind to intracellular proteins and can be exported from cells (Svardal *et al*, 1986). However, unlike SAH, Hcy can be taken up by many tissues including the liver (Finkelstein, 1998). Plasma Hcy, therefore, represents Hcy that has been exported from the cells of origin, to be transported to other cells/tissues that would catabolize it.

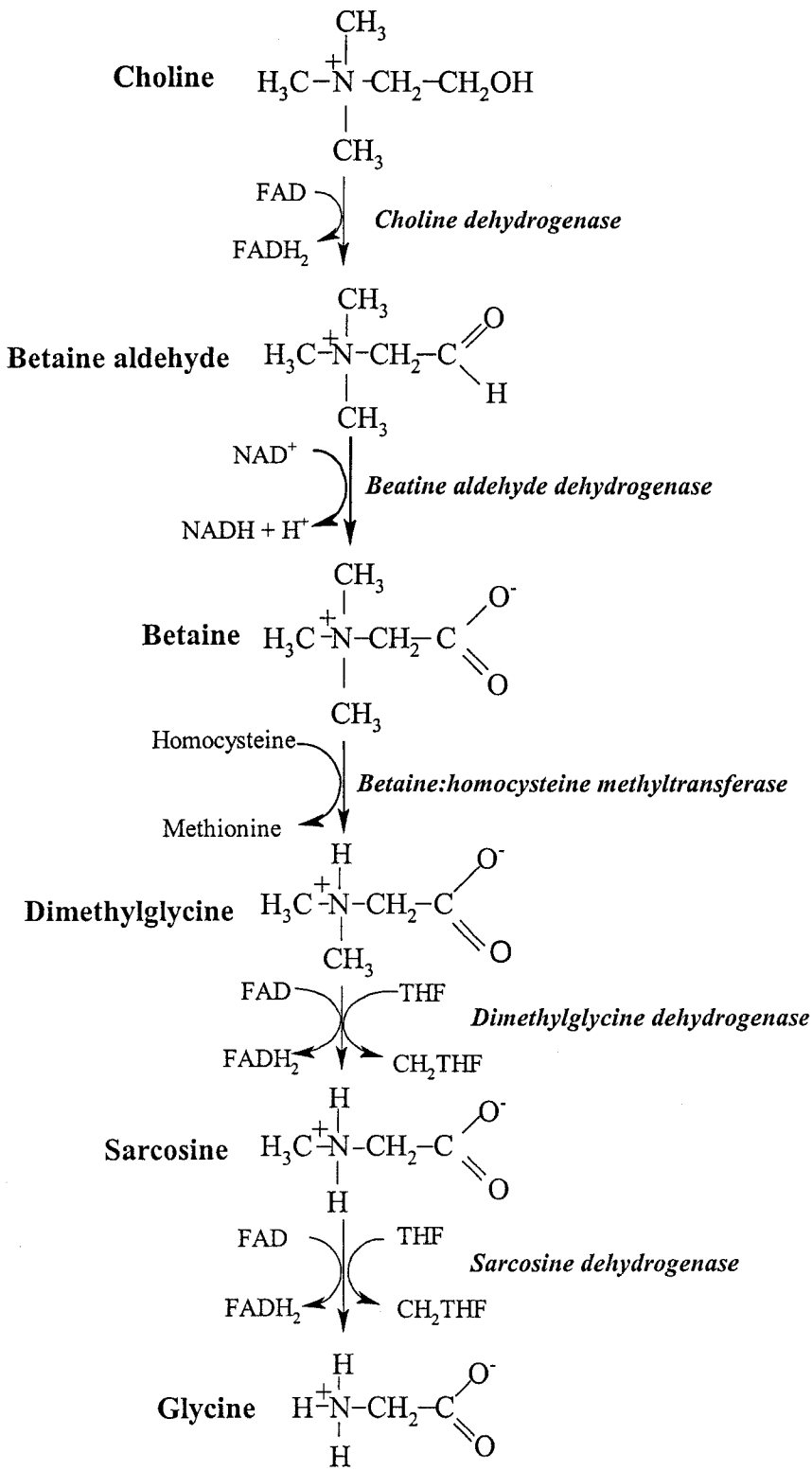
The remethylation of Hcy to methionine completes the methionine cycle and in turn functions to conserve the carbon skeleton of methionine. Interestingly, the methylation of Hcy to methionine is one exception where SAM does not provide the methyl group. Two different methyl donors, N^5-methyltetrahydrofolate or betaine, provide the methyl group necessary to convert Hcy to methionine by two independent pathways. Methionine synthase which transfers a methyl group from N^5-methyltetrahydrofolate to Hcy is widely distributed in mammalian tissue and contains methylcobalamin as an essential co-factor. The methyl group of N^5-methyltetrahydrofolate is synthesized by the folate coenzyme system using serine as the major donor of the one carbon units (Davis *et al*, 2004). N^5-methyltetrahydrofolate is formed by the irreversible reduction of $N^{5,10}$-methylenetetrahydrofolate, catalyzed by the flavoenzyme $N^{5,10}$-methylenetetrahydrofolate reductase (MTHFR) (EC 1.1.1.68) which uses NADPH as the reducing agent.

Betaine:homocysteine methyltransferase (BHMT) catalyzes a second route by which Hcy is converted to methionine, using pre-formed methyl groups from betaine, an intermediate in the pathway of choline oxidation (Finkelstein *et al*, 1972). BHMT, a zinc metalloenzyme (Breksa and Garrow, 1999), has a more limited distribution, being present only in liver, kidney and lens in humans (Finkelstein *et al*, 1972). BHMT is feed-back inhibited by its product N,N-dimethylglycine (Finkelstein *et al*, 1983). Dimethylglycine is converted to glycine in a series of reactions by which the methyl groups are oxidized and provide the methylene groups of $N^{5,10}$-methylenetetrahydrofolate (Figure 1.3)

If the carbon chain of methionine is not to be conserved, or if cysteine is required Hcy can be irreversibly converted to cysteine through the transsulfuration pathway which is catalyzed by two pyridoxal 5'-phosphate (PLP)-containing enzymes, cystathionine β-synthase (CBS) (EC 4.2.1.22) and cystathionine γ-lyase (CGL) (EC 4.4.1.1). CBS catalyzes the condensation of Hcy with serine to form cystathionine and commits the Hcy moiety to the transsulfuration pathway (Finkelstein, 1998). Cystathionine is hydrolyzed by CGL to form cysteine, α-ketobutyrate and ammonium, thereby making cysteine a non-essential amino acid. Both the pyruvate dehydrogenase complex and the branched-chain α-keto acid dehydrogenase complex can catalyze the oxidative decarboxylation of α-ketobutyrate to propionyl CoA, which enters the Krebs' cycle at succinyl CoA (Stead *et al*, 2000). Cysteine is a precursor of several essential molecules (e.g. glutathione, taurine, coenzyme A) as well as inorganic sulphate.

The transsulfuration pathway has a limited tissue distribution in rat tissues; only liver, kidney, pancreas and small intestine are capable of synthesizing cysteine from methionine or Hcy (Mudd *et al*, 1965). CBS is absent from heart, lung, testes, adrenal and

Figure 1.3. The choline oxidation pathway


Choline
FAD
FADH2
Choline dehydrogenase
Betaine aldehyde
NAD+
NADH + H+
Beatine aldehyde dehydrogenase
Betaine
Homocysteine
Methionine
Betaine:homocysteine methyltransferase
Dimethylglycine
FAD
THF
FADH2
CH2THF
Dimethylglycine dehydrogenase
Sarcosine
FAD
THF
FADH2
CH2THF
Sarcosine dehydrogenase
Glycine

spleen in rats [Finkelstein, 1998]. In humans, CBS was shown to be absent from heart muscle and primary aortic endothelial cells (Chen *et al*, 1999). The highest levels of CBS mRNA expression in human tissues have been observed in adult liver and fetal liver and fetal brain (Quere *et al*, 1999). Although CBS is present in the brain and adipose tissue of rats, CGL is absent from these tissues (Finkelstein, 1998). In agreement with this finding, very high levels of cystathionine have been found in human brains (Tallan *et al*, 1958). It may be that the function of CBS in the brain is to catalyze the production of H_2S, a neuromodulator and a smooth muscle relaxant (Chen *et al*, 2004).

1.4 Factors influencing methionine and Hcy metabolism

1.4.1 Gender and Age

Plasma Hcy has been shown to increase throughout life in both sexes (Refsum *et al*, 1998). In early childhood, both males and females have similar low plasma Hcy levels, but sex differences become apparent at puberty (Schneede *et al*, 2000). The skewed distribution among the genders continues from puberty into adulthood with plasma Hcy levels being 1-2 μmole/liter higher in men than in women, and with a steady increase in both sexes with increasing age (Schneede *et al*, 2000).

The gender differences could arise in a number of ways. It is possible that the differences are due to the higher formation of Hcy in connection with increased creatine synthesis. Creatine synthesis is proportional to muscle mass and, therefore, is higher in men than in women (Mudd and Poole, 1975). A second possibility could be effects of sex steroids, as estrogens have been shown to lower plasma Hcy levels (Kang *et al*, 1986). This is also consistent with the observation that, after menopause, the gender difference in

plasma Hcy concentrations is attenuated. Post-menopausal women have been shown to have higher levels of plasma Hcy than pre-menopausal women (Boers *et al*, 1983). The changes in the plasma Hcy levels that have been observed in the male-to-female and female-to-male transsexuals treated with sex steroids also show the effect of sex hormones on plasma Hcy levels (Giltay *et al*, 1998 A). Plasma Hcy was reduced in male–to-female transsexuals treated with ethinyl estradiol in combination with an anti-androgen while the female-to-male transsexuals treated with testosterone esters displayed an elevation in their plasma Hcy (Giltay *et al*, 1998 A). Women who are pregnant have significantly reduced plasma Hcy levels with tHcy decreasing between the first and the second trimesters and remaining low through the rest of the pregnacy (Andersson *et al*, 1992). Malinow *et al* (1998) suggested an increased uptake of maternal Hcy by the developing fetus as the reason for this lowered plasma Hcy in the mother.

A number of studies, conducted in different ethnic populations, have all demonstrated an age-related increase in plasma Hcy, the increase being most marked in the oldest age-group (Selhub *et al*, 1993). Plasma Hcy is about 30% lower in children than in adults (Schneede *et al*, 2000). The age-dependent increase in plasma Hcy could arise in a number of ways. Renal function is known to decline with age (Norlund *et al*, 1998). It is well established that renal disease is associated with increased plasma Hcy (Bostom *et al*, 1995 A). This may be due to metabolism of Hcy by the kidneys (Bostom *et al*, 1995 B), although the clear–cut data obtained in rats are not evident in humans (van Guldener *et al*, 1998). Sub-optimal vitamin status, in particular impaired folate status (Koehler *et al*, 1997) and cobalamin deficiency (Lindenbaum *et al*, 1994), may also play major roles.

1.4.2 Lifestyle

Several lifestyle factors are also known to affect plasma Hcy levels. Chronic, high ethanol consumption (Cravo *et al*, 1996), smoking (Nygard *et al*, 1995) and consumption of caffeinated coffee (Nygard *et al*, 1997 B) are all associated with an increase in the concentration of plasma Hcy. Interestingly, a moderate consumption of alcohol appears to lower the plasma Hcy concentration (Vollset *et al*, 1997). The consumption of beer has also been shown to lower plasma Hcy, probably because beer contains folate (Mayer *et al*, 2001). HHcy associated with chronic alcoholism may arise due to impaired folate, vitamin B_{12} and B_6 intake (Lindenbaum *et al*, 1994). Acute alcohol intoxication in alcoholics leads to a transient increase in plasma Hcy, which may be a direct result of inhibition of methionine synthase by acetaldehyde (Kenyon *et al*, 1998). In the Hordaland Hcy Study, individuals who consumed more than 6 cups of coffee per day exhibited 2 to 3 μmol/liter higher level of Hcy than individuals who did not consume coffee (Nygard *et al*, 1997 B). Decaffeinated coffee does not have the same effect on plasma Hcy as does regular coffee (Nygard *et al*, 1997 B).

Physical training has been shown to play a positive role in the reduction of plasma Hcy (Nygard *et al*, 1995). The difference in Hcy levels between subjects doing heavy training and those having a sedentary lifestyle was greater in an older age group (65-67 years) where exercise was associated with an approximately 1 μmole/liter reduction in Hcy whereas those aged 40-42 years exhibited a reduction of 0.76 μmole/liter. Acute exercise however, does not have any effect on the plasma Hcy levels (Wright *et al*, 1998).

1.4.3 Diet

SAM and SAH play a major role in the regulation of plasma Hcy levels by affecting the distribution of Hcy among competing pathways. Both these metabolites activate CBS (Finkelstein *et al*, 1974, Finkelstein *et al*, 1975). Finkelstein *et al* (1974) also showed that SAH is capable of inhibiting BHMT. Kutzbach and Stokstad (1971) demonstrated that SAM is a strong allosteric inhibitor of MTHFR while SAH functioned to partially reverse this inhibition. This inhibition will lead to reduced synthesis of N^5-methyltetrahydrofolate which itself is a modulator of methionine metabolism by its ability to bind and inhibit GNMT (Wagner *et al*, 1985).

These mechanisms can combine to regulate methionine and Hcy metabolism with regard to nutritional status, especially in response to protein and methionine intake, since increased methionine concentrations in the liver will lead to increased SAM levels. The fact that SAM activates MAT III (Cabrero and Alemany, 1988) makes this system highly sensitive to methionine concentrations. The increased SAM will promote disposal via the transsulfuration pathway by activation of CBS and will inhibit remethylation by inhibiting both BHMT and MTHFR. A quantitative study of the methionine cycle *in vivo*, in healthy young men who were supplied with methionine at a rate of 198 μmoles/kg/day, revealed a Hcy synthesis through transmethylation of 238 μmoles/kg; 38% of this Hcy was channeled into remethylation and 62% underwent transsulfuration (Storch *et al*, 1988). These data agreed with the labile methyl balances conducted by Mudd and Poole (1975) who showed that remethylation could vary from 70% to 40% when changing from a restricted methionine intake to a diet containing increased amounts of methionine and choline.

1.4.4 Methylation Demand

The contribution of the different reactions involved in the formation of Hcy must also be considered. SAM is the methyl donor of virtually all known biological methylation reactions, ranging from such diverse processes as metal detoxification, biosynthesis of many small molecules and gene regulation via DNA methylation. Thirty nine different species of SAM-dependent methyltransferases have been well characterized in mammals (Clarke and Banfield, 2001), with the possibility that many more will be identified by means of genomic approaches which use conserved sequence motifs to detect methyltransferases in genomic open reading frames (Katz *et al*, 2003). In fact, Katz *et al* (2003) calculated that class I methyltransferases make up ~0.6-1.6% of the genes in the yeast, human, mouse, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Arabidopsis thaliana*, and *Escherichia coli* genomes.

The first demonstration of the importance of methylation to plasma Hcy levels was provided by studies in patients with Parkinson's disease undergoing treatment with L-3, 4-dihydroxyphenylalanine (L-DOPA). Parkinson's disease is characterized by a severe depletion of nigro-striatal dopaminergic neurons resulting in the deficiency of dopamine in the basal ganglia and melanin in the substantia nigra (Hague *et al*, 2005). These patients are treated with L-DOPA alone or in combination with a peripheral decarboxylase inhibitor (Cheng *et al*, 1997). The dopamine deficiency is relieved by the decarboxylation of L-DOPA in the brain. Parkinson's patients undergoing treatment with L-DOPA have been shown to have plasma Hcy levels that are about 50% higher than controls [Muller *et al*, 2001]. This increase in plasma Hcy arises from the wasteful methylation of L-DOPA by catechol-O-methyltransferase (COMT) which catalyzes the

SAM-dependent methylation of aromatic hydroxyl groups (Cheng *et al*, 1997). Due to the removal of the administered L-DOPA through this reaction, patients need to be given quite high doses (up to several grams per day) to achieve therapeutic levels of L-DOPA. This phenomenon was also evident in rats; the increased plasma tHcy was accompanied by decreased tissue levels of SAM (Miller *et al*, 1997). Pre-treatment with a COMT inhibitor was shown to alleviate or attenuate this response. These studies showed that plasma Hcy levels were sensitive to the methylation of an exogenous substance.

Thereafter, studies conducted by the Brosnan group addressed the issue of the role of methylation demand imposed by physiological substrates on plasma Hcy. Creatine synthesis, where SAM is used to methylate guanidinoacetic acid (GAA), has been considered to use more SAM than all of the other physiological methyltransferases combined (Mudd and Poole, 1975) accounting for about 75% of Hcy formation. Creatine synthesis is an inter-organ pathway in which the two enzymes involved in creatine synthesis occur in two different organs: L-arginine:glycine amidinotransferase occurs in the kidney (AGAT; EC 2.1.4.1) and guanidinoacetate methyltransferase (GAMT; EC 2.1.1.2) is localized to the liver. Stead *et al* (2001) studied the effect of changes in the methylation demand by modulating the rate of creatine synthesis by feeding rats GAA- or creatine- supplemented diets for 2 weeks. They found that Hcy was significantly elevated in the plasma of rats fed GAA and that it was decreased by ~25% in the rats on a creatine-supplemented diet (Stead *et al*, 2001). Supplementation by either GAA or creatine resulted in a decrease in the activity of kidney AGAT. Incubation of hepatocytes with GAA resulted in the export of significantly higher amounts of Hcy, giving further evidence for the dependence of the plasma Hcy level on the demand for methylation by

physiological substrates. A separate series of studies showed that creatine supplementation was able to lower the plasma level of GAA as well as its arterio-venous difference across the kidney (Edison EE, Brosnan ME and Brosnan JT unpublished observations).

Noga *et al* (2003) demonstrated the importance of another physiological methylation reaction on the plasma Hcy level. Phosphatidylethanolamine *N*-methyltransferase (PEMT), a liver-specific enzyme, is involved in the conversion of phosphatidylethanolamine (PE) to phosphatidylcholine (PC) (Vance and Ridgeway, 1988). 70% of hepatic PC is produced from choline by the activity of the enzymes of the Kennedy pathway; PEMT is responsible for the formation of the remaining 30% (DeLong *et al*, 1999). The synthesis of one molecule of PC through the PEMT pathway requires three successive SAM-dependent methylations. Plasma Hcy in *pemt*$^{-/-}$ mice was decreased by about 50% (Noga *et al*, 2003). Hepatocytes isolated from the *pemt*$^{-/-}$ mice secreted ~50% less Hcy than the cells isolated from the wild type mice. McArdle RH7777 (rat hepatoma) cells, over-expressing PEMT, produced significantly more Hcy than the wild-type cells. These results clearly demonstrated PC synthesis through the PEMT pathway to be a major contributor to the plasma Hcy levels.

1.4.5 Hormonal regulation of plasma Hcy

Most of the early literature on Hcy metabolism focused on traditional factors such as nutrition and genetics. However, studies conducted in many laboratories have now added to our understanding of the hormonal regulation of Hcy metabolism. Diabetes mellitus, both Type 1 and Type 2, leads to increased plasma levels of Hcy when

accompanied by renal insufficiency (Hoogeveen *et al*, 1998, Hultberg *et al*, 1991). However, Type 1 diabetic patients with normal plasma creatinine levels have been shown to have plasma tHcy levels significantly lower than healthy subjects (Robillon *et al*, 1994). Studies conducted in our laboratory confirmed this observation in streptozotocin-diabetic rats (Type 1 model) (Jacobs *et al*, 1998). In these rats the decreased Hcy was accompanied by concomitant increases in the activities of the hepatic transsulfuration enzymes, CBS and CGL, which were restored to normal by insulin treatment, suggesting regulation of plasma Hcy by insulin. Administration of glucagon to rats was shown to reduce plasma Hcy and was accompanied by increased flux of methionine through the transsulfuration pathway and by increases in the activities of the transsulfuration enzymes (Jacobs *et al*, 2001). The concurrent increase in the activity and the mRNA of CBS suggested that glucagon may act at the level of gene transcription to alter Hcy metabolism. This was directly confirmed by the experiments of Ratnam *et al* (2002). Glucocorticoids or cAMP increased, and insulin attenuated, the CBS mRNA and protein levels in H4IIE cells, a rat hepatoma cell line, while insulin treatment of HepG2 cells, a human hepatoma cell line, led to a decreased level of CBS protein. The 70% reduction in CBS-1b promoter activity after insulin treatment further confirmed the effect of insulin, while the nuclear run-on experiments provided definitive evidence that both insulin and glucocorticoids act at the level of gene transcription.

MAT is also under hormonal regulation. Adrenalectomy results in a 3-fold decrease in its enzyme activity, immunoreactive protein and mRNA levels in the liver; these effects could be reversed by triamcinolone treatment (Gil *et al*, 1997). MAT mRNA content was increased, in a time- and dose-dependent manner, by both triamcinolone and

dexamethasone., which effect was blocked by insulin. A direct effect of triamcinolone on the transcription of this gene was evident from experiments in which a luciferase reporter gene was driven by 1.4 kb of the 5'-flanking region of the hepatic MAT gene. Triamcinolone treatment resulted in a 3-fold increase in the promoter activity (Gil *et al*, 1997).

A recent study described the modulation of GNMT by insulin and glucocorticoids [Nieman *et al*, 2004]. Pre-treatment with insulin prevented the induction of GNMT by dexamethasone in rat pancreatic AR42J cells or in hepatoma H4IIE cells. It was also found that induction of diabetes by injection of streptozotocin leads to increased activity and abundance of GNMT.

Early studies conducted by Finkelstein *et al* (1971) showed that hydrocortisone increased the activity of BHMT three-fold, whereas thyroxin treatment significantly reduced its activity. Methionine synthase was also shown to be responsive to hormones in tissues other than the liver; the kidney enzyme was increased by 151% by estrogen and decreased by 60% by growth hormone. Hydrocortisone treatment led to a 2-fold increase in the specific activity of methionine synthase in the pancreas (Finkelstein *et al*, 1971).

It is evident therefore, that glucagon and glucocorticoids function to increase the disposal of methionine and/or Hcy, whereas insulin counteracts these effects.

1.4.6 Redox Regulation

CBS is a unique enzyme, in that it depends on both heme and pyridoxal 5'-phosphate for its function (Kery *et al*, 1994). The 63 Kd CBS subunit binds a molecule each of heme and PLP, and the presence of heme is required for PLP binding. Recently,

Taoka *et al* (1998) found evidence for redox-linked regulation of CBS, dependent on heme. Under reducing conditions generated by the addition of titanium citrate they observed a 41% decrease in the activity of CBS. Re-oxidation of the ferrous enzyme with ferricyanide reversed the inhibition. Transsulfuration, catalyzed by the two enzymes, CBS and CGL, leads to the synthesis of cysteine, which is required for glutathione synthesis. The Hcy-dependent transsulfuration pathway plays a major role in the maintenance of the intracellular glutathione pool under oxidative stress conditions and, indeed, the flux through the transsulfuration pathway responds to the oxidant load (Mosharov *et al*, 2000). Approximately 50% of the intracellular glutathione pool in human liver cells is derived from cysteine produced by the transsulfuration pathway; thus the cellular redox environment may affect plasma Hcy concentrations (Mosharov *et al*, 2000).

Two other enzymes in the Hcy metabolic pathway have also been shown to be responsive to redox changes. S-nitrosylation by nitric oxide, under conditions of septic shock or hypoxia, leads to the inactivation of both isoforms of MAT, MAT I and MAT III (Avila *et al*, 1997); this inactivation can be reversed by millimolar concentrations of glutathione. H_2O_2 also inactivates MAT by reversibly and covalently oxidizing cysteine 121, located at a "flexible loop" over the active site cleft of MAT (Sanchez-Gongora *et al*, 1997). The GSH/GSSG ratio also modulates the redox state of MAT (Pajares *et al*, 1992). The second enzyme responsive to redox conditions, methionine synthase, is inhibited by oxidative conditions, potentially due to the oxidation of the cob(I)alamin form of the cobalamine cofactor (Chen *et al*, 1995) This curtailment of remethylation can be understood as a means of increasing the conversion of Hcy to cysteine for GSH synthesis.

1.5 Non-physiological causes of HHcy

1.5.1 Vitamin Deficiency

The original report linking HHcy to the occurrence of cardiovascular disease involved a patient who developed homocysteinuria due to a defect in cobalamin metabolism, now recognized as Cb1 C deficiency (Gaull, 1972). Since then, many studies have confirmed the relationship between vitamins and plasma Hcy levels. Inadequate plasma concentrations of one or more of the B vitamins are responsible for the HHcy in about two thirds of hyperhomocysteinemic cases (Selhub *et al*, 1996).

The ability of folate supplementation to decrease urinary Hcy excretion was documented in patients with homocystinuria and mental retardation as early as 1968 (Carey *et al*, 1968). Kang *et al* (1987) showed that depletion of tissue folate leads to HHcy in non-homocystinuric subjects. Isolated deficiency of vitamin B_{12} also leads to moderate to intermediate fasting HHcy, with Hcy levels exceeding that found in the obligate heterozygotes for CBS deficiency (Brattstrom *et al*, 1988). Vitamin B_6, the cofactor for both enzymes of the transsulfuration pathway, is important in determining post-methionine load plasma Hcy levels (Brattstrom *et al*, 1990). Data from the Framingham Heart Study cohort showed that plasma Hcy exhibits a strong inverse association with plasma folate and weaker associations with plasma vitamin B_{12} and pyridoxal-5'-phosphate (Selhub *et al*, 1996). Vitamin intervention therapy with the three B vitamins alone and various combinations of them has been shown to reduce both basal plasma Hcy as well as levels after a methionine load (Brattstrom, 1996).

A few recent studies have tried to explain the mechanisms behind the Hcy lowering effects of these B vitamins. Supplementation with vitamin B_{12} was shown to

increase the activity of methionine synthase, an effect ascribed by Gulati *et al* (1996) to a posttranslational regulation of methionine synthase by vitamin B_{12} since the induction of activity of this enzyme correlates with increased levels of protein. They were later able to show that B_{12} supplementation induces translational up-regulation by shifting the mRNA from the ribonucleoprotein to the polysome pool. The B_{12}-responsive element was localized to the 70 bp region located at the 3' end of the 5'-untranslated region of methionine synthase mRNA (Oltean and Banerjee, 2003). Miller *et al* (1994) postulated that deficiency of folate leads to increased plasma Hcy not only through the impairment of Hcy remethylation but also by impairing Hcy removal via the transsulfuration pathway due to lowered tissue SAM levels which are inadequate to stimulate CBS.

1.5.2 Genetic Disorders

The most frequently described genetic defects of HHcy are associated with the enzymes of transsulfuration. Plasma Hcy levels are quite elevated in patients with homozygous CBS deficiency resulting in homocystinuria (Mudd *et al*, 1985). However, this is a rare disorder with a frequency estimated between 1:58,000 and 1:1,000,000 in newborns and a worldwide birth prevalence of 1:300,000. Heterozygosity for CBS in the general population is less than 1% (Mudd *et al*, 1995). The fasting Hcy level in these individuals appears to be normal or only slightly elevated, although post-methionine load levels may be elevated (Tsai *et al*, 1996). Genetically-determined defects have also been described in CGL which leads to cystathioninuria (Frimpter, 1965).

On the other side of the spectrum are the patients with Down syndrome. Down syndrome or trisomy 21, is characterized by the failure of chromosome 21 to segregate

normally during meiosis (Epstein, 1995). The gene for CBS is located on chromosome 21 and as a result is overexpressed in children with Down syndrome. A 157% increase in CBS enzyme activity in patients with Down syndrome is associated with reduced levels of plasma Hcy (Chadefaux, 1985). However, the reduction in plasma Hcy leads to a concurrent reduction in the folate-dependent re-synthesis of methionine, creating a functional intracellular folate deficiency known as the "methyl trap" (Pogribna *et al*, 2001).

MTHFR mutations are also associated with HHcy. Severe MTHFR deficiency (less than 2% of normal enzyme activity) is rare (Rozen, 1996). However, Kang *et al* (1991 A) identified a new variant of MTHFR, which displayed a distinctive thermolability with about 50% of the enzyme activity. This variant is associated with significantly higher levels of plasma Hcy and is inherited as an autosomal recessive trait (Kang *et al*, 1991A). Patients who were postulated to be compound heterozygotes of the allele for the severe mutation and the allele for the thermolabile mutation have also been identified (Kang *et al*, 1991 B).

Two common functional polymorphisms of the MTHFR gene were later identified. C677T polymorphism in exon 4, resulting in an alanine to valine substitution at codon 222 (Frosst *et al*, 1995) gives rise to the thermolability. Individuals homozygous for the C677T polymorphism (TT genotype) have about 30% of normal enzyme activity and results in intermediate HHcy (Kang *et al*, 1988); heterozygotes have about 65% of normal activity (Frosst *et al*, 1995). The phenotypic expression of the TT genotype appears to relate to the folate status. In the Hordeland Hcy study, a majority of subjects with intermediate HHcy exhibited the TT genotype; 88% of these were folate deficient.

(Guttormsen *et al*, 1996). The C677T mutation displays ethnic variability. Caucasians have a high (40%) allele frequency (van der Put *et al*, 1997) while it is almost absent in African Americans (McAndrew *et al*, 1996). A second polymorphism, A1298C in exon 7, results in a substitution of glutamate with alanine at codon 429 (van der Put *et al*, 1998). Individuals with 1298CC genotype display 60% of the activity of those with AA genotype but it does not seem to give rise to HHcy.

Cobalamin is bound to methionine synthase and acts as a methyl carrier between methyltetrahydrofolate and Hcy. The methyl group of methyltetrahydrofolate is first transferred to the cobalamine cofactor to form methylcob(III)alamin which in turn becomes cob(I)alamin after transfer of the methyl group (Banerjee, 1997). Over time, cob(I)alamin may be oxidized to cob(II)alamin which renders the enzyme inactive. Cob(II)alamin needs to undergo a reductive methylation with SAM acting as the methyl donor to be converted back to its active state (Ludwig and Matthews, 1997).

Two forms of methionine synthase deficiency are known which lead to the development of HHcy (Harding *et al*, 1997). Patients from the cblG complementation group of folate/cobalamin metabolism have mutations in the methionine synthase gene (Leclerc *et al*, 1996). The second complementation group, cblE, shows reduced methionine synthase activity, due to a defect in the reducing system which keeps the enzyme in the functional state (Gulati *et al*, 1997). cblE patients have been shown to have defective NADPH-dependent reducing activity. Leclerc *et al* (1998) isolated the cDNA corresponding to the reductive activation enzyme which they named methionine synthase reductase and also identified a number of mutations in 3 cblE patients, a 4-bp frame shift in two and a 3-bp deletion in one.

1.5.3 Renal Disorders

Patients with diabetes mellitus, either Type 1 or Type 2, have decreased plasma Hcy levels when kidney function is normal (Robillon *et al*, 1994, Jacobs *et al*, 1998, Wijekoon *et al*, 2005). However, with decreasing kidney function the concentration of plasma Hcy changes and is elevated compared to controls. This has been observed in both Type 1 and Type 2 diabetes mellitus (Hoogeveen *et al*, 1998, Hultberg *et al*, 1991). Patients with end-stage renal disease with no diabetes also exhibit elevated plasma Hcy (Bostom *et al*, 1995 A). These observations agree with the finding of Bostom *et al* (1995 B) that the rat kidney is a major organ involved in Hcy metabolism. They showed a positive renal arteriovenous difference for Hcy slightly greater than 20% of the mean arterial plasma Hcy concentration, which suggested that the loss of the Hcy metabolizing capacity of the kidneys may be a major cause of the increased plasma Hcy in renal patients. The transsulfuration pathway was shown to be the major route of catabolism of Hcy taken up by rat kidneys, accounting for 78% of the disappearance of Hcy (House *et al*, 1997). The important role played by the kidneys in maintaining plasma Hcy homeostasis was shown by the ability of the kidneys to handle acute increases in plasma Hcy (House *et al*, 1998). Kidneys were able to significantly increase the uptake of Hcy from the plasma and still manage to metabolize it with no change in urinary excretion, demonstrating the capacity of the kidneys for Hcy catabolism.

1.5.4 Pharmacological intervention

Intervention with a variety of pharmacological agents has been shown to disrupt plasma Hcy metabolism. Many of these drugs act through their disruption of the

absorption or the metabolism of the various vitamins of Hcy metabolism. Methotrexate, used in a variety of diseases, interferes with folate metabolism by inhibiting dihydrofolate reductase (Bertino, 1993), thereby reducing methyltetrahydrofolate levels in cells. The time course for the development of HHcy depends on the dose of methotrexate used; thus it varies in patients with different diseases such as cancer (Refsum *et al*, 1991), psoriasis (Refsum *et al*, 1989) and rheumatoid arthritis (Morgan *et al*, 1998). Anticonvulsants also interfere with folate metabolism, thereby leading to HHcy (James *et al*, 1997). They are thought to deplete liver folate stores through inhibition of polyglutamation (Carl *et al*, 1991). Bile acid sequestrants interfering with the absorption of folate also cause HHcy. This has been observed in coronary patients treated with niacin and colestipol as they exhibit significantly higher Hcy levels when compared with similar patients receiving a placebo (Blankenhorn *et al*, 1991). Cholestyramine (Tonstad *et al*, 1998), a bile acid sequestrant, and the antidiabetic drug metformin (Carlsen *et al*, 1997) interfere with the absorption of cobalamine and folate, leading to the development of HHcy with long term use of these drugs.

The anesthetic, nitrous oxide, is a well known pharmacological agent that causes rapid (within 90 minutes) elevations in plasma Hcy (Ermens *et al*, 1991). It is known to oxidize cob(I)alamin to cob(II)alamin, thereby inactivating methionine synthase (Drummond and Matthews, 1994). Azuridine, which is no longer in use, caused HHcy in humans as well as animals probably through its interference with pyridoxal 5-phosphate (Drell and Welch, 1989). Isoniazid (Krishnaswamy, 1974), niacin (Basu and Mann, 1997) and theophylline (Ubbink *et al*, 1996) also cause increases in plasma Hcy by their interference with vitamin B_6 metabolism. The treatment of Parkinson's disease with L-

DOPA leads to increased plasma Hcy, the mechanism of which was discussed in the section on methylation demand.

Treatment with some other drugs has been shown to lead to reduction in plasma Hcy. Oral penicillamine has been shown to reduce both free and plasma protein-bound Hcy in homocystinuria patients (Kang *et al*, 1982). Patients with acute lymphoblastic leukemia treated with 2-deoxycoformycin also have markedly reduced plasma Hcy levels (Kredich *et al*, 1981). 2-deoxycoformycin indirectly inhibits S-adenosylhomocysteine hydrolase by blocking adenosine deaminase. The use of estrogen-containing oral contraceptives has also been shown to lower plasma Hcy levels (Steegers-Theunissen *et al*, 1992). Cyclic variations in plasma Hcy have been shown in oral contraceptive users with the variation in hormone levels in the contraceptives.

1.6 Diabetes Mellitus

Diabetes mellitus is one of the most common metabolic disorders affecting over 100-million people worldwide (Spruce *et al*, 2003) and is regarded as a major risk factor for cardiovascular disease (Wilson, 1998). The prevalence of atherosclerosis is 2-6 fold greater in diabetic patients compared to non-diabetic patients (Brand *et al*, 1989). The 7-year incidence for myocardial infarction (MI) for diabetic patients who have not experienced MI previously was similar to that of non-diabetic patients who have gone through a previous MI (Haffner *et al*, 1998). Half of all diabetic patients are also likely to die within a year of their first cardiac event, giving rise to a higher mortality rate than non diabetics (Miettinen *et al*, 1998).

Diabetes mellitus generally can be divided into two categories. Insulin-dependent diabetes mellitus or Type 1 diabetes mellitus usually develops in childhood or adolescence and results from the destruction of pancreatic beta cells by an auto-immune process that may be precipitated by a viral infection (Fajans, 1996). The second form of diabetes, Non-insulin dependent diabetes or Type 2 diabetes usually occurs in middle or later life and results from a highly complex pathogenetic process which has components that still defy biochemical explanation. Despite this complexity of its etiology the disease has two hallmark features: (i) insulin resistance, which is defined as an impaired ability of insulin to suppress hepatic glucose output and promote glucose entry into peripheral tissues and (ii) impaired function of pancreatic β-cells leading to insufficient production of insulin to overcome the insulin resistance (McGarry, 2002). Insulin resistance precedes the development of fulminant diabetes and is characterized by a hyper-secretion of insulin by the β cells to maintain normoglycemia (McGarry, 2002).

1.7 Insulin resistance

J. Dennis McGarry's 1992 article in Science titled "What if Minkowski had been ageusic? an alternative angle on diabetes" draws our attention to an important aspect of this disease (McGarry, 1992). In calling for a more "lipocentric" approach to the diseases as opposed to the traditional "glucocentric" approach, McGarry drew attention to the fact that Type 2 diabetes is almost invariably accompanied by increased levels of circulating free fatty acids (FFA) and triglycerides (TG), and excessive deposition of fat in tissues, in addition to the hyperglycemia (McGarry, 2002). One of the major current

hypotheses as to the etiology of insulin resistance concerns the deranged lipid metabolism.

Specific measurement of intramyocellular lipid (IMCL) with the use of 1^{H}-magnetic resonance spectroscopy (1^{H} MRS) was able to uncover a very tight negative correlation between IMCL and whole-body insulin sensitivity (Stein *et al*, 1997, Krssak *et al*, 1999). This finding is interesting especially in the light of the tight association of Type 2 diabetes with obesity. Artificial elevation of free fatty acids (FFA) in healthy human volunteers with concomitant increases in IMCL was also shown to reduce insulin mediated glucose disposal, clearly strengthening the cause and effect case between increased IMCL and insulin resistance (Brechtel *et al*, 2001, Boden *et al*, 1995). Roden *et al* (1996) showed that the FFA induced reduction of insulin sensitivity lead to a reduction in both oxidative glucose disposal and muscle glycogen synthesis by some 40-50% as measured by ^{13}C-MRS in healthy humans.

With the performance of a hyperglycemic, hyperinsulinemic clamp in healthy individuals and Type 2 diabetic patients, the rate of insulin-stimulated muscle glycogen synthesis as assessed by the incorporation of [^{13}C] glucose into gastrocnemius/soleus muscle glycogen, was shown to be decreased by >50% in Type 2 diabetic patients compared with healthy age and weight-matched individuals (Shulman *et al*, 1990). Defects in insulin-stimulated muscle glycogen synthesis therefore could be attributed to almost all of the impaired glucose removal component of insulin resistance seen in Type 2 diabetic patients (Petersen and Shulman 2006).

The measurement of the intracellular concentration of glucose, glucose-6-phosphate and glycogen in muscle of Type 2 diabetic patients and age and weight-

matched controls with ^{13}C and ^{31}P MRS studies to identify the rate control step in this defective glycogen synthesis, revealed an approximately 0.1 mM increase in intracellular glucose-6-phosphate concentration in normal individuals but no change in patients with Type 2 diabetes under similar conditions of hyperglycemia and hyperinsulinemia (Rothman *et al*, 1992). Very interestingly, lean, normoglycemic but insulin-resistant offspring of parents with Type 2 diabetes also showed a 50% reduction in the rate of whole body glucose metabolism mainly due to reduced rates of muscle glycogen synthesis along with reduced increments of intramuscular glucose-6-phosphate in response to insulin (Rothman *et al*, 1995). These data show the primary nature of this effect with regards to insulin resistance and could arise due to either defects in glucose transport into muscle or hexokinase II activity. Measurement of intracellular glucose concentration with a ^{13}C NMR method under hyperglycemic hyper-insulinemic conditions revealed that the intracellular glucose concentration was far lower in the diabetic subjects than the concentration expected if hexokinase II was the primary rate-limiting step for glycogen synthesis (Cline *et al*, 1999). These data lead to the conclusion that glucose transport into muscle cells by GLUT 4 is the primary defect leading to the impaired glycogen synthesis by muscle tissue.

These data stand in contrast to the classic Randle mechanism which suggested that fatty acid induced insulin resistance is brought about by the increased oxidation of fatty acids at the expense of glucose (Randle *et al*, 1963, Randle *et al*, 1965, Randle *et al*, 1964). According to this hypothesis increased fatty acid oxidation leads to increased intramitochondrial acetylCoA/CoA and $NADH/NAD^+$ ratios with subsequent inactivation of pyruvate dehydrogenase. The resultant increase in citrate inhibits phosphofructokinase

which then lead to the accumulation of glucose-6-phosphate and subsequent inhibition of hexokinase II leading to the accumulation of intracellular glucose.

