Impaired response of protein synthesis and turnover to insulin in men with type 2 diabetes mellitus

by

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Abstract

Although insulin resistance of glucose and fat metabolism in type 2 diabetes mellitus (T2DM) is firmly established, that of protein remains controversial for methodological reasons. A hyperinsulinemic (40mU/m^2·min) euglycemic (5.5 mmol/L) isoaminoacidemic (postabsorptive concentrations) clamp was combined with [3-^{3}H]glucose and [1-^{13}C]leucine kinetics to concurrently assess protein and glucose metabolism in 10 hyperglycemic men with T2DM and 11 men without (all BMI=29±1 kg/m^2), matched also for age, body composition, and waist circumference. In response to hyperinsulinemia, protein turnover and synthesis were stimulated in controls, but not in T2DM. Both insulin-stimulated total and non-oxidative glucose disposal were diminished in T2DM *vs.* controls. There was a robust positive correlation between the change in synthesis and glucose disposal. Hence, there is an additive effect of T2DM, beyond that of having excess fat, on insulin resistance of whole body protein turnover and synthesis. Furthermore, protein sensitivity to insulin parallels that of glucose, establishing this as an important concern in T2DM management.

Altérations du turnover et de la synthèse protéiques en réponse à l'insuline chez des hommes souffrant de diabète de type 2

Sandra M. Pereira

Résumé

Quoique l'insulino-résistance du glucose et des lipides soit clairement reconnue dans le diabète de type 2 (DMT2), celle des protéines demeure controversée, en partie pour des raisons méthodologiques. Dix hommes hyperglycémiques avec DMT2 ont été jumelés à onze hommes sans DMT2 (tous : IMC=29±1 kg/m^2) de même âge, composition corporelle et tour de taille pour être soumis à un clamp hyperinsulinique ($40mU/m^2 \cdot min$), euglycémique (5.5 mmol/L) et isoaminoacidémique (valeurs à jeun) et à des perfusions de [3-^{3}H]glucose et de [1-^{13}C]leucine pour évaluer simultanément leur métabolisme du glucose et des protéines. En réponse à l'hyperinsulinémie, le turnover et la synthèse protéiques ont augmenté chez les sujets contrôle, mais non chez les DMT2. La stimulation par l'insuline de l'utilisation du glucose par le corps était moindre chez les DMT2 *vs.* sujets non-diabétiques. On a trouvé une corrélation positive entre l'augmentation de synthèse et le taux d'utilisation du glucose. Nos données indiquent que le DMT2 aggrave l'insulino-résistance des protéines associée à l'excès de gras. De plus, l'insulino-résistance du métabolisme des protéines est parallèle à celle du glucose, d'où l'importance de s'en soucier dans la gestion du DMT2.

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List of Abbreviations

Δ	- Difference between clamp and baseline values for a given variable
A1C	- Glycosylated hemoglobin
AA	- Amino Acid
ADA	- American Diabetes Association
AMDR	- Acceptable Macronutrient Distribution Ranges
APE	- Atom Percent Excess
B	- Breakdown; rate at which an amino acid or nitrogen (depending on the amino acid tracer) is released from protein
BCAA	- Branched Chain Amino Acid
BCKDH	- Branched Chain α-Keto Acid Dehydrogenase
BIA	- Bioelectric Impedance Analysis
BMI	- Body Mass Index
BW	- Body Weight
CDA	- Canadian Diabetes Association
CIU	- Clinical Investigation Unit
CNS	- Central Nervous System
CT	- Computer Tomography
DXA	- Dual X-ray Absorptiometry
E	- Excretion; rate at which nitrogen is excreted in urine
ECG	- Electrocardiogram
EGP	- Endogenous Glucose Production
eIF4E	- Eukaryotic Initiation Factor 4E
eIF4E-BP1	- Eukaryotic Initiation Factor 4E Binding Protein 1
FFA	- Free Fatty Acid
FFM	- Fat Free Mass
FPG	- Fasting Plasma Glucose
G/I	- Glucagon to Insulin ratio
Hot ginf	- Hot glucose infusion; indicates the presence of a glucose tracer in a glucose infusate

HPLC	- High Pressure Liquid Chromatography
I	- Infusion or Ingestion; rate at which an amino acid is infused or ingested
IFG	- Impaired Fasting Glucose
IGT	- Impaired Glucose Tolerance
K_m	- Insulin concentrations at half-maximal response
LBM	- Lean Body Mass
LBO	- Lower Body Obese
MDC	- Metabolic Day Centre
MGH	- Montreal General Hospital
MRI	- Magnetic Resonance Imaging
mTOR	- Mammalian Target of Rapamycin
MUHC	- McGill University Health Centre
O	- Oxidation; rate at which an amino acid is oxidized
OGTT	- Oral Glucose Tolerance Test
$p70^{S6k}$	- 70 kDa ribosomal protein S6 kinase
PBF	- Percent Body Fat
PET	- Positron Emission Tomography
PI3K	- Phosphatidylinositol 3-kinase
PIBW	- Percent Ideal Body Weight
Q	- Turnover or Flux; rate of amino acid or nitrogen (depending on the tracer) turnover or flux
R_a	- Rate of appearance; in the context of glucose, endogenous R_a equals EGP
R_d	- Rate of disappearance; in the context of glucose, it is the rate of glucose disposal
RDA	- Recommended Dietary Allowance
REE	- Resting Energy Expenditure
RIA	- Radioimmunoassay
RQ	- Respiratory Quotient
RVH	- Royal Victoria Hospital
S	- Synthesis; rate at which an amino acid or nitrogen (depending on the amino acid tracer) is incorporated into protein

SA	- Specific Activity
S-B	- Net balance
SSF	- Suprailiac Skinfold thickness
T2DM	- Type 2 Diabetes Mellitus
UBO	- Upper Body Obese
UrN	- Excretion of Nitrogen in Urine
VCO_2	- Rate at which CO_2 is generated
V_{max}	- Maximal response to insulin
VO_2	- Rate at which O_2 is used up
WHO	- World Health Organization
WHR	- Waist to Hip Ratio
α-KIC	- α-Ketoisocaproate

1. INTRODUCTION

Protein originates from the Greek word *proteion*, which signifies "'first in rank' among the material substrates of life" (Kim 2005). Proteins are ubiquitous in the body with numerous functions, including signaling, structure, transport, movement, and immunity. Moreover, the central dogma, which describes the unidirectional conversion of information in DNA to RNA and from RNA to protein, is now seen as simplistic because proteins can control certain aspects of transcription and translation (Kim 2005). Proteins are constantly being synthesized and catabolized, a process known as protein turnover; whole body protein turnover refers to the sum of all the individual protein turnovers in the body (Waterlow et al. 1978). The rate of protein turnover regulates the amount and function of proteins in cells (Wolfe et al. 2005). A protein's rate of turnover depends on its function, its location, the dietary status of the organism, and endocrine as well as nervous systems (Liu et al. 2002). Protein synthesis and catabolism, the latter mainly by the ubiquitin-proteosome pathway, are both ATP-requiring processes and protein turnover accounts for 5-35% of resting energy expenditure (REE) (Kumar et al. 2005; Liu & Barrett 2002; Wolfe & Chinkes 2005). In healthy humans, most of the amino acids (AAs) released from protein breakdown are reused to synthesize proteins, but approximately 35g of protein per day cannot be recovered (Bender 1985; Wolfe & Chinkes 2005). Since proteins cannot be stored and since they perform vital bodily functions, it is not surprising that excessive body protein losses as a result of disease increase the risk of morbidity and mortality (Wilmore 1991).