Since the measurement of both intra-cellular glucose and glucose-6-phosphate using the novel methods indicated a reduction in these molecules rather than an increase as suggested by the Randle mechanism the defect in glucose transport appears to be the more likely cause of the fatty acid induced insulin resistance. Dresner *et al*, (1999) found that elevation of plasma fatty acid concentration abolished insulin stimulated, IRS-1 associated PI_3-kinase activity compared to a four-fold increase observed with infusion of glycerol. This effect of fatty acids on the activity of PI_3 kinase could result from the effect of fatty acids on upstream insulin signaling as infusion of lipids into rats have been shown to reduce the insulin stimulated IRS-1 tyrosine phosphorylation associated with the activation of protein kinase C θ (Griffin *et al*, 1999). Protein kinase C θ is a serine kinase known to be activated by diacylglycerol (Chalkley *et al*, 1998).

With the help of these experiments a unifying hypothesis for the common forms of human insulin-resistance was proposed where increased intracellular fatty acid metabolites such as long chain fatty acyl CoA, diacylglycerol or ceramides activate protein kinase C θ leading to the serine/threonine phosphorylation of IRS-1. Unlike the tyrosine phosphorylated forms of IRS, the serine/threonine phosphorylated forms fail to activate PI_3 kinase with the result of failure of activation of GLUT 4 transporters in response to insulin (Shulman, 2000). Consistent with this hypothesis is the severe insulin-resistance seen in a mouse model of severe lypodystrophy almost totally devoid of white adipose tissue. The intracellular fatty acyl-CoA levels in these mice are almost 2-fold higher in both muscle and liver and show defects in insulin activation of IRS-1 and IRS-2

associated PI_3 kinase activity in muscle and liver respectively (Kim *et al*, 2000). Also in support of this hypothesis is the finding by Bell *et al* (2000) that the reversal observed in insulin-resistance in high fat fed rats after a single meal of high carbohydrate is associated with a reduction in muscle LCACoA levels and loss of membrane bound PKCθ activity.

Accumulation of intracellular fat metabolites in the liver has also been shown to activate a serine kinase cascade involving PKC–ε, which leads to the decreased tyrosine phosphorylation of IRS-2- a key mediator of insulin action in the liver (Previs *et al*, 2000, Samuel *et al*, 2004). Studies with mitochondrial glycerol phosphate acyl CoA transferase knockout mice indicates that diacylglycerol, an activator of PKC–ε is responsible for these effects (Neschen *et al*, 2005).

All these findings emphasize the importance of the location of lipid deposition in the development of insulin resistance and indicate that it is not obesity *per se* that is the culprit. The ability of PPAR-γ agonists to restore insulin sensitivity also bears witness to this fact, as discussed later.

Age is also a factor in the development of insulin resistance (Petersen *et al*, 2003). It was noted that lean and otherwise healthy elderly people have increased fat accumulation in muscle and liver tissue and the increased intramyocellular lipid accumulation is accompanied by a 40% reduction in their mitochondrial oxidative phosphorylation activity compared with BMI and activity matched young individuals (Petersen *et al*, 2003). The insulin resistant offspring of parents with Type 2 diabetes were also shown to have impaired mitochondrial function with a 30% reduction in mitochondrial ATP synthesis and an 80% increase in intramyocellular lipid content (Petersen *et al*, 2004). Loss of mitochondrial function therefore also appears to be an

important factor in the development of insulin resistance and especially may be involved with the heritability of Type 2 diabetes (Petersen and Shulman, 2006).

Despite playing a minor role in the insulin-stimulated glucose uptake, insulin action and glucose transport in adipose tissue are also important determinants of systemic insulin sensitivity and glucose homeostasis (Abel *et al*, 2001, Bluher *et al*, 2002). Similar to skeletal muscles the rate limiting step for utilization of glucose by adipose tissue is the trans-membrane transport of glucose by GLUT 4 (Cline *et al*, 1999). GLUT 4 gene expression is down-regulated in adipocytes but not in skeletal muscles from humans with Type 2 diabetes and/or obesity (Carvalho *et al*, 1999, Carvalho *et al*, 2001). The importance of this finding was emphasized by the adipose-specific GLUT 4 knockout mice that developed insulin resistance and had an increased risk of Type 2 diabetes (Abel *et al*, 2001). Although the genetic defect was limited to adipose tissue, they developed insulin resistance secondarily in muscle and liver. Mice that over- express GLUT 4 selectively in adipose tissue have enhanced glucose tolerance, insulin sensitivity, fasting hypoglycemia, and hypo-insulinemia (Shepherd *et al*, 1999). Mice lacking GLUT 4 selectively in muscle are insulin-resistant and diabetic and the insulin resistance confined initially to the muscle spreads later on to adipose tissue and liver (Kim *et al*, 2001, Zierath *et al*, 1996). Breeding muscle GLUT 4 knockout (MG4KO) mice to mice over-expressing GLUT 4 in adipose tissue (AG4Tg) was able to normalize the fasting hyperglycemia and reverse the whole body insulin resistance in MG4KO mice although it had no effect on the glucose transport in muscle (Carvalho *et al*, 2005). The crossbred animals were also able to suppress the hepatic glucose production in response to insulin (Carvalho *et al*, 2005). Although the adipose-selective deletion of GLUT 4 leads to secondary defects in

glucose action in muscle and liver, these muscles have a normal response to insulin ex-vivo, indicating a circulating factor(s) as the cause of insulin resistance in these mice (Abel *et al*, 2001).

In recent years our view of adipose tissue has deviated from its traditional role as an energy storage depot and we are seeing it as an organ with a secretory function expressing and secreting various peptide hormones and cytokines, including TNF-α, plasminogen activator inhibiter-1, angiotensinogen, leptin (Kahn and Flier, 2000), along with resistin and adiponectin (Scheen, 2003). TNF-α and resistin have clearly been shown to inhibit insulin action in rodents (Greenberg and McDaniel, 2002). Their effects in humans however have still not been fully elucidated. Adiponectin, synthesized exclusively in adipose tissue, is found at lower levels in the plasma in obesity, in contrast to the other adipocytokines and appears to be a major modulator of insulin action with low adiponectin levels contributing to insulin resistance (Scheen, 2003). Leptin, the other adipocytokine involved in enhancing insulin action, will be discussed in detail in the next section. Retinol binding protein-4 (RBP-4) is a novel adipocyte derived molecule which is elevated in the adipose tissue of adipose-*Glut* $4^{-/-}$ mice and the serum of several insulin resistant states in mice and humans (Yang *et al*, 2005). Transgenic over-expression of human RBP4 or injection of recombinant RBP4 in normal mice causes insulin resistance while genetic deletion of RBP4 enhances insulin sensitivity (Yang *et al*, 2005). Increased serum RBP 4 was shown to induce the hepatic expression of the gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) and impair insulin signaling in muscle (Yang *et al*, 2005).

1.8 Leptin

Leptin was first discovered in 1994 by positional cloning of a single gene mutation in the *ob/ob* mouse, a model of obesity and diabetes (Zhang *et al*, 1994).The obesity in these mice is caused by the deficiency of the *ob* gene product, leptin, and is reversible with the administration of the peptide (Pellymounter *et al*, 1995). These findings led to the categorization of leptin as an anti-obesity hormone. However, considering the fact that over-weight individuals have higher levels of leptin than non-obese individuals it is now recognized that although leptin deficiency does lead to obesity the primary role of leptin is not the prevention of obesity (Unger *et al*, 1999). Leptin now is regarded to play a role in permitting over-nutrition and deposition of body fat to occur without causing metabolic injury to non-adipose tissue (Unger *et al*, 1999). This is achieved by confining the storage of excess calories to adipose tissue and reducing ectopic fat accumulation and the resulting metabolic trauma or lipotoxicity of these non-adipocytes (Shimabukuro *et al*, 1997, Muoia *et al*, 1997).

1.9 Treatment of insulin resistance and diabetes with peroxisome proliferator activated receptor γ (PPAR-γ) agonists

The peroxisome proliferator-activated receptors (PPAR) comprise a subfamily of nuclear receptors that heterodimerizes with the retinoid X receptor to regulate the transcription of many genes involved in lipid and glucose homeostasis in a ligand dependent manner (Jones *et al*, 2005). PPAR-γ, a member of this subfamily, is mainly expressed in white and brown adipose tissue and is expressed to a lesser extent in macrophages, colon, kidney and liver (Fajas *et al*, 1997). PPAR-γ has been shown to take

part in many biological processes such as adipocyte differentiation, glucose homeostasis, atherogenesis, inflammation and tumor suppression (Braissant *et al*, 1996).

A number of fatty acid metabolites including oxidized linoleic acid and arachidonic acid metabolites like 15-deoxy-$\Delta^{12,14}$ prostaglandin J_2 act as endogenous ligands for PPAR-γ (Nagy *et al*, 1998, Forman *et al*, 1995). Thiazolidinediones (TZD), a class of compounds known to enhance insulin sensitivity and improve metabolic control in patients with Type 2 diabetes act through the activation of PPAR γ receptors (Saltiel and Olefsky, 1996). These agents have been shown to reduce plasma glucose as well as plasma insulin levels, thereby clearly improving insulin sensitivity in a variety of insulin-resistant or diabetic rat and mice models (Olefsky 2000). The improvement in insulin sensitivity has also been directly demonstrated using the euglycemic glucose clamp technique where treatment with TZDs was shown to improve insulin-stimulated glucose disposal as well as insulin inhibition of hepatic glucose production in a wide array of insulin resistant and diabetic rat models (Olefsky, 2000). These studies clearly demonstrate the efficacy of TZDs in improving insulin sensitivity, however, the mechanisms by which the TZDs bring about this improvement of insulin sensitivity is unclear.

Mutations in human PPAR receptors provide mixed results as to its function. Complete loss-of-function mutations cause insulin resistance, which suggests that these receptors are involved in insulin signaling (Barroso *et al*, 1999). However, quite in contrast to what was expected, individuals carrying the Pro12Ala mutation have been found to have greater insulin sensitivity although having decreased receptor activity (Deeb *et al*, 1998). Similarly, heterozygous PPAR-γ null mice are more insulin sensitive

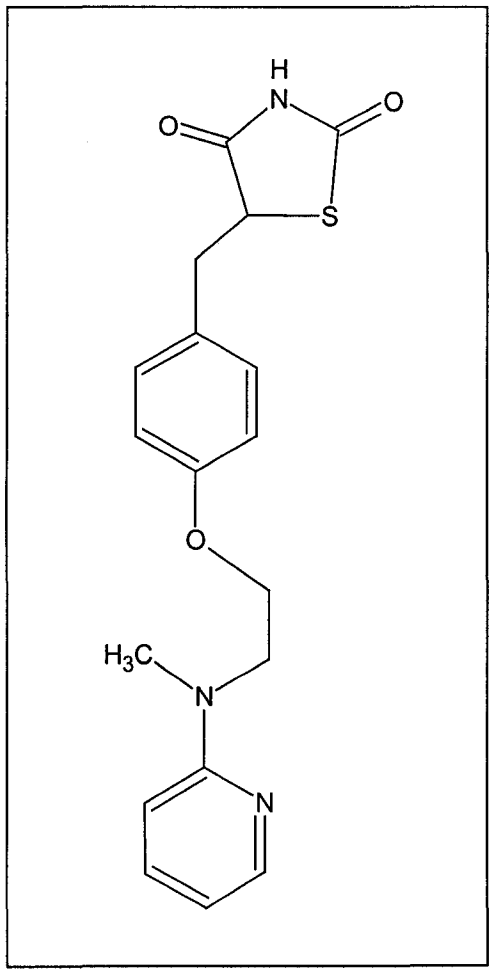
than their wild type littermates and are also protected from high-fat diet-mediated insulin resistance (Miles *et al*, 2000, Kubota *et al*, 1999). Jones *et al* (2005) used the Cre/loxP strategy to develop an adipose specific PPAR-γ knockout mouse, which was protected from developing insulin resistance, or glucose intolerance, probably due to the increased insulin stimulated glucose disposal by the liver in the face of an induction of hepatic PPAR-γ. These results have led to the suggestion that the normal role of PPAR-γ receptors and their endogenous ligands is to dampen insulin action, promoting a state of insulin resistance (Miles *et al*, 2000) and the insulin sensitizing effect of TZDs may reflect their ability to inhibit this dampening effect of the endogenous ligands (Olefsky, 2000).

Rosiglitazone (RSG) (Figure 1.4), one of the TZDs being currently used to treat human Type 2 diabetes, has been shown to reduce insulin resistance and prevent the loss of β-cell mass in obese ZDF rats by maintaining β-cell proliferation and preventing net β-cell death (Finegood *et al*, 2001). The insulin-sensitizing effect of RSG is also accompanied by a partitioning of fatty acid uptake into adipose tissue and away from the liver and muscle and a reduction in liver fatty acyl CoA accumulation (Ye *et al*, 2004).

1.10 Cardiovascular disease, diabetes and insulin resistance

The risk of CVD and mortality increases greatly with diabetes mellitus with an estimated 75% of Type 2 diabetic patients in the United States dying of cardiovascular complications (Audelin *et al*, 2001). They are also likely to develop CVD at a younger age and have a higher rate of diffuse multi-vessel disease with

Figure 1.4 Chemical structure of Rosiglitazone


H
N
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S
O
H_3C
N
N

impaired coronary vasodilatory reserve and develop congestive heart failure (Audelin *et al*, 2001).

Glycation of extracellular matrix proteins leads to the formation of advanced glycosylation end products which then induce cross linking of collagen and extra-cellular matrix proteins in many tissues including blood vessel walls (Brownlee, 1992, Vlassara and Bucala, 1996). This glycation process results from exposure to hyperglycemia over a period of time as is the case with diabetic patients. Generation of reactive oxygen species (ROS) leading to increased oxidative stress together with a decreased synthesis and an increased inactivation of nitric oxide (NO) derived from the endothelium is also a result of the glycosylation process. NO plays a major role in the endothelium-dependent vasorelaxation of blood vessels. In addition, oxidative damage to the arterial wall also leads to increased blood vessel stiffness, oxidized LDL production and its uptake into macrophages, with the production of foam cells, the secretion of platelet derived growth factor and proliferation of vascular smooth muscle cells. Increased platelet adhesion and aggregation, increased fibrinogen and increased plasminogen activator inhibitor-1 (PAI-1) concentrations also result from theses changes, giving rise to a pro-thrombotic state (Audelin *et al*, 2001).

Insulin resistance *per se*, without the added complications brought along by frank Type 2 diabetes is also associated with increased risk for CVD. The dyslipidemia of insulin resistance appears to be a major player in the increased risk for CVD. Whether the increase in plasma FFA led to the development of insulin resistance or insulin resistance at the level of adipose tissue with increased hydrolysis of TGs led to the release of FFA into the circulation, insulin resistance is associated with a hypertriglyceridemia. Increased

entry of these FFA into the liver results in increased release of VLDL by the liver. The progression of insulin resistance towards CVD begins with this VLDL-associated hypertriglyceridemia, which then leads to low HDL cholesterol and increased small dense LDL particles due to the action of cholesteryl ester transfer protein (CETP) where collision of VLDL with HDL leads to the exchange of cholesteryl ester from HDL for TG from VLDL. The TG of HDL is then hydrolyzed by hepatic lipases and probably by LPL which generates a small HDL that sheds some of its surface, including apo A-1. Apo A-1 is excreted through the kidneys making HDL unavailable for reverse cholesterol transport. In a similar fashion small dense LDL particles are formed with the exchange of LDL CE for VLDL TG in the presence of CETP (Bruce *et al*, 1998, Horowitz *et al*, 1993)

As reviewed by Ginsberg (2000) these changes can increase an individual's risk for CVD in several ways. First, the increased VLDL particles can enter the blood vessel wall and accumulate in atherosclerotic plaques. Due to the action of CETP, these VLDL contain more CE and therefore deliver more cholesterol per particle to the vessel wall. In addition, increased VLDL secretion competes for the chylomicron clearance pathways, leading to post-prandial hyperlipidemia, which is independently associated with coronary artery disease. Second, the diminished reverse cholesterol transport reduces the delivery of cholesterol to the liver along with an inability of HDL to fulfill its proposed functions as a direct antiatherogenic substance at the vessel wall. Scavenger receptor B1 (SRB1) is involved in selective delivery of cholesteryl esters to the liver for excretion via the biliary pathway without the endocytosis and delivery of the whole HDL particle (Acton *et al*, 1996). The reduction in CE due to the action of CETP hinders this reverse cholesterol pathway. Third, the small dense LDL may be more likely to undergo oxidation or may

more readily enter the artery wall and therefore be more atherogenic than an equal number of larger more CE rich LDL particles.

Fibrinogen, factor VII, and PAI-1, all of which are factors involved in clotting and fibrinolysis were shown to be increased in individuals with insulin resistance (Ginsberg, 2000). Although the connection between insulin resistance and some of these factors is still under investigation PAI-1 levels seem to increase with increased levels of insulin as well as VLDL (Erikssen *et al*, 1998). These effects on clotting definitely add to the CVD risk posed by insulin resistance.

1.11 Hcy levels in insulin resistance and diabetes

Despite the 4-6 fold increase in CVD risk found in diabetic patients compared to their non-diabetic counterparts a clear relationship between Hcy levels and diabetes has not been established. Araki *et al* (1993) observed a significantly higher level of plasma Hcy in Type 2 diabetic patients with macroangiopathy than in diabetic patients without macroangiopathy or non-diabetic subjects, with no difference in the plasma Hcy levels between the two latter groups. Studying both Type 1 and Type 2 diabetic patients, Munshi *et al* (1996) did not observe any differences between plasma Hcy levels in normal volunteers, diabetics without vascular disease or diabetics with vascular disease. However, after a methionine load they observed a higher frequency of HHcy in patients with Type 2 diabetes compared with age matched controls; HHcy was more common in those with macrovascular disease. Measurement of fasting plasma Hcy levels in 122 Type 2 diabetic patients identified 31% of the cohort as being hyperhomocysteinemic while the other 69% had normal values (Buysschaert *et al*, 2000). Here again the prevalence of

macroangiopathy was greater in the hyperhomocysteinemic group while they also exhibited impaired renal function evidenced by decreased creatinine clearance.

A study conducted with 50 Type 1 and 30 Type 2 diabetic patients on the other hand, found the mean plasma Hcy concentration in all diabetic patients to be significantly lower than in normal controls (Wollesen, *et al*, 1999). In Type 1 diabetic patients with no renal complications, plasma Hcy levels were found to be lower than in healthy people while those patients with nephropathy had elevated plasma Hcy levels (Matteucci *et al*, 2002). Robillon *et al* (1994) also found decreased levels of plasma Hcy in Type 1 diabetic patients. This decrease was also shown in an animal model of Type 1 diabetes, the streptozotocin-induced diabetic rat (Jacobs *et al*, 1998).

Wollesen *et al* (1999) were also able to show that the reduction in plasma Hcy seen in the diabetic patients was accompanied by relative urinary hyperfiltration with the plasma Hcy levels closely and independently associating with the glomeruler filtration rate (GFR). This association between plasma Hcy and GFR could also explain the age-related increases observed in plasma Hcy. On the other hand the reduced plasma Hcy seen in streptozotocin diabetic rats was associated with an increase in the removal of Hcy via an accelerated transsulfuration pathway giving a metabolic perspective to the reduced plasma Hcy in Type 1 diabetes (Jacobs *et al*, 1998).

Overt nephropathy however gives rise to increased plasma Hcy levels. Chico *et al* (1998) found Type 2 diabetic patients to have increased plasma Hcy compared to non-diabetic control subjects. They found the albumin excretion rate to show the strongest independent association with Hcy. Patients with both types of diabetes, with nephropathy, had higher plasma Hcy concentrations and the increase in Hcy was related to the severity

of nephropathy. Ozmen *et al* (2002) also found HHcy in Type 2 diabetes to be associated with deteriorating renal function. Several other studies describing increased plasma Hcy levels in Type 2 diabetes show an association between HHcy and decreased renal function (Emoto *et al*, 2001; Stabler *et al*, 1999; Davies *et al*, 2001). It is clear therefore, that plasma Hcy in diabetes varies depending on the presence or the absence of nephropathy with levels being lower or normal with no nephropathy and higher when there is nephropathy.

The literature on plasma Hcy levels in the insulin resistant state, before the onset of frank diabetes is quite conflicting. Pergola *et al* (2001) found the plasma Hcy level to be independently associated with insulin resistance in normal weight, overweight and obese pre-menopausal women. They observed a gradual increase in plasma Hcy levels across the quartiles into which the study group was divided according to their degree of insulin resistance as assessed by the Homeostatic model assessment (HOMA) (Pergola *et al*, 2001). In agreement with this finding Giltay *et al* (1998 B) also observed a significant association between insulin resistance and plasma Hcy levels in healthy, non-obese subjects. The degree of insulin resistance was determined according to the hyperinsulinemic euglycemic clamp method in this study. The Framingham offspring study with 2011 subjects with no CVD or diabetes found HHcy and an abnormal urinary albumin excretion to be associated with hyperinsulinemia (Meigs *et al*, 2001). Rats made hyperinsulinemic and insulin resistant by feeding a high fat sucrose diet were examined at 6 months and 2 years and were found to have increased plasma levels of Hcy compared to rats fed a low fat complex carbohydrate diet (Fonseca *et al*, 2000). Similarly, patients

with polycystic ovary disease who were exhibiting insulin resistance were also found to have increased plasma Hcy levels (Schachter *et al*, 2003).

In contrast to the above studies Abbasi *et al* (1999) found the Hcy levels to be similar in healthy volunteers who were classified as either insulin resistant (SSPG > 180 mg/dl) or insulin sensitive (SSPG <1000 mg/dl) according to the steady-state plasma glucose (SSPG) concentration during the insulin suppression test. A negative correlation between plasma Hcy and plasma insulin where hyperinsulinemic subjects had lower plasma Hcy concentration compared to normoinsulinemic subjects has also been reported (Bar-on *et al*, 2000). A similar negative relationship between insulin resistance and plasma Hcy were obtained by Rosolva *et al* (2002) in healthy volunteers categorized according to their insulin sensitivity as quantitated by the SSPG concentrations after an insulin suppression period.

These differences in plasma Hcy levels in insulin resistance may arise due to the differences in the methods employed to measure insulin resistance and/or the degree of insulin resistance of the subjects at the time of experimentation.

1.12 Hcy and cardiovascular disease in diabetes

Total plasma Hcy, lipids and parameters of fibrinolytic activity was measured in a group of 145 Japanese patients undergoing routine coronary angiography to assess chest pain or suspected coronary artery disease (Okada *et al*, 1999). The presence of diabetes was identified and the severity of the coronary artery stenosis (CAD scoring) was graded as non stenotic, stenotic single-vessel, stenotic two-vessel, or stenotic three-vessel. They found plasma Hcy to be significantly higher in patients with stenotic vessels than in

patients who were non-stenotic. Very interestingly thereafter they found that the number of stenotic coronary arteries was related to tHcy level only in patients with diabetes mellitus. 39% of the patients studied exhibited HHcy with the CAD score being highest in the diabetic patients with HHcy. After adjustment for age, HHcy was associated with an odds ratio (OR) for coronary artery stenosis of 2.01 for all patients which increased up to 6.6 in patients with diabetes. They concluded that plasma Hcy level was independently associated with the CAD score only in the Diabetes mellitus group.

The Hoorn study, a cross-sectional survey of glucose tolerance and other cardiovascular risk factors in a 50-75 year old general white population, found the OR per 5μmol/liter increment in Hcy for any cardiovascular disease was 1.38 in subjects with normal glucose tolerance, 1.55 in subjects with impaired glucose tolerance and 2.33 in subjects with Type 2 diabetes mellitus (Hoogeveen *et al*, 1998). They concluded that high serum Hcy may be a stronger risk factor (1.6-fold) for CVD in subjects with Type 2 diabetes mellitus than in non-diabetic subjects. Another study conducted using the same cohort showed after adjustment for major cardiovascular risk factors, serum albumin and HbA1c, the OR for 5-year mortality was 1.56 for HHcy and 1.26 per 5 μmol/liter increment of Hcy (Hoogeveen *et al*, 2000). They found that the OR for 5-year mortality for HHcy was 1.34 in non-diabetic subjects and 2.51 in diabetic subjects and that it is a stronger risk factor (1.9 fold) for mortality in Type 2 diabetic patients than in non diabetic subjects. Similarly, another prospective study based on a cohort of 211 Type 2 diabetic patients showed that plasma tHcy concentration is a significant predictor of six-year all-cause and cardiovascular mortality, both in patients with normoalbuminuria and in those with microalbuminuria or macroalbuminuria (Stehouwer *et al*, 1999). Becker *et al,*

(2003), using the samples collected in the Hoorn study, showed that the incidence rate of coronary events was 2.63 per 100 person-years among diabetic and 1.29 among non diabetic individuals. The risk of coronary events increased 28% for each 5 μmol/L increment of Hcy among the diabetic subjects while among the non diabetic participants there was no such association.

All of the above mentioned studies are concerned with Type 2 diabetes. Hoffman *et al*, (1998) were able to show that Type 1 diabetic patients with HHcy had higher plasma levels of thrombomodulin (TM) and a higher prevalence of macroangiopathy among other late diabetic complications. TM, a receptor for thrombin, is regarded as an important anticoagulant and is predominantly expressed on vascular endothelial cells. The form of TM that is found in plasma probably accounts for damaged blood vessels (Hoffman *et al*, 1998). The effect of increased plasma Hcy on atherosclerosis has also been studied in insulin resistance. Rats that were made insulin resistant by feeding a high fat sucrose diet (HFS) were found to have higher plasma Hcy levels along with higher post-endarterectomy intimal hyperplasia causing luminal stenosis compared to rats that had normal insulin sensitivity (Brown *et al*, 2003). In contrast to these studies Mazza *et al* (1999, 2000) reported that in 130 Type 2 diabetic patients without nephropathy, there was no association between the basal level of Hcy or the MTHFR polymorphism and carotid intima-media thickness.

These studies suggest an independent association between plasma levels of Hcy and CVD. Interestingly, the association shown is much stronger in diabetic than non-diabetic subjects. The association between all cause mortality and HHcy was also shown to be greater in diabetic than in non-diabetic patients. HHcy therefore, may play a major

role in the development of cardiovascular disease in diabetic patients along with the other known risk factors.

1.13 HHcy and pathogenesis of atherosclerosis

A unifying hypothesis for the mechanism of Hcy mediated vascular injury has not yet been established. However, a few theories have been put forward in the literature. The most frequently described mechanism involves oxidative damage caused by Hcy. Hcy rapidly auto-oxidizes in plasma with its sulfhydryl (-SH) group undergoing oxidation to disulfide (RSSR). In this process Hcy gets converted to homocystine and mixed disulfides with the production of various ROS, including super oxide radicals, hydrogen peroxide and under certain circumstances hydroxyl radicals (Welch, 1998). It has been proposed that H_2O_2 mediated vessel injury exposes the underlying matrix and smooth muscle cells which in turn proliferate and promote the activity of platelets and leukocytes (Harker *et al*, 1974). Superoxide anion in particular is involved in lipid peroxidation at the level of the endothelial plasma membrane and within lipoprotein particles as well as in the generation of oxidized low density lipoproteins (LDL) (Loscalzo, 1996, Heinecke *et al*, 1987). However, cysteine, with a plasma concentration 25 to 30 times greater than Hcy, is not considered a risk factor for cardiovascular disease despite 94-95% of total plasma cysteine being present in the oxidized form (Jacobsen, 2001).

Another mechanism that has been proposed is the creation of a pro-thrombotic environment by Hcy. Hcy has been shown to enhance the activities of factor XII (Ratnoff, 1968) and factor V (Rodgers and Kane, 1986) and reduce the activation of protein C (Rodgers and Conn, 1990). Hcy also induces the expression of tissue factor (Fryer *et al*,

1993), and suppresses the expression of TM (Lentz and Sadler, 1991) and heparin sulfate (Nishinaga *et al*, 1993) by the endothelium, thereby altering the normal antithrombotic properties of the endothelium.

Hcy also has several effects on the endothelium-derived vasoactive small molecule, NO. Formation of S-nitroso-Hcy by the nitrosation of the SH group of Hcy by NO is regarded as a way the normal endothelial cell deals with the problems posed by accumulating Hcy (Stamler *et al*, 1993). In addition to inhibiting the generation of ROS, S-nitroso-Hcy is also regarded as a potent platelet inhibitor and vasodilator (Stamler *et al*, 1992). Long term exposure to high levels of Hcy resulting in endothelial damage will limit the production of NO, compromising the protective effect of NO. Hcy has also been shown to decrease the synthesis of NO (Welch *et al*, 1997, Loscalzo, 1996), decrease the expression of NO synthase and directly degrade NO (Chin *et al*, 1992, Liao *et al*, 1995, Blom *et al*, 1995). Evidence is also there to show that Hcy suppresses the expression of glutathione peroxidase, a key player in the cellular anti oxidative machinery (Upchurch *et al*, 1997). Hcy is also a potent mitogen for vascular smooth muscle cells as shown in experiments where infusion of Hcy into baboons leads to the formation of atheroma (Harker *et al*, 1983). *In-vitro* experiments have shown exposure to Hcy to cause increased vascular smooth muscle cell proliferation brought about partly by increased expression of cyclin D1 and cyclin A (Tsai *et al*, 1994).

Hcy thiolactone, a cyclical oxidative product of Hcy is also considered a potential mechanism involved in vascular pathology. Aggregates formed by the combination of Hcy thiolactone with LDL were shown to be taken up by intimal macrophages to form foam cells and be incorporated into atheromatous plaques (Naruszewicz *et al*, 1994). Hcy

thiolactone is also incorporated into cellular and secretory proteins through lysine acylation leading to the dysfunction of the proteins (Jakubowski, 1997).

Another hypothesis that may account for the mechanism of vascular damage by Hcy is known as the molecular target hypothesis. This mechanism suggests that Hcy itself may interact with specific molecular targets thereby modulating their activity (Jacobsen, 2000). Treatment of rat aortic smooth muscle cells with DL Hcy (0-500 μM) was shown to decrease glutathione peroxidase activity, increase superoxide dismutase in a dose-dependent manner while catalase activity was unchanged (Nishio ad Watanabe, 1997). Hcy was also able to inactivate purified bovine hepatic glutathione peroxidase in a dose-dependent manner (Nishio and Watanabe, 1997). Upchurch *et al* (1997) also found Hcy to decrease the expression of the cellular isoform of glutathione peroxidase in cultured bovine aortic endothelial cells. Hcy was found to decrease the steady state mRNA for glutathione peroxidase by 90%.

The members of the anti-oxidant machinery of the cell are not the only molecules that could be targeted by Hcy. Hcy is able to inhibit the conversion of plasminogen to plasmin by forming a mixed disulfide with cys 9 on annexin II, thereby inhibiting the binding of tissue plasminogen activator (Hajjar *et al*, 1998). Hcy also forms a mixed disulfide with prometalloprotease 2, which activates the protease through a cysteine switch mechanism (Bescond *et al*, 1999). Contrary to the favorable picture painted by formation of nitrosothiol by the reaction of Hcy with NO, this same mechanism is also described to reduce the bioavailability of NO by trapping it as nitrosothiol intracellularly (Jacobsen, 2000). Once entering the cell Hcy can also act to modify intracellular targets (Jacobsen, 2000).

1.14 Endothelial dysfunction

The endothelium, a single layer of cells lining the inner wall of the blood vessels lies in between the blood stream and the vessel wall and is considered the largest organ in the body. The endothelium is able to sense mechanical stimuli, such as pressure and shear stress and hormonal stimuli such as vasoactive substances and to release various agents involved in vasomotor function, inflammation and hemostasis (Endemann and Schiffrin, 2004). NO, prostacyclin, endothelium-derived hyperpolarizing factor (EDHF), bradykinin, adrenomedullin, C-natriuretic protein are vasodilators released by the endothelium while endothelin-1, angiotensin-II, thromboxane A2, prostaglandins and ROS are vasoconstrictors (McGuire *et al*, 2001). NO, intercellular adhesion molecule -1 (ICAM-1), vascular adhesion molecule-1 (VCAM-1), E-selectin, and NF-κB are inflammatory mediators released by the endothelium while modulators of hemostasis include plasminogen activator, tissue factor inhibitor, von Willebrand factor, NO, prostacyclin, thromboxane A2, plasminogen activator inhibitor-1, and fibrinogen (Endemann and Schiffrin, 2004). In addition to these functions the endothelium also plays a role in mitogenesis, angiogenesis, vascular permeability and fluid balance (Endemann and Schiffrin, 2004).

Since the discovery of the requirement of the endothelium to exert the vasodilatory effects of acetylcholine (Furchgott and Zawadzki, 1980) to the first description of endothelial dysfunction in a human in 1990 (Panza *et al*, 1990), our understanding of the role of the endothelium in vascular homeostasis has greatly increased. Endothelial dysfunction is generally described as impaired vasodilation to specific stimuli such as acetylcholine or bradykinin but also includes impairment of

endothelial dependent regulation of hemostasis and inflammation (Endemann and Schiffrin, 2004). Endothelial dysfunction is implicated in the pathophysiology of several forms of cardiovascular disease such as hypertension, coronary artery disease, chronic heart failure, peripheral arterial disease, diabetes and chronic renal failure (Endemann and Schiffrin, 2004). Some studies have shown endothelial dysfunction to precede development of cardiovascular disease; for example offspring of hypertensive patients, despite being normotensive, have displayed endothelial dysfunction (Taddei *et al*, 1996). Similarly, symptom-free children and young adults at high risk for atherosclerosis have also displayed endothelial dysfunction (Celermajer *et al*, 1992).

Saenz *et al*, 1989 reported endothelial dysfunction in human penile corpura cavernosa for both Type 1 and Type 2 diabetic patients. Endothelial dysfunction has also been described in the peripheral circulation of Type 1 (Johnstone *et al*, 1993) and Type 2 diabetic patients (Ting *et al*, 1996). Endothelial dysfunction has also been reported in non-diabetic but insulin- resistant obese individuals (Steinberg *et al*, 1996). Studies done using 3 different models of Type 2 diabetes and insulin resistance have shown hyperglycemia to be the major risk factor for endothelial dysfunction in diabetes. The study used db/db, ob/ob and TallyHo mice and endothelial dysfunction was only observed in the db/db and TallyHo mice, which displayed both hyperglycemia and dyslipidemia, and not in ob/ob mice that only exhibit dyslipidemia (Triggle *et al*, 2003). Oxidative stress, brought about by the production of various ROS in response to the hyperglycemia, is believed to be the cause of this endothelial dysfunction as ROS decrease the bioavailability of the nitric oxide synthase cofactor, tetrahydrobiopterin

(BH_4) leading to "uncoupled eNOS". Uncoupled eNOS produces superoxide instead of NO (Triggle *et al*, 2003).

1.15 Pathophysiology of endothelial dysfunction

Endothelial dysfunction can be brought about by multiple mechanisms. Reduction in the levels of NO due to reduced activity of eNOS (due to exogenous or endogenous inhibitors of eNOS or due to unavailability of its substrate, L-arginine) or reduced bioavailability of NO leads to endothelial dysfunction. Under oxidative stress, ROS react with NO to produce the cytotoxic oxidant peroxynitrite, which causes nitration of proteins leading to their dysfunction and therefore to endothelial dysfunction (Koppenol *et al*, 1992). Peroxynitrite is also responsible for oxidation of LDL (Griendling and Fitzgerald, 2003) as well as the degradation of the eNOS cofactor BH_4 leading to the "uncoupling" of eNOS (Milstein and Katusic, 1999). Oxidative stress also causes the reduction of BH_4 to BH_2 (Endemann and Schiffrin, 2004). "Uncoupling" of eNOS involves the reduction of its oxygenase activity, which is responsible for forming NO, and an increase in its reductase activity leading to the production of further ROS (Endemann and Schiffrin, 2004).

Along with the reduced levels of NO, the oxidative environment also leads to a pro-inflammatory state in the vessel wall with up-regulation of adhesion (VCAM-1 and ICAM-1) and chemotactic molecules (macrophage chemoattractant peptide-1 [MCP-1]) (Griendling and Fitzgerald, 2003). NAD(P)H oxidase is the enzyme responsible for most cellular ROS with xanthine oxidase, mitochondria and uncoupled eNOS also giving rise to some (Endemann and Schiffrin, 2004).

Reduction in the bioavailability of NO may also be caused by the endogenous competitive inhibitor of eNOS, asymmetric dimethylarginine (ADMA). ADMA is a product of protein turnover and is either excreted through the kidneys or degraded to citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Therefore increased levels of ADMA can be seen in both chronic renal failure as well as in hepatic disease (Nijveldt *et al*, 2003).

HHcy has also been implicated in the pathophysiology of endothelial dysfunction as shown by many studies involving both animals and humans (Lentz, 2000, Faraci and Lentz, 2004). It has also been shown that HHcy causes endothelial dysfunction to a degree similar to other risk factors such as hypercholesterolemia and hypertension (Lentz, 2000). One of the mechanisms by which HHcy has been shown to cause endothelial dysfunction is through the reduction in NO bioavailability by oxidative excess of several types of ROS like superoxide, hydrogen peroxide and peroxynitrite (Faraci and Lentz, 2004). In addition to the direct production of ROS by the auto-oxidation of Hcy, Hcy also mediates a specific decrease in the expression of the cellular isoform of glutathione peroxidase (Gpx-1), as was shown in vitro as well as in vivo (Upchurch *et al*, 1997, Outinen *et al*, 1999). To complement these studies, Weiss *et al*, (2001) was able to show that over-expression of Gpx-1 compensates for the adverse effects of Hcy on endothelial function.

Another potential mechanism by which Hcy may exert a deleterious effect is via the elevation of ADMA, as has been demonstrated in a non-human primate model of HHcy (Boger *et al*, 2000). Hcy may cause elevation of ADMA by the inhibition of its catabolism through DDAH (Stuhlinger *et al*, 2003). Endoplasmic reticulum stress

(Outinen *et al*, 1999, Outinen *et al*, 1998), stimulation of pro-inflammatory processes (Ungvari *et al*, 2003), and alterations in the methylation of regulatory proteins such as Ras (Wang *et al*, 1997) have also been suggested as mechanisms by which Hcy can activate endothelial apoptotic pathways leading to endothelial dysfunction.

HHcy therefore appears to cause endothelial dysfunction through a multiplicity of mechanisms. These mechanisms may explain the increased cardiovascular risk posed by elevations in plasma Hcy levels.

1.16 Pathophysiological effects of endothelial dysfunction

Endothelial dysfunction is regarded as an early event in the pathophysiology of the atherosclerotic process as reduced bioavailability of NO appears to lead to a number of proatherogenic processes (Celermajer *et al*, 1992, Suwaidi *et al*, 2000). Low NO bioavailability has been shown to upregulate VCAM-1 in the endothelial cell through the induction of NF-κB expression (Khan *et al*, 1996). This plays a role in the inflammatory process as VCAM-1 binds monocytes and T-lymphocytes, the first step in the invasion of the vessel wall by these cells (Libby, 2002). Reduction in NO also leads to the induction of MCP-1 expression leading to the recruitment of mononuclear phagocytes (Zeiher *et al*, 1995). Lectin-like oxidized LDL receptor-1 (LOX-1) which is highly expressed in blood vessels in hypertension, diabetes and dyslipidemia (Mehta and Li, 2002) scavenges oxidized LDL leading to a reduction in eNOS expression (Mehta *et al*, 2001) and further stimulation of adhesion molecule expression (Chen *et al*, 2001). Vascular smooth muscle cell growth and interstitial collagen synthesis gets stimulated by the growth factors secreted by macrophages found in the plaque (Libby, 2002). In addition to creating this

favorable environment for plaque formation, decreased NO may also be implicated in the rupture of the fibrous cap of the plaque with the resultant formation of a thrombus, as it was shown to activate matrix metalloproteinases MMP-2 and MMP-9 which weakens the fibrous cap (Uemura *et al*, 2001, Eberhardt, 2000). Platelet aggregation may also be enhanced in the face of reduced NO as NO is a known inhibitor of platelet aggregation (Radomski *et al*, 1987).

1.17 Perspective

It is clear that plasma Hcy plays a major role in the etiology of several chronic diseases one of the most important being atherosclerotic cardiovascular disease. Cardiovascular disease is also recognized as the leading cause of death in diabetic patients. Is there any additional role played by plasma Hcy in the development of CVD in diabetes? The many studies that have been published dealing with plasma Hcy levels in both Type 1 and Type 2 diabetes as well as insulin resistance have yielded a multiplicity of results. This may very well reflect the many mechanisms involved in the regulatory process of plasma Hcy. We strongly believe in the metabolic regulation of plasma Hcy and have comprehensively shown the hormonal regulation of two major enzymes involved in the removal of plasma Hcy; CBS and BHMT. Although not quite fully explained as yet there also appears to be a relationship between kidney function and plasma Hcy. The kidney has been shown to be a major organ in the removal of Hcy in rats but the same has not been able to be displayed in humans. However, most studies have been able to show a positive correlation between plasma Hcy and plasma creatinine levels indicating the importance of the kidney in the regulation of plasma Hcy.

Renal function in Type 2 diabetes appears to change with the progress of the disease; hyper-filtration in the early stages and progressive deterioration with the progression of diabetes. Diabetes therefore provides an interesting situation with changes in kidney functions being superimposed on the already existing changes in the metabolic milieu.

1.18 Problems of investigation and objectives

The main focus of this work was the examination of Hcy metabolism in Type 2 diabetes and insulin resistance before the impairment of kidney function in order to gain further understanding of the metabolic regulation of plasma Hcy. We were also interested in identifying the changes that occur in other amino acids under these conditions as the available data on this subject deals mostly with Type 1 diabetes. The specific questions that we sought to find answers through this work include:

1. The effects of Type 2 diabetes and insulin resistance on plasma Hcy levels and Hcy metabolism in the liver.
2. The effects of Type 2 diabetes and insulin resistance on amino acid metabolism
3. Effects of rosiglitazone treatment on Hcy metabolism in insulin resistance and Type 2 diabetes
4. Vascular function in insulin resistance and Type 2 diabetes and the effects of rosiglitazone treatment on vascular function.

Chapter 2

Materials and Methods

2.1 Animals

All procedures were approved by Memorial University's Institutional Animal Care Committee and were in accordance with the guidelines of the Canadian Council of Animal Care. Age-matched (5 and 11 weeks old) male ZDF (ZDF fa/fa) and (ZDF +/?) rats purchased from Charles River Laboratories (Indianapolis, Indiana, USA) were used as a model of Type 2 diabetes. Rats were given water and Purina 5008 chow *ad libitum* unless otherwise stated. Rats were housed in the Animal Care Facility using a 12 hour light: 12 hour dark cycle which began with lights on at 8:00 am. In vascular assays, data were obtained from ZDF fa/+ and ZDF fa/fa rats at 6, 12 and 18 weeks of age. In the study of the effects of Rosiglitazone on Hcy metabolism, data were obtained from ZDF fa/+ and ZDF fa/fa rats at 6 and 12 weeks of age.

2.2 Rosiglitazone Treatment Protocol

ZDF fa/fa and the ZDF fa/+ rats were assigned randomly by drawing lots to equal groups of untreated and rosiglitazone-treated (RSG) at 6 weeks of age. Untreated rats continued to receive their unaltered diet (described above). RSG-treated rats were given *ad libitum* access to water and Purina 5008 chow containing rosiglitazone maleate (received as a gift from GlaxoSmithKline, West Sussex, UK) which was estimated to give a dose of 10 μmol/kg body weight /day (Finegood *et al*, 2001). A set of untreated ZDF fa/+ and ZDF fa/fa rats were sacrificed at 6 weeks of age to obtain metabolism and vascular function data at baseline (time 0). Then equal

groups of untreated and RSG-treated rats were sacrificed after 6 and 12 weeks of treatment.

2.3 Metabolism Assays

Animals were anaesthetized with sodium pentobarbital (65 mg/kg i.p.) on the day of the experiment. After a midline abdominal incision, blood was drawn into a heparinized syringe from the abdominal aorta. Before withdrawal of the syringe from the abdominal aorta, a piece of the liver was rapidly removed and freeze-clamped in liquid nitrogen. The rest of the liver was then removed and placed in ice-cold 50 mM potassium phosphate buffer (pH 7.0). The freeze-clamped tissues were stored at -70^{0}C until further use. The blood was centrifuged in a clinical centrifuge at 3700 g for 15 minutes. Plasma thus separated was kept at -20^o C until further use. Fresh liver was diluted 1:5 (w/v) with 50 mM potassium phosphate buffer (pH 7.0) and homogenized using a polytron (Brinkmann Instruments, Toronto, Canada) for 20s at 50% output. The homogenate was centrifuged at 18000 g for 30 min at 4^{0}C and the supernatant was carefully separated. All enzyme assays were performed on this 18000g supernatant except for the activity of choline dehydrogenase which was measured using the 20% homogenate.

2.4 Analytical procedure:

2.4.1 Plasma Hcy:

Plasma tHcy (which consists of protein-bound Hcy and free, which is both in the reduced and the oxidized form) was determined according to the method described

by Vestor and Rasmussen (1991). 150µl of sample (plasma or hepatocytes) was first incubated at 4^0C for 30 min with 20µl 10% tri-n-butyl phosphine in dimethylformamide to reduce disulfide bonds and liberate protein-bound Hcy. After the reduction period, 125µl of 0.6M perchloric acid was added to deproteinize along with 50 µl of 0.2 mM 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) which is used as an internal standard. The tubes were left at room temperature for 10 min and centrifuged at 14000 g (Brinkman instruments, Rexdale, ON, Canada) for 5 min. 100µl of the resultant supernatant was collected and 200µl of 2 M Potassium Borate containing 5 mM EDTA (pH 10.5) and 100µl of 7-Fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid (1mg/ml solution) in 2 M Potassium Borate (pH 9.5) were added. After a thorough mixing, the Eppendorf tubes were covered in aluminum foil and incubated at 60^0C for 60 min. The tubes were then allowed to cool for 5 min in an ice bath and were centrifuged at 14000 g for 5 min. The samples were filtered through a 0.45µm syringe filter in preparation for analysis by HPLC.

A Shimadzu High Performance Liquid Chromatography system from Man-Tech Associates Inc., consisting of a SIL-10 A (Shimadzu auto-injector), LC-10AD (Liquid chromatograph Solvent Delivery Module), SCL-10A (system controller module), FCV-10 AL (solvent selector), GT-104 (degasser) and a Shimadzu RF 535 Fluorescence HPLC monitor fitted with a Hypersil ODS 5µm C18 column (150 mm x 4.6 mm) (Supelco Inc), was used to measure Hcy.

0.1 M Sodium Acetate buffer (pH 4.0) containing 2% v/v or 20% v/v methanol was used as buffer A or B respectively. The buffers were filtered through a 0.45 µm Millipore filter before use. A 20 min linear gradient running from 100%

buffer A to 100% buffer B over 10 min, back to 100% buffer A over 5 min and a final 5 min equilibration period at 100% buffer A was used. 20µl of the sample was injected on to the column and the fluorescence signal was detected with excitation at 385 nm and emission at 515 nm and integrated using a Man-Tech Shimadzu CR 501 HPLC data processor. The standard curve for Hcy is shown in Fig. 2.1.

2.4.2 Plasma Glucose, creatinine and insulin:

Plasma glucose was determined enzymatically as described by Bergmeyer *et al.* (1974). Plasma creatinine (Chapter 3) was measured using a kit (catalogue No. 555A) from Sigma Diagnostics (St. Louis, MO), which uses the Jaffe reaction with some modifications. Plasma creatinine (Chapter 5) was measured by Dr. Ed Randell using SYNCHRON creatinine (CREm) reagent in conjunction with SYNCHRON LX AQUA CAL 1 and 2 as calibration standards on a SYNCHRON LX system. This method too is based on the Jaffe reaction. Plasma insulin was measured with a rat insulin ELISA kit (catalogue No. INSKR020) from Crystal Chem Inc., Downers Grove, IL, USA using anti rat /mouse insulin antibody, and standard rat insulin.

2.5 Enzyme analysis

Standard conditions of protein and time are given for each enzyme assay in Table 2.1. In all cases, protein and time were chosen to fall in the linear portion of the protein and time curves for each assay (see Figures 2.2 to 2.9). Protein concentrations were determined by the Biuret method using BSA as a standard.

Figure 2.1: Standard Curve for Hcy.

A typical graph is shown.

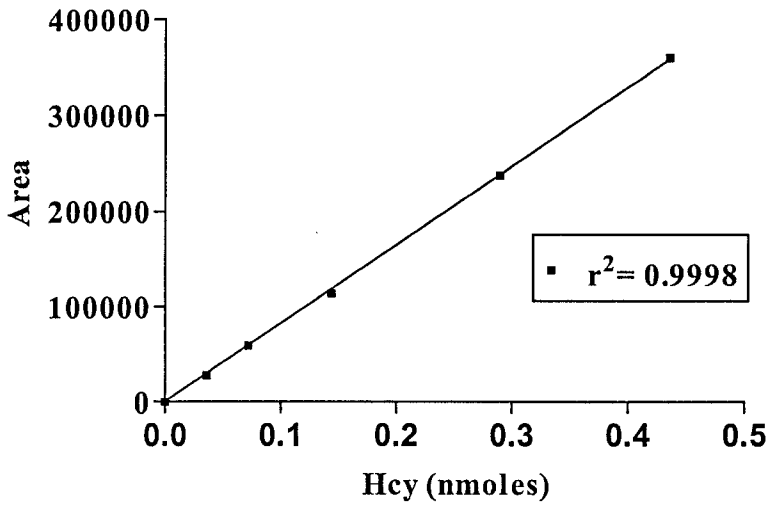

400000
300000
200000
100000
0
Area
0.0
0.1
0.2
0.3
0.4
0.5
Hcy (nmoles)
r2= 0.9998

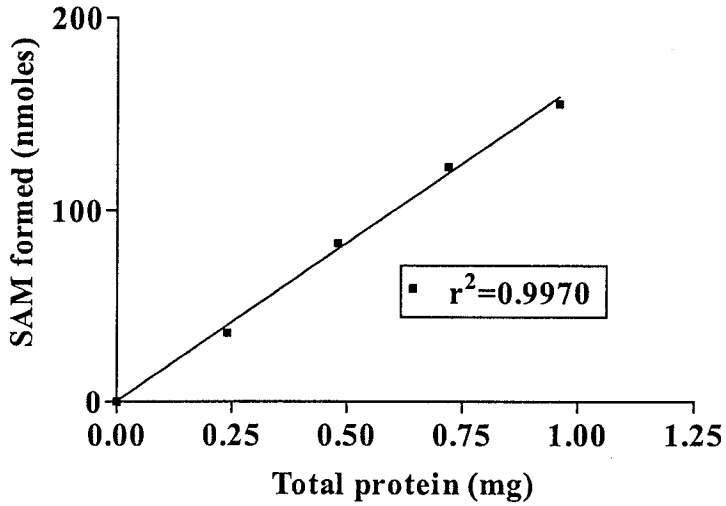
Table 2.1 Experimental conditions used in assaying enzyme activities

	Protein (mg)	Time (min)
Methionine adenosyltransferase	0.5	30
Glycine-N-methyltransferase	0.075	30
Cystathionine β-synthase	2	60
Cystathionine γ-lyase	1	10
Methionine synthase	1	30
Methylenetetrahydrofolate reductase	0.1	20
Betaine:homocysteine methyltransferase	0.3	60
Choline dehydrogenase	0.15	7.5

Linearity with time and protein was established for each assay and the protein and time to be used in the experiments were chosen from the linear portion of the curve.

Figure 2.2: Dependence of hepatic methionine adenosyltransferase activity on protein and time.

A 30 min incubation period was used to obtain the protein curve while 0.5 mg of protein was used in each incubation to determine the linearity with respect to time. A typical graph is shown for each condition.


200
100
0
SAM formed (nmoles)
r^2=0.9970
0.00
0.25
0.50
0.75
1.00
1.25
Total protein (mg)

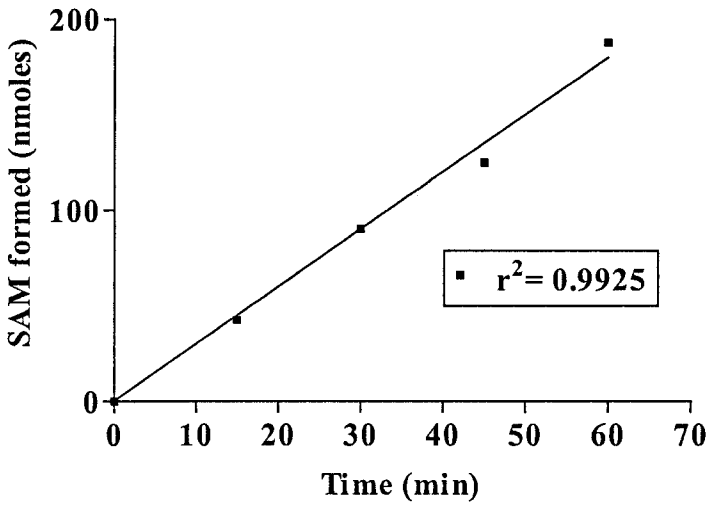
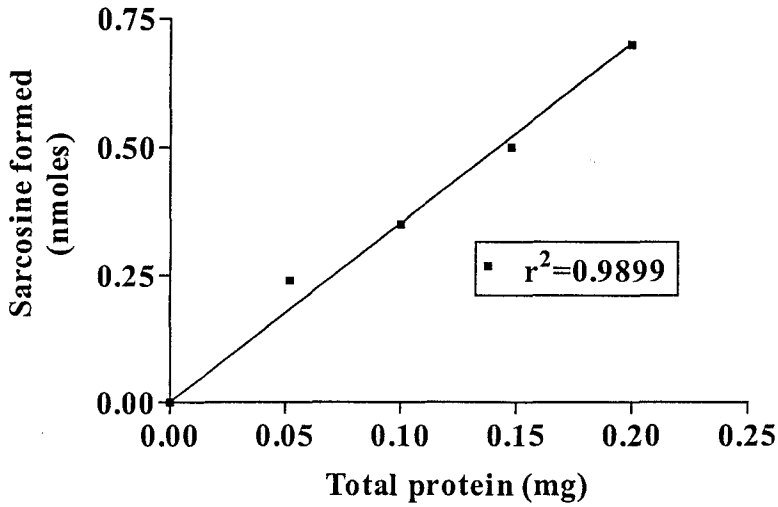

200
100
0
SAM formed (nmoles)
r^2= 0.9925
0
10
20
30
40
50
60
70
Time (min)

Figure 2.3: Dependence of hepatic glycine *N*-methyltransferase activity on protein and time.

A 30 min incubation period was used to obtain the protein curve while 0.075 mg of protein was used in each incubation to determine the linearity with respect to time. A typical graph is shown for each condition.


0.75
0.50
0.25
0.00
Sarcosine formed (nmoles)
0.00
0.05
0.10
0.15
0.20
0.25
Total protein (mg)
r²=0.9899

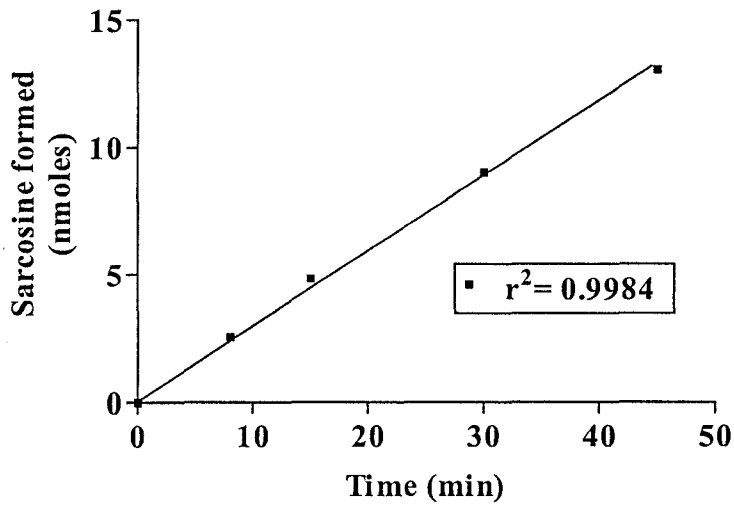
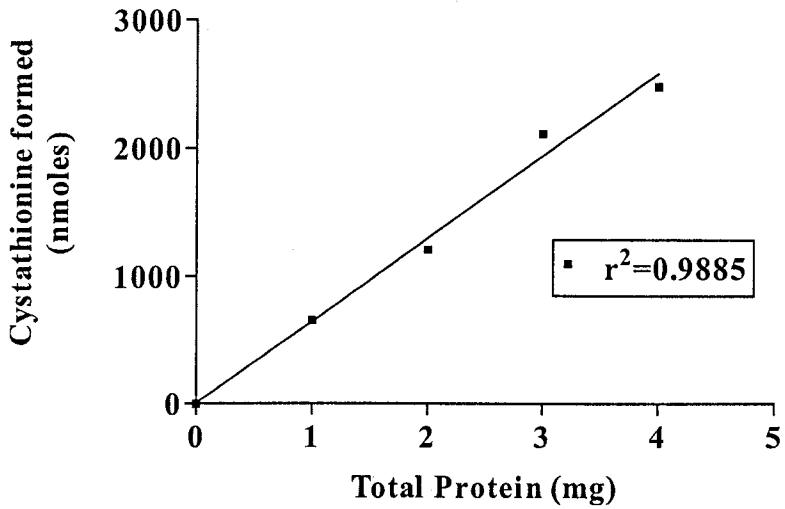

15
10
5
0
Sarcosine formed (nmoles)
0
10
20
30
40
50
Time (min)
r²= 0.9984

Figure 2.4: Dependence of cystathionine production by hepatic cystathionine β-synthase on time and protein.

A 60 min incubation period was used to obtain the protein curve while 2 mg of protein was used in each incubation to determine the linearity with respect to time. A typical graph is shown for each condition.


Cystathionine formed (nmoles)
3000
2000
1000
0
0
1
2
3
4
5
Total Protein (mg)
r²=0.9885

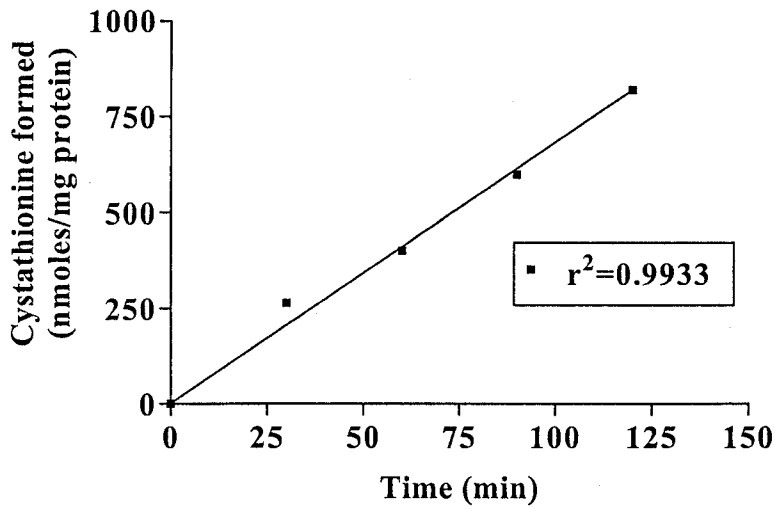

Cystathionine formed (nmoles/mg protein)
1000
750
500
250
0
0
25
50
75
100
125
150
Time (min)
r²=0.9933

Figure 2.5: Dependence of hepatic cystathionine γ-lyase activity on protein.

A typical graph is shown.

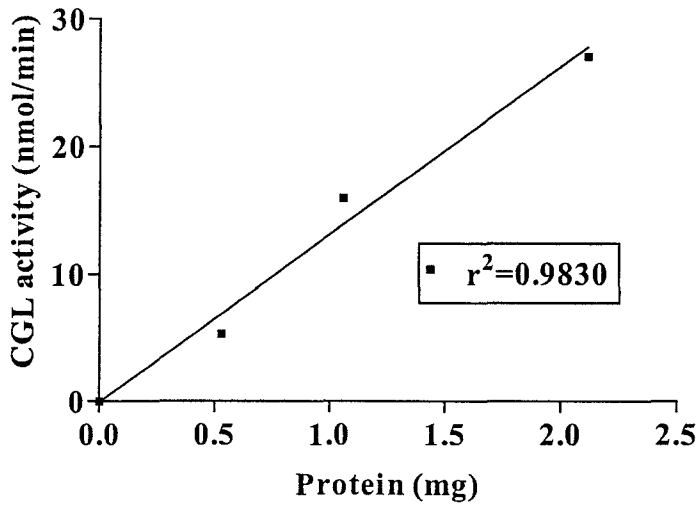
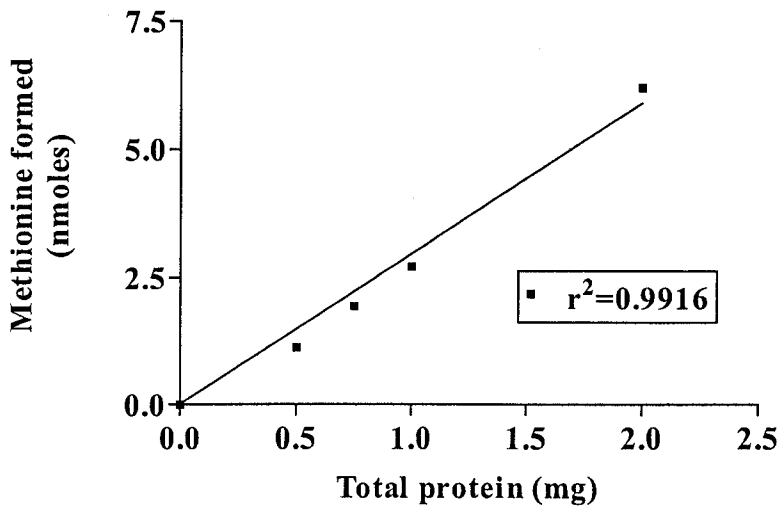

CGL activity (nmol/min)
30
20
10
0
0.0
0.5
1.0
1.5
2.0
2.5
Protein (mg)
r²=0.9830

Figure 2.6: Dependence of hepatic methionine synthase activity on time and protein.

A 30 min incubation period was used to obtain the protein curve while 1 mg of protein was used in each incubation to determine the linearity with respect to time. A typical graph is shown for each condition.


Methionine formed (nmoles)
7.5
5.0
2.5
0.0
0.0
0.5
1.0
1.5
2.0
2.5
Total protein (mg)
r2=0.9916

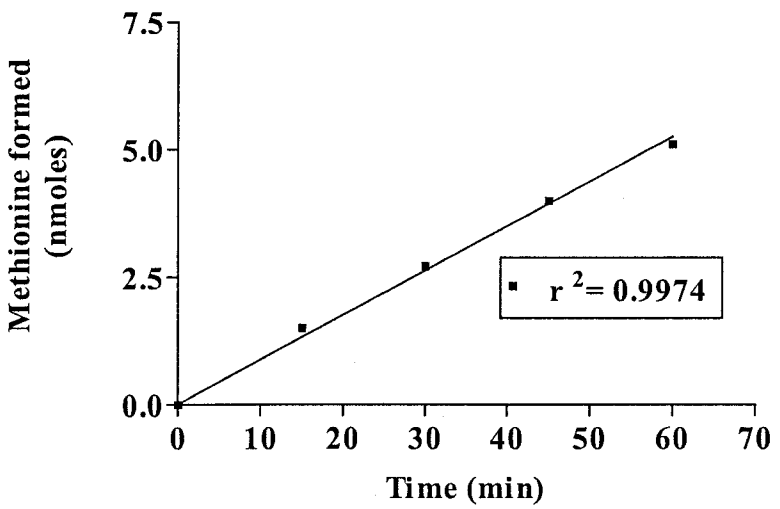
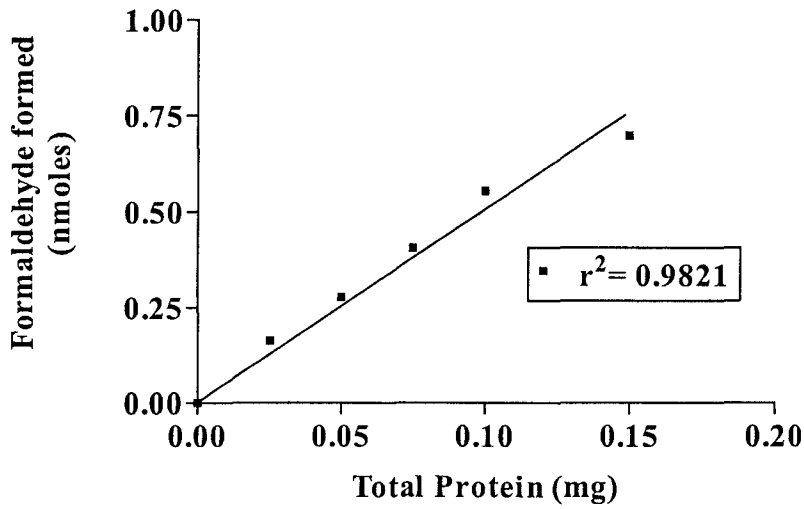

Methionine formed (nmoles)
7.5
5.0
2.5
0.0
0
10
20
30
40
50
60
70
Time (min)
r 2= 0.9974

Figure 2.7: Dependence of liver Methylenetetrahydrofolate reductase activity on time and protein.

A 20 min incubation period was used to obtain the protein curve while 0.1 mg of protein was used in each incubation to determine the linearity with respect to time. A typical graph is shown for each condition.


Formaldehyde formed (nmoles)
1.00
0.75
0.50
0.25
0.00
0.00
0.05
0.10
0.15
0.20
Total Protein (mg)
r²= 0.9821

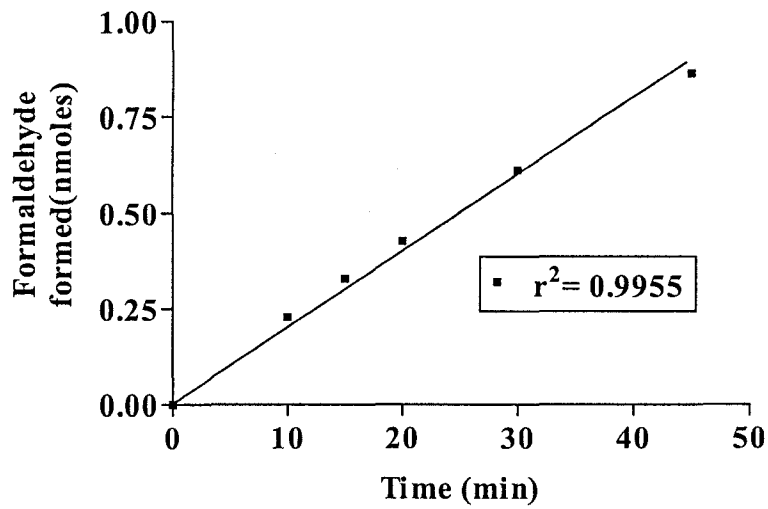
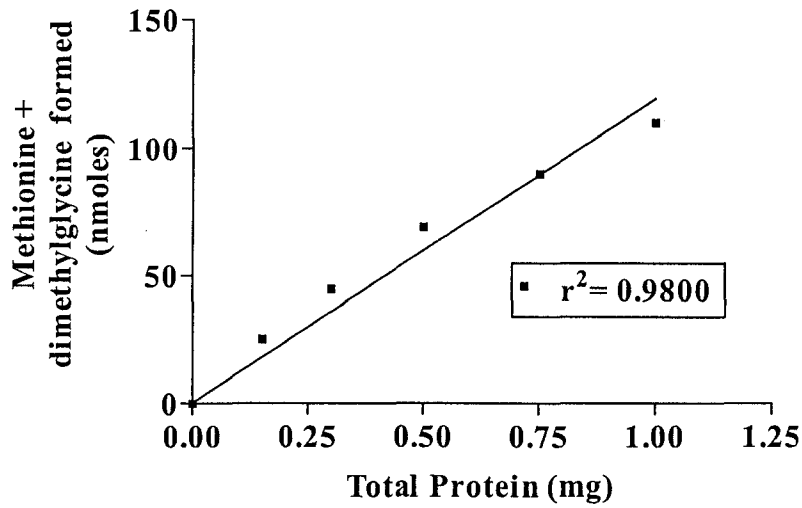

Formaldehyde formed(nmoles)
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
Time (min)
r²= 0.9955

Figure 2.8: Dependence of hepatic betaine:homocysteine methyltransferase on protein and time.

A 60 min incubation period was used to obtain the protein curve while 0.3 mg of protein was used in each incubation to determine the linearity with respect to time. A typical graph is shown for each condition.


Methionine + dimethylglycine formed (nmoles)
150
100
50
0
0.00
0.25
0.50
0.75
1.00
1.25
Total Protein (mg)
r2= 0.9800

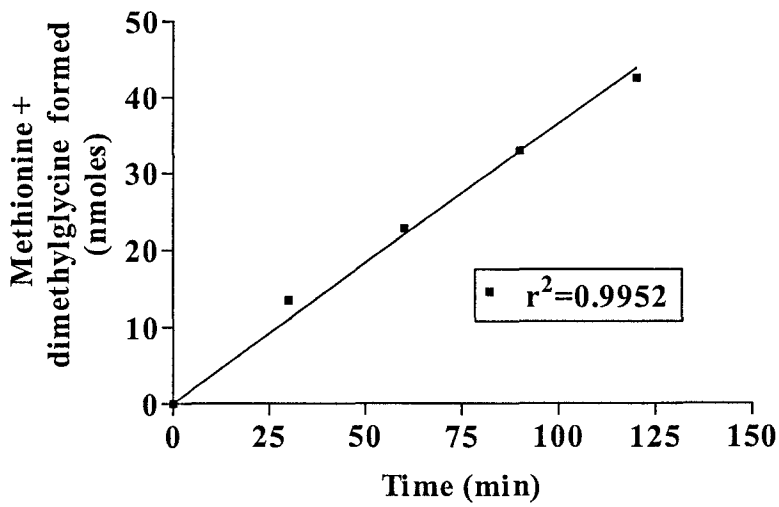
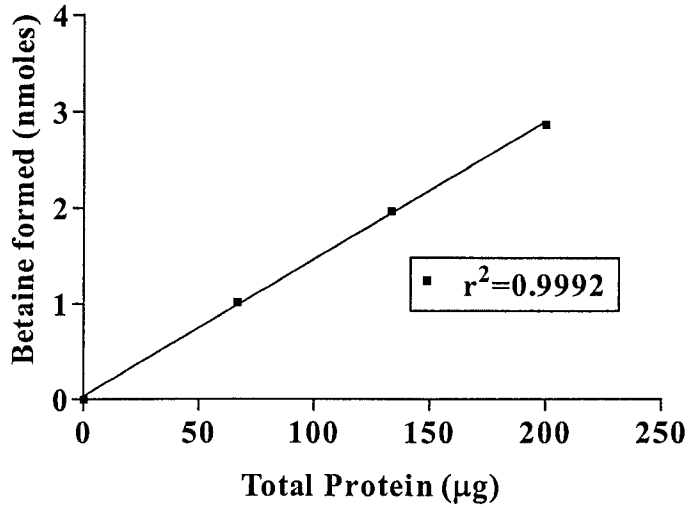
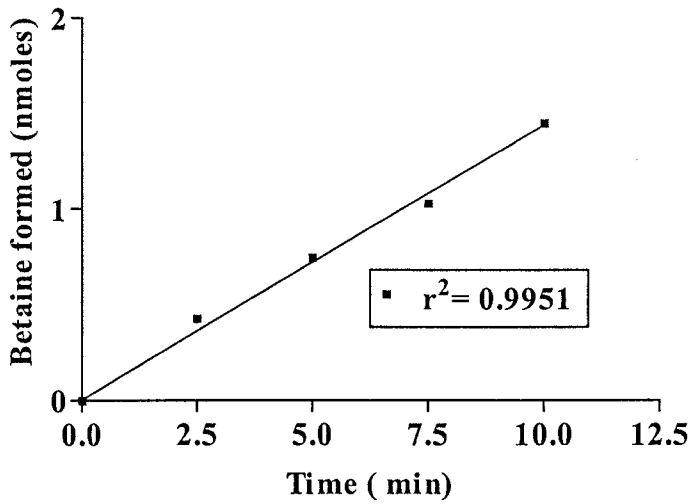

Methionine + dimethylglycine formed (nmoles)
50
40
30
20
10
0
0
25
50
75
100
125
150
Time (min)
r2=0.9952

Figure 2.9: Dependence of hepatic choline dehydrogenase on protein and time.

A 7.5 min incubation period was used to obtain the protein curve while 0.15 mg of protein was used in each incubation to determine the linearity with respect to time. A typical graph is shown for each condition.


Betaine formed (nmoles)
4
3
2
1
0
0
50
100
150
200
250
Total Protein (μg)
r²=0.9992


Betaine formed (nmoles)
2
1
0
0.0
2.5
5.0
7.5
10.0
12.5
Time (min)
r²= 0.9951

2.5.1 Methionine adenosyltransferase:

Methionine adenosyltransferase activity was measured using a method based on Mudd *et al* (1965) and Duce *et al* (1988). The assay included 100 mM Tris HCl (pH 7.8), 200 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, 5 mM 1-^{14} CH_3-Methionine (0.04 μCi) and 0.5 mg of liver protein in a final volume of 250 μl. Incubations were started with the addition of 1-^{14}C Methionine and were stopped with the addition of 750 μl of ice-cold H_2O after an incubation period of 30 min at 37^{0}C. 700 μl of the stopped incubate was added to a column containing Biorad 50W-X4 200-400 mesh H^+-form resin with the counter-ion changed to NH_4^+. The columns were washed with 5 x 4 ml of H_2O and labeled SAM was eluted with 2 x 3 ml of 3 M NH_4OH. 10 ml of Scintiverse (Fisher Scientific, ON, Canada) was added to each vial and the radioactivity was measured with a LKB-1214 Rackbeta liquid scintillation counter.

2.5.2 Glycine *N*-methyltransferase:

The activity of Glycine *N*-methyltransferase was measured according to a method described by Rowling *et al.* (2002). 100 mM Tris HCL (pH 9.0), 2 mM Glycine, 5 mM DTT, 0.2 mM Methyl -^{3}H -SAM in 50 mM potassium phosphate buffer (pH 7.0) and 0.15 mg of protein were incubated for 15 min at 25^{0}C in a final volume of 200 μl. The incubations were stopped by the addition of 100 μl of ice-cold 10% TCA. 500 μl of acid washed charcoal suspension (38 mg/ml in 0.1 M Acetic Acid) was added to the tubes and they were allowed to stand on ice for 15 min. The

tubes were then centrifuged at 3200 g for 10 min and 500 µl of the supernatant was added to 10 ml Scintiverse to determine radioactivity as described before.

2.5.3 Cystathionine β synthase

Cystathionine β synthase activity was measured using an assay described by Mudd *et al* (1965) and Taoka *et al.* (1998). The assay included 125 mM Tris HCL (pH 8.3), 2.1 mM EDTA, 0.42 mM pyridoxal-5'-phosphate, 0.146 mM L-cystathionine, 41.7 mM D,L-Hcy, 0.316 mM SAM, 2.1 mM propargylglycine and 25 mM 1-^{14}C Serine (0.1 µCi) in a final volume of 0.6 ml. 2 mg of protein were used in each assay. Incubations were carried out for 1 hr at 37^{0}C and were stopped with the addition of 300 µl of 15% TCA. The tubes were centrifuged at 1000 g for 10 min and 0.5 ml of the de-proteinized sample was added to a moist column containing 2 ml of BioRad 50 W X 4 200-400 mesh H^+ form resin. The columns were washed with 2 X 4 ml of H_2O and then 6 x 4 ml 1M HCL and finally with 4 x 4ml H_2O. Radiolabelled cystathionine was eluted with 5 ml of 3 M NH_4OH. 1 ml of the eluent was used to measure radioactivity after mixing with 10 ml of Scintiverse as described before.

2.5.4 Cystathionine γ-lyase

This assay was conducted as described by Stipanuk (1979) with the spectrophotometric measurement of the disappearance of NADH at 340 nm at 37^0 C for 10 min. The assay cocktail included 100 mM Potassium Phosphate buffer (pH

7.5), 0.125 mM pyridoxal-5-phosphate, 0.32 mM NADH, 4 mM L-Cystathionine, 1.5 units of Lactate dehydrogenase and 1 mg liver protein in a final volume of 2 ml. A blank was run for each sample with the above cocktail with no cystathionine.

2.5.5 Methionine synthase

The reaction mixture consisted of 64 mM potassium phosphate buffer (pH 7.5), 29 mM dithiothreiotol (DTT), 7 mM mercaptoethanol, 10 μM cyanocobalamine, 7.5 mM D, L-homocysteine, 0.25 mM SAM and 0.5 mM [5-^{14}C] methyl tetrahydrofolate (0.25 μCi) in 1.064 mM sodium ascorbate (pH 7.0). This reaction mixture was incubated at 37^0 C in the dark for 30 min with 1 mg of protein in a final volume of 200 μl. The reaction was stopped with 400 μl of ice-cold H_2O and the tubes were immediately transferred to an ice bath. After centrifugation, ^{14}C methionine was separated from ^{14}C methyltetrahydrofolate by adding the supernatant to an ion exchange column (BioRad AG 1-X8, 200-400 mesh, Cl^- form, 0.8 x 4 cm). The columns were washed with 3 x 500μl of H_2O, the effluent was collected and the radioactivity was counted after adding 10 ml of Scintiverse as described before (Koblin *et al.* 1981).

2.5.6 Methylenetetrahydrofolate reductase

Methylenetetrahydrofolate reductase activity was measured as described by Engbersen *et al.* (1995). The reaction mixture for this assay consisted of 0.024 mM 1-^{14} C methyltetrahydrofolate (0.3 μCi), 11.6 mM ascorbic acid, 0.18 M potassium phosphate buffer (pH 6.8), 54 μM FAD, 0.54 mM EDTA, 3.5 mM menadione and 0.1

mg of cytosolic protein. The reaction was started with the addition of menadione sodium bisulfate. The tubes were incubated at 37^0 C for 20 min in the dark and was stopped by the addition of 100 µl of 3 M potassium acetate (pH 4.5), 10 µl of 1 M formaldehyde and 200 µl of 250 mM dimedone in ethanol: H_2O 1:1 sequentially. They were then heated at 95 ^{0}C for 15 min and were cooled on ice for 10 min before adding 3 ml of toluene. After mixing well, the tubes were centrifuged at low speed for 5 min. 2 ml of the toluene phase was removed and mixed with 10 ml of Scintiverse to measure radioactivity as described before.

2.5.7 Betaine:homocysteine methyltransferase

The assay was conducted as described by Garrow (1996). The assay contained 5 mM D, L,Hcy, 2 mM 1-^{14}C betaine (0.05-0.1 µCi), 50 mM Tris HCL (pH 7.5) and 0.3 mg of cytosolic protein in a final volume of 0.5ml. The tubes were incubated at 37^0 C for 1 hr and were stopped with the addition of 2.5 ml of ice-cold H_2O. After centrifugation, 2 ml of the stopped reaction mixture was added to Dowex 1-X4 (Cl^-) 200-400 mesh resin in which the counter-ion had been changed to OH^-. The columns were washed with 3 X 5 ml of H_2O and radiolabelled methionine was eluted with 3 ml of 1.5 M HCL. The radioactivity in the eluant was measured as described before.