The existence of diabetes mellitus has been known for a long time; Aretaeus, the Cappadocian described it as a "melting down of the flesh and limbs into urine" almost two thousand years ago (Reed 1954). Through the discovery of insulin in the first quarter of the twentieth century, the pivotal role of the hormone was emphasized in diabetes mellitus. Subsequent *in vitro* as well as *in vivo* animal and human studies have shown the many actions of insulin, which include increase in glucose disposal in muscle and fat, inhibition of glucose production by the liver, decrease in adipose tissue lipolysis, increase in lipid storage in adipose tissue, increase in protein net balance (synthesis minus breakdown), and stimulation of growth (Ganong 2001). It is now clear that diabetes mellitus is a heterogeneous disorder and that early accounts of the disease refer mostly,

but not exclusively, to type 1 diabetes mellitus, an autoimmune disorder characterized by β cell destruction and insufficient insulin (American Diabetes Association 2006a). The other main subgroup of diabetes mellitus, type 2 diabetes mellitus (T2DM), affects approximately 90-95% of all diabetic persons (American Diabetes Association 2006a). It is characterized by insulin resistance, which is diminished insulin action in target organs, and inadequate insulin production by the β cell to offset the insulin resistance. The majority of patients with T2DM are obese and obesity-related insulin resistance appears to be an important component in the pathogenesis of T2DM (American Diabetes Association 2006a; Kahn et al. 2000). In 2000, there were 171 million people with T2DM globally and by 2030, 366 million will have the disease (Wild et al. 2004). T2DM prevalence is increasing due to longer life expectancies, minimal physical activity, and increases in the rates of obesity (Colagiuri et al. 2005; Gregg et al. 2004; Katzmarzyk et al. 2000). It is important to note that not all obese patients develop T2DM, indicating that other genetic and environmental factors play a role in pathogenesis (Mokdad et al. 2001).

T2DM is mainly defined in the context of glucose metabolism, especially insulin's reduced ability to stimulate glucose disposal in skeletal muscle and fat and inhibit endogenous glucose production (EGP) in the liver. Indeed, two classical symptoms of diabetes, polyuria and glycosuria, gave rise to the name of the disease; *diabetes* means "flow of fluid through a siphon" (Silverthorn 2001) and *mellitus* refers to the sweetness of the urine (Kahn et al. 2005). An emerging body of literature, however, places substantial importance on aberrations in lipid and protein metabolism in T2DM. Abnormal lipid metabolism is evidenced by elevated fasting and diminished insulin-mediated suppression of plasma free fatty acid (FFA) concentrations. Although some studies indicate that protein metabolism is altered in T2DM, the study of insulin resistance of protein metabolism *in vivo* has been hindered, especially for methodological reasons, but also by study group selection criteria that are not sufficiently stringent. At the level of the whole body, leucine isotopes labeled on the first carbon have been combined with the hyperinsulinemic euglycemic clamp, the gold standard for the study of insulin resistance, in an attempt to assess insulin resistance of protein metabolism. The induced decline in plasma AAs during this protocol is a potential confounding factor; numerous *in vivo* studies in healthy humans have shown the differential effects of insulin and AAs on

protein metabolism (Castellino et al. 1987; Chevalier et al. 2004). To examine the effects of insulin on whole body kinetics of protein metabolism independently of AAs, postabsorptive plasma AA concentrations should remain constant throughout the clamp by varying the infusion rate of an appropriate AA solution. Based on a thorough literature search, it appears that the current study is the first to apply a hyperinsulinemic euglycemic isoaminoacidemic clamp in conjunction with [1-^{13}C]leucine to study the kinetics of protein metabolism in T2DM.

2. INSULIN ACTION AND INSULIN RESISTANCE

An elevation in plasma glucose concentrations is a more potent stimulator of insulin release from the β cells in the pancreas than either elevated FFAs or AAs (Ganong 2001). Insulin secretion, however, is also controlled by the endocrine and nervous systems. The insulin receptor, a tetramer with an extracellular insulin binding site and intracellular tyrosine phosphorylation capability, is found on cell membranes of muscle, adipose, and liver tissues making these organs the primary targets for insulin (Ganong 2001; Kido et al. 2001). Binding of insulin to its receptor causes a phosphorylation cascade resulting in a variety of effects on glucose, fat, and protein metabolism through alterations in the concentration, activity, transcription, translation, and ubiquitin-proteosome pathway mediated degradation of a myriad of molecules, much of which is still not completely understood (Kido et al. 2001; Kumar & O'Rahilly 2005). An example of insulin's effects on glucose metabolism in muscle and fat is the translocation of GLUT4 glucose transporters to the plasma membrane, thereby augmenting the entry of glucose into the cell via facilitated diffusion (Ganong 2001). In the liver, glucokinase is stimulated to maintain a concentration gradient for glucose across the cell membrane, allowing a net glucose influx (Ganong 2001).

Insulin resistance occurs "whenever normal concentrations of hormone produce less than a normal biologic response" (Kahn 1978). The gold standard for quantifying insulin resistance of glucose is the hyperinsulinemic euglycemic clamp. Insulin is infused at a constant rate to obtain a steady concentration of insulin and the rate at which glucose must be infused to maintain euglycemia (5.5 mmol/L) is an index of insulin resistance; the lower the glucose infusion rate (adjusted for indices of body composition), the greater

the insulin resistance. The causes of insulin resistance can be broadly grouped into two categories: receptor and postreceptor (Felber et al. 1993; Olefsky et al. 1982). The former is essentially due to a decrease in the number of insulin receptors on the cell surface, which is caused by hyperinsulinemia (Olefsky et al. 1982). Postreceptor abnormalities include alterations in the concentrations and/or activity levels of the numerous molecules involved in the insulin signal transduction pathway such that signaling is diminished (Olefsky et al. 1982; Schinner et al. 2005). Dose-response curves obtained using various hyperinsulinemic euglycemic clamps have been suggested to define postreceptor abnormalities as decreases in V_{max}, the maximal response, and receptor abnormalities as augmented K_m, the insulin concentration for half-maximal response; an example of a response to insulin would be glucose disposal (Ferrannini et al. 1998; Kahn 1978; Olefsky et al. 1982).

Obese subjects show abnormalities at the receptor level and in some cases, also at the postreceptor level (Olefsky et al. 1981; Olefsky et al. 1982). In T2DM fasting hyperglycemia, receptor and postreceptor abnormalities are present, but the latter become more prominent as T2DM progresses (Kolterman et al. 1981; Olefsky & Kolterman 1981; Olefsky et al. 1982). Since V_{max} requires binding to only a small fraction of the total receptors, the importance of receptor abnormalities in T2DM has been questioned (Felber et al. 1993). Clark et al. (1983) also see the above interpretations of insulin dose-response curves as simplistic because both receptor and postreceptor abnormalities can lead to increases in K_m and decreases in V_{max}. Indeed, postreceptor abnormalities have been the focus of intense insulin resistance research in the last two decades, leading to the identification of several candidate molecules (Schinner et al. 2005). Diminished translocation of skeletal muscle and adipose tissue GLUT4 to the cell membrane in response to insulin is an important aspect of insulin resistance of glucose metabolism (Kahn 1996; Kotani et al. 2004). The majority of glucose disposal during the hyperinsulinemic hyperglycemic clamp is directed towards glycogen synthesis in skeletal muscle (Shulman et al. 1990). In T2DM, the reduced rate of glycogen synthesis in skeletal muscle is a result of blunted glucose uptake (Cline et al. 1999; Shulman et al. 1990).

3. TYPE 2 DIABETES MELLITUS

T2DM is characterized by both insulin resistance and inadequate insulin production by the β cell to offset the insulin resistance (American Diabetes Association 2006a; DeFronzo 1988). The contribution of each of these features to T2DM should be seen as a spectrum, depending on the individual patient (American Diabetes Association 2006a; DeFronzo et al. 1992). Indeed, insulin sensitivity is not altered in some T2DM patients, especially among the non-obese (Banerji et al. 1989; Bonora et al. 1998; García-Estévez et al. 2002; Gerich 2000), although the study by Banerji et al. (1989) clearly included obese T2DM. Hence, T2DM is not a uniform disorder; it depends upon numerous genetic and environmental factors that act at the level of various organs (Gerich 1998). Diagnosis of T2DM is based on abnormalities of carbohydrate metabolism, but detection often occurs many years after the disease starts (American Diabetes Association 2006a). Specific criteria for the diagnosis of T2DM have been established (American Diabetes Association 2006a; Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003), and include cut-off points for fasting glycemia (≥ 7 mmol/L), 2 hour Oral Glucose Tolerance Test (OGTT) (≥ 11.1 mmol/L), random plasma glucose (≥ 11.1 mmol/L), and symptoms associated with high blood glucose such as polyuria. Poor diabetes management, based on continuously elevated fasting glycemia (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003) and glycosylated hemoglobin (A1C) greater than 7% (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003), is a risk factor for micro- and macro-vascular diabetes complications such as retinopathy, nephropathy, neuropathy, and cardiovascular disease (American Diabetes Association 2006b; Turner et al. 1998).

Although various risk factors for developing T2DM have been identified (American Diabetes Association 2006b), including obesity and first degree relatives with the disease, a detailed picture of the pathogenesis of T2DM is far from being complete. Longitudinal and cross-sectional studies have shown that the initial abnormality is insulin resistance (Martin et al. 1992), compromised function at the level of the β cell (O'Rahilly et al. 1986), or both (Tripathy et al. 2004; Weyer et al. 1999; Weyer et al. 2001). Even if the original problem is insulin resistance, much debate exists about which organ is first targeted, either adipose tissue (Lewis et al. 2002), muscle (Petersen et al. 2004; Vaag et

al. 1992b), or even liver (DeFronzo et al. 1992; Tripathy et al. 2004). Furthermore, in skeletal muscle, evidence for dysfunction in mitochondrial oxidation (Petersen et al. 2004) and glycogen synthase (Vaag et al. 1992b) have been proposed to be the initial triggers. Hence, the pathogenesis of T2DM is complex and it results from the interplay between genetic and environmental factors (Gerich 1998).

4. ABDOMINAL OBESITY

According to the World Health Organization (WHO), obesity is defined as a surplus of fat that is associated with adverse health outcomes, including insulin resistance, metabolic syndrome, T2DM, and cardiovascular disease (Abate et al. 1995; Alberti et al. 2006; Chan et al. 1994; Goodpaster et al. 1997; World Health Organization 2000). The most commonly used anthropometric indicators of obesity are Body Mass Index (BMI) for overall obesity, which is calculated by dividing body weight by the square of the height in meters, and waist circumference as well as Waist to Hip Ratio (WHR) for abdominal obesity (Chan et al. 2003; Janssen et al. 2002; World Health Organization 2000). Hip circumference reduces the risk of cardiovascular events in women, but not in men in longitudinal studies (Heitmann et al. 2004) and WHR has been shown to be a worse predictor of adverse health outcomes than BMI and waist circumference (Chan et al. 1994; Wang et al. 2005; World Health Organization 2000).

Cut-off points for BMI, waist circumference, and WHR have been established. While a BMI between 18.50 and 24.99 is considered normal, risk of adverse health outcomes augments with BMI categories in the following order: overweight (25.00-24.99), class I obese (30.00-34.99), class II obese (35.00-39.99), and class III obese ($\geq$ 40) (World Health Organization 2000). There are two cut-off points for waist circumference in Caucasian populations: greater than or equal to 94 cm and 102 cm for men and greater than or equal to 80 and 88 cm for women (World Health Organization 2000). The lower cut-off points for waist circumference in both men and women correspond to a BMI greater than or equal to 25, while the higher cut-off points correspond to a BMI equal to or greater than 30 in both sexes (Lean et al. 1995). For WHR, the cut-off points are greater than 1.0 for men and greater than 0.85 for women (World Health Organization 2000).

Abdominal fat includes intra-abdominal or visceral fat, deep subcutaneous fat, and superficial subcutaneous fat (Garg 2004; Kelley et al. 2000b). Almost two decades ago, Björntorp (1990) proposed the portal FFA hypothesis, which selectively portrayed visceral fat as the culprit of various metabolic abnormalities, including hyperinsulinemia, insulin resistance, and T2DM. Visceral fat has an elevated rate of lipolysis and since veins from visceral fat are connected to the portal vein, it was believed that in cases of augmented visceral fat high FFA concentrations reached the liver, thereby disturbing hepatic and peripheral metabolism (Björntorp 1990; Garg 2004). Indeed, Yoshii et al. (2006) recently showed in animal models that high portal levels of FFAs result in elevated plasma insulin systemically. Various studies with non-diabetic subjects implicate visceral adiposity, quantified using Magnetic Resonance Imaging (MRI) or Computerized Tomography (CT), in blunting insulin-mediated glucose disposal (Abate et al. 1995; Garg 2004; Goodpaster et al. 1997; Kelley et al. 2000b). Nevertheless, the portal hypothesis theory has been questioned because subcutaneous abdominal fat has been documented to blunt insulin sensitivity of glucose in healthy and obese subjects and cytokines produced by adipose tissue may have an adverse role in insulin resistance (Abate et al. 1995; Garg 2004; Goodpaster et al. 1997; Kelley et al. 2000b; Kershaw et al. 2004; Snijder et al. 2006).

5. INSULIN RESISTANCE OF GLUCOSE AND FAT METABOLISM IN TYPE 2 DIABETES MELLITUS

The plasma insulin concentrations obtained during a hyperinsulinemic clamp can be broadly grouped into physiological or supra-physiological categories. Postabsorptive and postprandial plasma insulin levels are altered by a variety of factors, including obesity, abnormalities in carbohydrate metabolism, ethnicity, meal energy and macronutrient content, and the insulin assay used (Blom et al. 2006; Blom et al. 2005; Cozma et al. 2005; Dickinson et al. 2002; Marques-Lopes et al. 2001; Rask et al. 2001; Wolever et al. 2004). The approach taken in the current study was to determine the maximal postprandial insulin concentrations obtained in healthy adults in previous studies: they were approximately 600-800 pmol/L (Dickinson et al. 2002; Timlin et al. 2005). Hence, insulin levels at or below this range are considered physiological, while

levels above it are considered supra-physiological. Although this terminology simplifies the interpretation of results, actual insulin levels or the multiple by which they increased above baseline will be explicitly stated when ambiguity arises.

The following discussion of glucose and fat metabolism in T2DM will focus on *in vivo* human studies. Unless otherwise stated, study groups were matched for obesity, either BMI or Percent Ideal Body Weight (PIBW) or both and the type of clamp used was a hyperinsulinemic euglycemic clamp. In certain protocols, insulin was administered the night before the clamp study and this may have altered insulin sensitivity in the liver and periphery. Hence, for these studies, postabsorptive glucose and lipid kinetic results are not reported although some clamp results are described and identified when necessary.

5.1. Insulin Resistance of Glucose Metabolism in Type 2 Diabetes Mellitus

5.1.1. Hepatic Insulin Resistance in Type 2 Diabetes Mellitus

EGP is the result of gluconeogenesis and glycogenolysis. While EGP occurs mostly in the liver, the kidneys are also involved (Meyer et al. 2004). EGP, measured using glucose tracers, has not been consistently shown to be elevated in T2DM, either in the postabsorptive state or during the hyperinsulinemic euglycemic clamp. While some studies indicate that postabsorptive EGP is elevated in T2DM (Basu et al. 2005; Campbell et al. 1988; DeFronzo et al. 1985; Firth et al. 1987; Gastaldelli et al. 2000; Groop et al. 1989; Meyer et al. 2004; Rooney et al. 1993; Vaag et al. 1992a), others have found EGP to be similar in T2DM and controls (Abate et al. 1996; Firth et al. 1987; Hother-Nielsen et al. 1991), or even smaller in T2DM (Pigon et al. 1996). Gluconeogenesis in the postabsorptive state is higher in T2DM (Basu et al. 2005; Boden et al. 2001b; Gastaldelli et al. 2000), but glycogenolysis is either similar between T2DM subjects and controls of comparable obesity (Basu et al. 2005; Gastaldelli et al. 2000) or it is diminished in T2DM (Boden et al. 2001b). In T2DM, EGP is elevated mainly because of increased gluconeogenesis and glycogenolysis does not change with fasting glucose (Gastaldelli et al. 2002). Unchanged glycogenolysis in the presence of hyperinsulinemia and hyperglycemia is inappropriate, however, because glucose and insulin inhibit glycogenolysis in healthy individuals (Basu et al. 2005).