2.5.8 Choline dehydrogenase

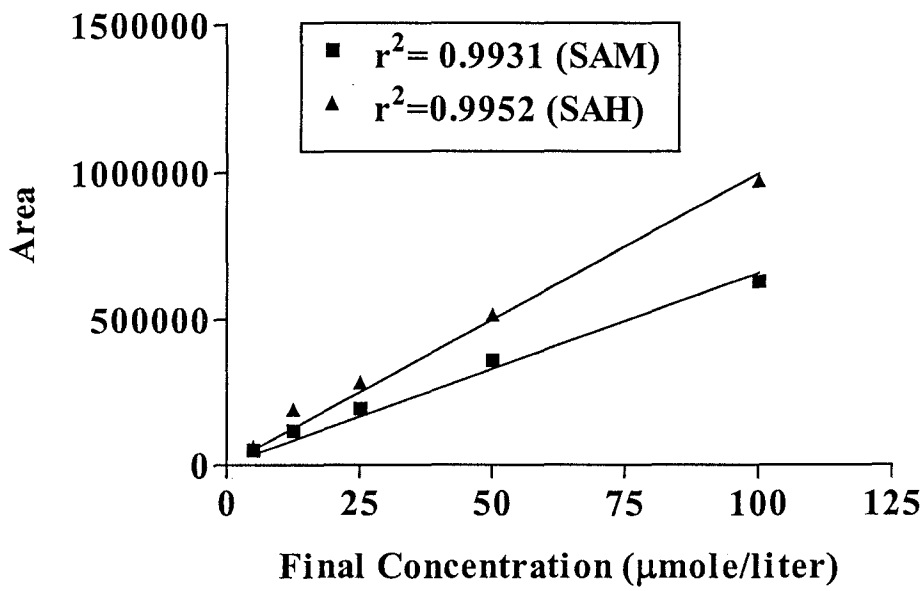
Choline dehydrogenase activity was measured using a method modified from Grossman and Hebert (1989) and Haubrich and Gerber (1981). Each incubation

included 3.5 mM tris HCL (pH 7.6), 0.35 mM EDTA, 0.7 mM β mercaptoethanol, 1.08 mM phenazine methosulfate (PMS)/0.35 mM $CaCl_2$, 0.5 mM 1-^{14} C choline chloride (0.2 μCi) and 15 μl of liver homogenate containing 0.15 mg of protein. The tubes were incubated in the dark at 37^0 C for 7.5 min and were stopped with the addition of 20 μl 1M NaOH and were transferred to an ice bath. After mixing well, 15 μl of 30% H_2O_2 was added to the tubes and they were incubated at Room temperature for 1 hr. 415 μl of cold ddH_2O was added to each tube and the contents were loaded on to columns containing Dowex 50W-X4 (100-200 mesh) H^+-form resin with the counter-ion changed to Li^+ by washing with LiOH. The columns were washed with 6 X 833μl of H_2O and the collected effluent was mixed with 10 ml of Scintiverse to be measured for ^{14}C betaine in the scintillation counter.

2.6 S-adenosylmethionine and S-adenosylhomocysteine

For the determination of SAM and SAH, freeze clamped liver was quickly homogenized in 5 volumes of 8% ice-cold trichloroacetic acid. The samples were then centrifuged at 12,500 g for 5 min. After filtration, the supernatant was analyzed by HPLC using a Vydac C18 column (model 2187P54) with 50 mM NaH_2PO_4 containing 10 mM heptanesulfonic acid (pH 3.18-3.2) and 100% acetonitrile as buffers A and B respectively. A 15 min gradient from 96% A, 4% B to 80% A and 20% B was used. UV absorbance at 258 nm was measured and the peaks were quantified using a Waters 743 data module integrator. Standard curves for SAM and SAH are given in Fig. 2.10.

Figure 2.10: Standard curves for SAM and SAH. ■ SAM, ▲ SAH.


1500000
1000000
500000
0
Area
r2= 0.9931 (SAM)
r2=0.9952 (SAH)
0
25
50
75
100
125
Final Concentration (μmole/liter)

2.7 Reverse Transcriptase-PCR:

Total RNA was isolated from fresh liver samples as previously described (Chomczynski and Sacchi, 1987). 2μg of RNA was reverse transcribed using a one step reverse transcription kit (Qiagen, Mississauga, Canada) and was amplified by 15 cycles. An upstream primer (5'- GCCTTCAGGACATCCAGTGT-3') and a downstream primer (5'- TCTTTCCGGGTCTGCTCACGGGC-3') corresponding to the type 3 rat CBS cDNA (Kraus *et al*, 1998) were used to amplify a 1644-bp PCR fragment. For BHMT, an upstream primer (5' ACCGATTGCCGGCAAGAA-3') and a down stream primer (5'- CTGTGCGGATTTGAATTTTTG-3') corresponding to the rat BHMT cDNA was used to amplify a 1216 bp PCR-fragment. 768 bp fragment of the rat β actin was co-amplified using amplimer set primers (CLONTECH laboratories, Palo Alto, CA). Once amplified the PCR products were separated on 0.8% (w/v) agarose. The bands were stained with ethidium bromide and were visualized under UV illumination. In the case of CBS mRNA, the gel was immersed in 0.25 M HCL, denatured in denaturing buffer, rinsed with dH_2O, soaked in neutralization buffer, incubated in 10x SSC, and transferred to an S and S Nytran SuPer charge nylon membrane. After transfer, the DNA was immobilized by UV cross-linking in a Hoefer UVC 500 UV cross-linker (Amersham Biosciences). The blots were simultaneously probed with ^{32}P- labeled CBS cDNA and Radiolabeled β- actin cDNA probes (CLONTECH Laboratories, Palo Alto, Ca). Finally autoradiography was carried out using Kodak XAR or Biomax film. Autoradiographs were scanned on ChemiImager^{TM} 4000, and RNA

levels were quantitated with Alpha Ease software (Alpha Innotech Corporation, San Leandro, CA).

2.8 Isolation of Hepatocytes

Hepatocytes were isolated according to the method of Berry *et al* (1991). 0.1 ml of heparin (1000 units/ml) was injected into the femoral vein of an anaesthetized rat. After a mid-line incision, ties were placed around the inferior vena cava above the renal artery and several around the portal vein. A cannula was inserted into the portal vein and tied in place. Flow-through perfusion with 500 ml of "Ca^{+2}-free" Krebs-Henseleit medium (144 mM Na^{+}, 6 mM K^{+}, 1.2 mM Mg^{+2}, 126 mM Cl^{-}, 1.2 mM $H_2PO_4^{-}$, 1.2 mM SO_4^{2-}, 25 mM HCO_3^{-}) containing 2 mM EGTA, 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate (pH 7.4) was started immediately at a flow rate of 40 ml/min. The Krebs-Henseleit medium was gassed with 95/5% O_2/CO_2 for at least 20 min before use. To start flow through the liver the inferior vena cava was severed below the kidney. The flow through of Ca^{+2} free medium was followed by about 500 ml of "Ca^{+2}-plus" medium (1.3 mM Ca^{+2}) containing 20 mM glucose, 2.1 mM lactate, and 0.3 mM pyruvate. A second cannula was inserted through the right atrium and into the inferior vena cava after opening the chest just above the heart and was tied off above the renal artery. With the completion of the second flow-through, recirculation of Krebs–Henseleit Ca^{+2} medium containing 0.25 % (w/v) BSA and 30 mg/100 ml collagenase (Boehringer # 103586) was begun and the perfusion was continued until the liver became soft. The liver was removed into a Petri dish containing collagenase medium and gently teased with fingers to release

cells. The released cells were incubated at 37^0C in a shaking water bath for 10 min while gassing with 95/5% O_2/CO_2, filtered through a strainer and centrifuged at 600 rpm for 2 min. The supernatant was poured off and the cells were resuspended in fresh Krebs-Henseleit Ca^{+2} medium, centrifuged again and the process repeated. Finally the cells were washed with Krebs-Henseleit Ca^{+2} medium containing 2.5% BSA. The supernatant was poured off and the cells were resuspended in Krebs-Henseleit Ca^{+2} 2.5% BSA medium (diluted 1:18). 3 ml of the resulting cell suspension and 3 ml of the 2.5% BSA Krebs-Henseleit medium were poured into separate tared petri dishes and were dried at 50^0 C for 24 hrs to measure dry weight. Cell viability was measured by 0.2% Trypan Blue exclusion with at least 95% of the cells being viable in all cases.

2.9 Transsulfuration Flux

Hepatocytes were isolated from ZDF fa/fa and ZDF +/? rats as described above. The transsulfuration pathway provides an outlet for methionine to leave the methionine metabolic cycle and get converted to cysteine and consists of the cystathionine β-synthase reaction, which converts Hcy to cystathionine and α-ketobutyrate, and cystathionine γ-lyase, which converts cystathionine to cysteine. 1-^{14}C methionine gives rise to 1-^{14}C α-ketobutyrate, which is metabolized via pyruvate dehydrogenase to produce $^{14}CO_2$. 1-^{14}C α-ketobutyrate that does not get metabolized through this pathway will be decarboxylated to give $^{14}CO_2$ in the presence of hydrogen peroxide. The total transsulfuration flux was determined as the sum of the ^{14}C present in α-ketobutyrate and in ^{14} CO_2, as described by Stead *et al.* (2000). 1 ml

of hepatocytes and an appropriate volume of Krebs-Henseleit medium were pre-incubated for 20 min with no betaine or 0.1, 0.3 or 1 mM betaine. The zero time flasks had 0.6 ml of 30% $HCLO_4$ added to them at the beginning of the assay. 20 µl of 100 mM or 10 mM 1-^{14}C methionine were added to the flasks (final volume 2 ml) and incubated for 30 min. At the end of 30 min the flasks were covered with a plastic septum, equipped with a centre-well containing 0.6 ml of NCS-II tissue solubilizer (Amersham International, Oakville, ON, Canada). 0.6 ml of 30% $HCLO_4$ (w/v) was added via a syringe through the center-well to stop the incubation, after which $^{14}CO_2$ was collected for 1 hr. At the end of the hour, centre-wells were added to scintillation vials containing 10 ml of scintillation fluid (Toluene Omnifluor) and radioactivity was measured. A second rubber septum, equipped with a centre-well was fitted to the flasks and 0.3 ml of 30% H_2O_2 (w/v) was added through the septum by means of a syringe. $^{14}CO_2$ was collected again for an hour and the radioactivity in the second centre well measured.

2.10 Hcy export from isolated hepatocytes

In a separate series of experiments, hepatocytes were incubated with 0.1 mM methionine ± 0.3 mM betaine to measure the rate of Hcy export from isolated hepatocytes. After a 20 min pre-incubation of hepatocytes with betaine and Krebs-Henseleit medium, methionine was added and the flasks were incubated for 30 min at 37^0C. At the end of 30 min the contents of the flasks were quickly transferred to Eppendorf tubes and were centrifuged at 14000 g (Brinkman Instruments, Rexdale, ON, Canada) for 2 min to sediment the cells. An aliquot of the supernatant was

collected and stored at -20 ^{0} C for the analysis of exported Hcy. Hcy was analyzed as described for plasma.

2.11 Amino acid analysis:

0.3 ml of plasma was deproteinized using 0.1 ml of ice-cold 10% (w/v) sulfosalicylic acid and the precipitated proteins were removed by centrifugation. The pH of the supernatant was adjusted to 2.2 with lithium citrate buffer. For the analysis of liver and muscle amino acids, a piece of the previously frozen tissue was ground to a fine powder in liquid nitrogen. 4 ml of 6% (w/v) $HClO_4$ was added to 1 g of frozen powder and immediately homogenized with a motorized homogenizer. The samples were centrifuged at 18900 g for 20 minutes. To the resulting supernatant 50% (w/v) K_2CO_3, to a final concentration of 2.25% and 0.02 ml of universal indicator were added and the pH was brought to neutral with the drop-wise addition of 20% KOH. Aliquots of these neutralized samples were frozen at -20^{0} C and the pH was adjusted to 2.2 with concentrated HCl on the day of amino acid analysis. These samples along with the plasma samples were analyzed on a Beckman model 121-MB amino acid analyzer, using Benson D-X8, 0.25 cation Xchange resin and a single column 3 buffer lithium method as described in Beckman 121MB-TB-017 application notes. The quantification of the results was done using a Hewlett Packard Computing Integrator Model 3395 A.

2.12 Calculation of intracellular concentrations of amino acids:

The intracellular concentrations of amino acids (ICAA) in the liver were calculated using the following equation:

Hepatic content of AA (nmoles/g) = {[plasma AA] nmoles/ml x ECF (ml/g liver)}
+
{[ICAA] nmoles/ml x ICF (ml/g liver)}

The extracellular fluid volume (ECF) in the liver was taken as 0.25 ml/g while the intra- cellular fluid volume (ICF) was taken to be 0.45 ml/g liver, in accordance with previous studies from our laboratory (Qian and Brosnan 1998). The same equation was used to calculate the ICAA concentrations in skeletal muscle. The ECF volume was taken as 0.09 ml/g while the ICF volume was taken as 0.68 ml/g of skeletal muscle (Hundal *et al.* 1992).

2.13 Isolated blood vessel assays

2.13.1 Conditions for isometric tension measurements

Rats were sacrificed by cervical dislocation. In preliminary studies, both aorta and mesenteric arteries were isolated and used in vascular function assays. Arteries were obtained from control-treated and RSG-treated rats on the same day (2 rats/day). Thoracic aorta and the intestines containing the mesenteric arterial arcade each were isolated quickly and then placed in ice-cold Krebs solution (pH 7.4) consisting of 114 mM NaCl, 4.7 mM KCl, 0.8 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM D-glucose and 25 mM $NaHCO_3$ which was bubbled with 95% O_2

/5% CO_2 gas. With the aid of a light dissection microscope, surrounding fatty and loose connective tissues were cleaned from the aorta and second order mesenteric arteries. Two rings each ~ 2mm in length were cut from the lengths of the cleaned blood vessels. Mesenteric arterial rings were suspended between a micropositioner and a force transducer using a pair of tungsten wires (40 μm diameter) whereas aortic rings were suspended between two metal hooks (200 μm diameter) in a Mulvany- Halpern–style (Mulvaney, 1988) organ bath chamber (10 ml volume; 610 Multi- Myograph system; J.P. trading, Copenhagen, Denmark) containing 95% O_2- 5% CO_2 gas-bubbled Krebs solution at 37^0 C. Aortic rings set at a resting tension of 20 mN and mesenteric arteries set at 1.5 mN were equilibrated for 1 h prior to obtaining data from the vascular function protocol. Drugs and compounds were added directly to the myograph chambers and changes to isometric tension were recorded on-line via a serial connection from the 610 myograph to a computer hard drive at a rate of 1 Hz. Software for data acquisition and analysis (Myodaq 2.01/Myodata 2.02) were designed by J.P. Trading for use with the 610 Multi-Myograph system.

2.13.2 Vascular function protocol

To assess viability and obtain an internal standard of contractile activity (isometric tension in mN) for individual blood vessels, arteries were first contracted with high concentration of KCl (120 mM). Following a washout period, cumulative concentration-contractile response relationships were obtained using the α-adrenoreceptor agonist cirazoline (10 nM- 10 μM). Such relationships were used to

determine the concentrations of cirazoline to be used to produce contractions at submaximum values (~ 50-70% of E_{max}). To assess vascular smooth muscle relaxation activity, cumulative concentration-relaxation response relationships for acetylcholine (ACh; 10 nM – 10 μM), sodium nitroprusside (SNP; 10 nM-10 μM), bradykinin (10 nM-3μM), 2-furoyl-LIGRLO-amide (2fly; 10 nM-3μM), and arachidonic acid (AA; 10 nM-30μM) were obtained in each artery contracted submaximally with cirazoline (30-100 nM) in series. After each treatment in this series, the artery was washed with fresh Krebs solution until the recorded tensions returned to initial resting tension values followed by an additional 5 min in fresh Krebs solution before starting the next treatment.

2.13.3 Relaxation inhibitor studies

In 6 week old rats, the ACh-induced relaxation responses of contracted arteries were also tested after treatment for 15 min with NO synthases inhibitor L-NAME (300 μM). In 12 and 18 week old rats, this L-NAME treatment was combined with various inhibitors including indomethacin (cylcooxygenases), apamin (small conductance Ca^{2+}-activated K^+ channels; SK_{Ca}) and TRAM-34 (intermediate conductance Ca^{2+}-activated K^+ channel; IK_{Ca}). Following these treatments, the cumulative concentration-relaxation response to ACh was determined.

2.13.4 Data analyses

Values are represented by the mean ± standard error of the mean (error bars) unless otherwise indicated. N = number of rats (2 arteries per rat). Maximal

effect (E_{max}) of cirazoline is expressed as % of the contraction by 120 mM KCl. E_{max} for each vasodilator is expressed as % relaxation; 100% relaxation = complete reversal of cirazoline-induced contracted tone. For each compound, EC_{50} values were determined from the concentration-response relationship in individual arteries by intrapolation from the graph data. Potency is expressed by the negative logarithm of the EC_{50} values for each compound. The data were analyzed separately within each age category. Student's unpaired t test was used to compare artery response of rat strains at 6 weeks of age. 2-way ANOVA were used to determine the effects of strain, treatment and interactions at 12 and 18 weeks of age and were followed by 1-way ANOVA with Newman-Keuls post hoc test for multiple comparisons of groups within each age category. A P of < 0.05 was taken to be a significant difference.

2.14 Phospholipid metabolism

These experiments were conducted using freeze clamped hepatic tissue by Dr. René Jacobs (University of Alberta).

2.14.1 Cytosol and membrane preparation

Livers were homogenized in 2 ml of buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 1mM DTT and 0.1 mM phenylmethylsulfonyl fluoride) with a mini-polytron. Homogenization was followed by sonication for 30 sec. Samples were centrifuged at 99 K for 15 min and the supernatant (cytosol) was removed and the pellet (membranes) were resuspended in homogenization buffer.

2.14.2 CTP:phosphocholine cytidyltransferase

CT activity was measured according to a method described previously (Choy *et al*, 1977). Separated fractions (homogenate, cytosol and microsome) were incubated with 1 mM [^{3}H] phosphocholine (20 μCi/μmol), 1 mM CTP, 12 mM magnesium acetate, and 20 mM tris/succinate at 37 ^{0} C for 15 min at a final volume of 0.1 ml (pH 6.4). The reactions were stopped by immersing the tubes in boiling H_2O for 2 min. 20 μl of CDP-choline (50mg/ml) was added to the tubes as a carrier. CDP-choline produced was separated by thin layer chromatography with a solvent system of CH_3OH:0.6% NaCl:NH_4OH (50:50:5, v/v/v) for 30 min. The plates were sprayed with 0.1% 2,7-dichlorofluroesien and the CDP-choline band was visualized under ultraviolet light and scraped into vials. The radioactivity was determined using a liquid scintillation counter.

2.14.3 Choline kinase

Choline kinase activity was measured with minor modifications to a previously described method (Ishidate and Nakazawa, 1992). Cytosols were incubated in a final volume of 100 μl reaction buffer containing 0.1 M Tris-HCl (pH 8.75), 2 μM ATP, 15 mM $MgCl_2$, and 0.25 mM [^{3}H] choline chloride (10.5 μCi/ml) at 37^{0}C for 20 min. The phosphocholine produced was separated using a column filled with Bio-Rad AG1-X8 (200-400 mesh, OH- form) resin.

2.14.4 Phosphatidylethanolamine *N*-methyltransferase

PEMT activity was measure using the method described by Ridgway and Vance (1992). Homogenates were incubated in a final volume of 100 µl reaction mixture containing 125 mM Tris-HCl (pH 9.2), 5 mM DTT, 1 mM Triton X-100, and 0.4 mM PDME. The assay was initiated by the addition of [methyl-^{3}H] SAM (21mCi/mmol) to a final concentration of 200 µM, and the tubes were transferred to a 37°C shaking water bath and incubated for 30 min. The assay was terminated by the addition of 2 ml of choloroform-methanol (2:1 v/v). The samples were vortexed, centrifuged (2000 rpm, 10 min) and the upper (aqueous) phase discarded. The lower phase was washed 3x 2 ml with 0.5%NaCl:methanol:$CHCl_3$ (50:50:4). 200 µl of lower phase was aliquoted into scintillation vials, dried down, and the radioactivity was counted.

2.14.5 Phosphatidylethanolamine and phosphatidylcholine mass in liver and plasma

0.01 mg of PE was added to 0.5 mg of liver homogenate or 100 µl of plasma as an internal standard. Samples were extracted as described by Folch *et al* (1957). Phospholipids were separated and quantified using an HPLC system equipped with a 4.8 x 100 mm silica column coupled to an evaporative light scattering detector. A mobile phase consisting of isopropyl alcohol/*n*-hexane/water run as a ternary gradient (58:40:8, v/v/v) in 10 min was followed by a stationary phase (52:40:8, v/v/v) for 35 min (Bergo *et al*, 2002)

2.15 Triacylglycerol

Plasma and liver TG were measured by Adam Byrne.

2.15.1 Extraction of lipid from liver

50 mg of liver was homogenized in 2 ml of $CHCl_3$/methanol 2:1 using a Teflon homogenizer at 4^0C. The homogenate was transferred to a glass tube and was mixed with 400 µl of 50 mM NaCl and was kept at 4^0C overnight to separate. The lower lipid-containing layer was transferred to a glass vial and was evaporated under nitrogen.

2.15.2 Plasma and hepatic Triacylglycerol

Lipids were extracted from the liver as stated above. 100 µl of isopropanol was added to the extract and TG was quantified using a triglyceride assay kit # 2150L101 from Stanbio laboratories, TX, USA. Plasma TG was measured using the same kit.

2.16 Glomerular filtration rate

Glomerular filtration rate (GFR) was measured by measuring the excretion and plasma level of creatinine. Therefore,

$$GFR = \frac{\text{Urinary concentration of creatinine (µmole/liter) x Urine flow (ml/min)}}{\text{Arterial plasma concentration of creatinine (µmoles/liter)}}$$

Chapter 3

Homocysteine metabolism in ZDF (Type 2) diabetic rats

3.1 Background

Patients with coronary, cerebrovascular or peripheral arterial disease have mean plasma tHcy levels significantly higher than control subjects (Kang *et al*, 1992). Over the last decade, these findings led to the identification of HHcy as an independent risk factor for vascular disease (Kang *et al*, 1992). In addition, HHcy has also been identified as a risk factor for Alzheimer's disease (Seshadri *et al*, 2002) and osteoporotic fractures (van Meurs *et al*, 2004).

Hcy is formed after the donation of a methyl group from S-adenosylmethionine. It can then be metabolized by one of three enzymes. Methionine synthase and betaine:homocysteine methyltransferase (BHMT) catalyze the remethylation of Hcy to methionine, and cystathionine β-synthase (CBS) forms part of the transsulfuration pathway where Hcy condenses with serine to form cystathionine. Figure 1.2 shows the pathway of methionine metabolism.

Diabetes mellitus, whether Type 1 or Type 2, is associated with an increased risk of cardiovascular mortality, with the prevalence of atherosclerosis being 2-to 6-fold higher in diabetic patients than in people without diabetes (Brand *et al*, 1989). Insulin resistance, which precedes the development of Type 2 diabetes, is also associated with an increased risk of coronary artery disease (Rosolova *et al*, 2002). HHcy has been shown to be a stronger risk factor for cardiovascular disease and for mortality in patients with Type 2 diabetes mellitus than in subjects without diabetes (Hoogeveen *et al*, 1998).

Plasma tHcy in diabetic patients is known to be dependent on the presence or absence of nephropathy. Both Type 1 and Type 2 diabetic patients with nephropathy have elevated levels of tHcy (Hoogeveen *et al*, 1998, Hultberg *et al*, 1991). However,

Type 1 diabetic patients with no renal complications have plasma tHcy levels lower than controls (Robillon *et al*, 1994). This decrease was also shown in an animal model of Type 1 diabetes (Jacobs *et al*, 1998).

We, therefore, examined the effect of insulin resistance and Type 2 diabetes on plasma tHcy and its metabolism in the liver. We used the leptin-receptor defective Zucker Diabetic Fatty rat (ZDF), an excellent model for Type 2 diabetes (Peterson *et al*, 1990). Before developing frank diabetes, they go through a phase of insulin resistance, thereby giving us an opportunity to study the effects of both insulin resistance and of Type 2 diabetes.

3.2 Statistical Analysis:

Data are presented as means ± SD. Means were compared using Student's unpaired t test, or one way analysis of variance as appropriate. $P < 0.05$ was taken to indicate a significant difference. Significant differences across the age groups are only reported for ZDF +/? rats as any age-related change in ZDF fa/fa rats will be confounded by the diabetes.

3.3 Results

Table 3.1 shows the body weight, hepato-somatic index, plasma insulin, creatinine and glucose concentrations. At 5 weeks, the concentrations of plasma glucose (12.1 ± 1.7 mmole/liter vs. 8.4 ± 1.3 mmole/liter) and insulin (8.8 ± 2.9 ng/ml vs. 0.83 ± 0.4 ng/ml) in the ZDF fa/fa and ZDF +/? rats, respectively, showed that the ZDF fa/fa rats had become insulin resistant. At 11 weeks, the concentrations of plasma glucose (30.3 ± 1.6 mmole/liter vs. 10.9 ± 0.8 mmole/liter) and insulin (3.5 ± 0.8 ng/ml vs. 3.9 ± 0.6 ng/ml)

in the ZDF fa/fa and the ZDF +/? rats, respectively, showed that the ZDF fa/fa rats had become diabetic. Creatinine levels of ZDF fa/fa and control rats at 5 weeks were similar (32.4 ± 3.8 μmole/liter vs. 28.9 ± 3.1 μmole/liter), but at 11 weeks were lower in the ZDF fa/fa rats (18.9 ± 2.7 μmole/liter vs. 29.7 ± 6.1 μmole/liter)

3.3.1 Plasma homocysteine and hepatic enzymes of homocysteine metabolism

At 5 weeks, plasma tHcy was about 25% lower in the ZDF fa/fa rats, while at 11 weeks it was reduced by about 60% (Figure 3.1). The plasma tHcy level in the ZDF +/? rats showed an increase with age. The reduced plasma tHcy at 5 weeks in the ZDF fa/fa rats, was accompanied by changes in several enzymes that metabolize methionine (Table 3.2). Of the enzymes responsible for producing Hcy, MAT and GNMT showed slight, but significant, increases. Of the enzymes responsible for removing Hcy, CBS, CGL and BHMT were increased respectively by about 54%, 18% and 36%. Methionine synthase showed a slight but significant decrease in the ZDF fa/fa rats. At 11 weeks, MAT, GNMT, CBS, CGL and BHMT all showed significant increases in activity.

In addition to the differences in enzyme activity between the control and the experimental animals at each age group, MAT, CBS and GNMT all showed significant increases in their activity with age in the ZDF +/? rats.

3.3.2 mRNA levels

At 5 weeks the increased activity of CBS in the ZDF fa/fa rats was accompanied by a comparable increase in CBS mRNA level (Fig 3.2 A). The increased BHMT activity

Table 3.1. Body weight, plasma insulin, glucose and creatinine levels and the hepato-somatic index of ZDF +/? and ZDF fa/fa rats at 5 and 11 weeks.

	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
Body Weight (g)	134 ± 5	178 ± 7*	306 ± 11	348 ± 10*
Insulin (ng/ml)	0.83 ± 0.4	8.8 ± 2.9*	3.9 ± 0.6	3.5 ± 0.8
Creatinine (μM)	28.9 ± 3.1	32.4 ± 3.8	29.7 ± 6.1	18.9 ± 2.7*
Glucose (mM)	8.4 ± 1.3	12.1 ± 1.7*	11.0 ± 0.8	30.3 ± 1.6*
Hepato-Somatic Index[a]	4 ± 0.1	4.5 ± 0.2*	3.8 ± 0.2	5. 1 ± 0.2*

Data are given as means ± SD and were analyzed using Student's unpaired t test. * indicates $P<0.05$ when compared with lean controls (ZDF +/?) in each age group. n=6 for each group. [a] Liver (g) x 100/ body weight (g).

Figure 3.1. Plasma Homocysteine

Values are expressed as μmole/liter. Differences within each age group were compared using Student's unpaired t test. One way ANOVA followed by Newman-Keuls post-hoc test was used to compare the differences between the ages. Means ± SD for 6 rats are shown. At each age group differing letters indicate significant difference from each other. ¶ indicates significant difference in ZDF +/? rats at 11 weeks compared to 5 weeks. *($P < 0.05$)*

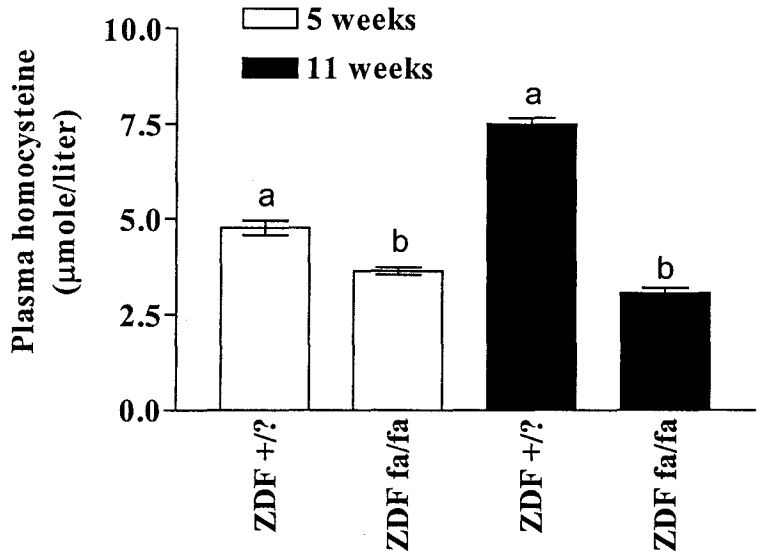

5 weeks
11 weeks
Plasma homocysteine (µmole/liter)
10.0
7.5
5.0
2.5
0.0
a
b
a
b
ZDF +/?
ZDF fa/fa
ZDF +/?
ZDF fa/fa

Table 3.2. Hepatic activities of enzymes of homocysteine metabolism

	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
Methionine adenosyltransferase	124 ± 4	131 ± 6*	323 ± 20¶	380 ± 11*
Glycine-*N*-methyltransferase	130 ± 14	160 ± 4*	168 ± 20¶	220 ± 20*
Cystathionine β-synthase	1000 ± 90	1540 ± 220*	1670 ± 270¶	2200 ± 370*
Cystathionine γ-lyase	4120 ± 500	4870 ± 520*	4380 ± 210	7550 ± 890*
Methylenetetrahydrofolate reductase	23 ± 2	24 ± 2	22 ± 4	22 ± 4
Methionine synthase	8 ± 1	6 ± 0.4*	8 ± 1	8 ± 1
Betaine:homocysteine methyltransferase	100 ± 8	157 ± 14*	92 ± 17	150 ± 18*
Choline dehydrogenase	666 ± 131	700 ±145	527 ± 97	608 ± 108

Data are given as means ± SD for six rats. Differences within each age group were compared using Student's unpaired t test. One way ANOVA followed by Newman-Keuls post-hoc test was used to compare the differences between the ages. In each group enzyme activities are expressed as *nmoles of product synthesized per minute per gram liver.* * signify significant differences within each age group. ¶ indicates significant difference in enzyme activity in ZDF +/? rats at 11 weeks compared to 5 weeks. *($P< 0.05$)*

observed in the ZDF fa/fa rats at 5 and 11 weeks, were also accompanied by comparable increases in mRNA levels (Fig 3.2 B).

3.3.3 S-adenosylmethionine, S-adenosylhomocysteine and SAM/SAH ratio

The hepatic levels of SAM and SAH and the SAM/SAH ratio are shown in Table 3.3. At both 5 and 11 weeks SAM was significantly increased in the ZDF fa/fa rats. The SAM/SAH ratio was significantly higher in the ZDF fa/fa rats at 5 weeks. Both SAM and the SAM/SAH ratio showed increases with age in ZDF +/? rats, consistent with the age-related increase in MAT activity.

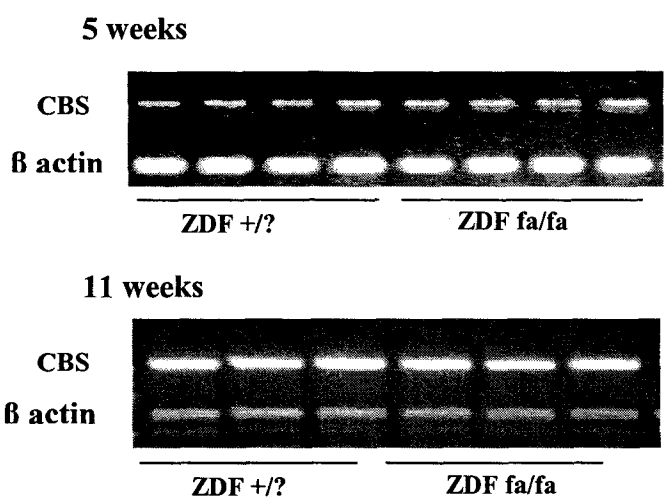
3.3.4 Transsulfuration Flux

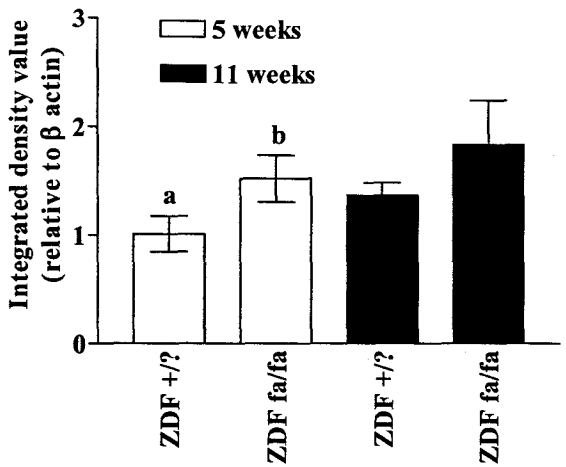
In a preliminary study, we investigated the effect of betaine on the transsulfuration flux, as measured by the conversion of L-[1-^{14}C] methionine to $^{14}CO_2$ and ^{14}C-α-ketobutyrate, by isolated hepatocytes from Sprague Dawley rats. All concentrations of betaine used (0.1, 0.3 or 1 mM) significantly reduced the catabolism of methionine, compared to incubations of hepatocytes with methionine alone (Figure 3.3). We then conducted studies with hepatocytes from ZDF rats, using 0.1mM L-[1-^{14}C] methionine and 0.3 mM betaine. Incubation with 0.3 mM betaine significantly decreased the transsulfuration flux in hepatocytes of ZDF fa/fa rats at both ages, but the reduction seen in the hepatocytes of ZDF +/? rats was significant only at 11 weeks of age (Table 3.4).

Figure 3.2. mRNA abundance

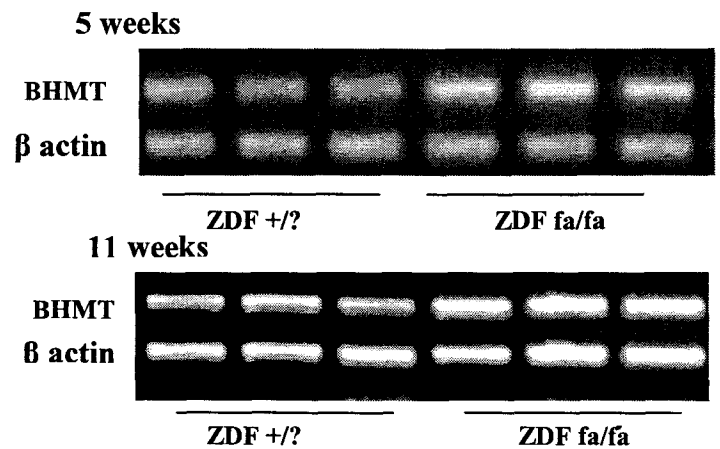
RT-PCR analysis of hepatic CBS mRNA (A) and BHMT mRNA (B). The relative amounts of each mRNA, measured by spot densitometry, are graphically represented. Both CBS and BHMT mRNA were normalized to β-actin mRNA. Values are Means ± SD for six rats. Differing letters at each age group indicate significant difference from each other. $P<0.05$

A. CBS


5 weeks
CBS
ß actin
ZDF +/?
ZDF fa/fa
11 weeks
CBS
ß actin
ZDF +/?
ZDF fa/fa


Integrated density value
(relative to β actin)
5 weeks
11 weeks
a
b
ZDF +/?
ZDF fa/fa
ZDF +/?
ZDF fa/fa

B. BHMT


5 weeks
BHMT
β actin
ZDF +/?
ZDF fa/fa
11 weeks
BHMT
ß actin
ZDF +/?
ZDF fa/fa

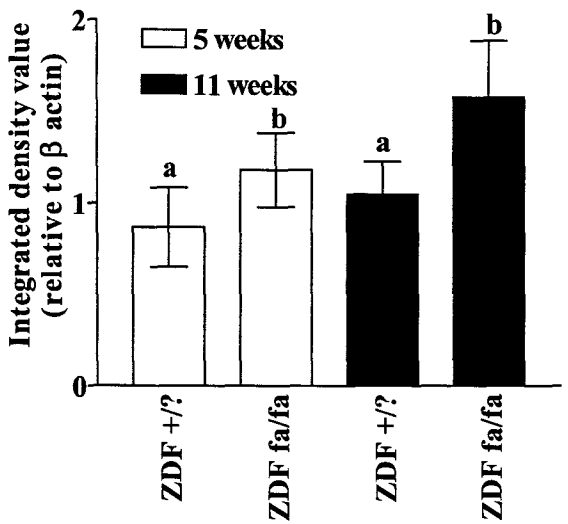

Integrated density value
(relative to β actin)
5 weeks
11 weeks
a
b
b
a
ZDF +/?
ZDF fa/fa
ZDF +/?
ZDF fa/fa

Table 3.3 Hepatic SAM, SAH and SAM/SAH ratio.

	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
SAM	88 ± 8.4	142.1 ± 11.3*	172.9 ± 0.0¶	199.9 ± 15.5*
SAH	12.3 ± 1.2	11.6 ± 1.3	11.9 ± 2.3	11.7 ± 2.2
SAM:SAH	7.2 ± 0.7	12.3 ± 2.0*	15.1 ± 4.7¶	17.5 ± 3.5

Values for SAM and SAH are expressed as nmoles/g liver. Means ± SD for six rats in each group are shown. Differences within each age group were compared using Student's unpaired t test. One way ANOVA followed by Newman-Keuls post-hoc test was used to compare the differences between the ages. At each age group, * indicates significant difference from the ZDF +/? rats. ¶ indicates significant difference in ZDF +/? rats at 11 weeks compared to 5 weeks. *($P < 0.05$)*

Figure 3.3. Effect of betaine on transsulfuration flux from isolated hepatocytes.

Hepatocytes were incubated with 0.1 mM L [1-^{14} C] methionine alone or with 0.1, 0.3 and 1 mM betaine. * indicates a significant difference from the result with no betaine. Means ± SD for 3 rats are shown (*P<0.05).*

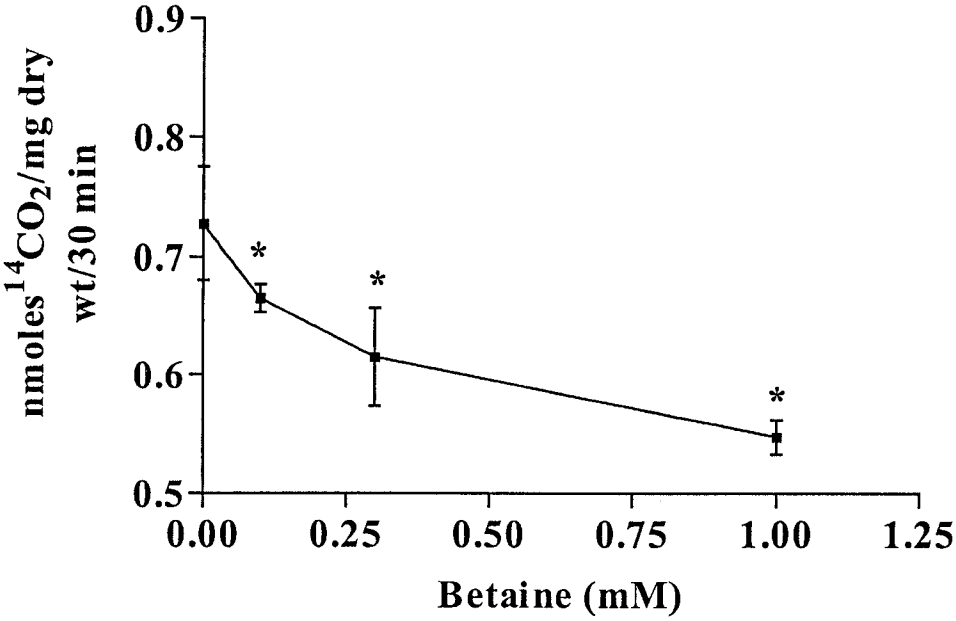

0.9
0.8
0.7
0.6
0.5
nmoles $^{14}CO_2$/mg dry wt/30 min
*
*
*
0.00
0.25
0.50
0.75
1.00
1.25
Betaine (mM)

Table 3.4

Transsulfuration flux in isolated hepatocytes.