Insulin inhibits EGP both through direct and indirect mechanisms (Lewis et al. 1996). The latter include diminished plasma FFA and glucagon concentrations (Lewis et al. 1998). In T2DM, the direct insulin action on the liver is blunted, resulting in elevated EGP (Lewis et al. 1999). Campbell et al. (1988) used different rates of insulin infusions to achieve three different levels of clamp insulinemia: the K_m for EGP was greater in T2DM than controls and EGP was augmented in T2DM *vs.* controls during the two physiological clamps, but with supraphysiological insulin concentrations EGP was entirely inhibited in T2DM and control subjects. These results are supported by others using analogous experimental designs (Butler et al. 1990; Firth et al. 1987; Groop et al. 1989; Rooney et al. 1993), although EGP was still elevated in T2DM at supraphysiological insulin in Butler et al. (1990). Clamps performed at physiological insulin concentrations have also revealed a blunted ability of insulin to diminish EGP during the clamp plateau (Abate et al. 1996; Basu et al. 2005; DeFronzo et al. 1985), but no differences were found by Pigon et al. (1996), both Vaag et al. (1995) and Kelley et al. (1993) failed to find a difference at an infusion of $40mU/m^2 \cdot min$, and Del Prato et al. (1993) found a similar EGP at $20mU/m^2 \cdot min$. In addition, both Staehr et al. (2001) and Turk et al. (1995) showed a higher EGP in T2DM at lower insulin infusions, although no differences were found at more elevated physiological insulin levels. Nevertheless, in the studies that found similar EGP at higher physiological insulin levels (Kelley et al. 1993; Staehr et al. 2001; Turk et al. 1995; Vaag et al. 1995), except Pigon et al. (1996), insulin was administered before the clamp procedure to normalize fasting plasma glucose (FPG) and this may have decreased EGP in T2DM subjects. When EGP, gluconeogenesis and glycogenolysis were simultaneously assessed during hyperinsulinemic euglycemic clamps, gluconeogenesis and glycogenolysis were found to be elevated in T2DM (Basu et al. 2005).

Fasting hyperglycemia is believed to result from insulin resistance in the liver and in the periphery (Boden et al. 2001b; Campbell et al. 1988; Gastaldelli et al. 2000; Staehr et al. 2001). DeFronzo (1988) has suggested that at lower fasting glucose concentrations, the main abnormality is at the level of insulin resistance in the periphery, while elevated EGP becomes the main abnormality at higher fasting glucose levels. FPG correlates positively with EGP in the postabsorptive state (Boden et al. 2001b; Bogardus et al. 1984;

Campbell et al. 1988; DeFronzo 1988; Jeng et al. 1994), but FPG threshold values have been proposed for this relationship (DeFronzo 1988; Jeng et al. 1994), including 7.8 mmol/L (DeFronzo 1988). Similarly, poor T2DM control, as indicated by FPG, is associated with elevated EGP during the clamp (DeFronzo 1988; DeFronzo et al. 1985).

5.1.2. Peripheral Insulin Resistance in Type 2 Diabetes Mellitus

Postabsorptive glucose disposal is elevated in T2DM (Baron et al. 1985; Campbell et al. 1988; Ciaraldi et al. 2005; Groop et al. 1989; Thorburn et al. 1990). Firth et al. (1987) showed that while obese T2DM had higher rates of postabsorptive glucose disposal than obese controls, postabsorptive glucose disposal was similar in lean T2DM and lean controls. Hother-Nielsen & Beck-Nielsen (1991) did not find any differences between T2DM and controls using a similar experimental design with four groups. Most postabsorptive glucose disposal is independent of insulin, occurring through facilitated diffusion (Baron et al. 1988; Baron et al. 1985). In turn, most of the insulin-independent glucose disposal occurs in the central nervous system (CNS) in healthy humans, although it is unclear if this also extends to T2DM (Baron et al. 1988). In the fasting state, *in vivo* skeletal muscle glucose disposal has been found to be increased (DeFronzo et al. 1985), the same (Williams et al. 2001), or decreased (Ciaraldi et al. 2005) in T2DM *vs.* control subjects. It is unclear whether glucose disposal in the splanchnic region is abnormal in T2DM (DeFronzo et al. 1985).

Insulin increases glucose disposal, as assessed with hyperinsulinemic euglycemic clamps. At the higher end of physiological insulinemia, for example a 40mU/m^2·min clamp, and during supraphysiological hyperinsulinemia, insulin-mediated glucose disposal has consistently been shown to be diminished in T2DM during the clamp (Abate et al. 1996; Bavenholm et al. 2003; Bonadonna et al. 1996; Bonadonna et al. 1993; Butler et al. 1990; Campbell et al. 1988; DeFronzo et al. 1985; Firth et al. 1987; Golay et al. 1988; Groop et al. 1989; Hother-Nielsen & Beck-Nielsen 1991; Iozzo et al. 2003; Kelley et al. 1992; Pigon et al. 1996; Rooney et al. 1993; Staehr et al. 2001; Turk et al. 1995; Vaag et al. 1995). At lower physiological levels, in the presence of euglycemia, studies have also shown lower rates of insulin-stimulated glucose disposal in T2DM (Bavenholm et al. 2003; Campbell et al. 1988; Firth et al. 1987; Pigon et al. 1996; Rooney et al. 1993;

Turk et al. 1995), including Abate et al. (1996), Staehr et al. (2001), and Del Prato et al. (1993) at 20mU/m^2·min; however, Groop et al. (1989) found no group differences at 20mU/m^2·min. The K_m for glucose disposal is also increased in T2DM *vs.* controls (Campbell et al. 1988).

Insulin-mediated glucose disposal is diminished in skeletal muscle of T2DM (Bonadonna et al. 1996; Bonadonna et al. 1993; DeFronzo et al. 1985; Iozzo et al. 2003; Kelley et al. 1992; Kelley et al. 2001), as determined using Positron Emission Tomography (PET) or the arterial-venous balance technique in legs or arms in conjunction with the hyperinsulinemic euglycemic clamp. Since about 90% (DeFronzo et al. 1985) of the total insulin-stimulated glucose disposal occurs in skeletal muscle of T2DM and controls, most of the decrease in insulin-stimulated glucose disposal in T2DM is due to insulin resistance in skeletal muscle. Kelley et al. (1993) found that leg insulin-mediated glucose disposal did not differ in lean T2DM vs. control subjects, which is in contrast to their previous findings, using a similar protocol, with obese T2DM subjects (Kelley et al. 1992). Even though these results could be interpreted as different skeletal muscle insulin sensitivity in lean T2DM compared to obese T2DM subjects, the insulin given prior to the clamp in both studies may have been a confounder. A small fraction of glucose disposal during the hyperinsulinemic euglycemic clamp occurs in adipose and splanchnic tissues (DeFronzo et al. 1985). For the latter, this represents less than 5% of glucose disposal in both T2DM and control groups (DeFronzo et al. 1985) and although DeFronzo et al. (1985) concluded that insulin does not affect glucose disposal in the splanchnic region in T2DM and control subjects, Iozzo et al. (2003) have shown that the liver in T2DM is resistant to insulin-stimulated glucose disposal. The rest of the glucose disposal during a hyperinsulinemic euglycemic clamp occurs in the CNS in an insulin-independent manner (DeFronzo et al. 1985).

Glucose disposal can be divided into two categories, oxidative and non-oxidative, using indirect calorimetry. Non-oxidative glucose disposal includes non-oxidative glycolysis, such as lactate production, and glycogen synthesis (Del Prato et al. 1993; Kelley et al. 1993; Vaag et al. 1995; Young et al. 1988); during the clamp, the latter is by far the larger contributor to overall non-oxidative glucose disposal (Pratipanawatr et al. 2002; Shulman et al. 1990). Postabsorptively, it has been reported that non-oxidative

disposal is elevated (Groop et al. 1989; Thorburn et al. 1990) and that oxidative disposal is augmented (Golay et al. 1988), similar (Groop et al. 1989), or diminished (Thorburn et al. 1990) in T2DM compared with controls. During the hyperinsulinemic euglycemic clamp, oxidative glucose disposal has been found to be lower (Butler et al. 1990; Del Prato et al. 1993; Golay et al. 1988; Vaag et al. 1995) or similar (Yokoyama et al. 2006), non-oxidative glucose disposal to be lower (Butler et al. 1990; Golay et al. 1988; Vaag et al. 1995), glycogen synthesis to be lower (Del Prato et al. 1993; Vaag et al. 1995), and non-oxidative glycolysis to be higher in T2DM *vs.* controls (Del Prato et al. 1993; Vaag et al. 1995). While both oxidative and non-oxidative glucose disposal were lower in T2DM at insulin infusion rates of 40 and 100 $mU/m^2 \cdot min$, no differences were found at $20mU/m^2 \cdot min$ (Groop et al. 1989). Bavenholm et al. (2003) also found lower oxidative glucose disposal rates only at higher insulin concentrations.