	Transsulfuration Flux (nmoles/mg of dry weight/30 min)			
	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
L- [1-^{14}C] Methionine	1.00 ± 0.25	1.08 ± 0.05	1.18 ± 0.21	1.08 ± 0.15
L- [1-^{14}C] Methionine + Betaine	0.91 ± 0.32	0.87 ± 0.12*	0.96 ± 0.15*	0.86 ± 0.13*

Hepatocytes were incubated with 0.1 mM L- [1-^{14}C] methionine ± 0.3 mM betaine to determine the transsulfuration flux. Data are means ± SD. n = 3 for 5 weeks and n = 5 for 11 weeks. * indicates a significant difference from the corresponding incubations without betaine *($P<0.05$)*.

3.3.5 Hcy Export

Hepatocytes from ZDF fa/fa and ZDF +/? rats, at both ages, showed a significant reduction in Hcy export when incubated with 0.3 mM betaine (Table 3.5). However, at both ages the decrease observed in the ZDF fa/fa rats was about 2-fold greater than that of the ZDF +/? rats.

3.3.6 Choline and related metabolites

Table 3.6 shows the liver concentrations of choline and related metabolites. The liver betaine concentration was lower in the ZDF fa/fa rats at both 5 and 11 weeks while there was no difference in the choline concentration. One of the most remarkable findings was the high level of betaine found in the liver of the young animals and the reduction that occurs with age. Hepatic glycerophosphorylcholine (GPC) was significantly higher in the ZDF fa/fa rats at 5 weeks though at 11 weeks it was significantly lower. Phosphorylcholine (PCho) was significantly higher in the ZDF fa/fa rats at both ages. Both GPC and PCho levels increased with age in the ZDF +/? rats. Phosphatidylcholine was significantly decreased in ZDF fa/fa rats at 11 weeks while sphingomyelin was significantly reduced at both 5 and 11 weeks.

Table 3.7 shows choline and related metabolite concentrations in the plasma. The only significant difference in plasma metabolites between the different rats was an increased phosphatidylcholine level in the ZDF fa/fa rats at 11 weeks. However, the plasma betaine concentration showed a decrease with age in ZDF +/? rats.

Table 3.5

Homocysteine export from isolated hepatocytes.

	Homocysteine export (nmoles/mg dry weight/30 min)			
	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
Methionine	1.17 ± 0.23	1.21 ± 0.25	1.64 ± 0.61	2.03 ± 0.92
Methionine + Betaine	0.86 ± 0.12*	0.52 ± 0.08*	0.89 ± 0.45*	0.32 ± 0.26*

Hcy export was measured in hepatocytes incubated with 0.1 mM methionine ± 0.3 mM betaine. Data are means ± SD. n = 3 for 5 weeks and n = 5 for 11 weeks. * indicates a significant difference from corresponding incubations without betaine *(P<0.05)*.

Table 3.6

Choline and related metabolite concentrations in the liver of ZDF +/? and ZDF fa/fa rats aged 5 and 11 weeks.

Metabolite	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDf fa/fa
Betaine	17.6 ± 3.6	9.9 ± 1.4*	5.3 ± 0.7¶	2.5 ± 0.7*
Choline	0.06 ± 0.03	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01
Glycerophosphorylcholine	0.6 ± 0.04	1.0 ± 0.2*	1.5 ± 0.1¶	1.0 ± 0.1*
Phosphorylcholine	1.2 ± 0.27	2.1 ± 0.3*	1.9 ± 0.3¶	2.9 ± 0.9*
Phosphatidylcholine	28.1 ± 2.8	29.2 ± 1.4	25.3 ± 5.2	19.3 ± 2.0*
Sphingomyelin	3.3 ± 0.3	2.9 ± 0.2*	3.4 ± 0.4	2.8 ± 0.3*

The data are expressed as means ± SD (μmol/g) for six rats. Differences within each age group were compared using Student's unpaired t test. One way ANOVA followed by Newman-Keuls post-hoc test was used to compare the differences between the ages. At each age group, * indicates significant difference from the ZDF +/? rats. ¶ indicates significant difference in ZDF +/? rats at 11 weeks compared to 5 weeks. *(P< 0.05)*

Table 3.7

Choline and related metabolite concentrations in the plasma of ZDF +/? rats and ZDF fa/fa rats at 5 and 11 weeks of age.

Metabolite	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDf fa/fa
Betaine	0.17 ± 0.04	0.17 ± 0.02	0.12 ± 0.03¶	0.09 ± 0.03
Choline	0.08 ± 0.02	0.08 ± 0.03	0.07 ± 0.01	0.08 ± 0.02
Phosphatidylcholine	0.86 ±0.27	0.95 ± 0.23	0.80 ± 0.29	1.69 ± 0.48*

Data are expressed as means ± SD (μmol/ml) for six rats. Differences within each age group were compared using Student's unpaired t test. One way ANOVA followed by Newman-Keuls post-hoc test was used to compare the differences between the ages. At each age group, * indicates significant difference from the ZDF +/? rats. ¶ indicates significant difference in ZDF +/? rats at 11 weeks compared to 5 weeks. *(P< 0.05).*

3.4 Discussion

This study describes the altered metabolism of methionine and Hcy that occurs in insulin resistance and in early Type 2 diabetes. It is clear that the hormonal changes that are prevalent in these two disease states act at several sites in the pathway of methionine metabolism. Hyperphagia in the diabetic rats may also contribute to these metabolic changes. Indeed, we observed a 2-fold increase in food intake per 100g body weight at the diabetic stage. However, there was no difference at 5 weeks. The increased hepatic CBS and BHMT activity at 5 weeks cannot, therefore, be attributed to increased protein intake. In addition, we have shown a direct effect of insulin in repressing CBS expression in both human and rat cultured hepatocytes (Ratnam *et al*, 2002). Our finding of lowered plasma tHcy in the 5 week ZDF fa/fa rats shows that insulin-resistance alone can decrease plasma tHcy levels.

The most striking finding in the present study concerns the role of betaine and BHMT in determining plasma tHcy. Not only were BHMT activity and mRNA levels elevated but the hepatic betaine concentration was remarkably reduced in the diabetic rats. Furthermore, this increased BHMT was clearly effective in decreasing hepatic Hcy output. This is evident from the data showing that addition of betaine to hepatocytes from ZDF fa/fa rats virtually eliminated Hcy export from cells, compared to a lesser effect in hepatocytes from ZDF +/? rats (Table 3.4). Increased remethylation of Hcy by BHMT also reduced the Hcy available for flux through the transsulfuration pathway, which decreased in these cells (Table 3.3). Although the importance of the BHMT-catalyzed remethylation reaction in determining Hcy levels has not received as much attention as the folate-dependent remethylation pathway, it should be recalled that Schwahn *et al*

(2003) found a highly significant negative correlation between plasma betaine and plasma tHcy concentrations in humans. Supplementation with betaine was shown to lower plasma tHcy in humans (Steenge *et al*, 2003) and in *mthfr* +/+, +/- and -/- mice (Schwahn *et al*, 2003). Because of the elevated BHMT activity, betaine may be particularly efficacious in reducing plasma tHcy in the later stages of Type 2 diabetes, when renal complications bring about HHcy.

Our data suggest that there is enhanced utilization of betaine in the ZDf fa/fa rat. The observed decrease in hepatic phosphatidylcholine concentration (Table 3.6) is consistent with enhanced utilization of choline to form betaine in the livers of these rats. Sphingomyelin, which is derived from phosphatidylcholine, is also reduced in fa/fa liver. The increase in phosphorylcholine concentrations may reflect increased activity of the pathway for phosphatidylcholine biosynthesis that occurs when tissue phosphatidylcholine concentrations fall. The significant increase in plasma phosphatidylcholine concentrations in ZDF fa/fa rats at 11 weeks (Table 3.6) probably reflects increased secretion of lipoproteins from liver.

Another remarkable feature of betaine metabolism is the extraordinarily high hepatic betaine concentration found in young rats. In the control animals (ZDF +/?) the hepatic betaine level was 17.6 μmol/g at 5 weeks; this decreased to 5.3 μmol/g at 11 weeks. Assuming that hepatic betaine is evenly distributed in intracellular water and that rat liver contains 0.45 ml of cell water per gram (Qian and Brosnan, 1996), we can calculate a betaine concentration, in the 5 week-old control animals, of 39 mM. This should be compared with total osmolarity of about 305-310 mOsM in mammalian tissues (except for the renal medulla).

Davies *et al* (1988) have reported a very large urinary excretion of betaine in normal neonates and in young rats after weaning. High betaine concentrations are found in mammalian kidneys, particularly in the renal medulla. Renal betaine has been characterized as a "compatible osmolyte" (together with other trimethylamines and polyols) where it can offset the high extracellular osmolarity that arises as a result of the urinary concentrating mechanisms. In addition, these osmolytes may protect these cells from adverse effects of urea, which is present at high concentrations (Beck *et al*, 1998).

Wettstein *et al* (2000) have shown that betaine plays a role in cell volume homeostasis in perfused livers of adult rats. Whether this is also the function of the high betaine concentrations in neonatal liver is a focus of our current work. We must also comment on the ratio of hepatocellular to plasma betaine. Plasma betaine was fairly constant, in the 90 μM to 170 μM range, in all of the rats (Table 3.6). The liver/plasma concentration ratios for betaine were about 230, 130, 100 and 60, respectively, for ZDF +/? and ZDF fa/fa rats at 5 weeks and at 11 weeks of age. How such gradients are produced and maintained are important issues for future work.

Diabetes is characterized not only by the lack of insulin or the resistance to the action of insulin, but also by increases in the counter-regulatory hormones, glucocorticoids and glucagon (Stubbs and York, 1991, Consoli *et al*, 1990). Hepatic MAT activity has been shown to be increased by glucocorticoid administration (Gil *et al*, 1997). Finkelstein *et al* (1971) examined the effects of administration of a number of hormones on BHMT activity. The principal findings were that hydrocortisone increased BHMT activity whereas thyroxin decreased it. This group also demonstrated increased activity in alloxan-diabetic rats. This latter finding has been confirmed by Nieman *et al* (2004) in

streptozotocin-diabetic rats. We are currently exploring the possibility that insulin may regulate BHMT expression. Insulin treatment of streptozotocin-induced diabetic rats was shown to restore the increased activity of CBS to normal levels (Jacobs *et al*, 1998), by acting at the level of CBS gene transcription to repress CBS promoter activity (Ratnam *et al*, 2002). Cyclic AMP, the intracellular messenger of glucagon, and glucocorticoids, increases CBS expression in rat hepatoma cells (Goss, 1986). The increased activity of CBS, brought about both by allosteric activation by the increased hepatic SAM levels (Finkelstein *et al*, 1975) and by increased expression, does not imply an increased transsulfuration flux. This flux is determined by the rate at which methionine enters into hepatic metabolism which will be largely determined by dietary methionine consumption. It seems that the major effect of increased CBS activity is to decrease the steady state Hcy concentration at which transsulfuration occurs (Reed *et al*, 2004). In this way it contributes to the decreased plasma tHcy concentration. Similarly, the increased activity of BHMT does not necessarily imply an increased remethylation flux in vivo but, rather, it decreases the steady state Hcy concentration at which remethylation via BHMT occurs.

It is also possible that alterations in renal function may contribute, somewhat, to the decreased plasma tHcy, at least when the rats are diabetic. The kidney is a major site for the removal and subsequent metabolism of Hcy (Bostom *et al*, 1995 A). Hyperfiltration is a characteristic sign of renal dysfunction in early diabetes (Thomson *et al*, 2004). Such hyperfiltration has been reported to occur in ZDF fa/fa rats from about 7 weeks of age and to continue until they are about 3 months old (Hoshi *et al*, 2002). Such an occurrence probably accounts for the decreased plasma creatinine seen at 11 weeks. The delivery of an increased quantity of Hcy to the kidney via filtration could result in

increased renal catabolism of Hcy and contribute to its reduced plasma concentration. However, this cannot contribute to the decreased Hcy at the insulin-resistant stage where creatinine levels were unchanged.

It is now clear that plasma tHcy levels decrease in both Type 1 and Type 2 diabetes when there is no renal damage. The present work showed that this is true even at the pre-diabetic, insulin-resistant stage. However, literature reports on the phenomenon show a variety of responses of Hcy to insulin resistance (Rosolova *et al*, 2002, Meigs *et al*, 2001, Abbasi *et al*, 1999). This variability is likely to result from different degrees of insulin resistance as well as other factors such as impaired renal function. Our results at 5 weeks were obtained in an animal model in which there was no evidence of impaired renal function as well as a consistent degree of insulin resistance, agree remarkably well with Rosolova's study of the relationship between Hcy and insulin resistance in healthy human subjects (Rosolova *et al*, 2002).

In summary, this study shows a decreased plasma tHcy level in both insulin-resistant and Type *2* diabetic rats. We show increased activities of BHMT and CBS in these states, as well as increased mRNA levels for these enzymes. We also show increased hepatic SAM levels, which will activate CBS. We report experiments in hepatocytes that directly demonstrate the role of betaine metabolism in enhanced Hcy removal. These results emphasize the importance of BHMT in regulating Hcy metabolism.

Chapter 4

Amino Acid Metabolism in the Zucker Diabetic Fatty Rat: Effects of Insulin Resistance and of Type 2 Diabetes

4.1 Introduction

The pathophysiology of Type 2 diabetes involves defects in three organ systems (Saltiel 2000). Metabolic defects in liver, in peripheral target tissues such as adipose tissues and skeletal muscle and in pancreatic β-cells all play major roles in the development of this disease. Higher than normal levels of circulating insulin are common, indicating that insulin resistance, rather than an absolute lack of insulin, is a major feature. The hormonal changes in diabetes, whether due to a lack of insulin (Type 1) or a resistance to insulin action (Type 2), and the subsequent changes in the counter-regulatory hormones not only lead to the well-characterized derangements in glucose and lipid metabolism but also to changes in protein metabolism. Withdrawal of insulin treatment in patients with Type 2 diabetes has been shown to be associated with increased plasma levels of branched-chain amino acids (BCAA), increased urinary N loss and increased whole body protein turnover (Halvatsiotis *et al.* 2002). Bloxam *et al* (1972) reported on plasma and liver amino acids in streptozotocin-diabetic rats. The concentrations of many plasma amino acids were reduced whereas there was an increase in the level of BCAA. They also found increased levels of BCAA and proline in the liver of these rats. These findings were confirmed in our laboratory. Brosnan *et al* (1980) and Brosnan *et al* (1983) also observed a reduction in gluconeogenic amino acids in the plasma and liver of streptozotocin-diabetic rats. They found a decreased concentration of histidine and increased levels of proline, valine, isoleucine and leucine in muscles. The brain had an increase in the BCAA with reduced concentrations of a number of other amino acids.

In contrast to the wealth of knowledge concerning the free amino acids in Type 1 diabetes, data on Type 2 diabetes are lacking. Several studies have shown increased urinary N loss and net whole body protein loss in Type 2 diabetic patients, even when hyperinsulinemic (Gougeon *et al.* 1994; 1997; 1998). Halvatsiotis *et al.* (2002) found no difference in plasma amino acid concentrations between Type 2 diabetic patients from whom insulin treatment was withdrawn transiently and patients who were given intensive insulin treatment for 11 days, except for a reduction in the glycine levels in the untreated patients.

The leptin receptor-defective Zucker diabetic fatty (ZDF fa/fa) rat is considered to be an excellent model for the study of Type 2 diabetes (Peterson *et al.* 1990; Unger 1997). In this model, diabetes develops, spontaneously, in all male rats and most females. The rats proceed from impaired glucose tolerance, through insulin resistance (normoglycemia, hyperinsulinemia) to frank Type 2 diabetes (Etgan and Oldham 2000) Diabetes in these rats is associated with the usual clinical symptoms of hyperphagia, polyuria, polydypsia, and impaired weight gain (Finegood *et al.* 2001). We, therefore, employed ZDF rats to explore amino acid metabolism both in the pre-diabetic insulin- resistant stage and in frank Type 2 diabetes.

4.2 Results:

Table 3.1 provides data on body weight, plasma insulin, creatinine and glucose concentrations and the hepato-somatic index. At 5 weeks of age the ZDF fa/fa rats had a ten-fold elevation of plasma insulin compared to the ZDF +/? controls. Despite the hyperinsulinemia, plasma glucose levels were already significantly elevated in the ZDF

fa/fa rats, clearly demonstrating insulin resistance. By 11 weeks, plasma glucose was about 30 mM in the ZDF fa/fa rats while insulin levels had fallen and were no longer different from the controls. Therefore, at 5 weeks the ZDF fa/fa rats can be classified as insulin-resistant, but not diabetic. At 11 weeks, the similar insulin levels, together with the profound hyperglycemia indicate that they are frankly diabetic in addition to showing a resistance to the action of insulin.

Plasma creatinine levels were measured in the rats so as to determine whether there was any indication of renal insufficiency. Although there was no significant difference between the creatinine levels of ZDF fa/fa and control rats at 5 weeks, there was a reduction in the plasma creatinine level of the ZDF fa/fa rats at 11 weeks. Hoshi *et al* (2002) have reported that the GFR is significantly elevated at 3 months in ZDF fa/fa rats and this may provide an explanation for the reduced plasma creatinine levels in our animals. The hepato-somatic index was significantly higher in the ZDF fa/fa rats at both 5 and 11 weeks of age, indicating a relative increase in liver weight.

4.2.1 Plasma amino acid concentration:

Plasma amino acid concentrations are shown in Table 4.1. The plasma amino acid concentrations in the control group (ZDF +/?) were similar to those reported by Brosnan et al (1983) in non-diabetic animals. At 5 weeks of age, aspartate, serine, glutamine, glycine and histidine (all gluconeogenic amino acids) were lower in the plasma of ZDF fa/fa rats. Taurine, α-amino adipic acid, methionine, isoleucine, leucine, valine, phenylalanine, and tryptophan were significantly higher in the plasma. At 11 weeks of

Table 4.1: Plasma amino acid concentrations (μmole/liter)

Amino Acid	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
Taurine	66 ± 6	133 ± 10*	136 ± 9	147 ± 21
Aspartic Acid	22 ± 2	18 ± 2*	15 ± 1	15 ± 2
Hydroxy proline	55 ± 10	48 ± 5	44 ± 2	24 ± 3*
Threonine	219 ± 23	220 ± 23	272 ± 59	137 ± 15*
Serine	187 ± 19	135 ± 9*	180 ± 9	122 ± 9*
Asparagine	33 ± 5	31 ± 4	40 ± 4	27 ± 2 *
Glutamic acid	60 ± 7	64 ± 7	97 ± 7	134 ± 22*
Glutamine	556 ± 12	411 ± 14*	601 ± 53	354 ± 26*
α- amino adipic acid	16 ± 1	22 ± 3*	18 ± 1	24 ± 3*
Proline	174 ± 34	211 ± 26	190 ± 20	166 ± 8*
Glycine	289 ± 20	142 ± 18*	240 ± 15	109 ± 11*
Alanine	374 ± 37	396 ± 44	425 ± 44	449 ± 61
Citrulline	61 ± 6	58 ± 4	60 ± 7	47 ± 4*
Valine	105 ± 11	190 ± 29*	162 ± 15	263 ± 34*
Cystine	BLD	BLD	4 ± 0.2	4 ± 0.3
Methionine	28 ± 3	35 ± 3*	54 ± 3	40 ± 4*
Isoleucine	49 ± 5	90 ± 16*	79 ± 10	116 ± 18*
Leucine	80 ± 8	150 ± 26*	134 ± 16	202 ± 42*
Tyrosine	70 ± 11	67 ± 7	115 ± 13	75 ± 12*
Phenylalanine	34 ± 3	53 ± 7*	59 ± 5	65 ± 10
Tryptophan	60 ± 8	75 ± 9*	117 ± 9	82 ± 10*
Ornithine	53 ± 27	56 ± 9	62 ± 14	63 ± 21
Lysine	270 ± 24	276 ± 20	341 ± 20	250 ± 22*
Histidine	53 ± 3	46 ± 4*	55 ± 4	41 ± 3*
Arginine	97 ± 24	116 ± 21	136 ± 13	91 ± 13*
TOTAL	**3011 ± 206**	**3040 ± 218**	**3634 ± 195**	**3047 ± 196***

Values are means ± SD. * $P < 0.05$ when compared with lean controls (ZDF +/?) in each age group. BLD: Below the limits of detection by our system. n=6 for each group

age, diabetic rats had a significantly lower plasma concentration of hydroxyproline, threonine, serine, aspartate, glutamine, proline, glycine, citrulline, methionine, tyrosine, tryptophan, lysine, histidine and arginine and significantly elevated concentrations of glutamic acid, α-amino adipic acid and the 3 BCAAs.

4.2.2 Hepatic amino acids:

The amino acid levels in the liver are shown in Table 4.2. At 5 weeks the livers of ZDF fa/fa rats showed lower levels of serine, asparagine, glutamic acid and glutamine with increased levels of taurine and proline. At 11 weeks diabetic rats showed lower levels of threonine, serine, glutamine, and glycine and increased levels of glutamic acid, valine and leucine.

Hepatic/plasma concentration ratios for amino acids are shown in Table 4.3. The ratios for aspartate and glycine were increased at 5 weeks of age in the ZDF fa/fa rats while the ratios for glutamic acid, isoleucine, leucine and phenylalanine were decreased. At 11 weeks proline, glycine, methionine and tyrosine showed an increased ratio in the diabetic rats while the rest of the amino acids showed no difference.

4.2.3 Skeletal muscle amino acids:

The amino acid levels in the skeletal muscle are shown in Table 4.4. Levels of taurine, aspartate, proline, alanine, isoleucine and leucine were all higher in skeletal muscles while many of the gluconeogenic amino acids were significantly lower in the ZDF fa/fa rats, at 5 weeks of age. Concentrations of lysine, anserine and carnosine were also lower. At 11 weeks of age, tyrosine and lysine together with all the

Table 4.2: Hepatic amino acid levels (nmole/g)

Amino Acid	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
Taurine	1243 ± 231	2146 ± 616*	3422 ± 1233	2808 ± 849
Aspartic Acid	1324 ± 529	1596 ± 464	2829 ± 811	2225 ± 347
Threonine	409 ± 108	320 ± 81	276 ± 56	170 ± 32*
Serine	760 ± 194	433 ± 86*	285 ± 34	213 ± 33*
Asparagine	70 ± 17	46 ± 13*	67 ± 28	50 ± 12
Glutamic acid	1324 ± 298	950 ± 240*	1297 ± 345	2498 ± 556*
Glutamine	3916 ± 891	2651 ± 381*	4932 ± 1277	3496 ± 738*
Proline	270 ± 61	382 ± 85*	591 ±171	867 ± 269
Glycine	1430 ± 432	1366 ± 272	1848 ± 219	1539 ± 240*
Alanine	1359 ± 262	1522 ± 387	2199 ± 658	3001 ± 1470
Citrulline	53 ± 24	29 ± 14	55 ± 48	16 ± 13
Valine	121 ± 47	139 ± 34	163 ± 37	293 ± 36*
Cystine	3 ± 3	1 ± 2	59 ± 13	73 ± 25
Methionine	39 ± 9	36 ± 9	94 ± 18	97 ± 21
Isoleucine	77 ± 19	88 ± 16	105 ± 20	159 ± 57
Leucine	115 ± 38	143 ± 24	168 ± 31	249 ± 44*
Tyrosine	56 ± 23	38 ± 9	69 ± 28	66 ± 10
Phenylalanine	43 ± 13	40 ± 14	70 ± 18	67 ± 13
Ornithine	142 ± 43	103 ± 25	154 ± 37	139 ± 23
Lysine	378 ± 106	393 ± 68	525 ± 168	495 ± 143
Histidine	412 ± 88	384 ± 79	506 ± 114	497 ± 123
Arginine	30 ± 19	23 ± 9	80 ± 25	53 ± 22
TOTAL	**13574 ± 2989**	**12832 ± 2391**	**19794 ± 3814**	**19071 ± 4013**

Values are means ± SD. * $P < 0.05$ when compared with lean controls (ZDF +/?) in each age group. n=6 for each group.

Table 4.3: Intracellular/extracellular concentration ratios for hepatic amino acids

Amino Acid	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
Taurine	42 ± 10	36 ± 11	57 ± 24	41 ± 9.4
Aspartic Acid	130 ± 51	197 ± 46*	412 ± 111	347 ± 80
Threonine	3.7 ± 1.3	2.6 ± 0.6	1.8 ± 0.7	2.2 ± 0.4
Serine	8.5 ± 2.4	6.6 ± 1.2	3.0 ± 0.5	3.3 ± 0.4
Asparagine	4.4 ± 1.8	2.7 ± 0.8	3.2 ± 1.6	3.5 ± 0.8
Glutamic acid	50 ± 14	32 ± 5.0*	29 ± 8.4	42 ± 12
Glutamine	15 ± 3.5	14 ± 2.0	18 ± 4.4	22 ± 5.7
Proline	3.0 ± 1.2	3.5 ± 0.5	6.5 ± 2.4	11 ± 3.5*
Glycine	10 ± 3.3	21 ± 3.2*	17 ± 2.7	31 ± 7.1*
Alanine	7.6 ± 1.5	7.9 ± 1.5	11 ± 2.7	14 ± 5.7
Citrulline	1.3 ± 0.8	0.6 ± 0.6	1.4 ± 1.5	0.2 ± 0.6
Valine	2.0 ± 1.0	1.1 ± 0.3	1.7 ± 0.5	1.9 ± 0.4
Cystine	-	-	30 ± 6.1	40 ± 13
Methionine	2.7 ± 0.8	1.9 ± 0.7	3.3 ± 0.6	4.9 ± 1.4*
Isoleucine	2.9 ± 0.8	1.6 ± 0.4*	2.4 ± 0.5	2.5 ± 1.1
Leucine	2.6 ± 0.8	1.6 ± 0.3*	2.2 ± 0.5	2.2 ± 0.6
Tyrosine	1.2 ± 0.6	0.7 ± 0.3	0.8 ± 0.5	1.4 ± 0.3*
Phenylalanine	2.3 ± 0.6	1.1 ± 0.6*	2.1 ± 0.7	1.8 ± 0.6
Ornithine	6.2 ± 3.0	3.8 ± 1.4	5.2 ± 1.8	4.6 ± 1.1
Lysine	2.6 ± 1.1	2.5 ± 0.4	2.9 ± 1.0	3.9 ± 1.3
Histidine	16.6 ± 3.5	18 ± 2.5	20 ± 5.0	26 ± 6.7
Arginine	0.2 ± 0.5	-0.1 ± 0.2	0.8 ± 0.4	0.8 ± 0.8

Values are means ± SD. * $P < 0.05$ when compared with lean controls (ZDF +/?) in each age group. – could not be calculated from available data. n=6 for each group.

gluconeogenic amino acids that were lower at 5 weeks showed reduced concentrations in skeletal muscles of diabetic rats while the level of alanine, valine and leucine were significantly elevated.

Intracellular/extracellular ratios for muscle amino acids are shown in Table 4.5. At 5 weeks of age, the ratios for taurine, threonine, serine, glutamine, citrulline, valine, ornithine, lysine, histidine and arginine were decreased in the ZDF fa/fa rats while those for aspartate and alanine were increased. At 11 weeks, glycine and alanine showed an increase in the ratio of intracellular/ extracellular concentration. The rest of the amino acid ratios did not show any significant difference.

4.2.4 Branched-Chain Amino Acids:

Figure 1 shows the excellent correlation of the plasma level of the BCAAs to one another. The plasma concentrations of these three amino acids change in parallel. This is to be expected as they share a common transaminase (the branched-chain aminotransferase) followed by a common α–keto acid dehydrogenase (the branched-chain α-ketoacid dehydrogenase complex). These close relationships among the three BCAAs are similar to those previously found, in this laboratory, in streptozotocin-diabetic rats (Brosnan *et al*, 1983).

Table 4.4: Skeletal muscle amino acid levels (nmole/g)

	5 weeks		11 weeks	
Amino Acid	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
Taurine	8987 ± 256	14115 ± 1015*	12557 ± 732	13702 ± 2227
Aspartic Acid	375 ± 25	560 ± 87*	615 ± 175	752 ± 258
Hydroxy proline	306 ± 39	302 ± 53	259 ± 46	225 ± 99
Threonine	743 ± 60	590 ± 46*	602 ± 86	374 ± 103*
Serine	917 ± 119	543 ± 70*	641 ± 81	466 ± 139*
Asparagine	137 ± 29	94 ± 30*	150 ± 22	99 ± 32*
Glutamic acid	1637 ± 56	1661 ± 145	1438 ± 188	1497 ± 503
Glutamine	4546 ± 125	3026 ± 268*	3517 ± 547	2152 ± 612*
Proline	421 ± 50	523 ± 42*	388 ± 76	475 ± 151
Glycine	6073 ± 375	2798 ± 354*	4731 ± 797	3282 ± 926*
Alanine	1460 ± 112	2036 ± 181*	1509 ± 239	3351 ± 891*
Citrulline	262 ± 18	204 ± 33*	182 ± 18	170 ± 76
Valine	129 ± 36	168 ± 25	142 ± 24	264 ± 90*
Cystine	BLD	BLD	36 ± 4	32 ± 6
Methionine	14 ± 10	31± 17	85 ± 16	66 ± 22
Isoleucine	40 ± 13	72 ± 8.8*	74 ± 23	105 ± 43
Leucine	57 ± 11	103 ± 18*	111 ± 21	167 ± 53*
Tyrosine	112 ± 15	103 ± 21	170 ± 46	111 ± 36*
Phenylalanine	BLD	BLD	70 ± 17	72 ± 28
Ornithine	122 ± 15	108 ± 12	83 ± 11	76 ± 18
Lysine	1850 ± 74	1201 ± 98*	1501 ± 269	935 ± 257*
Histidine	256 ± 25	160 ± 16*	141 ± 26	97 ± 42
Anserine	1559 ± 160	1247 ± 114*	5181 ± 599	5285 ± 1595
Carnosine	3316 ± 126	2970 ± 122*	4425 ± 598	4492 ± 1146
Arginine	706 ±76	604 ± 64*	744 ± 137	542 ± 149*
TOTAL	**34473 ± 587**	**33579 ± 1958**	**39352 ± 4099**	**38791 ± 9135**

Values are means ± SD. * P< 0.05 when compared with lean controls (ZDF +/?) in each age group. BLD: Below the limits of detection by our system. n=6 in each group.

Table 4.5: Intracellular/extracellular concentration ratios for skeletal muscle amino acids

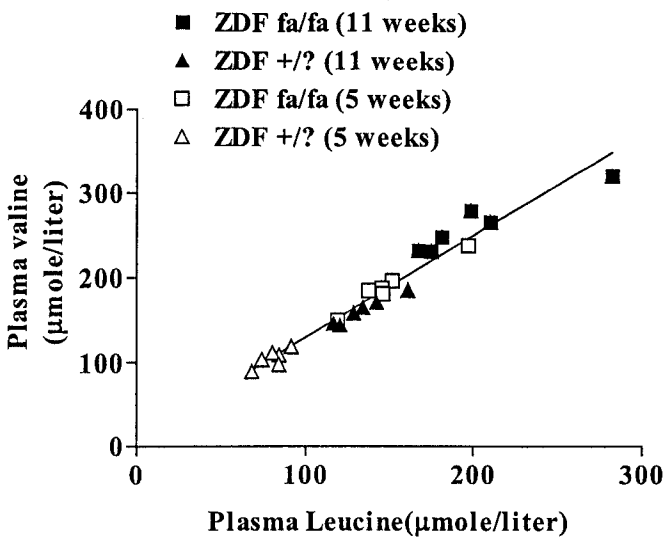
Amino Acid	5 weeks		11 weeks	
	ZDF +/?	ZDF fa/fa	ZDF +/?	ZDF fa/fa
Taurine	203 ± 23	157 ± 20*	137 ± 15	140 ± 36
Aspartic acid	24 ± 2	46 ± 7*	59 ± 16	78 ± 32
Hydroxy proline	8 ± 2	9 ± 2	9 ± 1	14 ± 6.2
Threonine	5 ± 0.4	4 ± 0.04*	3 ± 1	4 ± 1
Serine	7 ± 1	6 ± 1*	5 ± 1	6 ± 2
Asparagine	6 ± 1	4 ± 2	5 ± 1	5 ± 2
Glutamic acid	41 ± 6	38 ± 4	21 ± 4	16 ± 5
Glutamine	12 ± 1	11 ± 1*	9 ± 2	9 ± 2
Proline	4 ± 0.4	4 ± 0.4	3 ± 1	4 ± 1
Glycine	31 ± 3	29 ± 3	29 ± 5	44 ± 12*
Alanine	6 ± 0.4	8 ± 1*	5 ± 1	11 ± 4*
Citrulline	6 ± 1	5 ± 1*	4 ± 1	5 ± 2
Valine	2 ± 1	1 ± 0.2*	1 ± 0.2	1 ± 0.4
Cystine	-	-	12 ± 1	11 ± 2
Methionine	1 ± 1	1 ± 1	2 ± 1	2 ± 1
Isoleucine	1 ± 1	1 ± 0.1	1 ± 1	1 ± 1
Leucine	1 ± 0.2	1 ± 0.1	1 ± 0.3	1 ± 0.3
Tyrosine	2 ± 0.1	2 ± 0.2	2 ± 1	2 ± 1
Phenylalanine	-	-	2 ± 1	2 ± 1
Ornithine	4 ± 1	3 ± 1	2 ± 1	2 ± 1
Lysine	10 ± 1	6 ± 1*	6 ± 1	5 ± 2
Histidine	7 ± 0.4	5 ± 0.4*	3 ± 2	3 ± 1
Arginine	11 ± 3	8 ± 2*	8 ± 2	9 ± 3

Values are means ± SD. * $P < 0.05$ when compared with lean controls (ZDF +/?) in each age group. - could not be calculated from available data. n=6 for each group.

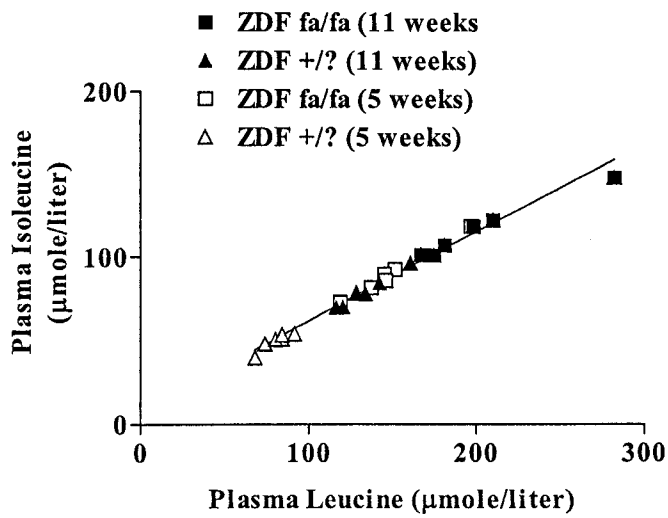
Figure 4.1.

Concentration of Valine (A) and Isoleucine (B) in arterial blood as a function of leucine concentration and the concentration of Isoleucine (C) in arterial blood as a function of Valine concentration. For (A) r^2=0.9578, P<0.0001, (B) r^2=0.9705, P<0.0001, (C) r^2=0.9853, P<0.0001

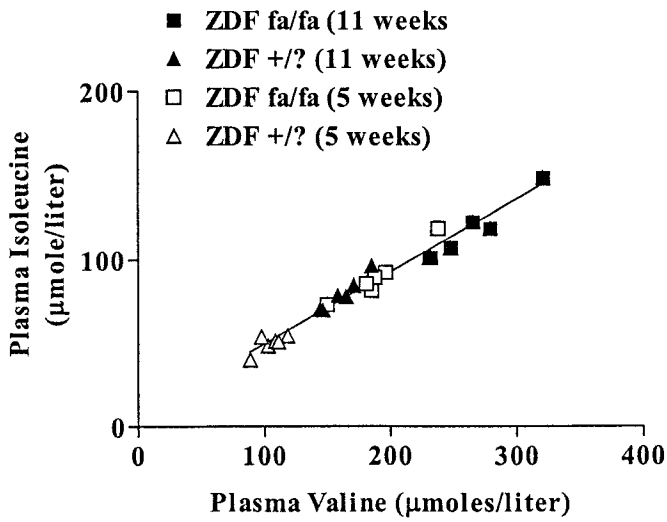
A.


ZDF fa/fa (11 weeks)
ZDF +/? (11 weeks)
ZDF fa/fa (5 weeks)
ZDF +/? (5 weeks)
Plasma valine (µmole/liter)
400
300
200
100
0
0
100
200
300
Plasma Leucine(µmole/liter)

B.


ZDF fa/fa (11 weeks
ZDF +/? (11 weeks)
ZDF fa/fa (5 weeks)
ZDF +/? (5 weeks)
Plasma Isoleucine (µmole/liter)
200
100
0
0
100
200
300
Plasma Leucine (µmole/liter)

C.


ZDF fa/fa (11 weeks
ZDF +/? (11 weeks)
ZDF fa/fa (5 weeks)
ZDF +/? (5 weeks)
Plasma Isoleucine (µmole/liter)
200
100
0
0
100
200
300
400
Plasma Valine (µmoles/liter)

4.3 Discussion:

Both at 5 weeks and at 11 weeks, all three branched-chain amino acids (BCAA) were significantly elevated in the plasma of ZDF fa/fa rats. In skeletal muscle and in the liver, also, the BCAA were increased at both ages although the difference was not always statistically significant. The increase in BCAA in this model of Type 2 diabetes is consistent with the reports on Type 1 diabetes by Bloxam et al. (1972), Brosnan *et al.* (1980), and Brosnan *et al.* (1983) in streptozotocin-diabetic rats.

Isoleucine, leucine and valine are essential amino acids. A reversible transamination step, catalyzed by the branched-chain aminotransferase is the first step in the catabolism of all three BCAAs. Removal of the carboxyl group of the keto acid thus formed is also catalyzed by a common enzyme-complex, the branched-chain α-ketoacid dehydrogenase complex (BCKDC). After these two common initial steps, the three BCAAs take different routes of catabolism, with isoleucine yielding acetyl CoA and succinyl CoA, valine yielding succinyl CoA and leucine yielding acetoacetyl CoA and acetyl CoA. Thus, despite the initial common steps in their catabolism, valine is glucogenic, leucine is ketogenic and isoleucine is both glucogenic and ketogenic. However, Figure 4.1 explicitly shows that the plasma concentrations of these three amino acids correlate with each other extremely well, providing strong evidence for the common metabolic regulation of the catabolism of these three amino acids.

The activity of the BCKDC has been shown to be regulated by nutritional and hormonal stimuli. Gibson *et al.* (1993) have reported a reduction in the activity of BCKDC, which was reversed by insulin treatment in streptozotocin-induced severe

ketotic diabetes in rats. However, there are also studies that have shown an increase in the activity state of BCKDC in the liver (Lombardo *et al.* 1998; Li *et al.* 2001) and in skeletal muscles (Lombardo *et al.* 1999) of rats with experimentally induced-diabetes.

The elevation of BCAA in Type 1 diabetes has been attributed to the increased proteolytic state of body tissues and hyperphagia in the diabetic animals (Rodriguez *et al.* 1997; Crandall and Fernstrom 1983). There is evidence for both of these factors in ZDF rats. Increased gluconeogenesis is likely to demand increased proteolysis. In addition ZDF rats are also known to be hyperphagic (Finegood *et al.* 2001).

One important consequence of the increase in BCAA could be a possible effect on brain uptake of large neutral amino acids. Brosnan *et al.* (1984) have shown that in Type 1 diabetes the decreased brain uptake and the decreased brain level of tyrosine is due to the high circulating levels of BCAA. The brain uptake of tyrosine is proportional to [Tyr] / $\sum$ [LNAA], where [Tyr] represents the plasma concentration of tyrosine and $\sum$ [LNAA] represents the sum of the plasma concentrations of the other large neutral amino acids that share this transporter (phenylalanine, tryptophan, methionine, histidine, isoleucine, leucine and valine) (Pardridge, 1983). This ratio is 0.17 ± 0.02 and 0.10 ± 0.02, respectively for the control and the ZDF fa/fa rats at 5 weeks, and 0.17± 0.02 and 0.09±0.02 respectively, for the control and the ZDF fa/fa rats at 11 weeks. At both ages these numbers were significantly different from each other. Thus, the elevated BCAA in this model also, may compromise tyrosine uptake by the brain.