Glucose transport in skeletal muscle of T2DM subjects is diminished during the hyperinsulinemic euglycemic clamp (Bonadonna et al. 1993; Williams et al. 2001). Moreover, Williams et al. (2001) showed that, during the clamp, the reduced rate at which glucose is phosphorylated to glucose 6-phosphate in the skeletal muscle of T2DM can be overcome with higher insulin concentrations. Two key enzymes in glycogen formation and glucose oxidation are glycogen synthase and pyruvate dehydrogenase, respectively; their activities have been shown to be diminished in the skeletal muscle of T2DM during the clamp (Kelley et al. 1992; Kelley et al. 1993; Vaag et al. 1995).

In addition to the hyperinsulinemic euglycemic clamp, clamps characterized by isoglycemia, where glucose infusion rates are altered such that each individual's baseline glycemia is maintained during the clamp plateau, have been performed to quantify insulin resistance. When the results of hyperinsulinemic euglycemic clamps in controls were compared to those of hyperinsulinemic isoglycemic clamps in T2DM subjects, no group differences were found in total glucose disposal (Vaag et al. 1992a) or glucose infusion rates (Kelley et al. 1990). In both studies, isoglycemia in T2DM subjects meant hyperglycemia, approximately 10 mmol/L. Despite the comparable glucose disposal rates, a greater proportion of the glucose disposal in T2DM subjects was insulin-independent (Del Prato et al. 1993) and thus, the mass action of glucose presents a potential confounder in this experimental design. When both T2DM and control subjects

were studied using hyperinsulinemic hyperglycemic clamps of comparable insulin and glucose concentrations (approximately 10 mmol/L), T2DM subjects showed lower rates of total glucose disposal (Shulman et al. 1990). Thus, it appears that insulin resistance of total glucose disposal is still evident in T2DM subjects when hyperinsulinemic hyperglycemic clamps are performed in both T2DM and control subjects. Henceforth, the focus of my discussion will continue to be the hyperinsulinemic euglycemic clamp, as this is the type of clamp used in my study protocol.

5.2. Insulin Resistance of Fat Metabolism in Type 2 Diabetes Mellitus

In healthy individuals, insulin promotes net storage of triglycerides in adipose tissue, but not in skeletal muscle (Kumar & O'Rahilly 2005). FFA and glycerol tracers have been utilized to obtain detailed information on whole body lipid metabolism. Postabsorptively, it has been shown that lipolysis is elevated (Puhakainen et al. 1992) or FFA turnover is comparable in T2DM *vs.* controls (Groop et al. 1989; Groop et al. 1991b), while FFA oxidation is similar (Groop et al. 1989; Groop et al. 1991b). Groop et al. (1989) found insulin resistance of lipid metabolism in T2DM, as evidenced by higher FFA turnover and oxidation in T2DM during clamps at various insulin levels, from just above postabsorptive to supraphysiological. Groop et al. (1991b) showed that the K_m's for FFA turnover, re-esterification, and FFA oxidation are not different between T2DM and controls, but V_{max}'s for all three variables are higher in T2DM. In both studies, the elevated clamp re-esterification rates among T2DM may be the result of higher plasma FFAs (Groop et al. 1989). Since neither FFA nor glycerol tracers were used in my protocol, the ensuing discussion is limited to the effects of T2DM on plasma FFA concentrations and whole body fat oxidation.

Concentrations of FFAs in plasma at any given time are the result of lipolysis in adipose tissue, FFA movement into cells, triglyceride formation within cells, and FFA oxidation (Groop et al. 1989; Groop et al. 1991b; Lewis et al. 2002). Since some of these processes are regulated by insulin, plasma FFA concentrations can be seen as an index of insulin sensitivity of lipid metabolism (Kumar & O'Rahilly 2005; Lewis et al. 2002). In the postabsorptive state, many studies have shown elevated plasma FFAs in T2DM (Del Prato et al. 1993; Meyer et al. 2002; Nurjhan et al. 1992; Rooney et al. 1993), but some

have found no differences between groups (Basu et al. 2005; Bavenholm et al. 2003; Groop et al. 1989; Iozzo et al. 2003; Thorburn et al. 1990; Vaag et al. 1992a). The response of plasma FFA concentrations to insulin is more dramatic than that of plasma glucose (Kumar & O'Rahilly 2005). Using a series of hyperinsulinemic euglycemic clamps, from low physiological to supraphysiological insulin, Groop et al. (1989) and Rooney et al. (1993) showed that the ability of insulin to diminish plasma levels of FFAs is blunted in T2DM. Most other researchers have also found higher FFAs in T2DM during hyperinsulinemic euglycemic clamps (Bavenholm et al. 2003; Del Prato et al. 1993; Golay et al. 1988; Groop et al. 1991b), but no differences have also been reported (Iozzo et al. 2003). The K_m for plasma FFAs has been shown to be similar in T2DM and controls (Groop et al. 1989; Groop et al. 1991b).

While FFA oxidation only refers to oxidation of plasma FFAs, whole body lipid oxidation includes lipid oxidation within cells and in plasma (Groop et al. 1991b). Lipid oxidation depends on FFA concentrations as well as other metabolic aspects, such as insulin action in adipose tissue and oxidation efficiency (Devlin 2002; Groop et al. 1991a; Kelley et al. 2000a; Lewis et al. 2002; Petersen et al. 2006). Indeed, fasting and clamp FFAs show a positive correlation with lipid oxidation (Felber et al. 1987). In the postabsorptive state, whole body lipid oxidation has been shown to be similar in T2DM *vs.* controls (Bavenholm et al. 2003; Golay et al. 1988; Groop et al. 1989; Groop et al. 1991b; Thorburn et al. 1990; Vaag et al. 1992a). During the hyperinsulinemic euglycemic clamp, the results for lipid oxidation in T2DM are more heterogeneous. While no group differences have been found throughout a range of hyperinsulinemia (Golay et al. 1988; Groop et al. 1989), Del Prato et al. (1993) found elevated lipid oxidation during a $20mU/m^2 \cdot min$ clamp in T2DM. Conversely, Bavenholm et al. (2003) found that at a lower physiological clamp, there were no differences in lipid oxidation, but that at a higher physiological clamp, T2DM showed elevated rates of fat oxidation. The K_m for lipid oxidation is similar between T2DM and controls (Groop et al. 1991b).

5.3. The Effects of Free Fatty Acids on Insulin Sensitivity of Glucose Metabolism in Type 2 Diabetes Mellitus

Over four decades ago, it was shown that elevated FFAs in muscle decrease glucose movement into cells and glucose oxidation by altering the activity of key enzymes involved in glucose metabolism; this is the glucose fatty acid cycle (Kelley & Mandarino 2000a; Randle et al. 1963). Inverse correlations between whole body lipid oxidation and oxidative glucose disposal as well as non-oxidative glucose disposal during fasting and the clamp have been shown in controls and T2DM (Felber et al. 1987) and in each group separately (Groop et al. 1989). These relationships remained during the clamp after taking into account age and PIBW (Felber et al. 1987). Nevertheless, the glucose fatty acid cycle cannot totally describe the abnormalities associated with FFA-induced insulin resistance (Boden 1997; Boden et al. 1995; Boden et al. 1991; Kelley & Mandarino 2000a). Recent results indicate that FFAs diminish insulin sensitivity at the level of the muscle through products of FFA metabolism, such as long chain fatty acyl-CoAs and diacylglycerol, which can directly downregulate the insulin signaling pathway (Boden 2003; Boden et al. 2002; Lewis et al. 2002). Moreover, triglycerides are elevated in T2DM muscle (Goodpaster et al. 2002; Kelley 2002; Kelley & Mandarino 2000a), possibly due to a diminished ability of the mitochondria to oxidize fats (Goodpaster & Kelley 2002; Petersen & Shulman 2006).

The consequences of elevated FFAs on hepatic insulin sensitivity are less clear. In the postabsorptive state, plasma FFAs are positively correlated with gluconeogenesis (Boden et al. 2001a; Nurjhan et al. 1992) in T2DM and control subjects, but not all studies show this (Puhakainen et al. 1992). Plasma FFA concentrations also show a positive correlation with EGP in the postabsorptive and insulin-stimulated states (Basu et al. 2005). Based on positive correlations between EGP and lipid oxidation, Groop et al. (1989) also suggested that liver oxidizes FFAs to provide energy for the costly process of gluconeogenesis. Lastly, it appears that FFAs can also have adverse effects on the insulin signaling pathway in the liver (Lam et al. 2003; Lam et al. 2002).

6. PROTEIN METABOLISM

Nitrogen balance is indicative of the overall status of nitrogenous compounds in the body, but it is limited by assumptions that can be erroneous and it cannot specifically assess protein kinetics (Bier 1989; Kumar & O'Rahilly 2005). Similarly, plasma AA levels are the net result of various complex pathways in multiple tissues, including protein synthesis and breakdown as well as AA oxidation and transport in and out of the cell (Cynober 1995). Hence, AA tracers have been used to reveal detailed mechanisms of whole body protein metabolism in healthy and diseased states for over 50 years (Bier 1989).

Protocols with glycine and leucine tracers are broadly defined as end-product and precursor methodologies, respectively (Abrams et al. 2003; Cynober 1995; El-Khoury 1999). The isotopes ^{15}N-glycine (stable) and leucine labeled on the first carbon, mostly L-[1-^{13}C]leucine (stable) but also L-[1-^{14}C]leucine (radioactive), are commonly used to study whole body kinetics of protein metabolism. The underlying model consists of two pools: a free AA pool and a larger protein pool (Abrams & Wong 2003; El-Khoury 1999). The tracers follow the same metabolic pathways as the AAs (El-Khoury 1999). From the AA pool, AAs can be incorporated into protein (S) or oxidized (O); conversely, AAs released from protein breakdown (B) and ingested or infused (I) will contribute to the AA pool. At steady state, when enrichment and physiological equilibria are reached, the following equation applies (Abrams & Wong 2003; Cynober 1995; El-Khoury 1999):

$$Q = B + I = S + O$$

Q, which is flux or turnover, I, B, S, and O are all rates. When ^{15}N-glycine is used as a tracer, the AA pool specifically represents amino nitrogen (Abrams & Wong 2003; El-Khoury 1999); Q refers to nitrogen flux, B represents the release of nitrogen from protein, I is nitrogen intake, S is the incorporation of nitrogen into protein, and O is substituted by E, the rate of total nitrogen excretion in urine (Abrams & Wong 2003; Cynober 1995; El-Khoury 1999; Gougeon et al. 1997; Wolfe & Chinkes 2005). In ^{15}N-glycine methodologies, the enrichment of ^{15}N-urea in urine is necessary for the calculation of Q (Abrams & Wong 2003; Cynober 1995). Calculations of leucine Q and O use plasma ^{13}C-α-ketoisocaproate (^{13}C-α-KIC) enrichment, which is the reciprocal model, instead of plasma ^{13}C-leucine enrichment, known as the primary model, because the reciprocal

model reflects more accurately intracellular leucine enrichment (Matthews et al. 1982; Thompson et al. 1988). A similar rationale applies in studies using L-[1-^{14}C]leucine as a tracer. Since Q and O (or E) are calculable and I is known, S and B are determined by subtraction.

There are differences between the glycine and leucine tracer methodologies mentioned above: 1) leucine is an essential AA, but glycine is not, 2) the leucine tracer is administered intravenously, but ^{15}N-glycine is usually given orally (Abrams & Wong 2003; Bier 1989), and 3) while L-[1-^{13}C]leucine and L-[1-^{14}C]leucine can be used to determine postabsorptive and insulin-stimulated kinetics of protein metabolism during clamp protocols, glycine provides information on fasted and fed states as a sum (Gougeon et al. 1997). Moreover, although ^{15}N-glycine methodology is not invasive, it has certain limitations and currently, the best approach to studying kinetics of protein metabolism at the level of the whole body is using the ^{13}C-leucine tracer (Cynober 1995; Wolfe & Chinkes 2005).

Even though the focus of the ensuing discussion is on studies using leucine as a tracer, research with other tracers, such as isotopes of phenylalanine, is utilized to illustrate certain points. Like leucine, phenylalanine is an essential AA and during steady state the above equation, $Q = B + I = S + O$, applies, but O represents the rate at which phenylalanine is metabolized to tyrosine (Devlin 2002; Thompson et al. 1989). Throughout the literature on kinetics of protein metabolism, kinetic variables are often expressed per m^2 of surface area, per kg Body Weight (BW), per kg of Fat Free Mass (FFM), or per kg of Lean Body Mass (LBM) in an attempt to take into account the size of the body or of some bodily compartments.

6.1. Insulin and its Effects on Protein Metabolism in Healthy Subjects

6.1.1. Effects of Insulin on Plasma Amino Acids

Various studies using the hyperinsulinemic euglycemic clamp have confirmed insulin's ability to diminish plasma AAs in healthy subjects. Specifically, insulin has been shown to consistently diminish plasma concentrations of leucine (Castellino et al. 1987; Flakoll et al. 1989; Fukagawa et al. 1985; Petrides et al. 1994; Tessari et al. 1987; Tessari et al. 1986), isoleucine (Castellino et al. 1987; Flakoll et al. 1989; Fukagawa et al.

1986; Petrides et al. 1994; Tessari et al. 1987; Tessari et al. 1986), total branched chain amino acids (BCAAs) (Castellino et al. 1987; Petrides et al. 1994), and total AAs (Castellino et al. 1987; Petrides et al. 1994). Compared to other AAs, BCAAs demonstrate the greatest response to insulin, as their levels are most severely affected by the hormone (Flakoll et al. 1989; Fukagawa et al. 1986). Insulin causes a decline in plasma valine concentration in most (Flakoll et al. 1989; Fukagawa et al. 1986; Petrides et al. 1994; Tessari et al. 1987; Tessari et al. 1986), but not all studies (Castellino et al. 1987). The reduction in plasma BCAAs is proportional to the insulin concentrations reached during the clamp, ranging from 3 to 400 times basal insulin (Flakoll et al. 1989; Fukagawa et al. 1985; Fukagawa et al. 1986). Interestingly, Fukagawa et al. (1986) showed that the K_m for glucose disposal was comparable to the K_m for plasma leucine and valine concentrations and greater than the K_m for isoleucine concentrations. Alanine, however, does not decrease in response to insulin (Castellino et al. 1987; Fukagawa et al. 1985; Fukagawa et al. 1986; Petrides et al. 1994; Tessari et al. 1986). Glycine levels dropped in response to insulin in some studies (Castellino et al. 1987; Tessari et al. 1986), but not in others (Fukagawa et al. 1986; Petrides et al. 1994).

During the hyperinsulinemic euglycemic clamp, insulin inhibits protein breakdown, thereby diminishing plasma AAs (Castellino et al. 1987; Fukagawa et al. 1985; Petrides et al. 1994). Petrides et al. (1994) also contrasted these typical 3 hour long clamp results with those of a 24 hour long hyperinsulinemic euglycemic clamp. During the latter clamp, compared to baseline, BCAAs decreased while protein breakdown did not change. Other AAs went up to baseline levels and alanine, glycine, and total AA concentrations were greater at 24 hours than at baseline. The authors concluded that in the long-term AAs, in addition to insulin, may play a role in controlling protein breakdown and thereby plasma AAs, and that insulin may not influence all AAs in the same way.