Fujimoto *et al.* (2004) have reported that, at 8 weeks of age, ZDF fa/fa rats show markedly higher total glucose output compared to lean rats under basal conditions. They also showed that ZDF fa/fa rats have defects in the suppression of hepatic glucose

production and total glucose production in response to increased plasma glucose and insulin (Fujimoto *et al.* 2004). It is likely that amino acids are important substrates for hepatic gluconeogenesis in this situation. At five weeks of age, although the animals do exhibit insulin resistance, it is clear that there is some appreciable insulin action since blood glucose is only slightly elevated. The plasma concentrations of only five of the glucogenic amino acids (aspartate, serine, glutamine, glycine and histidine) are decreased. The plasma concentrations of five other glucogenic amino acids (valine, methionine, isoleucine, phenylalanine and tryptophan) are actually increased. However, at 11 weeks blood glucose is massively increased and it is clear that these animals experience very little insulin action. Under these circumstances one expects much higher rates of gluconeogenesis. Consistent with this expectation, we have observed that the plasma concentrations of twelve of the glucogenic amino acids (hydroxyproline, threonine, serine, asparagine, glutamine, proline, glycine, methionine, tyrosine, tryptophan, histidine and arginine) are decreased. On the other hand, only three glucogenic amino acids (glutamate, valine, and isoleucine) increase their plasma concentrations.

Specific mention must be made of the regulation of the metabolism of a number of glucogenic amino acids. Alanine and glutamine are the most important amino acid precursors for hepatic gluconeogenesis during starvation and severe illness. In our model of Type 2 diabetes, glutamine was decreased in plasma, liver and skeletal muscle while alanine concentrations were comparable with the controls in plasma and liver while showing increased levels in the muscle at both 5 and 11 weeks. In streptozotocin-diabetic rats Williamson *et al.* (1967) and Brosnan *et al.* (1980) had shown decreased levels of both alanine and glutamine in plasma. Hundal *et al.* (1992) observed a reduction in intra-

sarcolemmal glutamine levels in streptozotocin-diabetic rats. In the face of this reduced intramuscular glutamine level, and a low muscle/plasma distribution ratio (observed by us also in 5 weeks old rats) they also observed an increased efflux of glutamine from muscle, indicating an increase in the V_{max}/K_m ratio (i.e. the first order rate constant) of the glutamine transporter in muscle. Glutamine and alanine were shown to account for almost 70% of the amino acids released by muscle of streptozotocin-diabetic rats, while they comprised 56% of the gluconeogenic amino acids removed by the liver (Brosnan *et al.* 1983). It has also been shown that hepatocytes from streptozotocin-induced diabetic rats use glutamine more rapidly than do hepatocytes from normal or insulin-maintained diabetic rats (Squires *et al.* 1997). The activity of glutaminase itself was shown to be increased in livers of Type 1 diabetic rats (Squires *et al.* 1997). This may contribute to the reduced glutamine levels observed in the livers of the ZDF fa/fa rats and indicate increased gluconeogenic flux from glutamine. Although there is increased efflux of glutamine from the skeletal muscles, the increased uptake by the liver in the face of increased gluconeogenic flux will lead to the reduction in plasma levels of this amino acid. Interestingly, skeletal muscle glutamine showed a lower distribution ratio while alanine showed an increased distribution ratio, both in the insulin-resistant and the diabetic stages. This is consistent with the idea that glutamine loss is primarily driven by an alteration in the kinetics of its transport (Hundal *et al.* 1992), while increased alanine release is a result of increased tissue levels.

Serine, threonine and glycine are all gluconeogenic amino acids, which are converted to pyruvate. We saw a reduction in the levels of all three of these amino acids in the plasma, liver and the skeletal muscles at 11 weeks. At 5 weeks of age, all three

amino acids were decreased in the skeletal muscles, while serine and glycine were decreased in the plasma with only serine showing a decreased level in the liver. These decreases are in agreement with increased rates of gluconeogenesis. Serine/threonine dehydratase is induced in diabetes, as is serine: pyruvate aminotransferase (Freedland and Avary 1964; Kanamoto *et al.* 1991; Mak *et al.* 1981; Su *et al.* 1990; Rowsell *et al.* 1973). These effects may be brought about by glucagon (Jost *et al.* 1968; Su *et al.* 1990; Rowsell *et al.* 1973; Fukishima *et al.* 1978). Flux through the glycine cleavage system has also been shown to be stimulated by glucagon, epinephrine and norepinephrine (Brosnan *et al.* 1990). Glucagon has also been shown to increase oxidation of threonine as well as its transport into hepatocytes (House *et al.* 2001). Histidine:pyruvate aminotransferase has been reported to be identical with serine:pyruvate aminotransferase (Noguchi *et al.* 1978). Thus, the observed reduction in histidine too may be due to the increased activity of its degradative enzyme.

We also calculated the muscle/plasma and liver/ plasma distribution ratios for the amino acids. At 11 weeks, proline and methionine both showed an increased liver/plasma distribution ratio in the diabetic rats. This increase in the distribution ratio of proline was also observed in our laboratory in rats treated with glucagon (M. Haslett and M. Brosnan unpublished). Treatment with glucagon was also shown to increase the liver/plasma ratio of methionine, which was associated with a marked stimulation of methionine transport into liver cells (Jacobs *et al.* 2001).

In conclusion, this work describes, for the first time, plasma as well as tissue levels of amino acids in this model of Type 2 diabetes. It is clear that insulin resistance

alone caused alterations in amino acid metabolism. Many of the effects observed in frank Type 2 diabetes were similar to those found in models of Type 1 diabetes.

Chapter 5

Effect of Rosiglitazone on homocysteine metabolism in ZDF (Type 2) diabetic rats.

5.1 Background

Increased levels of plasma Hcy, an intermediate in the pathway of methionine metabolism, have been identified as an independent risk factor for vascular disease (Kang *et al*, 1992), Alzheimer's disease (Seshadri *et al*, 2002) and osteoporotic fractures (van Meurs *et al*, 2004). An elevation in plasma Hcy by even as small an amount as 5 μmol/liter can increase the risk of coronary artery disease by 60% in men and 80% in women (Boushey *et al*, 1995).

As discussed in the introduction, both Type 1 and Type 2 diabetes mellitus as well as the metabolic syndrome of insulin-resistance, are associated with an increased risk of coronary artery disease. The prevalence of atherosclerosis is reported to be 2-to 6-fold higher in diabetic patients than in people without diabetes (Brand *et al*, 1989). However, the association between HHcy and cardiovascular disease and mortality has been found to be strongest in Type 2 diabetes mellitus (Hoogeveen *et al*, 1998).

Our earlier study conducted in ZDF rats in the insulin-resistant and the frank diabetic stages showed that plasma tHcy is significantly lower in ZDF fa/fa rats when compared to Untreated rats. This reduction was brought about by several changes in the enzymes involved in Hcy metabolism.

RSG, a thiazolidinedione, is an agonist of the peroxisome proliferator-activated receptor γ (PPAR- γ) subfamily of nuclear receptors. It is an anti-diabetic agent used to treat patients with Type 2 diabetes and is known to enhance insulin sensitivity and to improve metabolic control in these patients. RSG has also been shown to reduce insulin resistance and prevent the loss of β-cell mass in ZDF rats by maintaining β-cell proliferation and preventing net β-cell death (Finegood *et al*, 2001).

In this study, we examined Hcy metabolism under conditions of improved insulin sensitivity after treatment with RSG with the aim of gaining more understanding of the regulation of Hcy metabolism.

5.2 Results

5.2.1 Body Weight, Hepato-somatic index, fat pad weight, food intake, water intake and urinary output

Body weight, hepato-somatic index, fat pad weight, food and water intake and urinary output are shown in Table 5.1. ZDF fa/fa rats were 47% heavier than the ZDF fa/+ rats at 6 weeks of age but by 12 weeks, although still being significantly heavier than the untreated rats the difference had dropped to 14%. The ZDF fa/fa (RSG) rats on the other hand were able to maintain the weight difference initially observed. The ZDF fa/fa (untreated) rats, had a significantly higher hepato-somatic index compared to their RSG treated counterparts as they had greatly enlarged livers. Fat pad weight was measured only at 12 weeks of age. Both groups of ZDF fa/fa rats had a significantly higher fat pad weight than the ZDF fa/+ rats. However, the fat pad weight of the ZDF fa/fa (RSG) rats was significantly higher than the ZDF fa/fa (untreated) rats. Very interestingly, the ZDF fa/fa (untreated) rats also had a much higher feed intake /100g body weight as well as a higher water intake and urinary output compared to the other 3 groups. The ZDF fa/fa (untreated) rats therefore are showing the classical features of diabetes mellitus of polyphagia, polydypsia and polyuria along with a failure to gain weight.

Table 5.1. Body Weight, Hepato-somatic index, fat pad weight, feed intake, water intake and urinary output of RSG treated and untreated ZDF fa/+ and ZDF fa/fa rats.

	ZDF fa/+		ZDF fa/fa	
	Untreated	**RSG-treated**	**Untreated**	**RSG-treated**
Body weight (g)				
6 weeks	127 ± 7	-	187 ± 12^{b}	-
12 weeks	299 ± 7^{a}	308 ± 21^{a}	339 ± 17^{b}	457 ± 23^{c}
Hepato-somatic index				
6 weeks	5 ± 1^{a}	-	6 ± 1^{a}	-
12 weeks	3 ± 0.2^{a}	$3 \pm 0.1^{a,c}$	5 ± 0.3^{b}	3 ± 0.3^{c}
Fat pad weight (g)				
6 weeks	0.3 ± 0.01^{a}		1.7 ± 0.2^{b}	
12 weeks	2 ± 1^{a}	2 ± 1^{a}	6 ± 0.4^{b}	13 ± 1^{c}
Feed Intake g/100 g BW				
6 weeks	12 ± 2^{a}		13 ± 2^{a}	
12 weeks	6 ± 1^{a}	$6 \pm 0.3^{a,c}$	12 ± 1^{b}	7 ± 0.3^{c}
Water Intake (ml/100 g BW)				
6 weeks	14 ± 1^{a}		17 ± 2.1^{a}	
12 weeks	7 ± 1^{a}	8 ± 1^{a}	39 ± 4^{b}	7 ± 1^{a}
Urinary output (ml/100g BW)				
6 weeks	5 ± 1^{a}		9 ± 1^{b}	
12 weeks	4 ± 1^{a}	4 ± 0.0^{a}	44 ± 3^{b}	5 ± 1^{a}

Mean ± SD for 6 rats are shown. Differing superscripts within each row indicate significant difference from each other

5.2.2 Plasma glucose, insulin, creatinine and the glomeruler filtration rate

Plasma levels of glucose, insulin and creatinine and the calculated GFR are shown in Table 5.2. The ZDF fa/fa rats were insulin-resistant at 6 weeks of age, as indicated by the 5- fold greater increase in plasma insulin needed to maintain a normal plasma glucose level. By 12 weeks, the ZDF fa/fa (untreated) rats have progressed to the diabetic stage with a 3-fold increase in their plasma glucose, while the plasma insulin level dropped and was no longer different from the ZDF fa/+ rats. This could probably be due to the loss of β cells that occur with the progression of diabetes as shown by Finegood *et al* (2001). The ZDF fa/fa (RSG) rats still maintained a significantly higher plasma insulin albeit much lower than the level seen at 6 weeks. Plasma glucose however, was normalized in the ZDF fa/fa (RSG) rats by the RSG treatment. These results could indicate the preservation of some β-cells as well as an increase in insulin sensitivity brought about by the RSG treatment.

Since renal function is one of the major determinants of plasma Hcy in humans we measured plasma and urinary creatinine levels and calculated the GFR from these values and the total urinary output per day. Plasma creatinine was significantly increased in the ZDF fa/fa (untreated) rats at 12 weeks and at both ages they excreted more creatinine in their urine. GFR however, was only increased at 6 weeks of age suggesting a state of hyper-filtration at this age. At 12 weeks, the increase seen in both plasma and urinary creatinine in the face of a normal GFR may indicate the increased plasma creatinine to be a result of a higher production and not be due to failing kidney function.

Table 5.2. Plasma glucose, insulin, creatinine, total urinary creatinine and GFR of RSG treated and ZDF fa/+ and ZDF fa/fa rats.

	ZDF fa/+		ZDF fa/fa	
	Untreated	**RSG-treated**	**Untreated**	**RSG-treated**
Plasma Glucose (mM)				
6 weeks	10 ± 1^a	-	11 ± 1^a	-
12 weeks	11 ± 1^a	11 ± 1^a	32 ± 3^b	11 ± 2^a
Plasma Insulin (ng/ml)				
6 weeks	4 ± 1^a	-	21 ± 9^b	-
12 weeks	4 ± 1^a	4 ± 1^a	6 ± 2^a	8 ± 1^b
Plasma Creatinine (μM)				
6 weeks	27 ± 3^a		28 ± 3^a	
12 weeks	27 ± 3^a	31 ± 5^a	46 ± 6^b	29 ± 4^a
Urinary creatinine loss (μMoles/day)				
6 weeks	56 ± 9^a		89 ± 17^b	
12 weeks	129 ± 9^a	127 ± 13^a	184 ± 13^b	138 ± 9^a
GFR (ml/min)				
6 weeks	1.4 ± 0.3^a		2.2 ± 0.2^b	
12 weeks	3.3 ± 0.5^a	2.9 ± 0.8^a	2.8 ± 0.3^a	3.4 ± 0.5^a

Mean ± SD for 6 rats are shown. Differing superscripts within each row indicate significant difference from each other.

5.2.3 Plasma Homocysteine

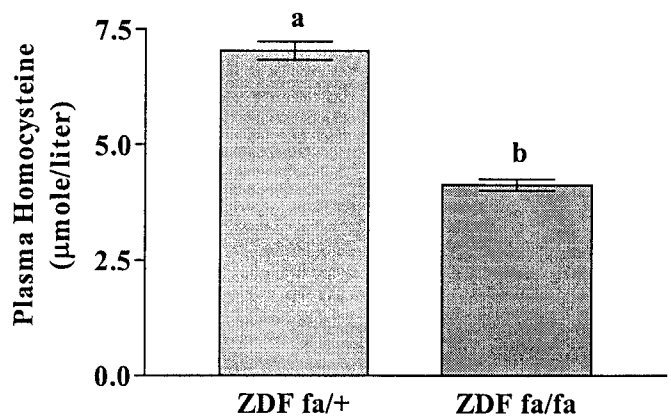
Plasma Hcy levels at 6 and 12 weeks are shown in Figure 5.1. Plasma tHcy was 41% lower in the ZDF fa/fa rats when compared to the ZDF fa/+ rats at 6 weeks of age. RSG treatment for 6 weeks returned the tHcy level in the ZDF fa/fa (RSG) rats towards normal.

5.2.4 Hepatic activities of enzymes of Homocysteine metabolism

Table 5.3 shows the activities of the enzymes involved in Hcy metabolism. We have already seen, in Chapter 3 of this thesis, the changes in enzyme activities that lead to decreased plasma Hcy levels in insulin resistance and early Type 2 diabetes. In this study we show that the RSG treatment is able to return the lowered plasma Hcy level towards normal. We were therefore interested in identifying the enzymes that respond to the increased insulin sensitivity brought about by the RSG treatment.

The baseline enzyme activities at the beginning of the study before the commencement of RSG treatment were similar to those in the first study (Table 3.2) except for the reduced MTHFR activity in the ZDF fa/fa rats. After 6 weeks of RSG treatment, methionine adenosyltransferase activity, which was increased in the ZDF fa/fa (untreated) rats, was normalized. Two-way ANOVA showed cystathionine β-synthase activity to be significantly higher in the ZDF fa/fa rats compared to the ZDF fa/+ rats but the difference was slight. Contrary to what we expected, the RSG treatment did not affect its activity.

A. 6 Weeks



B. 12 weeks

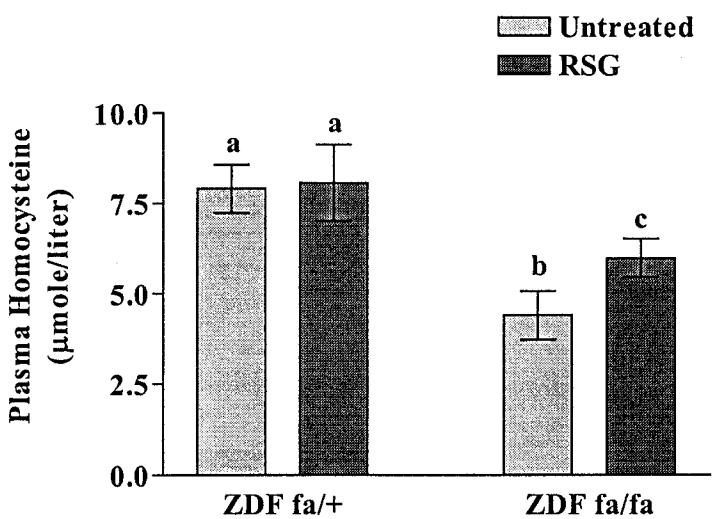


Table 5.3. Hepatic activities of enzymes of homocysteine metabolism

	ZDF fa/+		ZDF fa/fa	
	Untreated	RSG-treated	Untreated	RSG-treated
Methionine adenosyltransferase				
6 weeks	298 ± 38 [a]		360 ± 32 [b]	
12 weeks	232 ± 97 [a]	254 ± 99 [a]	484 ± 209 [b]	309 ± 58 [a]
Glycine-N-methyltransferase				
6 weeks	298 ± 38 [a]		360 ± 32 [b]	
12 weeks	416 ± 50 [a]	483 ± 30 [b]	538 ± 52 [b]	545 ± 59 [b]
Cystathionine β-synthase				
6 weeks	944 ±66 [a]		1073 ± 89 [b]	
12 weeks	1879 ± 202	2079 ± 282	2288 ± 194*	2160 ± 308*
Cystathionine γ-lyase				
6 weeks	3211 ±310 [a]		4284 ± 631 [b]	
12 weeks	4025 ± 784 [a]	4081 ± 673 [a]	6238 ± 849 [b]	4365 ± 808 [b]
Methylenetetrahydrofolate reductase				
6 weeks	17 ± 3 [a]		14 ± 2 [b]	
12 weeks	17 ± 3	17 ± 3	16 ± 6*	14 ± 5*
Methionine synthase				
6 weeks	12 ± 1[a]		9 ± 0.4 [b]	
12 weeks	14 ± 1	12 ± 2[§]	11± 2*	11 ± 1*[§]
Betaine:homocysteine methyltransferase				
6 weeks	170 ±52 [a]		377 ± 57 [b]	
12 weeks	170 ±38	222 ± 67[§]	259± 31*	351 ±52*[§]
Choline dehydrogenase				
6 weeks	981 ± 49 [a]		942 ± 78 [a]	
12 weeks	1217 ± 100 [a]	1202 ± 237 [a]	1428 ± 106 [b]	1395 ± 288 [b]

Data are given as mean ± SD for six rats. Enzyme activities are expressed as *nmoles of product synthesized per minute per gram liver*. Differences in superscript letters signify significant differences within each horizontal row *($P< 0.05$)* with one-way ANOVA. * and § signify significant difference due to genotype and RSG treatment respectively, with Two-way ANOVA.

On the other hand, the second transsulfuration enzyme, CGL showed a marked increase in the ZDF fa/fa (untreated) rats and was returned to normal by the RSG treatment, possibly contributing to the normalization of plasma Hcy. MTHFR activity was slightly, though significantly lower in ZDF fa/fa rats compared to ZDF fa/+ rats but the RSG treatment did not have any additional effects. In the case of methionine synthase, both RSG treatment and the genotype had significant effects on its activity, with the ZDF fa/fa rats showing a lower level of activity than the ZDF fa/+ rats while the RSG treatment was also effective in lowering its activity. The activity of BHMT was once again observed to be significantly higher in the ZDF fa/fa rats with the RSG treatment also acting to increase its activity.

5.2.5 S-adenosylmethionine, S-adenosylhomocysteine and SAM/SAH ratio

SAM, SAH and the SAM/SAH ratio are shown in Table 5.4. Hepatic SAM as well as SAH levels were significantly higher in ZDF fa/fa rats at 6 weeks of age. At 12 weeks, the ZDF fa/fa (untreated) rats still maintained a significantly higher level of SAM than the rest of the groups, which is consistent with the higher activity observed in MAT. Along with the normalization of MAT seen with RSG treatment we also observe a lowering of this increased SAM level in ZDF fa/fa (RSG) rats. However, the inability of SAM to completely return to normal could arise from the increase in the activity of BHMT seen in these rats. Analysis of the data using two-way ANOVA shows both the genotype and the interaction between the genotype and the treatment to be significant for SAM. However, the treatment itself did not have a significant effect. In the case of SAH, both RSG treatment and the genotype showed significant effects with the ZDF fa/fa rats

Table 5.4. S-adenosylmethionine, S-adenosylhomocysteine and the SAM/SAH ratio

	ZDF fa/+		ZDF fa/fa	
	Untreated	**RSG-treated**	**Untreated**	**RSG-treated**
S-adenosylmethionine (nmoles/g liver)				
6 weeks	107 ± 21^{a}		143 ± 26^{b}	
12 weeks	132.8 ± 10.1^{a}	$140.6 \pm 10.9^{a,c}$	169.5 ± 7.0^{b}	146.9 ± 9.2^{c}
S-adenosylhomocysteine (nmoles/g liver)				
6 weeks	12.4 ± 1.6^{a}		15.1 ± 1.9^{b}	
12 weeks	16.5 ± 0.9	$18.8 \pm 1.3^{\S}$	18.3 ± 0.9*	19.9 ± 2.3*§
SAM/SAH				
6 weeks	9.3 ± 1.1^{a}		11.7 ± 1.1^{b}	
12 weeks	8.1 ± 0.9^{a}	7.5 ± 0.5^{a}	9.3 ± 0.7^{b}	7.5 ± 0.9^{a}

Data are given as means ± SD for six rats. Differences in superscript letters signify significant differences within each row *($P < 0.05$)*. * and § signify significant difference due to genotype and RSG treatment respectively, with Two-way ANOVA.

having a higher level of SAH than the ZDF fa/+ rats and the RSG treatment also having a positive effect. At 6 weeks, the SAM/SAH ratio was significantly higher in the ZDF fa/fa rats and continued to remain so in the ZDF fa/fa (untreated) rats at 12 weeks. The RSG treatment normalized the ratio in the ZDF fa/fa (RSG) rats.

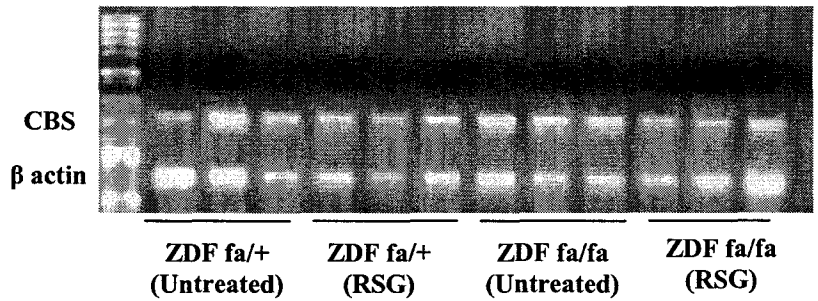
5.2.6 Relative mRNA levels of CBS and BHMT

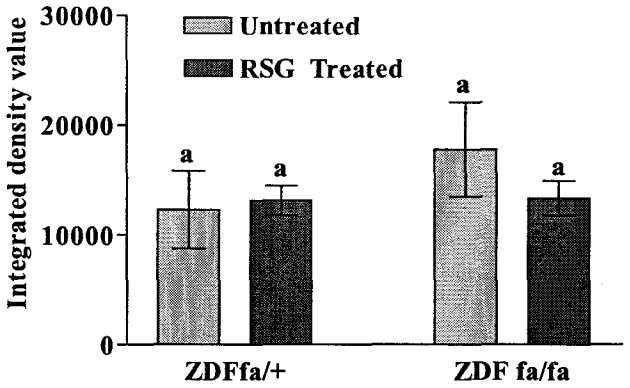
RT-PCR analysis of BHMT mRNA showed a significant increase in mRNA abundance in the ZDF fa/fa rats compared to the ZDF fa/+ rats (Figure 5.2 B) which is in agreement with the genotypic effect observed in the enzyme activity. However, CBS mRNA did not differ between the groups (Figure 5.2A).

5.2.7 Hepatic activities of enzymes of phospholipid metabolism

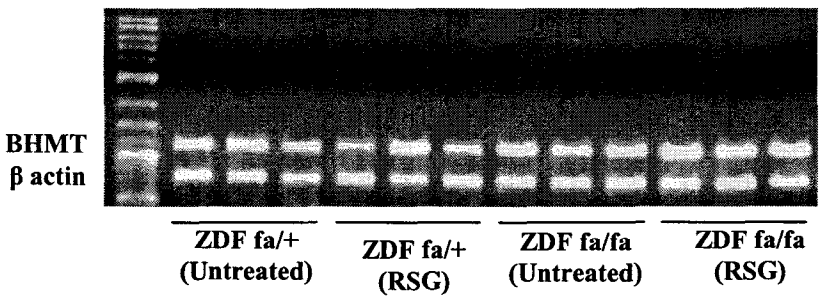
This work on phospholipid metabolism was done by Dr. Rene Jacobs of the University of Alberta. The activities of the enzymes involved in phospholipid metabolism are shown in Table 5.5. CTP: phosphocholine cytidylyltransferase (CT) is the rate limiting enzyme in the Kennedy pathway of PC synthesis. The activity of CT was measured in liver homogenates as well as in the cytosolic and microsomal fractions. At 6 weeks, CT activity did not show any significant differences between the ZDF fa/fa and the ZDF fa/+ rats in any of the fractions tested. However, at 12 weeks the liver homogenates from ZDF fa/fa rats showed a higher level of CT activity than the ZDF fa/+ rats; RSG-treated ZDF fa/fa rats showed the highest activity. Cytosolic CT activity, too, was highest in the ZDF fa/fa (RSG) rats with RSG treatment and the genotype having significant effects. The genotypic difference in CT activity was also evident in the

A. CBS mRNA





B. BHMT mRNA



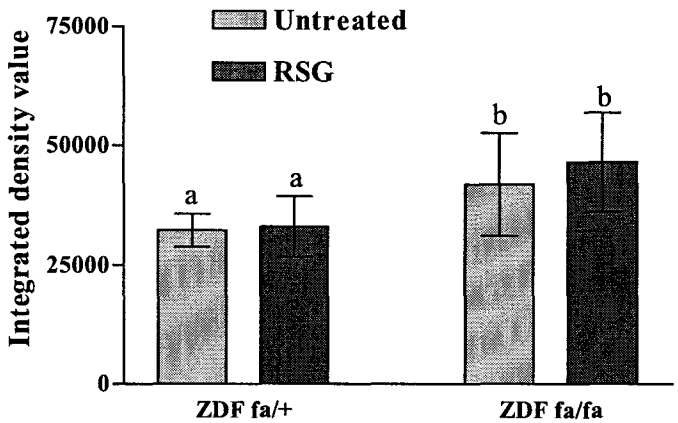


Table 5.5. Hepatic activities of enzymes of phospholipid metabolism

	ZDF fa/+		ZDF fa/fa	
	Untreated	RSG-treated	Untreated	RSG-Treated
CTP:phosphocholine cytidylyltransferase (Homogenate)				
6 weeks	1.8 ± 0.2		2.0 ± 0.3	
12 weeks	1.3 ± 0.1	1.5 ± 0.2[§]	1.9 ± 0.3*	2.3 ± 0.4*[§]
CTP:phosphocholine cytidylyltransferase (Cytosol)				
6 weeks	1.0 ± 0.2		1.2 ± 0.1	
12 weeks	0.6 ± 0.1[a]	0.7 ± 0.02[a,b]	0.7 ± 0.1[b]	0.9 ±0.1[b,c]
CTP:phosphocholine cytidylyltransferase (Microsome)				
6 weeks	2.5 ± 0.8		2.2 ± 0.6	
12 weeks	3.2 ± 0.2[a]	3.1 ± 0.2[a]	4.0 ± 0.4[b]	3.9 ± 0.4[b]
Choline kinase				
6 weeks	9.0 ± 2.1		8.0 ± 3.1	
12 weeks	7.6 ± 0.9	8.4 ± 1.8	7.7 ± 1.0	6.9 ± 2
Phosphatidylethanolamine *N*-methyltransferase				
6 weeks	0.7 ± 0.1		0.6 ± 0.1	
12 weeks	0.7 ± 0.1	0.7 ± 0.02	0.8 ± 0.1*	0.8 ± 0.1*

Data are given as means ± SD for six rats. Enzyme activities are expressed as *nmoles of product synthesized per minute per mg protein.* Differences in superscript letters signify significant differences within each row *($P< 0.05$).* * and § signify significant difference due to genotype and RSG treatment respectively, with Two-way ANOVA.

microsomal fraction but RSG treatment did not show any additional effects. Choline kinase activity did not show any differences between the groups either at 6 weeks or at 12 weeks. Phosphatidylethanolamine methyltransferase (PEMT) catalyzes the conversion of phosphatidylethanolamine to PC in an alternate pathway to the Kennedy pathway. At 12 weeks the activity of PEMT showed a small, though significant, increase in the ZDF fa/fa rats compared to the ZDF fa/+ rats; RSG treatment did not produce any additional effects.

5.2.8 Phosphatidylcholine and Phosphatidylethanolamine levels

Table 5.6 shows the levels of PC and PE in the liver and plasma. Hepatic PC and PE and plasma PC was similar in both groups of rats at 6 weeks of age. At 12 weeks, plasma PC was significantly higher in the ZDF fa/fa group; RSG treatment increased plasma PC further. Liver PC and PE both were significantly lower in the ZDF fa/fa rats compared to the ZDF fa/+ rats; RSG treatment had no significant effects.

5.2.9 Hepatic and plasma Triacylgycerol levels

Table 5.7 shows the hepatic and plasma Triacylglycerol (TG) levels. Both plasma and hepatic TG levels were greatly increased, at 12 weeks, in the ZDF fa/fa (untreated) rats. Two-way ANOVA revealed the genotypic and the treatment effects to be significant, with ZDF fa/fa rats showing a higher level of both plasma and hepatic TG than the ZDF fa/+ rats and RSG treatment acing to reduce it.

Table 5.6. Phosphatidylcholine and Phosphatidylethanolamine levels

	ZDF fa/+		ZDF fa/fa	
	Untreated	RSG-treated	Untreated	RSG-Treated
Plasma Phosphatidylcholine (nmoles/ml)				
6 weeks	1137 ± 191		1367 ± 557	
12 weeks	810 ± 123[a]	900 ± 152[a]	2332 ± 478[b]	3836 ± 495[c]
Hepatic Phosphatidylcholine (nmoles/mg protein)				
6 weeks	136 ± 41		128 ± 24	
12 weeks	138 ± 9[a]	138 ± 7[a]	103 ± 16[b]	112 ± 9[b]
Hepatic Phosphatidylethanolamine (nmoles/mg protein)				
6 weeks	90 ± 18		83 ± 10	
12 weeks	83 ± 6[a]	84 ± 15[a]	58 ± 16[b]	64 ± 15[b]

Data are given as means ± SD for six rats. Differences in superscript letters signify significant differences within each row *($P < 0.05$)*.

Table 5.7. Hepatic and plasma Triacylglycerol levels

	ZDF fa/+		ZDF fa/fa	
	Untreated	RSG-treated	Untreated	RSG-Treated
Hepatic Triacylglycerol (mg/mg protein) 12 weeks	0.04 ± 0.01	0.03 ± 0.00§	0.15 ± 0.04*	0.10 ± 0.02*§
Plasma Triacylglycerol (mmoles/ml) 12 weeks	0.20 ± 0.06^{a}	0.30 ± 0.04^{a§}	7.05 ± 3.9^{b*}	1.90 ± 0.4^{a§*}

Data are given as mean ± SD for six rats. Differences in superscript letters signify significant differences within each row *($P < 0.05$)* with one-way ANOVA. * and § signify significant difference due to genotype and RSG treatment respectively, with Two-way ANOVA.

found to be significant on the hepatic TG levels with ZDF fa/fa rats showing a higher level of TG and the RSG treatment acting to lower the level.

5.3 Discussion

This study reinforces previous work from this laboratory which showed the regulation of homocysteine metabolism by insulin and its counter-regulatory hormones. In the streptozotocin-induced diabetic (type 1) rat model we have shown that plasma homocysteine is reduced in the untreated diabetic rats and that this reduction is prevented when the diabetic rats are treated with insulin (Jacobs *et al*, 1998). This was the first demonstration of the involvement of insulin in the regulation of Hcy metabolism; this was followed by the demonstration in hyperglucagonemic rats of the contribution of the counter-regulatory hormone, glucagon (Jacobs *et al*, 2000). These two studies clearly illustrated the opposing effects of these metabolic hormones on the regulation of Hcy metabolism.

The current study deals with Type 2 diabetes mellitus, where insulin-resistance rather than the absolute lack of insulin is the major contributing factor. Therefore, we treated the rats with the thiazolidinedione, RSG, which along with other TZDs such as troglitazone and pioglitazone have been shown to improve insulin action in a variety of insulin resistant and diabetic rat models as well as in human Type 2 diabetic patients (Olefsky and Saltiel, 2000). RSG was clearly able to restore insulin sensitivity in ZDF fa/fa rats studied here as shown by their plasma glucose and insulin levels. The insulin level in the ZDF fa/fa (RSG) rats at 12 weeks of age, although greatly reduced from the level seen at 6 weeks was higher than in the ZDF fa/fa (untreated) rats. This may indicate

the prevention of β cell death and the maintenance of its mass by RSG treatment as previously shown in this rat model by Finegood *et al*, (2001).

Here, again, we observe that plasma Hcy is decreased in insulin-resistance as well as in early Type 2 diabetes. We also observe that the increase in insulin sensitivity by RSG treatment is able to restore the Hcy level in ZDF fa/fa (RSG) rats towards normal similar to what was reported in type 1 diabetic rats treated with insulin (Jacobs *et al*, 1998). However, unlike insulin treatment of Streptozotocin-diabetic rats, RSG treatment of ZDF fa/fa rats was unable to restore the Hcy levels to complete normalcy.

This discrepancy could be explained by a very interesting change observed in the activity of one of the enzymes involved in Hcy removal. Betaine:homocysteine methyltransferase (BHMT), catalyzes the reaction where Hcy is remethylated to methionine using a methyl group donated by betaine. Our earlier studies both in type 1 (Ratnam *et al*, 2006) and Type 2 (Wijekoon *et al*, 2005) diabetes showed that the reduction observed in plasma Hcy in both these types of diabetes was accompanied by increases in the activity of BHMT. Nieman *et al* (2004) have also found this increased activity of BHMT in streptozotocin-diabetic rats. The present study too was able to show an increase in hepatic BHMT activity at the insulin resistant as well as the diabetic stage. Surprisingly, we also observed a significantly positive effect of RSG treatment on BHMT activity: both ZDF fa/+ (RSG) and ZDF fa/fa (RSG) rats showed higher BHMT activity than the respective untreated groups. This is surprising in the light of the work done in our lab by Ratnam *et al* (2006) where, in the type 1 diabetic model, insulin treatment was shown to return the increased BHMT activity back to the level seen in the control rats. In a rat hepatoma cell line (H4IIE cells), Ratnam *et al* showed that treatment with

glucocorticoids increased the level of BHMT mRNA and the rate of BHMT mRNA synthesis while insulin reduced the abundance of BHMT mRNA as well as the de novo transcription rate of the gene (Ratnam *et al*, 2006). Thereby, they were able to show very convincingly the direct effects of both insulin and glucocorticoids on BHMT gene expression. However, in the present study, we observed a further increase in the activity of BHMT rather than a reduction that would be expected according to the above information.

This phenomenon could be explained by looking at how ZDF fa/fa rats become diabetic, the mode of action of RSG in modifying Type 2 diabetes and finally the contribution made by BHMT to phospholipid (PL) metabolism, in addition to its direct action in remethylating Hcy. It is now generally accepted that Type 2 diabetes is associated with obesity. More specifically, the ectopic accumulation of fat in muscle, liver as well as β cells is widely regarded to be instrumental in the etiology of insulin resistance and possibly in the ultimate destruction of pancreatic β cells, leading to the development of Type 2 diabetes (McGarry, 2002). The mutation in the leptin receptor, OB-R, is the only genetic abnormality that has been identified in ZDF rats (Unger, 1997). The ensuing leptin resistance results in obesity and a generalized intracellular fat overload in adipocytes as well as non adipocytes (Unger, 1997). The importance of intracellular TG accumulation in liver and skeletal muscle in the etiology of insulin resistance has been shown comprehensively in humans as well as in animals (McGarry, 2002). That ectopic accumulation of fat especially in the β cells, leading to their destruction, is the cause of diabetes in ZDF rats is supported by the finding that pair-feeding of pre-diabetic ZDF fa/fa rats to their lean counterparts leads to the reduction in islet TG content, and

prevents hyperglycemia, loss of β cell GLUT 2 and minimizes the loss of glucose-stimulated insulin secretion (Ohneda *et al*, 1995).

The importance of betaine as well as BHMT, overlooked over the years in favor of the more famous counterparts such as the B vitamins of Hcy metabolism and the transsulfuration enzymes, is beginning to gain recognition as a major player in regulation of Hcy metabolism. Betaine supplementation has been shown to be effective in preventing a rise in plasma Hcy after methionine intake in subjects with mild HHcy (Steenge *et al*, 2003), and doses of betaine in the range of dietary intake were shown to reduce fasting and post-methionine loading plasma Hcy concentrations in healthy men and women (Olthof *et al*, 2003). Schwahn *et al* (2003) showed betaine supplementation to lower plasma Hcy levels in mice heterozygous or homozygous for the disruption of the *mthfr* gene as well as in their wild type littermates. Very interestingly, they were also able to show a significant negative relationship between plasma Hcy and betaine concentrations in humans with cardiovascular disease. The same group also reported the ability of betaine to lower plasma Hcy in a mouse model of moderate HHcy due to a heterozygous disruption of the *Cbs* gene (Schwahn *et al*, 2004).

These effects on plasma Hcy levels by betaine feeding and the resultant increase in the flux through BHMT are not surprising given the role of BHMT in removal of Hcy. However, BHMT is also thought to play a role in phospholipid (PL) metabolism. In the mouse, the daily flux of PL through the liver is estimated to approximate the entire hepatic PL pool (Walkey *et al*, 1998). The CDP-choline or the Kennedy pathway occurs in all nucleated cells and accounts for 60-80% of hepatic phosphatidylcholine (PC) biosynthesis (Sundler and Akesson, 1975). The second mode of PC synthesis,

quantitatively significant only in the liver, converts phosphatidylethanolamine (PE) to PC with the consecutive transfer of three methyl groups from three SAM molecules, catalyzed by the enzyme phosphatidylethanolamine methyltransferase (PEMT). BHMT is regarded as the first enzyme in the three-enzyme pathway culminating in the conversion of PE to PC, the other two being MAT and PEMT (Sehayek *et al*, 2003). In line with this, both BHMT and PEMT have been found in the bile canalicular membrane of mice where they are believed to be involved in local canalicular PC biosynthesis and secretion of PL into bile (Sehayek *et al*, 2003). A recent study by Jacobs *et al* (2005) in liver specific *CT-α* knockout mice showed flux through PEMT to be increased two-fold and this increase was accompanied by increases in the activity of both BHMT and MAT.

In addition to being the predominant PL in bile and eukaryotic cell membranes, PC is also the primary PL in all classes of lipoproteins in mammals (Vance and Vance, 1985) and is required for the secretion of VLDL (Yao and Vance, 1988, Yao and Vance, 1989a, Yao and Vance, 1989b, Vermeulen *et al*, 1997). Although the Kennedy pathway has been shown to be essential for the maintenance of normal plasma VLDL levels (Vermeulen *et al*, 1997) PC derived from PE has also been shown to be necessary for normal VLDL secretion (Nishimaki-Mogami *et al*, 2002). Recently, hepatocytes isolated from male *Pemt*$^{-/-}$ mice were found to secrete 50% less TG in VLDL/LDL lipoprotein fractions than hepatocytes from *Pemt* $^{+/+}$ mice (Noga *et al*, 2002). They also observed a 70% reduction in the secretion of apoB100 in VLDL/LDL. Transfection of McArdle hepatoma cells lacking PEMT with PEMT cDNA was also shown to increase the secretion of TG in VLDL. Noga and Vance (2003) found *Pemt* $^{-/-}$ mice to have a defect in VLDL and apoB100 secretion compared with *Pemt*$^{+/+}$ mice when fed a high fat/high

cholesterol diet, but with a gender specificity. In addition PEMT knockout mice were shown to have hepatic steatosis and abnormal levels of hepatic choline metabolites despite ingesting the recommended dietary intake of choline (Zhu *et al*, 2003).