6.1.2. Assessing Insulin Sensitivity of Protein with the Hyperinsulinemic Euglycemic Clamp

The hyperinsulinemic euglycemic clamp has been used extensively to study insulin sensitivity of whole-body protein metabolism when combined with AA tracers. The clamp causes a decrease in B, as assessed by phenylalanine and leucine tracers (Castellino et al. 1987; Flakoll et al. 1989; Fukagawa et al. 1985; Meek et al. 1998; Petrides et al. 1994; Tessari et al. 1987; Tessari et al. 1986) that is proportional to the insulin concentration achieved during the clamp plateau (Flakoll et al. 1989; Fukagawa et al. 1985; Meek et al. 1998; Tessari et al. 1986). In the first three studies, the maximum decrease in B was 30-50% at insulin concentrations that were 200 to 400 times basal levels. Although Flakoll et al. (1989) suggest that the greatest possible reduction in B (45%) occurs when the insulin concentration is greater than 5740 pmol/L (about 80 times above baseline), decreases of 13-40% have been reported at high physiological levels of 570-720 pmol/L (about 10 times baseline) (Castellino et al. 1987; Tessari et al. 1987). Moreover, B is not affected by the level of glycemia or glucose disposal during the clamp (Heiling et al. 1993).

During the hyperinsulinemic euglycemic clamp, there is great variability in the response of leucine O to the clamp, especially in the physiological range of insulin. When clamp insulin concentrations are 110-720 pmol/L (3-13 times above baseline), O can be stable (Flakoll et al. 1989; Fukagawa et al. 1985; Petrides et al. 1994), decrease (Castellino et al. 1987; Tessari et al. 1987; Tessari et al. 1986), or increase (Fukagawa et al. 1985) compared to baseline. At higher clamp insulin levels, O either does not change (Fukagawa et al. 1985) or decreases (Flakoll et al. 1989; Tessari et al. 1986) compared to baseline.

The rate at which leucine or phenylalanine is incorporated into protein (S) has been repeatedly shown to decrease, compared to baseline, during a hyperinsulinemic euglycemic clamp (Castellino et al. 1987; Flakoll et al. 1989; Meek et al. 1998; Petrides et al. 1994; Tessari et al. 1987; Tessari et al. 1986). In addition, net leucine balance (S-B) either increased (Castellino et al. 1987) or did not change (Petrides et al. 1994). These results are perplexing because insulin stimulates protein synthesis *in vitro* (Manchester et al. 1958). Furthermore, Flakoll et al. (1989) noted that S decreased steadily as the level

of clamp hyperinsulinemia increased. Since the hyperinsulinemic euglycemic clamp is accompanied by decreases in plasma AAs and since AAs are the substrates for protein synthesis, limiting their availability was thought to at least partly explain the *in vivo* results for S (Castellino et al. 1987). Hence, the independent effects of insulin and AAs on protein metabolism were explored.

6.1.3. Effects of Insulin and Amino Acids on Whole Body Protein Metabolism

To differentiate the effects of insulin from those of AAs on kinetics of protein metabolism *in vivo*, hyperinsulinemic euglycemic clamps with or without AA infusions have been performed. Details of the study design are important because AAs and insulin share an intracellular signaling pathway (Wullschleger et al. 2006) and hence, the level at which one variable is held constant may affect the response of the other. Castellino et al. (1987) combined the ^{14}C-leucine tracer methodology with four clamps: 1) hyperinsulinemic (40mU/m^2·min) euglycemic, 2) hyperinsulinemic (40mU/m^2·min) euglycemic isoaminoacidemic, 3) hyperinsulinemic (40mU/m^2·min) euglycemic hyperaminoacidemic, and 4) basal insulinemia with hyperaminoacidemia. Constant infusions of the AA mixture 10% Travasol were used to achieve the desired AA concentrations during the clamp. In response to the hyperinsulinemic euglycemic isoaminoacidemic clamp, Q did not alter, B decreased, and O and S-B increased. The effect of insulin on S differed depending on which leucine model was used for enrichment; using the reciprocal model, S did not change in response to insulin. In slight contrast to clamp 2, Q, O, S, and S-B increased to a similar extent in response to hyperaminoacidemia during hyperinsulinemia (clamp 3) and hyperaminoacidemia during basal insulin (clamp 4). The results for B depended on the model used; using the reciprocal model, the decrease in B was comparable in the two protocols. Thus, insulin does not appear to have any additional effects on kinetics of protein metabolism when AA concentrations are increased from postabsorptive levels to hyperaminoacidemia. Similarly, Tessari et al. (1987) found that when a clamp with basal insulin and hyperaminoacidemia was compared to a hyperinsulinemic (650 pmol/L) euglycemic hyperaminoacidemic clamp, the percentage change in oxidation did not differ. Taken together, when determining the effects of insulin on kinetics of protein metabolism, it is

not only important to prevent plasma AAs from declining during the clamp, but also to compare protocols with similar AA concentrations. Hence, the ensuing discussion on the effects of insulin on leucine kinetics will focus on hyperinsulinemic euglycemic clamps that have attempted to maintain postabsorptive AA concentrations.

The direction of the effects of AAs on each variable for whole body kinetics of protein metabolism is fairly consistent across studies. In the protocol previously described for Castellino et al. (1987), in response to hyperaminoacidemia during basal insulin (clamp 4), Q, O, S, and S-B increased, while B decreased. Increasing plasma AA concentrations in the presence of hyperinsulinemia (clamp 1 *vs.* 2 *vs.* 3) causes a graded rise in Q, S, O, and S-B. During isoaminoacidemia and hyperaminoacidemia in the presence of hyperinsulinemia, B decreased to a similar extent; in both cases, B is lower than during the hyperinsulinemic euglycemic clamp. This indicates that AAs reinforce insulin's suppression of B, although excessive levels of AAs are not necessary for this effect. Similar findings were obtained by Tessari et al. (1987). In that study, leucine kinetics were calculated using the primary model, the reciprocal model, and their sum, but I will not focus on the latter approach because the results were sometimes at odds with the other two calculations. In the presence of basal insulin, hyperaminoacidemia increased Q (reciprocal and primary models), decreased B (primary model), and increased O (reciprocal model). During hyperinsulinemia (650-720 pmol/L), that is, comparing percentage changes due to a hyperinsulinemic euglycemic clamp to those of a hyperinsulinemic euglycemic hyperaminoacidemic clamp, AAs caused O to increase. Flakoll et al. (1989), however, found that when a series of hyperinsulinemic (physiological to supraphysiological) euglycemic clamps with AA infusions (attempted isoaminoacidemia) were compared to those without AA infusions, the only statistically significant effect of AAs on leucine kinetics was to diminish B, although the authors argue that AAs tended to augment O and S. Taken together, the effects of AAs on kinetics of protein metabolism include increases in Q, O, S, and S-B (Castellino et al. 1987; Flakoll et al. 1989; Tessari et al. 1987), although the effect on O and S did not reach statistical significance in Flakoll et al. (1989). It is not clear, however, to what extent these effects are altered by circulating insulin levels (Castellino et al. 1987; Flakoll et al. 1989).

Research groups have attempted to maintain isoaminoacidemia during the hyperinsulinemic euglycemic clamp in order to establish the effects of insulin on kinetics of protein metabolism independently of AAs. Castellino et al. (1987), as previously described, attempted to maintain isoaminoacidemia by infusing 10% Travasol at a constant rate during a hyperinsulinemic euglycemic clamp and they were successful with respect to BCAAs and total AAs, but some individual AA concentrations fluctuated. Similarly, Heslin et al. (1992) performed a hyperinsulinemic (510 pmol/L) euglycemic clamp where leucine and total BCAA concentrations did not differ from baseline as a result of infusing 10% Travasol at a constant rate. Nevertheless, the individual concentrations of other AAs increased during the clamp. In response to hyperinsulinemia, leucine B diminished, O and S-B augmented, and S did not change. These findings agree with those of Castellino et al. (1987). To improve the ability to maintain postabsorptive AAs, Flakoll et al. (1989) altered the rate of infusion of 8.5% Travasol, according to plasma leucine concentrations, when performing various hyperinsulinemic euglycemic clamps. Leucine and essential AA concentrations during the clamps did not differ from baseline, but total AAs increased. While O and Q did not change, B decreased and S either did not change or decreased (highest insulin infusion). With respect to the decrease in S, Flakoll et al. (1989) proposed that the decrease in B was large enough to cause a decline in intracellular AA concentrations, even though plasma AAs were comparable or higher than baseline. In a similar experiment, Russell-Jones et al. (1994) used a hyperinsulinemic (350 pmol/L) euglycemic clamp where the AA (Vamin 14EF) infusion rates were altered based on plasma leucine readings. Although leucine concentrations during the clamp did not differ from baseline, other individual AA concentrations changed. Leucine O increased, B decreased, while both Q and O were not altered. Lastly, Frexes-Steed et al. (1990) performed a series of experiments, after participants had fasted for 12 and 84 hours. Since physiological parameters change as fasting continues, including insulin sensitivity of protein (Frexes-Steed et al. 1990), and since my focus is on the response of protein metabolism to insulin after an overnight fast, I will only elaborate on the results for the 12 hour fast. There were two euglycemic clamps, one that achieved hyperinsulinemia of 800 pmol/L and another of 11510 pmol/L, while ensuring baseline leucine concentrations by altering the