BHMT, because of its association with PEMT and therefore with secretion of VLDL from the liver, appears to play a role in lipid mobilization and secretion. In support of this assumption is the large increase in apoB mRNA abundance and secretion seen in BHMT-transfected McArdle cells (Sowden *et al*, 1999). A recent publication by the same group was able to confirm these findings *in-vivo* where feeding rats a methionine-restricted betaine-supplemented diet resulted in a 4-fold induction of hepatic BHMT and a 3-fold induction of ApoB mRNA compared with methionine restriction alone (Sparkes *et al*, 2006). This group also observed an increased secretion of VLDL along with a 45% reduction in liver TG. These experiments are reinforced by the finding that ethanol feeding increases BHMT activity (Barak *et al*, 1987) as well as apoB mRNA and apo B secretion (Wang *et al*, 1994). Very interestingly, dietary betaine is known to promote the generation of hepatic SAM and protect the liver from ethanol-induced fatty infiltration (Barak *et al*, 1993). BHMTs involvement with fatty liver was further demonstrated by the finding that synthesis of BHMT is continuously enhanced in fatty livers of thyroidectomized chickens (Shibata *et al*, 2003).

Therefore, our finding of increased BHMT activity in ZDF fa/fa (untreated) rats comes as no surprise. What is interesting is the increased activity seen in the RSG-treated rats. As stated earlier, this finding appears to be at variance with our previous report where BHMT expression was shown to be negatively regulated by insulin (Ratnam *et al*, 2006). An exploration of the mode of action of TZDs suggests an explanation for this

phenomenon. One means by which TZDs improve insulin sensitivity is by increasing insulin-dependent glucose disposal and reducing hepatic glucose output (Saltiel and Olefsky, 1996). Another well documented effect of TZDs is adipocyte differentiation, in both white (Tontonoz *et al*, 1994) and brown (Tai *et al*, 1996) adipose tissue. Partitioning of circulating lipids away from muscle and liver into adipose tissue, known as "lipid steal" is attributed to TZDs (Ye *et al*, 2004). In favour of this mechanism, TZDs have been shown to reduce circulating levels of lipids as well as lowering their accumulation in ectopic locations such as liver, skeletal and cardiac muscle and pancreatic islets (Spiegelman 1998, Oakes *et al*, 1994, Oakes *et al*, 1997, Zhou *et al*, 2000, Shimabukuro *et al*, 1998, Higa *et al*, 1999). In addition, TZDs have been shown to directly increase FFA exchange between plasma and adipose tissue in ZDF rats (Oakes *et al*, 2001). Ye *et al* (2004) found a two-fold increase in adipose tissue fatty acid uptake after treatment of normal rats with RSG for 7 days. Our data also displayed a reduction in plasma as well as hepatic levels of TG with RSG treatment. Along with these reductions we observed a highly significant increase in the fat pad weight which implies a redistribution of lipids. Redistribution of lipids from the liver to the periphery is accomplished by the release of mainly TG-rich VLDL from the liver. Therefore, the involvement of BHMT with VLDL production, as outlined above, could very well account for the increased BHMT activity seen with RSG treatment. Although the rat BHMT promoter region is not yet mapped it would be interesting to find whether it contains a PPAR response element.

In the present study, the activity of CT, the rate limiting enzyme in the CDP-choline pathway of PC synthesis is significantly increased in the ZDF fa/fa rats compared to the ZDF fa/+ rats, which could be attributed to the increased hepatic TG

levels in the ZDF fa/fa rats. What would then be the cause for the increase in CT activity in the ZDF fa/fa (RSG) rats, as they had a decreased hepatic TG level? This may provide clues as to the means by which RSG brings about this reduction in hepatic TG. If this reduction is due to the increased flux of TG from the liver to the adipose tissue then there will be an increased need to produce VLDL, the major phospholipid of which is PC. The increase in CT activity seen with the RSG treatment favors this argument. In addition, we also see a significant increase in plasma PC in the ZDF fa/fa rats with the ZDF fa/fa (RSG) rats having a significantly higher level of PC than the ZDF fa/fa (untreated) rats. Hepatic PEMT however only showed a very slight, but significant, increase in the ZDF fa/fa rats compared to the ZDF fa/+ rats with no effects of the RSG treatment. We also see a significant reduction in both PE and PC in the liver in the ZDF fa/fa rats which may indicate an increased contribution from the PEMT pathway to the increased plasma PC level. The effect of RSG on PC production through the PEMT pathway is difficult to assess, however, without conducting flux studies, as RSG treatment was successful in increasing the activity of BHMT, decreasing the activity of MAT and had no effect on PEMT itself.

The increase in the activity of MAT in the ZDF fa/fa (untreated) rats and its normalization by insulin sensitization agrees with the findings of Gil *et al* (1997) where they showed the induction of MAT expression by glucocorticoids and its inhibition by insulin. We also observed an increase in the activity of the transsulfuration enzymes, CBS and CGL, in the ZDF fa/fa rats compared to the ZDF fa/+ rats at both 6 and 12 weeks similar to our earlier findings. Although significant, the increase in CBS activity in the ZDF fa/fa group was very small and was not corrected by the RSG treatment. However,

the RSG treatment was able to significantly reduce the liver weight of the treated ZDF fa/fa rats, which would reduce the total CBS activity in the treated rats. CGL, which converts cystathionine into cysteine on the other hand, was very effectively normalized by the RSG treatment. The possible regulatory role of CGL has been generally ignored. However, the importance of this enzyme in the transsulfuration flux is evident in premature infants, whose hepatic CGL activity is absent or low in concentration and who have low plasma cysteine levels and glutathione synthesis rates (Vina *et al*, 1995). The same group has also shown that in rats exposed to surgical stress, CGL activity is reduced by ~40%, leading to reduced rates of cysteine synthesis in isolated hepatocytes (Vina *et al*, 1992). Therefore, the normalization of CGL activity to the lower level seen in the ZDF fa/+ rats could play a role in the increase in plasma Hcy brought about by RSG treatment. It would be of interest to measure cystathionine levels in these situations.

Our data also indicate a reciprocal regulation of the two remethylation reactions. At 6 weeks of age the increase in the activity of BHMT in the ZDF fa/fa rats was accompanied by a significant decrease in the activity of methionine synthase which we also observed at 12 weeks of age. Remarkably the increase in BHMT activity that occurred with the RSG treatment was also accompanied by a reciprocal reduction in methionine synthase activity. This attempt to balance the rate of remethylation may also contribute towards the normalization of plasma Hcy but, without flux studies, firm conclusions cannot be drawn. Similar changes in the two enzymes have also been shown by Nieman *et al* (2004) in streptozotocin-induced diabetic rats and are also a common occurrence in alcoholic liver diseases where a reduction in the activity of methionine synthase is accompanied by increased BHMT activity (Barak *et al*, 1996).

The significantly higher food intake seen in the ZDF fa/fa (untreated) rats and its normalization upon RSG treatment may also be responsible for some of the changes observed in this study as it is well established that diet plays a major role in the regulation of Hcy metabolism.

In conclusion, it is clear that the hormonal changes that are prevalent in insulin-resistance and Type 2 diabetes act at several sites in the pathway of methionine metabolism. It would be interesting to further investigate the direct effects of RSG on BHMT activity as it will provide us with insight into the physiological functions of this remarkable enzyme.

Chapter 6

Type 2 Diabetes and vascular function: effects of Rosiglitazone

6.1 Background

The first evidence of the importance of the endothelium for vascular homeostasis was the discovery of the absolute necessity of an endothelial cell layer for acetylcholine-induced relaxation of rabbit aorta (Furchgott and Zawadzki, 1980). Since then endothelial dysfunction has been implicated in the pathophysiology of a number of forms of cardiovascular disease including hypertension, coronary artery disease, chronic heart failure, peripheral artery disease and chronic renal failure (Endemann and Schiffrin, 2004). Most often endothelial dysfunction in vitro has been described by a reduction in endothelium-dependent relaxation of vascular smooth muscle (Endemann and Schiffrin, 2004).

During the active manifestation of Type 1 and Type 2 diabetes, blood vessels have been reported to display the same *in vitro* characteristic of endothelial dysfunction as in cardiovascular diseases (De Vriese *et al*, 2000; Pannirselvam *et al*, 2003; Saenz *et al*, 1989; Johnston *et al*, 1993; Ting *et al*, 1996). In fact, insulin resistance prior to the development of Type 2 diabetes was found to be associated with endothelial dysfunction in blood vessels (Steinberg *et al*, 1996). Hyperglycemia *per se* has been suggested to be a major factor in the development of endothelial dysfunction in diabetes in both humans and animals (Williams *et al*, 1997, Triggle et *al*, 2003). One explanation of this endothelial dysfunction has been that an increased oxidative stress caused by hyperglycemia decreases the bioavailability of a co-factor of nitric oxide synthase 3 (endothelial NO synthase; eNOS), tetrahydrobiopterin. This leads to an uncoupling of the normal electron transfer to L-arginine during eNOS catalytic activity, which instead

produces super oxide; NO is identified as the primary mediator of ACh-induced relaxation in large caliber blood vessels (Triggle *et al*, 2003).

HHcy has been identified as an independent risk factor for atherosclerosis; up to 40% of patients diagnosed with premature coronary artery and peripheral vascular disease and venous thrombosis have increased plasma homocysteine levels (Clarke *et al*, 1991, den Heijer *et al*, 1996, Selhub *et al*, 1995). Homocysteine has also been shown to cause endothelial dysfunction and apoptotic cell death in endothelial cells (Hossaine *et al*, 2003, Zhang *et al*, 2001) as well as smooth muscle cells (Buemi *et al*, 2001). In apolipoprotein E (apo E)-deficient mice, the induction of hyperhomocysteinemia either by dietary or genetic means was shown to accelerate atherosclerosis (Zhou *et al*, 2001). Likewise a reduction of plasma Hcy with folic acid or a combination of B vitamins in these animals was shown to attenuate the development of atherosclerosis, and thus, implicated Hcy as being a causative agent (Zhou *et al*, 2003, Hofmann *et al*, 2001). The mechanism by which Hcy causes endothelial dysfunction has not been fully elucidated. A mechanism involving oxidative stress-induced reduction in the bioavailability of NO has been proposed for endothelial dysfunction associated with hyperglycemia, and may mediate some of the effects of Hcy-induced endothelial dysfunction (Faraci and Lentz, 2004).

In Chapter 3 we found that plasma Hcy levels were actually decreased in both insulin resistant (6 weeks of age ZDF fa/fa) and diabetic rats (12 weeks of age ZDF fa/fa). The purpose of this study was to determine if arteries from these rats exhibited *in vitro* characteristics of endothelial dysfunction in spite of the reduced plasma levels of Hcy. We hypothesized that if either hyperinsulinemia alone or hyperglycemia was sufficient to produce endothelial dysfunction then the arteries of ZDF fa/fa rats would exhibit less

endothelium-dependent relaxation of vascular smooth muscle than the untreated ZDF fa/+ rats in an age-dependent manner. Furthermore we expected that arteries from rosiglitazone-treated diabetic rats would be protected from such vascular dysfunction because the mechanism of action for this agonist of peroxisome-proliferator activated receptor gamma (PPAR-γ) includes an apparent ability to reduce oxidative stress along with normalization of plasma insulin and glucagon.

6.2 Results

6.2.1 Contractile function of arteries from ZDF rats

Contractions induced by 120 mM KCl were used to assess the viability of arteries from ZDF fa/+ and ZDF fa/fa rats. These KCl-induced increases in isometric tension were not different between these strains at 6, 12 and 18 weeks (Table 6.1). Likewise, KCl-induced contractions of arteries from RSG-treated rats were not different than untreated rats of each strain at 12 and 18 weeks of age (Table 6.1). Contractions of arteries induced by the α_1-adrenoreceptor agonist cirazoline were not significantly different between groups at 6, 12 and 18 weeks of age (Table 6.2, Fig. 6.1). The cirazoline-induced contractions of arteries from RSG-treated rats were not different from the contractions of arteries from untreated rats of each strain at 12 and 18 weeks of age (Table 6.2, Fig, 6.1).

Table. 6.1 Contractile responses of ZDF rat arteries induced by high extracellular concentration of KCl

Age (weeks)	6		12				18			
Strain	ZDF fa/+	ZDF fa/fa	ZDF fa/+		ZDF fa/fa		ZDf fa/+		ZDF fa/fa	
Treatment	Untreated	Untreated	Untreated	RSG	Untreated	RSG	Untreated	RSG	Untreated	RSG
Contraction (mN)	6.5 ± 0.6	6.7 ± 0.7	10.0 ± 0.4	9.6 ± 0.6	9.0 ± 0.6	8.2 ± 0.6	10.4 ± 0.4	8.7 ± 1.1	7.9 ± 1.1	10.6 ± 1.5

Rings of rat mesenteric arteries (2 mm lengths) were mounted in myograph chambers under isometric tension conditions using a resting baseline tension of 2.5 mN. Contractile activity was measured by the increase in force above resting tension caused by the application of high concentration of KCl (120 mM) to each artery. Data are means ± SEM (n = 4 for each group) and were analyzed separately within each age category. Student's unpaired t-test was used to compare artery responses of rat strains at 6 weeks of age. 2-way ANOVA was used to determine the effects of strain, treatment and interaction of strain with treatment at 12 and 18 weeks of age and was followed by 1-way ANOVA with Newman-Keuls post-hoc test for multiple comparisons of groups within each age category. $P>0.05$, all statistical comparisons.

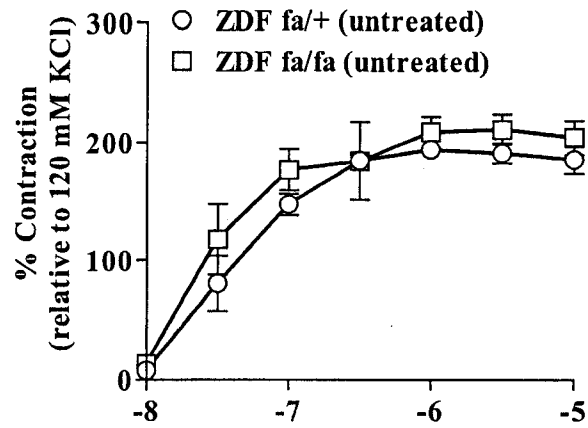
Table 6.2 Maximal contractile effect and potency for the α_1-adrenoreceptor agonist cirazoline in mesenteric arteries of untreated and RSG-treated ZDF rats at 6, 12 and 18 weeks of age.

Age (weeks)	6		12				18			
Strain	ZDF fa/+	ZDF fa/fa	ZDF fa/+		ZDF fa/fa		ZDf fa/+		ZDF fa/fa	
Treatment	Untreated	Untreated	Untreated	RSG	Untreated	RSG	Untreated	RSG	Untreated	RSG
E_{max} (%)	197 ± 5	211 ± 13	168 ± 7	173 ± 9	188 ± 12	206 ± 24	176 ± 23	175 ± 12	211 ± 17	167 ± 7
log EC_{50}	-7.5 ± 0.1	-7.7 ± 0.2	-7.5 ± 0.1	-7.6 ± 0.1	-7.5 ± 0.1	-7.6 ± 0.2	-8.1 ± 0.6	-7.9 ± 0.3	-8.1 ± 0.4	-8.1 ± 0.2

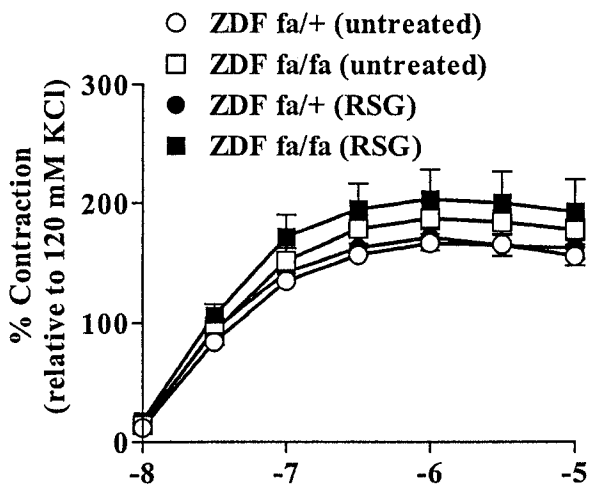
E_{max} values represent contractile responses standardized to % of contraction responses-induced by 120 mM KCl (see Table 6.1). Data are means ± SEM (n = 4 for each group) and were analyzed separately within each age category. Student's unpaired t test was used to compare artery response of rat strains at 6 weeks of age. 2-way ANOVA was used to determine the effects of strain, treatment and interactions at 12 and 18 weeks of age and was followed by 1-way ANOVA Newman-Keuls post hoc test for multiple comparisons of groups within each age category. $P > 0.05$, all statistical comparisons.

Figure 6.1. Cumulative concentration-contraction response relationships for the α_1-adrenoreceptor agonist cirazoline in mesenteric arteries of RSG-treated and untreated ZDF rats at 6, 12 and 18 weeks of age.

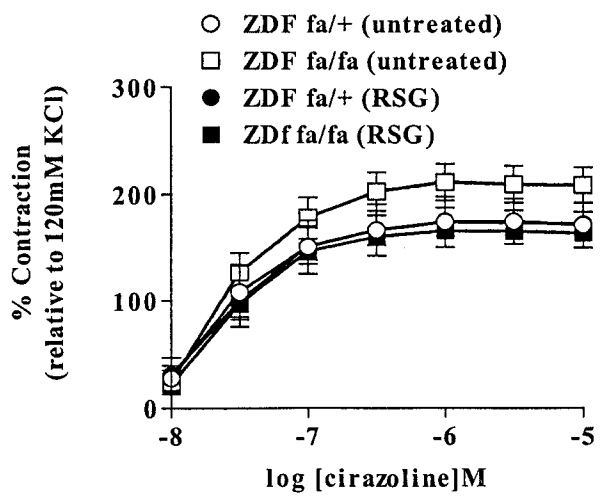
A. 6 weeks



B. 12 weeks



C. 18 weeks



6.2.2 Acetylcholine-induced Endothelial function of the small mesenteric arteries from ZDF rats

6.2.2.1 Acetylcholine-induced relaxations

Acetylcholine (ACh)-induced relaxations of arteries from ZDF fa/fa rats were not different than relaxations of arteries from ZDF fa/+ rats at 6, 12 and 18 weeks of age (Fig 6.2, Table 6.3). These ACh-induced relaxations were not different between RSG-treated and untreated rats of each strain at 12 and 18 weeks of age (Fig 6.2, Table 6.3).

The NO synthases inhibitor L-NAME (300 μM), was able to significantly reduce the ACh-induced relaxation at all ages (Fig 6.2). Arteries from 12 weeks old ZDF fa/+ (untreated) and ZDF fa/fa (untreated) were reduced by ~18 % and ~ 29 %, respectively, relative to the relaxation caused by Ach in the absence of L-NAME. The relaxation of arteries from ZDF fa/+ (RSG) and ZDF fa/fa (RSG) rats were reduced by ~39 % and 51 % respectively. At 18 weeks of age, L-NAME treatment of arteries from ZDF fa/+ (untreated) and ZDF fa/fa (untreated) rats reduced ACh-induced relaxations by ~ 32 % and ~ 39 %, respectively, relative to relaxation caused by Ach alone. The reduction seen in the ZDF fa/+ (RSG) and ZDF fa/fa (RSG) rats was ~ 56% and ~45 % respectively. However, ACh-induced relaxations of L-NAME-treated arteries from ZDF fa/+ and ZDF fa/fa rats were not statistically different than arteries that were not treated with L-NAME at 6 weeks of age (Table 6.4, Fig 6.2).

In the presence of the cyclooxygenases inhibitor indomethacin (10 μM) plus the presence of L-NAME (300 μM), ACh-induced relaxations of ZDf fa/+ (untreated), ZDF fa/fa (untreated), ZDF fa/+ (RSG) and ZDF fa/fa (RSG) rats were reduced by ~ 66 %, ~ 55 %, ~ 64 % and ~ 72 % respectively, relative to Ach alone at 12 weeks of age. This

combination of indomethacin + L-NAME reduced ACh-induced relaxations of arteries from ZDF fa/+ (untreated), ZDF fa/fa (untreated), ZDF fa/+ (RSG) and ZDF fa/fa (RSG) by ~ 66 %, ~66 %, ~ 85% and ~69% respectively, relative to Ach alone at 18 weeks of age (Table 6.4, Fig 6.3).

Combined treatment of arteries from ZDF fa/+ and ZDF fa/fa rats with IK_{Ca} inhibitor TRAM-34 (10 μmole/liter) plus L-NAME (300 μmole/liter) reduced ACh-induced relaxations by ~ 91%, ~85 %, ~91% and ~88% respectively for ZDF fa/+ (untreated), ZDF fa/fa (untreated), ZDF fa/+ (RSG) and ZDF fa/fa (RSG), respectively, relative to Ach induced relaxation in the absence of any inhibitors at 12 weeks of age. This combination of TRAM-34 and L-NAME reduced ACh-induced relaxations of arteries from ZDF fa/+ (untreated) and ZDF fa/fa (untreated) by ~ 81 % and ~ 66 %, respectively, and ZDF fa/+ (RSG) and ZDF fa/fa (RSG) by ~89% and ~78% respectively relative to Ach alone at 18 weeks of age (Table 6.4, Fig 6.3).

In the presence of the combination of SK_{Ca} inhibitor apamin (1 μmole/liter) + TRAM-34 (10 μmole/liter) + L-NAME (300 μmole/liter), ACh-induced relaxations of arteries from ZDF fa/+ (untreated) and ZDF fa/fa (untreated) rats were reduced by ~ 74 % and ~ 92 %, respectively, relative to Ach alone at 12 weeks of age. The reduction seen in the ZDF fa/+ (RSG) and ZDF fa/fa (RSG) rats were ~91% and ~93% respectively. The combination of apamin + TRAM-34 + L-NAME reduced ACh-induced relaxations of arteries from ZDF fa/+ (untreated), ZDF fa/fa (untreated), ZDF fa/+ (RSG) ZDF fa/fa (RSG) rats by ~84 %, ~ 78 %, ~98% and ~90% respectively, relative to Ach alone at 18 weeks of age (Table 6.4, Fig 6.3).

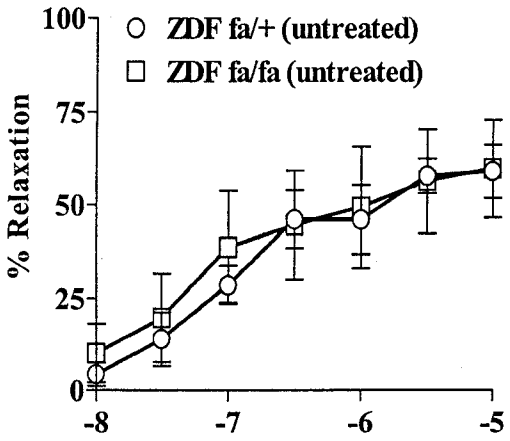
Table 6.3. Maximum relaxation effect and potency of acetylcholine on cirazoline-contracted mesenteric arteries of untreated and RSG-treated ZDF rats.

Age (weeks)	6		12				18			
Strain	ZDF fa/+	ZDF fa/fa	ZDF fa/+		ZDF fa/fa		ZDf fa/+		ZDF fa/fa	
Treatment	Untreated	Untreated	Untreated	RSG	Untreated	RSG	Untreated	RSG	Untreated	RSG
E_{max} (%)	59 ± 7	60 ± 13	74 ± 4	80 ± 5	85 ± 4	86 ± 1	74 ± 6	84 ± 2	68 ± 15	86 ± 2
log EC_{50}	-7.0 ± 0.2	-7.1 ± 0.2	-9.0 ± 0.5	-8.3 ± 0.2	-8.2 ± 0.2	-8.3 ± 0.1	-7.4 ± 0.3	-7.4 ± 0.1	-7.6 ± 0.3	-7.8 ± 0.1

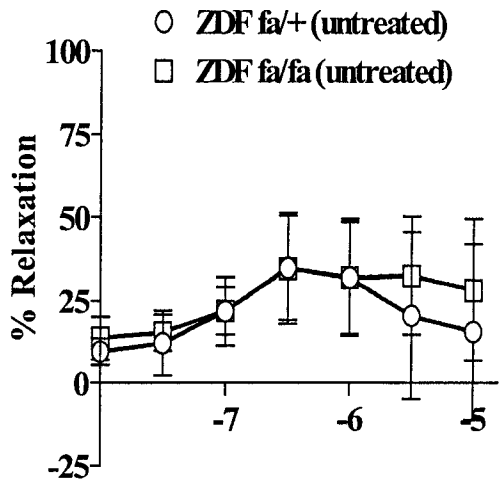
E_{max} values represent % relaxation responses standardized by the reversal of cirazoline-induced submaximal contraction (100 % relaxation = complete reversal of contraction). Data are means ± SEM (n = 4 for each group) and were analyzed separately within each age category. Student's unpaired t test was used to compare artery response of rat strains at 6 weeks of age. 2-way ANOVA was used to determine the effects of strain, treatment and interactions at 12 and 18 weeks of age and was followed by 1-way ANOVA with Newman-Keuls post hoc test for multiple comparisons of groups within each age category. $P > 0.05$, compared to untreated for all comparisons.

Figure 6.2: Effects of L-NAME on cumulative concentration-relaxation response relationships for acetylcholine in small mesenteric arteries of untreated and RSG-treated ZDF rats at 6, 12 and 18 weeks of age

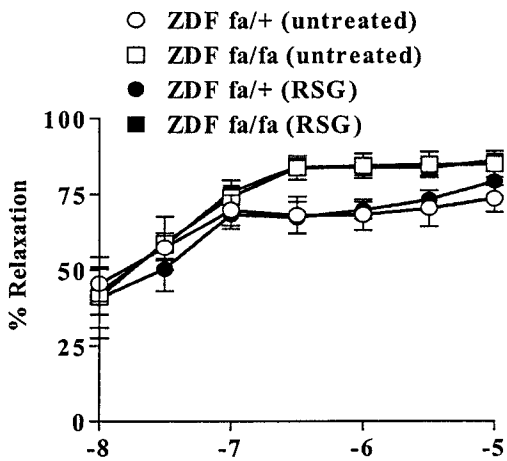
A. 6 weeks



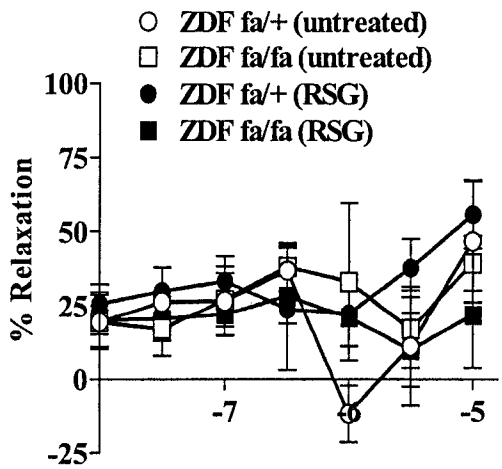
D. 6 weeks (+ L-NAME)



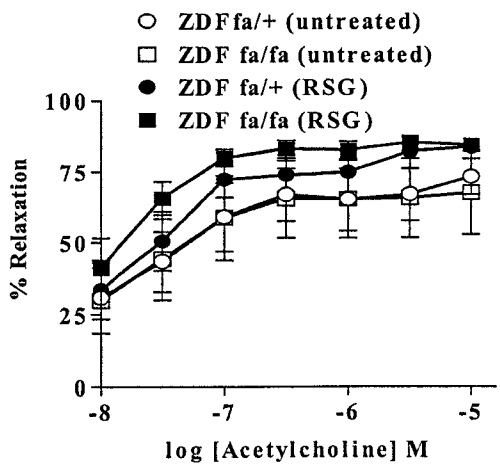
B. 12 weeks



E. 12 weeks (+ L-NAME)



C. 18 weeks



F. 18 weeks (+ L-NAME)

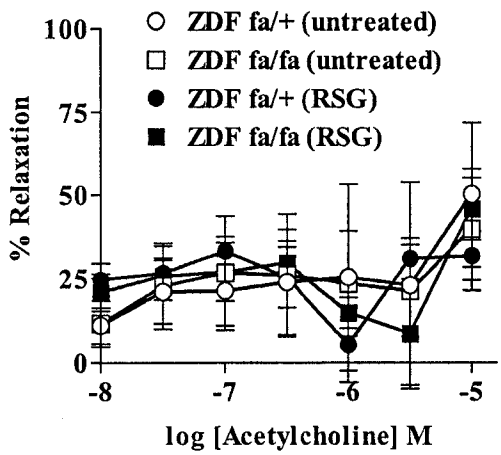


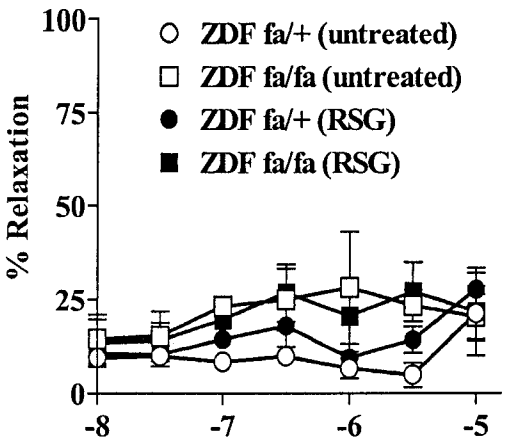
Table 6.4. Maximum ACh-induced relaxations of cirazoline-contracted mesenteric arteries from ZDF fa/+ and ZDF fa/fa rats in the presence of various combinations of inhibitors of NO synthases, cyclooxygenases, IK_{Ca} and SK_{Ca}.

Age (weeks)	6		12				18			
Strain	ZDF fa/+	ZDF fa/fa	ZDF fa/+		ZDF fa/fa		ZDF fa/+		ZDF fa/fa	
Treatment	Untreated	Untreated	Untreated	RSG	Untreated	RSG	Untreated	RSG	Untreated	RSG
L-NAME	42 ± 14	38 ± 17	61 ± 8	49 ± 3	60 ± 11	42 ± 9	50 ± 22	37 ± 6	41 ± 17	47 ± 9
L-NAME + Indomethacin	-	-	25 ± 8*	29 ± 4*	38 ± 11*	24 ± 9*	25 ± 11*	13 ± 5*	23 ± 9*	27 ± 6*
L-NAME + TRAM-34	-	-	7 ± 2*‡	7 ± 6*‡	13 ± 8*‡	10 ± 3*‡	14 ± 7*	9 ± 7*	23 ± 5*	19 ± 10*
L-NAME + TRAM-34 + Apamin	-	-	19 ± 7*‡	7 ± 4*‡	7 ± 2*‡	6 ± 1*‡	12 ± 8*‡	2 ± 2*‡	15 ± 4*‡	9 ± 6*‡

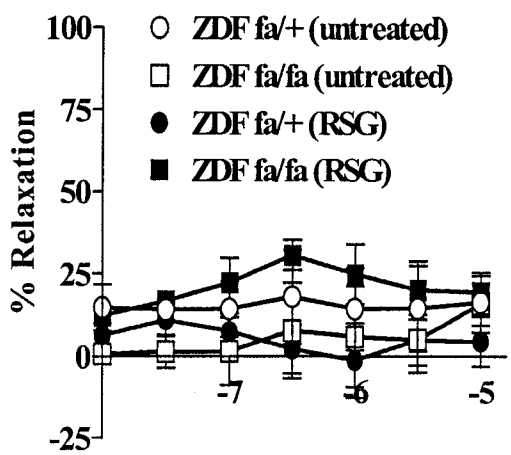
Rings of mesenteric arteries from ZDF rats were treated with various combinations of inhibitors of NO synthase (L-NAME), COX 1/2 (indomethacin), IK_{Ca} (TRAM-34) and SK_{Ca} (apamin) prior to obtaining ACh-induced relaxation responses of cirazoline-contracted tissues. These data were obtained during the same experiments described for the arteries in Table 6.2, but are presented separately. Data are presented as mean ± SEM (n = 4 for each experimental group) and were analyzed using 2-way ANOVA followed by 1-way ANOVA and Newman-Keuls post hoc test for multiple comparisons between groups. * $P<0.05$, compared to L-NAME treated arteries. ‡ $P<0.05$, compared to treatment with L-NAME + Indomethacin. A dash symbol indicates that the experimental group was not completed.

Figure 6.3. ACh-induced relaxations of mesenteric arteries from untreated and RSG-treated ZDF rats in the presence of L-NAME + indomethacin (A and D), L-NAME + TRAM-34 (B and E) and L-NAME + TRAM-34 + Apamin (C and F) at 12 and 18 weeks of age.

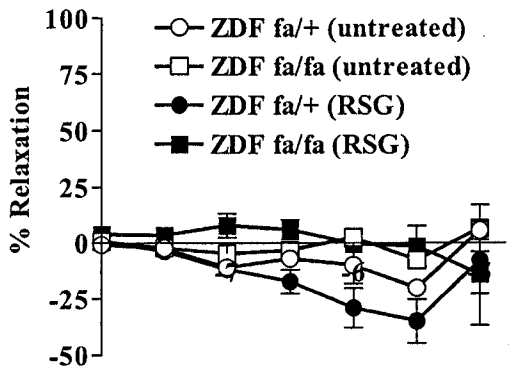
A. 12 weeks



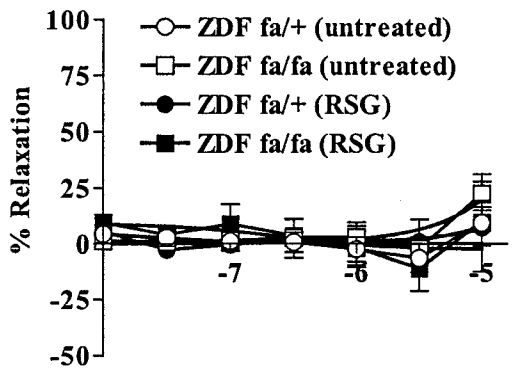
D. 18 weeks



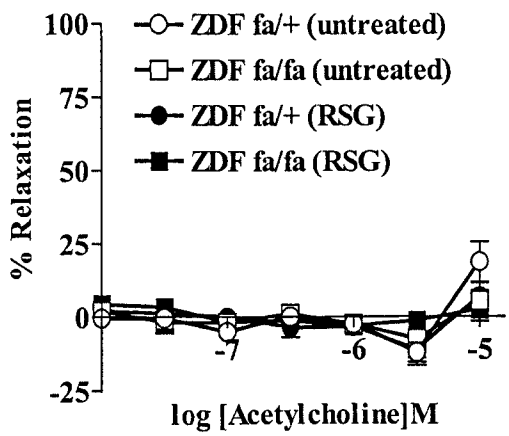
B. 12 weeks



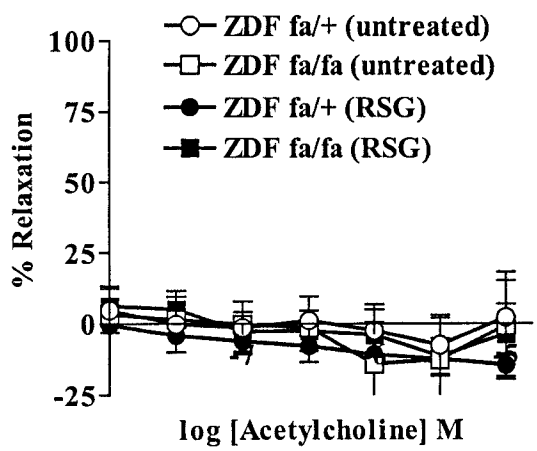
E. 18 weeks



C. 12 weeks



F. 18 weeks



6.2.3 SNP-induced relaxations

SNP-induced relaxation of arteries from untreated ZDF fa/fa rats was not significantly different than relaxation of arteries from untreated ZDF fa/+ rats at 6, 12 and 18 weeks of age (Table 6.5, Fig 6.4). However, SNP-induced relaxations of arteries from RSG-treated ZDF fa/+ and RSG-treated ZDF fa/fa rats, were increased by ~100 % and ~50 %, respectively, relative to the untreated rats at 12 weeks of age (Table 6.5, Fig. 6.4). Likewise SNP-induced relaxations of arteries from RSG-treated ZDF fa/+ and RSG-treated ZDF fa/fa rats, were increased by ~50 % and ~100 %, respectively, relative to untreated rats at 18 weeks of age.

In the presence of the NO synthase inhibitor L-NAME (300 μmole/liter) (Table 6.6, Fig 6.4) SNP-induced relaxations of arteries from the untreated ZDF fa/+ and ZDF fa/fa rats increased by ~80% and 67%, respectively, relative to arteries that were not treated with L-NAME at 12 weeks of age. L-NAME treatment of arteries from untreated ZDF fa/+ and ZDF fa/fa increased SNP-induced relaxation by ~136 % and ~123 %, respectively, relative to relaxation caused by SNP alone at 18 weeks of age.

SNP-induced relaxations of L-NAME-treated arteries from RSG-treated ZDF fa/+ rats increased by ~5% and ~32% respectively from the relaxation caused by SNP alone at 12 and 18 weeks of age. SNP-induced relaxations of L-NAME treated arteries from RSG-treated ZDF fa/fa rats increased by ~38% from the relaxation seen in the absence of L-NAME at both 12 and 18 weeks of age.

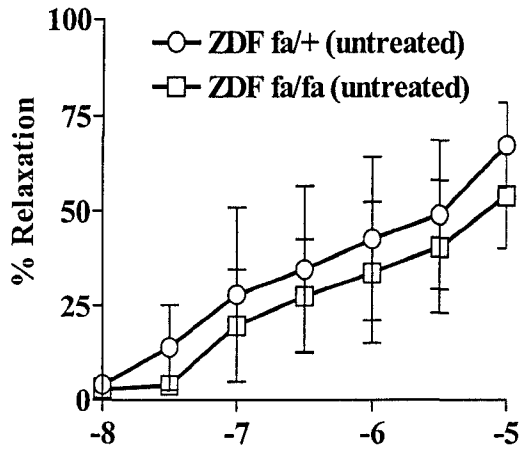
Table 6.5. Maximum relaxation responses and potency for SNP in cirazoline-contracted mesenteric arteries of untreated and RSG-treated ZDF rats.

Age (weeks)	6		12				18			
Strain	ZDF fa/+	ZDF fa/fa	ZDF fa/+		ZDF fa/fa		ZDf fa/+		ZDF fa/fa	
Treatment	Untreated	Untreated	Untreated	RSG	Untreated	RSG	Untreated	RSG	Untreated	RSG
E_{max} (%)	67 ± 11	54 ± 14	38 ± 5	78 ± 8*	43 ± 8	65 ± 6*	33 ± 7	48 ± 7*	26 ± 9	53 ± 6*
log EC_{50}	-6.3 ± 0.5	-6.2 ± 0.4	-7.1 ± 0.2	-6.7 ± 0.2	-7.4 ± 0.5	-6.7 ± 0.2	-7.0 ± 0.5	-7.4 ± 0.4	-6.2 ± 1.7	-6.0 ± 0.4

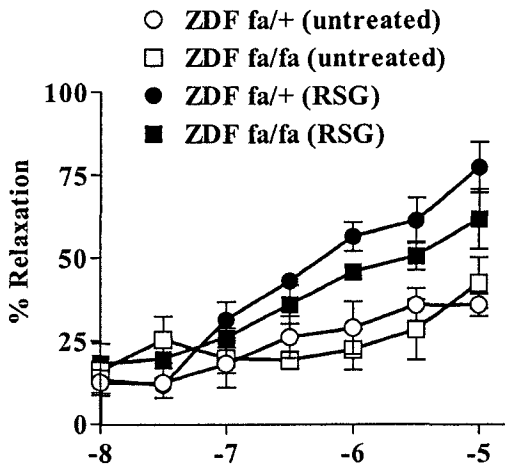
Data are means ± SEM (n = 4 for each group) and were analyzed separately within each age category. Student's unpaired t test was used to compare artery responses of the rat strains at 6 weeks of age. 2-way ANOVA was used to determine the effects of strain, treatment and the interaction of strain with treatment at 12 and 18 weeks of age. 1-way ANOVA with Newman-Keuls post hoc test was used for multiple comparisons of groups within each age category. * $P<0.05$, compared to untreated rat.

Figure 6.4. Effects of L-NAME on cumulative concentration-relaxation response relationships for sodium nitroprusside (SNP) in mesenteric arteries of untreated and RSG-treated ZDF rats at 6, 12 and 18 weeks of age.

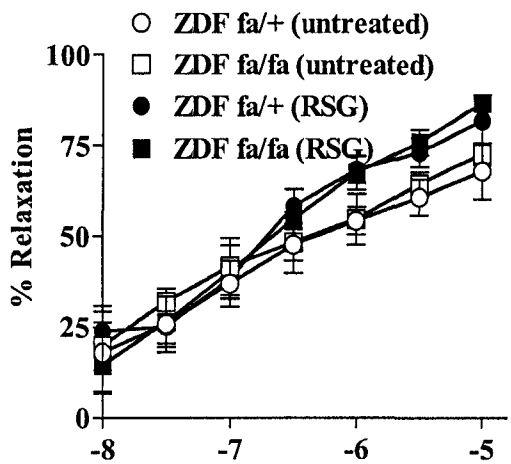
A. 6 weeks



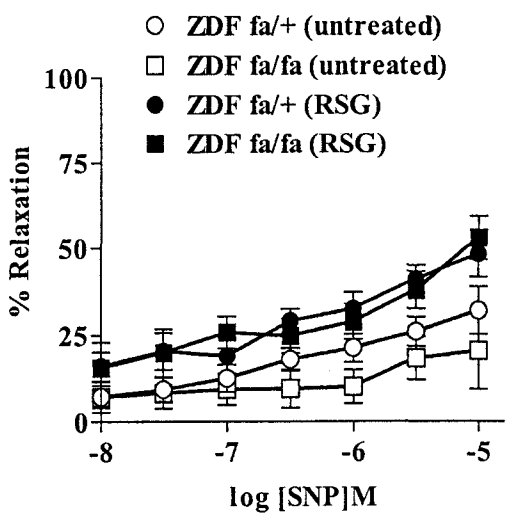
B. 12 weeks



D. 12 weeks (+ L-NAME)



C. 18 weeks



E. 18 weeks (+ L-NAME)

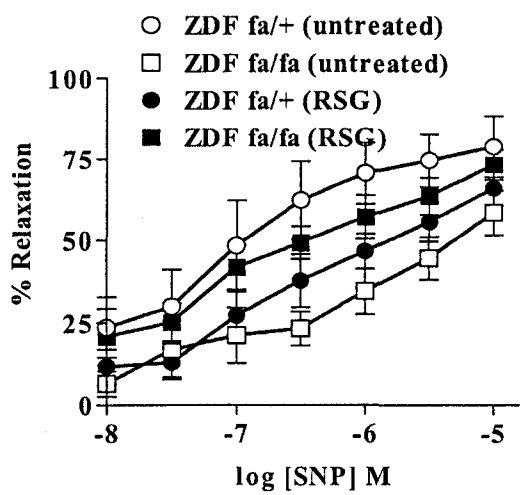


Table 6.6. Maximum SNP-induced relaxations of cirazoline-contracted mesenteric arteries from untreated and RSG-treated ZDF rats in the presence of the inhibitor of NO synthases L-NAME (300 μmole/liter).

Age (weeks)	12				18			
Strain	ZDF fa/+		ZDF fa/fa		ZDf fa/+		ZDF fa/fa	
Treatment	Untreated	RSG	Untreated	RSG	Untreated	RSG	Untreated	RSG
E_{max} %	68 ± 8	82 ± 7*	72 ± 3	86 ± 2*	78 ± 7	66 ± 5	58 ± 7	73 ± 5
log EC_{50}	-6.9 ± 0.4	-7.1 ± 0.3	-7.2 ± 0.2	-6.8 ± 0.2	-7.2 ± 0.3	-6.7 ± 0.5	-6.6 ± 0.4	-6.9 ± 0.2

These data were obtained in the same experiments described in Table 6.5. To make the presentation of data more clear, L-NAME treated arteries have been separated from the arteries represented in Table 6.5. E_{max} values represent % relaxation responses standardized by the reversal of cirazoline-induced sub-maximal contraction (100 % relaxation = complete reversal of contraction). Data are means ± SEM (n = 4 for each group) and were analyzed separately within each age category. 2-way ANOVA was used to determine the effects of strain, treatment and interactions and was followed by 1-way ANOVA with Newman-Keuls post-hoc test for multiple comparisons of groups. Group analyses included the data from Table 6.5. * $P < 0.05$, compared to untreated rats.

6.2.4 PAR2 agonist-induced relaxations

The relaxation responses of arteries to PAR2 activation were tested with the potent PAR2 agonist 2-furoyl-LIGRLO-amide (2fli) (McGuire *et al*, 2004). 2fli-induced relaxations of arteries from ZDF fa/fa rats were not different than relaxations of arteries from ZDF fa/+ rats at 6, 12 and 18 weeks of age (Fig 6.7, Table 6.5). These 2fli-induced relaxations were not different between RSG-treated and untreated rats of each strain at 12 and 18 weeks of age (Table 6.7, Fig. 6.5).

In the presence of the NO synthases inhibitor L-NAME (300 μmole/liter), 2fli-induced relaxations of arteries from untreated and RSG-treated ZDF fa/+ rats were reduced by ~16 % and ~30 %, respectively, relative to 2-fli alone at 12 weeks of age. This treatment with L-NAME reduced 2fli-induced relaxations of arteries from untreated and RSG-treated ZDF fa/fa by ~22 % and ~55 %, respectively, relative to 2-fli alone at 12 weeks of age (Fig. 6.5).

In the presence of the NO synthase inhibitor L-NAME (300 μmole/liter), 2fli-induced relaxations of arteries from untreated and RSG-treated ZDF fa/+ were reduced by ~30 % and ~66 %, respectively, relative to 2-fli alone at 18 weeks of age. This treatment with L-NAME reduced 2fli-induced relaxations of arteries from untreated and RSG-treated ZDF fa/fa by ~24 % and ~25 %, respectively, relative to 2-fli alone at 18 weeks of age (Fig. 6.5).

6.2.5 Arachidonic acid induced relaxations

The relaxation responses of arteries to treatment with arachidonic acid were tested. Arachidonic acid-induced relaxations of arteries from ZDF fa/fa rats were not

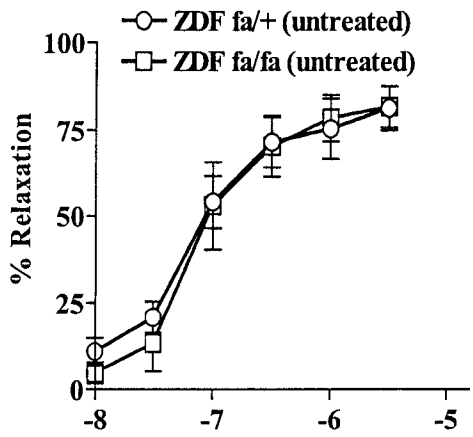
Table 6.7. Maximum relaxation responses and potency for 2fli in cirazoline-contracted mesenteric arteries of untreated- and RSG-treated ZDF rats.

Age (weeks	6		12				18			
Strain	ZDF fa/+	ZDF fa/fa	ZDF fa/+		ZDF fa/fa		ZDf fa/+		ZDF fa/fa	
Treatment	Untreated	Untreated	Untreated	RSG	Untreated	RSG	Untreated	RSG	Untreated	RSG
E_{max} %	81 ± 6	82 ± 6	81 ± 6	82 ± 2	83 ± 4	87 ± 3	78 ± 5	83 ± 4	73 ± 11	80 ± 5
log EC_{50}	-7.1 ± 0.1	-7.1 ± 0.1	----	----	----	----	----	----	----	----

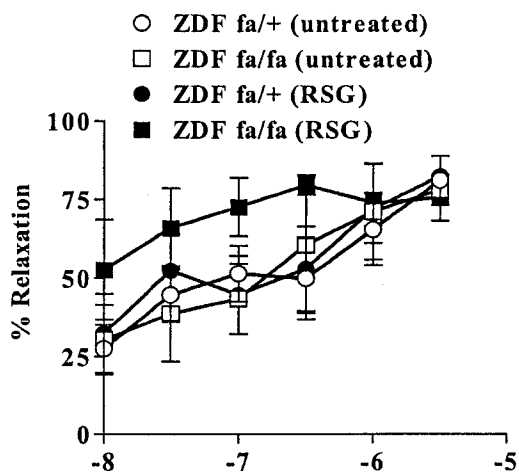
E_{max} values represent % relaxation responses standardized by the reversal of cirazoline-induced sub-maximal contraction (100 % relaxation = complete reversal of contraction). Data are means ± SEM (n = 4 for each group) and were analyzed separately within each age category. Student's unpaired t test was used to compare artery response of rat strains at 6 weeks of age. 2-way ANOVA was used to determine the effects of strain, treatment and interactions at 12 and 18 weeks of age and was followed by 1-way ANOVA for multiple comparisons of groups within each age category. $P > 0.05$, compared to untreated for all comparisons. ---- indicates that the values could not be determined from the concentration-response relationships.

Figure 6.5. Effects of L-NAME on cumulative concentration-relaxation response relationships for PAR2 agonist 2fli in mesenteric arteries of untreated and RSG-treated ZDF rats at 6, 12 and 18 weeks of age.

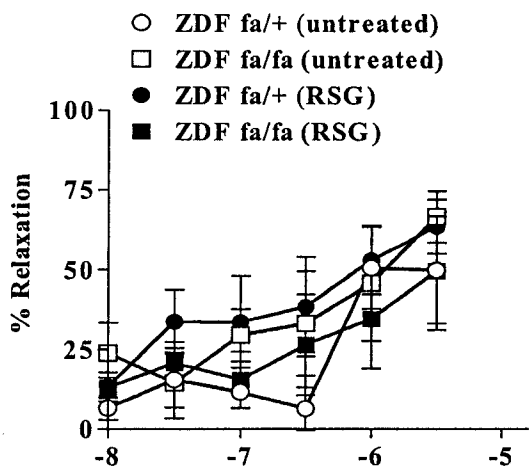
A. 6 weeks



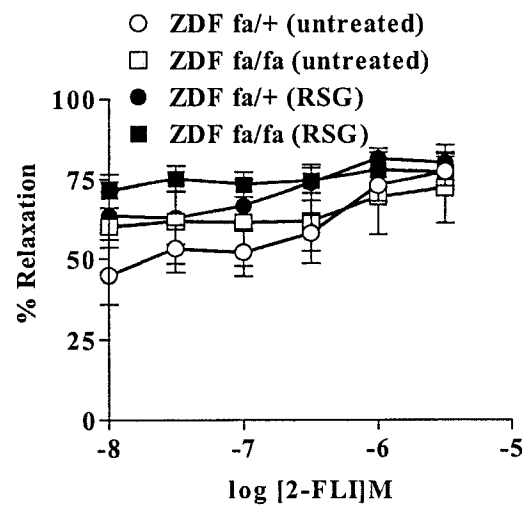
B. 12 weeks



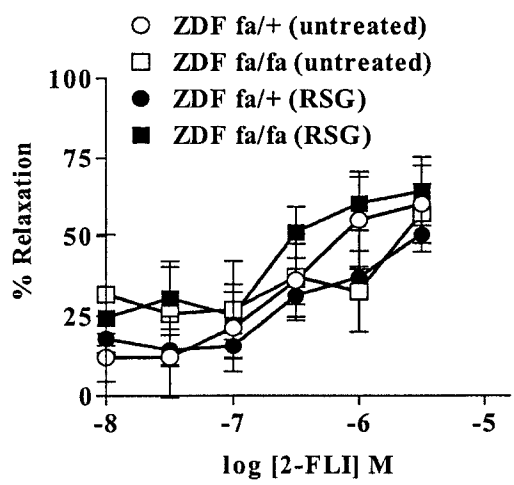
D. 12 weeks (+ L-NAME)



C. 18 weeks



E. 18 weeks (+ L-NAME)



different than relaxations of arteries from ZDF fa/+ rats at 6, 12 and 18 weeks of age (Fig 6.8, Table 6.6). These arachidonic acid-induced relaxations were not different between RSG-treated and untreated rats of each strain at 12 and 18 weeks of age (Table 6.8, Fig. 6.6).

6.3 Discussion

6.3.1 General Findings

The primary purpose of this study was to assess the arteries from insulin resistant-non-diabetic ZDF and diabetic ZDF rats for evidence of endothelial dysfunction in spite of low plasma levels of Hcy in these animals. In insulin-resistant ZDF fa/fa rats (6 weeks old) and fully diabetic ZDF fa/fa rats (12 and 18 weeks old), the endothelial-dependent relaxations of arteries by ACh and 2fli were found to be equivalent to those observed in age-matched untreated ZDF fa/+ rats. These endothelium-dependent relaxations were similar to those observed in age-matched RSG-treated ZDF fa/fa rats, whose plasma Hcy levels were brought towards normal by the RSG treatment. Thus, we did not find any evidence of endothelial dysfunction in small caliber arteries from ZDF fa/fa rats at either low or relatively normalized plasma levels of Hcy. These results suggest that the presence of insulin-resistance alone or hyperglycemia alone is not sufficient to produce endothelial dysfunction, which may require additional factors including those associated with the presence of HHcy.

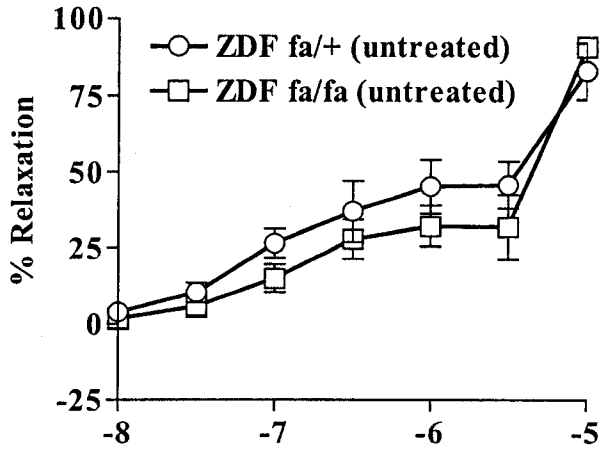
Table 6.8. Maximum relaxation responses and potency for arachidonic acid in cirazoline-contracted mesenteric arteries of untreated and RSG-treated ZDF rats.

Age (weeks)	6		12				18			
Strain	ZDF fa/+	ZDF fa/fa	ZDF fa/+		ZDF fa/fa		ZDf fa/+		ZDF fa/fa	
Treatment	Untreated	Untreated	Untreated	RSG	Untreated	RSG	Untreated	RSG	Untreated	RSG
E_{max} %	83 ± 9	91 ± 2	83 ± 3	73 ± 5	70 ± 7	75 ± 6	60 ± 7	68 ± 9	71 ± 7	75 ± 4
log EC_{50}	-6.2 ± 0.3	-5.6 ± 0.2	-5.9 ± 0.2	-5.7 ± 0.4	-6.2 ± 0.5	-5.5 ± 0.4	-5.3 ± 0.2	-5.3 ± 0.1	-5.1 ± 0.1	-5.7 ± 0.2

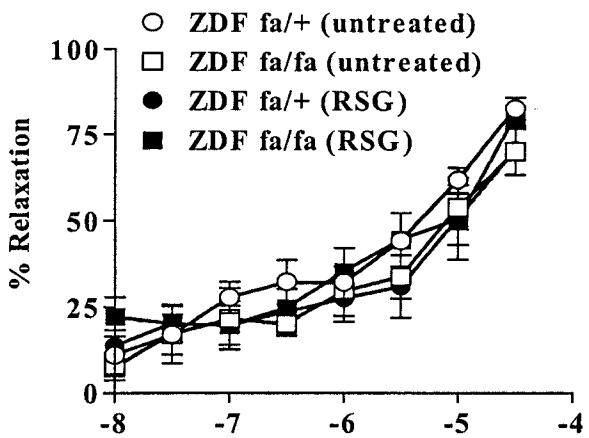
E_{max} values represent % relaxation responses standardized by the reversal of cirazoline-induced sub-maximal contraction (100 % relaxation = complete reversal of contraction). Data are means ± SEM (n = 4 for each group) and were analyzed separately within each age category. Student's unpaired t test was used to compare artery response of rat strains at 6 weeks of age. 2-way ANOVA was used to determine the effects of strain, treatment and interactions at 12 and 18 weeks of age and was followed by 1-way ANOVA with Newman-Keuls post-hoc test for multiple comparisons of groups within each age category. $P > 0.05$, compared to untreated for all comparisons.

Figure 6.6. Cumulative concentration-relaxation response relationships for arachidonic acid Na Salt in small mesenteric arteries of untreated and RSG-treated ZDF rats at 6, 12 and 18 weeks of age.

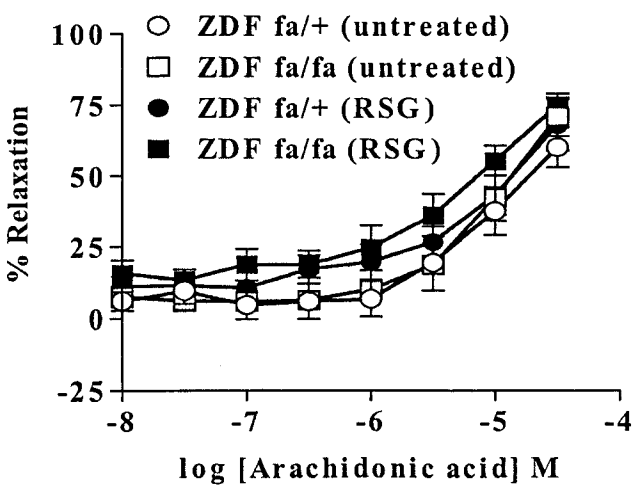
A. 6 weeks



B. 12 weeks



C. 18 weeks



6.3.2 Relaxations by ACh

The present study did not find any differences between the relaxations to ACh of small mesenteric arteries of the groups studied at any of the ages. These data do not support previous findings of general age-dependent development of endothelial dysfunction of arteries in the ZDF fa/fa rat. Brodsky *et al* (2004) and Oltman *et al.* (2006) have reported that Ach-induced relaxations of aortic rings from ZDF fa/fa rats were reduced relative to controls. In contrast to these studies, our study used primarily small caliber arteries. Oltman *et al.* (2005, 2006) reported that Ach-induced vasodilation of pressurized mesenteric arteries, epineurial arterioles and coronary arteries from hyperglycemic ZDF fa/fa rats were reduced relative to age-matched ZDF-lean (+/?) rats. Nevertheless, a negative finding relating to endothelial dysfunction which was similar to ours has been reported in small caliber arteries. Bohlen and Lash (1995) reported that vasodilations in the intestinal microvasculature of both 22-25 and 35-40 weeks old ZDF fa/fa rats were similar to lean littermates. Based on these studies, some types of arteries from ZDF fa/fa rats have appeared to display characteristics of endothelial dysfunction, while some have not. When our results have been included with these studies however, the data appear to favor a conclusion that ACh-induced relaxations of mesenteric arteries from ZDF fa/fa rats are not impaired.

6.3.2.1 Mechanisms of ACh relaxations:

Previous studies with ZDF fa/fa rats reported a loss of ACh-mediated endothelium-dependent hyperpolarization of mesenteric smooth muscle as characteristic of endothelial dysfunction (Burnham *et al.*, 2006a, 2006b). Whereas the L-arginine-nitric

oxide pathway is recognized as the major vasodilator pathway in response to ACh in large caliber arteries, other mechanisms collectively referred to as EDHFs (endothelium-derived hyperpolarization factors) cause vasorelaxation in small caliber arteries (McGuire et al, 2001). Indeed this distinction was evident by the inability of the NOS inhibitor, L-NAME to completely abolish vasorelaxation in response to ACh, since arteries were still able to display a considerable relaxation in the presence of L-NAME. We therefore, conducted further inhibitor studies based on similar studies with rat mesenteric arteries to identify the possible alternative mechanisms responsible for this vasodilatory process; primarily, COX-1/2 products (indomethacin) and Ca^{2+}-activated K-channels (TRAM-34 and apamin). Our results showed that both COX and the Kca channels were involved in NOS independent vasorelaxation as seen by the reductions in relaxation in the presence of their respective inhibitors. Kca channels have been shown to play an essential role in the actions of EDHF (McGuire et al., 2001 for review). This was also evident in the present study as inhibition of the Kca channels clearly had a greater effect on vasorelaxation than inhibition of COX. In contrast to the observations by Burnham *et al.*, (2006a, 2006b) of reduced ACh-induced membrane hyperpolarisations, the current isometric tension data failed to give any indication of a reduction in either NO or EDHF vasodilation mechanisms, which would have been taken as evidence for the presence of endothelial dysfunction in this model. However, it is possible that those observations by Burnham *et al* (2006a, 2006b) do not translate to functional deficiencies in vascular smooth muscle relaxation.

6.3.3 Relaxations by PAR2 agonist

In support of the data obtained using ACh, maximal relaxation of mesenteric arteries by activation of endothelial Proteinase-Activated Receptor 2 (PAR2; G protein coupled receptor 11) in ZDF fa/fa rats was not different than in the non diabetic rats. This is the first study to describe the effects of PAR2 activation in the ZDF fa/fa rat. PAR2 is an endothelial receptor whose activation either after the proteolytic cleavage of its N-terminus by several trypsin-like serine proteinases or sans enzyme, by specific activating peptides which mimic the proteolytic activation, causes vascular smooth muscle relaxation (Hollenberg and Compton, 2002). 2fli has been shown to be the most potent and selective activating peptide agonist for PAR2 (McGuire et al., 2004). Nevertheless we interpret these results with some caution because it would appear from the cumulative concentration-response relationships to PAR2 agonist that there was in fact an increase in sensitivity to 2fli for ZDF fa/fa rats after 6 weeks of age. PAR-2 expression has been shown to increase during stress to the vasculature and also in the presence of various cytokines and pro-inflammatory substances (McGuire, 2004). Increased PAR2 expression may be considered consistent with displayed increases in pro-inflammatory substances in the ZDF rat model also (Hotamisligil *et al*, 1993). Similarly, in non-obese diabetic (NOD) mice, a model of Type 1 diabetes, high or severe glycosuria was shown to provoke an increase in the vasodilatory response to PAR2 stimulation (Roviezzo *et al.* 2005). Notwithstanding these observations, an increased sensitivity to 2fli-induced vasodilation could indicate any number of upregulations involving signal transduction of PAR2. PAR2 vasodilator response in small caliber arteries is reported to be mediated by endothelium-dependent hyperpolarization of vascular smooth muscle via Ca^{2+}-activated K^{+} channels in

mice and rats (McGuire, 2004; McGuire et al, 2002a, 2004a, 2004b). In large caliber arteries NO is reported to mediate the vascular smooth muscle relaxation responses in humans, rats and mice (McGuire et al, 2002b; McGuire, 2004). Our current results were consistent with these former observations since inhibition of NOS by L-NAME resulted only in a partial inhibition of the response to 2fli. Together these data obtained with a PAR2 agonist are consistent with our conclusions that no dysfunction of endothelial vasodilator function by either NO mediated or EDHF mediated mechanisms was observed.

6.3.4 Relaxations by Arachidonic-Acid

We did not observe any differences in the vasorelaxation in response to exogenous application of AA in any of the groups of mesenteric arteries. These results are not consistent with the general results of 2 previous studies. In the first study it was reported that dilation of small mesenteric arteries by exogenous application of arachidonic acid appeared to be impaired in ZDF fa/fa rats (Zhou *et al.* 2005). Arachidonic acid is an important endogenous precursor of vasoactive eicosanoids and is converted to vasoactive mediators via three major enzymatic pathways; lipoxygenase (LOX), cytochrome P450 epoxygenase and cyclooxygenase (COX). The products formed by these pathways are important regulators of vasoreactivity with the cytochrome P450 pathway being responsible for AA mediated dilation of human coronary arterioles (Miura and Gutterman, 1998) while the LOX pathway is responsible for dilation of rat mesenteric arteries (Miller *et al*, 2003). Zhou *et al* (2005) identified a reduction of 12-lipoxygenase protein and activity as the cause of impairment. In a second study, dilation of small

coronary arteries of ZDF rats was also shown to be impaired in response to exogenous application of AA and was associated with impairment of the activation of the large conductance Ca^{+2} activated K^+ channels due to reduced PGI_2 synthase activity (Lu *et al*, 2005). Aside from different arteries being used in the second study, in both of the above mentioned studies, data were obtained from ZDF rats after ~ 4 weeks of hyperglycemia. This would make these rats approximately the same age as the 12 weeks old rats in the present study. We conclude that we could not reproduce the findings of these previous studies and that there was no evidence of a reduction in responsiveness to AA in the vascular smooth muscle of ZDF fa/fa rats.

6.3.5 Relaxations by SNP

The data obtained using SNP to cause relaxations did not provide any evidence of a reduction in vascular smooth muscle responsiveness to NO in arteries from ZDF fa/fa relative to arteries from ZDF fa/+ rats. However, both the ZDF fa/fa (untreated) and the ZDF fa/+ (untreated) rats showed a reduced relaxation responsiveness to SNP relative to these values reported in the literature (Zhou *et al*, 2005). Nonetheless, these data were consistent with previous studies which did not find any changes (Zhou *et al*, 2005).

6.3.6 Rosiglitazone effects on vascular function.

Rosiglitazone treatment *in vivo* failed to affect the relaxations of arteries from ZDF fa/+ and ZDF fa/fa rats by ACh, 2fli and AA relative to untreated animals. These data could be considered simply as being consistent with the lack of observed indicators for vascular dysfunction in our study of ZDF fa/fa rats. However, Walker *et al.* (1999) have reported

that RSG treatment was able to partially prevent the impairment of ACh induced relaxation in Zucker Fatty rats, a model of insulin resistance with hyperinsulinemia and normoglycemia. Ours is the first study to test the effects of RSG treatment on PAR2 and AA relaxations, but its implications are not clear to us. When SNP-induced relaxations were assessed in RSG-treated rats by way of assessing RSG action on vascular smooth muscle sensitivity to NO, we found that RSG treatment of rats enhanced SNP-induced maximum relaxations of arteries from all groups of rats. These enhanced SNP relaxations in arteries from RSG-treated rats at 12 weeks were similar to the relative values reported in untreated ZDF rats in other studies (Zhou *et al*, 2005). These data are consistent with a study which reported of improved endothelium-independent vasodilation in response to another NO donor, Nitroglycerine, in non-diabetic patients with the metabolic syndrome after treatment with RSG (Wang *et al*, 2004). SNP releases NO spontaneously and is independent of eNOS and thus, is able to bypass the requirement for the endothelium in causing vasorelaxation. Relaxation caused by SNP is regarded as endothelium-independent, in contrast to the endothelium-dependent vasorelaxation caused by ACh. NO generated from SNP is thought to follow the same downstream mechanisms as endothelial-derived NO such as by ACh in the vascular smooth muscle cells of large arteries to cause vasorelaxation. Since the response of these arteries to ACh mediated NO vasodilation appears to be preserved we suggest that the effect of RSG was not due to an action on the downstream mediators of NO i.e. guanylyl cyclase and cyclic GMP. One can only speculate that somehow the RSG action enables an exogenous source of NO to cause vascular smooth muscle relaxation differently than endothelial derived NO. We

deemed this effect of RSG to at least indicate that the selected dose of RSG had indeed caused a change in vascular function that was detected and measurable by the assay.

NOS inhibition by L-NAME caused the same degree of block of relaxations by ACh and 2fli in arteries from RSG-treated rats relative to untreated rats. These results would appear to support the evidence for normal endothelial derived NO and EDHF in the arteries from ZDF fa/fa rats. To our knowledge the effects of RSG treatment on ACh-induced EDHF relaxations have not been reported earlier. L-NAME treatment enhanced the relaxations by SNP of arteries from all groups of rats tested at both 12 and 18 weeks except for the already extra responsive arteries from rosiglitazone-treated ZDF fa/+ rats. L-NAME treatment of arteries has been known for some time to enhance SNP-induced relaxations (Moncada *et al.* 1991). The leading hypothesis remains that a decrease in the basal levels of cyclic GMP arising from eNOS inhibition (or physical removal of the endothelium) leads to greater fold-increases in cyclic GMP upon exogenous activation of soluble guanylyl cyclase (Moncada *et al.* 1991). The data did not inform us of any specific mechanistic explanation for the loss of this nitrovasodilator sensitization effect by L-NAME in RSG-treated ZDF fa/+ rats. We speculate that sensitization could have already been maximized prior to L-NAME treatment of arteries in these rats. This study did not have sufficient evidence to elucidate a possible mechanism, but has provided sufficient to warrant further investigation of this action of RSG treatment.

6.3.7 Hcy plasma levels, rosiglitazone, vascular function

ZDF fa/fa (untreated) rats had significantly lower plasma Hcy levels at all three ages which were studied. Lentz *et al* (2000) reported that relaxation of aortic rings in

response to acetylcholine did not differ in CBS $^{+/+}$ and CBS $^{+/-}$ mice fed a control diet, but greatly differed between the 2 groups when fed a folate-deficient diet with the relaxation being markedly impaired in CBS $^{+/-}$ mice. Plasma Hcy did not differ between the two groups on the control diet, but was greatly elevated in the CBS $^{+/-}$ mice on the folate deficient diet. These results would support the notion that plasma Hcy was a determinant of vascular relaxation. Diabetic hyperglycemia is also described as a causative determinant for the endothelial dysfunction observed in diabetes (Triggle *et al*, 2003). Initially it was simply thought that the reduction in Hcy that we observed in ZDF fa/fa rats may have been able to counterbalance the increase in plasma glucose and thus, normalized net endothelial function in the present study. This interpretation would be consistent with the data obtained in the ZDF rats studied by Bohlen and Lash (1995) which also displayed hyperglycemia despite their normal response to ACh. However, RSG treatment of ZDF fa/fa rats was able to normalize hyperglycemia while increasing the plasma Hcy level towards normal, but had no apparent effect on endothelial-dependent relaxations relative to untreated rats. Therefore, it seems reasonable to conclude that hyperglycemia alone was not sufficient to produce endothelial dysfunction in the mesenteric arteries of ZDF fa/fa rats. We propose that elevation of plasma levels of homocysteine in this model would lead to endothelial dysfunction.

6.3.8 Impact of information regarding vascular function, homocysteine, hyperglycemia, diabetes.

In conclusion endothelium-dependent and –independent vasorelaxation were preserved during insulin resistance as well as during frank diabetes. The normalizing

effects of RSG on plasma levels of glucose and Hcy and yet, its lack of enhancement effect on endothelial vasodilator function supports the idea that plasma Hcy may serve as a balance point for development of endothelial dysfunction in this model of Type 2 diabetes.

Chapter 7

General Discussion and conclusions

Mild hyperhomocysteinemia has been identified as a risk factor for many diseases, including cardiovascular disease. A 5μmol/liter increase in total plasma Hcy has been shown to be comparable to an increase in cholesterol of 0.5 mmol/liter, in increasing the risk of coronary artery disease.

Recently, however, uncertainty has arisen regarding the degree of risk associated with elevated plasma Hcy. This may be related to one form of Hcy being more important in causing vascular disease than total Hcy as well as to the specific location of Hcy (Stamm and Reynolds, 1999). It is also likely that the Type of cardiovascular disease is important. The recent Heart Outcome Prevention Evaluation (HOPE) 2 study, which used vitamin B therapy to reduce plasma Hcy found no beneficial effects on the risk of death from cardiovascular causes or myocardial infarction but there was a reduction in stroke incidence (Lonn *et al*, 2006). There are certain situations however, where the risk posed by elevated Hcy is undisputable. Type 2 diabetes in one of these and HHcy has been shown to be a stronger risk factor for cardiovascular disease and for mortality in Type 2 diabetic patients than in non-diabetics (Hogeveen *et al*, 1998).

The principal objective of our work was to identify the changes that occur in plasma Hcy in insulin resistance and Type 2 diabetes and to identify the metabolic steps that lead to these changes. For this, we used the leptin-receptor-defective Zucker Diabetic Fatty rats which are known to go through an initial insulin resistant phase before going on to develop frank Type 2 diabetes.

As discussed in Chapter 3 of this thesis, we were able to show that hormonal changes that occur in insulin resistance and Type 2 diabetes act at several points of the

methionine metabolic pathway leading to alterations in methionine and Hcy metabolism. Our study showed that, similar to what has been shown in Type 1 diabetic humans and rats, plasma total Hcy is reduced in insulin resistance as well as in Type 2 diabetes (Figure 3.1). We also saw that this reduction is brought about by increases in the transsulfuration as well as the remethylation routes of Hcy removal through increases in the activities of CBS, CGL and BHMT (Table 3.2). The increase in the transsulfuration enzymes did not come as a surprise as it has been demonstrated previously in glucagon-treated as well as streptozotocin diabetic rats (Jacobs *et al*, 2001, Jacobs *et al*, 1998). Work in our lab has also shown the direct effect of insulin and glucagon on CBS expression (Ratnam *et al*, 2002). What was interesting and novel was the effect that betaine and BHMT had on determining plasma Hcy. We saw that BHMT activity and mRNA levels were increased in both the insulin resistant and diabetic stages with a concomitant reduction in hepatic betaine concentration. These data suggest that increased BHMT activity is driving the Hcy levels down and is depleting hepatic betaine stores. In support of these findings, Schwahn *et al* (2003) have previously shown a negative correlation between plasma betaine and plasma tHcy in humans while supplementation with betaine has been shown to reduce plasma Hcy (Steenge *et al*, 2003). Furthermore, the fact that BHMT was more active in ZDF fa/fa rats was further evident in the virtual elimination of Hcy export from isolated hepatocytes incubated with methionine and betaine compared to the significantly lowered effect betaine had on Hcy export from cells isolated from ZDF fa/? rats (Table3.5). The extraordinarily high level of hepatic betaine found in the young rats was another remarkable finding, the function of which we are currently exploring (Table 3.6).

We then went on to explore the changes that occur in amino acid metabolism in insulin resistance and Type 2 diabetes. We measured plasma, liver and skeletal muscle amino acid levels and also calculated the intracellular/extracellular concentration ratios for hepatic and skeletal muscle amino acids. Consistent with previous reports on Type 1 diabetic models (Bloxam, 1972, Brosnan *et al*, 1980, 1983) we observed a significant increase in plasma BCAA in both insulin resistant and diabetic stages (Table 4.1). Similar changes were seen in the liver and skeletal muscle with valine and leucine being increased in both tissues at 11 weeks while isoleucine and leucine showed increases in skeletal muscle at 5 weeks (Tables 4.2 and 4.4). The literature on the activity of the rate limiting step in the catabolism of BCAA, the branched chain α-ketoacid dehydrogenase in diabetes is controversial with some studies showing a reduction (Gibson *et al*, 1993) and others showing increases (Lombardo *et al*, 1998, Li *et al*, 2001, Lombardo *et al*, 1999). However, it appears that increased proteolysis along with Hyperphagia could be the cause for this increase in BCAA, similar to what has been suggested for Type 1 diabetes (Rodriguez *et al*, 1997, Crandall and Fernstrom 1983). We were also able to provide strong evidence for a common metabolic regulation of these three amino acids, as shown by the almost perfect correlation between the plasma concentrations of the three amino acids (Figure 4.1).

A number of glucogenic amino acids showed decreased concentrations in plasma, liver and skeletal muscle in the ZDF fa/fa rats compared to ZDF fa/? rats (Tables 4.1, 4.2 and 4.4). This is consistent with their use as substrates for glucose synthesis in the ZDF fa/fa rats who have been shown to have defective suppression of hepatic glucose production and, therefore, an increased total glucose output (Fujimoto *et al*, 2004). The

reduction of one glucogenic amino acid in particular, serine, stands out in the context of this thesis as our central focus is Hcy. We see reductions in the level of serine in all three tissues studied at both ages. Since serine is an essential substrate for the cystathionine β-synthase reaction, this reduction may somewhat limit Hcy removal through the transsulfuration pathway. Interestingly, although our in-vitro studies did show an increased activity of CBS we did not find a difference in the transsulfuration flux in the presence of methionine alone between the ZDF fa/fa and the ZDF fa/? rats at either age. This will further highlight the importance of the BHMT reaction in the removal of Hcy under insulin resistant and diabetic conditions.

The study on the effect of Rosiglitazone on Hcy metabolism was able to validate the conclusion made at the end of the first study that changes in the level of or the sensitivity towards insulin and its counter-regulatory hormones are responsible for the changes observed in Hcy metabolism in insulin resistance and diabetes. The restoration of insulin sensitivity by Rosiglitazone brought the reduced Hcy level towards normal but was unable to completely normalize it. Here again the changes that were observed in the activity of BHMT proved to be very interesting. Although, BHMT has been shown to be under the regulation of insulin and the counter-regulatory hormones (as is CBS), RSG treatment and the resulting increases in the sensitivity towards insulin did not provide us with the expected results. We saw that the increased activity of BHMT observed in the ZDF fa/fa rats were further increased by the RSG treatment rather than the expected normalization. An explanation for this phenomenon could be obtained from the involvement of BHMT in lipid and lipoprotein metabolism. The insulin resistance and diabetes in the ZDF rats is known to be lipogenic; indeed, the accumulation of fat in liver,

muscle and pancreatic β cells is hypothesized to be pathogenic. RSG increases insulin sensitivity by causing adipocyte differentiation with increased FFA deposition in adipose tissue (Oakes *et al*, 2001). RSG therefore is able to lessen the accumulation of lipids in ectopic locations. The interesting involvement of BHMT in the PEMT pathway of PC synthesis, and therefore in VLDL secretion which needs to be increased to accomodate this relocation of FFA, may provide an explanation as to the increased activity of BHMT under the influence of RSG. The direct effect of RSG on BHMT activity as well as the possible role of betaine in VLDL secretion in the presence of RSG should be investigated in the future to gain a full understanding of the function of this enzyme.

Although the exact mechanism by which Hcy causes endothelial dysfunction has not been elucidated, hyperhomocysteinemia has been shown to be a causative agent for endothelial dysfunction (Hossaine *et al*, 2003) as well as atherosclerosis (Zhou *et al*, 2003). Diabetes mellitus and insulin resistance have also been shown to be associated with endothelial dysfunction. It was therefore very interesting to examine the effect of lowered plasma total Hcy on endothelial function in insulin resistance and diabetes.
The endothelium-dependent vasorelaxation was observed to be preserved in both insulin resistant and Type 2 diabetic rats as seen by the response of mesenteric arteries to acetylcholine, 2-fli and arachidonic acid. We speculate that the reduced Hcy levels may have been able to counterbalance the increase in plasma glucose in rescuing endothelial function especially in the diabetic stage.

In the face of this normal endothelium dependent vasorelaxation we were surprised to observe a defect in the endothelium independent vasorelaxation as seen by the response of mesenteric arteries to Na-Nitroprusside. Both groups of untreated rats

(ZDF fa/fa and ZDF fa/+) were defective in their response to SNP with the RSG treated rats responding significantly better. However, in the presence of the NOS inhibitor L-NAME, the vessels isolated from the untreated rats were able to respond similar to the RSG treated vessels. This led us to consider that the defective response to SNP arose from a sensitization of the vessels to NO as iNOS is known to be up-regulated in the ZDF rats. The response of the RSG treated vessels also complements this notion as PPAR-γ is a known anti-inflammatory agent with inhibition of the activation of iNOS as one of its functions.

In conclusion, our work was successful in demonstrating the importance of hormones in the regulation of Hcy metabolism. We saw that the changes in insulin and the counter-regulatory hormones that occur in insulin resistance and Type 2 diabetes were instrumental in lowering the plasma homocysteine level and that this was achieved through changes in the activity of several enzymes involved in methionine and Hcy metabolism. Specific attention was drawn by our work towards the enzyme BHMT which proved to be a multi-functional enzyme involved in both amino acid and lipid metabolism.

We were also able to describe the changes that occur in amino acid levels in plasma, skeletal muscle and the liver in insulin resistance and Type 2 diabetes. Finally, our work on endothelial function may indicate Hcy as an important determinant of cardiovascular function in diabetes.

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