rate of infusion of 8.5% Travasol, based on plasma leucine measurements. Nevertheless, the concentrations of a variety of other AAs fluctuated. Compared to baseline, using the reciprocal model, leucine Q decreased during the higher insulin clamp, but there was no change in the lower clamp; B decreased in both clamps; and both O and S did not change. Taken together, these clamp protocols, which are often limited by the ability to truly maintain isoaminoacidemia, indicate that physiological hyperinsulinemia does not change Q (Castellino et al. 1987; Flakoll et al. 1989; Frexes-Steed et al. 1990; Russell-Jones et al. 1994), decreases B (Castellino et al. 1987; Flakoll et al. 1989; Frexes-Steed et al. 1990; Heslin et al. 1992; Russell-Jones et al. 1994), S does not change (Castellino et al. 1987; Flakoll et al. 1989; Frexes-Steed et al. 1990; Heslin et al. 1992; Russell-Jones et al. 1994), O increases (Castellino et al. 1987; Heslin et al. 1992; Russell-Jones et al. 1994) or does not change (Flakoll et al. 1989; Frexes-Steed et al. 1990), and S-B increases (Castellino et al. 1987; Heslin et al. 1992).

A more recent study altered the rate of infusion of 10% TrophAmine based on frequent readings of plasma BCAAs in order to maintain isoaminoacidemia during a hyperinsulinemic ($40mU/m^2 \cdot min$) euglycemic clamp in men (Chevalier et al. 2004). Most AA concentrations remained at basal levels, including BCAAs and total AAs. Leucine B decreased, O did not change, and Q and S-B both increased like in some previous studies. Surprisingly, S was stimulated by insulin in the presence of isoaminoacidemia. The ability to maintain basal AAs and the fairly robust sample size partially explain why the results for S in other studies differ from this study.

Nygren et al. (2003) recently explored how insulin and AAs affect kinetics of protein metabolism in different organs. Various protocols were carried out, including hyperinsulinemic euglycemic clamps with isoaminoacidemia or hyperaminoacidemia, using 10% TrophAmine; the level of insulinemia reached was five times above baseline (<200 pmol/L). Stepwise linear regression revealed that in muscle, B was diminished by insulin only, while insulin and AAs increased S. In the splanchnic area, AAs increased S and decreased B, but insulin did not alter S or B. The response of S to insulin found in this study differs from earlier reports involving hyperinsulinemic euglycemic clamps (Meek et al. 1998). There, it was found that in muscle, insulin did not alter S, but in the splanchnic area, insulin inhibited S. This is yet another example of misleading

interpretation of protein kinetics that may occur when the confounding effect of decreasing plasma AAs is present during clamps.

6.2. Obesity

Studies of protein metabolism in obesity in the postabsorptive state, in response to insulin, and during hyperinsulinemia are sometimes conflicting with respect to whether or not protein kinetics are altered and which kinetics are altered. Overall, the evidence suggests that protein kinetics are impaired in obesity.

6.2.1. Postabsorptive State: Plasma Amino Acids

Postabsorptive concentrations of BCAAs were found to be elevated among obese subjects compared to lean controls in some studies (Caballero et al. 1991; Chevalier et al. 2005b; Felig et al. 1969), but not in others (Luzi et al. 1996). With respect to fasting plasma leucine levels, some studies indicate that they are not different between obese and control groups (Chevalier et al. 2005b; Jensen et al. 1991; Luzi et al. 1996; Solini et al. 1997), but Caballero et al. (1991) and Felig et al. (1969) found them to be higher among the obese. Studies that measured total AA concentrations failed to find a difference between groups (Chevalier et al. 2005b; Luzi et al. 1996; Solini et al. 1997).

6.2.2. Postabsorptive State: Leucine Kinetics

Studies have found statistically significant differences in leucine kinetics between obese and lean subjects (Bruce et al. 1990; Chevalier et al. 2005b; Jensen & Haymond 1991; Nair et al. 1983). Nair et al. (1983) measured and calculated all ^{13}C-leucine kinetic variables in 5 healthy obese (BMI>35) women and 5 healthy lean (BMI<25) men and found that Q and S per kg LBM were greater in the obese but that Q and S per kg BW were statistically smaller in the obese; S-B and O were not different between the two groups, regardless of how they were normalized. Bruce et al. (1990) compared the ^{13}C-leucine kinetics of 6 obese healthy women to those of 6 healthy women of normal BMIs; their age was not significantly different. S per kg FFM was greater in the obese group, but B and O per kg FFM were similar between the two groups. Jensen & Haymond (1991) studied 3 groups of 10 age-matched healthy women, namely, Upper Body Obese

(UBO), Lower Body Obese (LBO), and lean. Both groups of obese women had BMIs between 30-36 and the criteria for placement in the UBO group was WHR>0.85 and WHR<0.76 for the LBO. ^{13}C-Leucine B per kg LBM was the only protein kinetic variable calculated in this protocol and it was found to be significantly greater in both the UBO and LBO groups compared to the lean group. Chevalier et al. (2005b) compared 10 obese women (BMI>30), 4 of which had abnormal OGTTs but were otherwise healthy, to 9 healthy women (BMI<25) with respect to all the ^{13}C- leucine kinetic variables. When leucine kinetics were expressed per kg FFM, Q, S, and O were greater and S-B was more negative among women. Thus, most of the above studies were able to show that obesity is associated with increased rates of protein synthesis and protein breakdown postabsorptively.

Other researchers, however, did not find any abnormalities in protein metabolism among obese subjects in the fasting state (Caballero & Wurtman 1991; Luzi et al. 1996; Solini et al. 1997). It is noteworthy that all of the above studies showed significantly higher levels of fasting insulin among the obese (both UBO and LBO in the case of Jensen & Haymond (1991)). Luzi et al. (1996) have suggested that in the presence of greater insulin concentrations, even comparable rates of protein metabolism between obese and lean subjects may represent insulin resistance of protein in the obese.

6.2.3. Hyperinsulinemic Euglycemic Clamp: Leucine Kinetics

In order to specifically assess insulin sensitivity of protein metabolism, without the confounding of different insulin levels, researchers have used the insulin clamp technique (Caballero & Wurtman 1991; Chevalier et al. 2005b; Jensen & Haymond 1991; Luzi et al. 1996; Solini et al. 1997). The hyperinsulinemic euglycemic clamp, originally devised to assess insulin sensitivity of glucose metabolism (DeFronzo et al. 1979), was performed at different levels of hyperinsulinemia in obese subjects (Caballero & Wurtman 1991; Jensen & Haymond 1991; Luzi et al. 1996; Solini et al. 1997). Using mostly infusion rates of 10mU/m^2·min, 20mU/m^2·min, and 40mU/m^2·min, studies have achieved clamp insulin concentrations that remained within the physiological range. The level of hyperinsulinemia achieved was as follows: 2 times above baseline (70-130 pmol/L) for an infusion of 0.2mU/kgLBM·min (Jensen & Haymond 1991); 2-5 times

above baseline (140-170 pmol/L) for an infusion of 10mU/m^2·min and 5-16 times above baseline (500-570 pmol/L) for insulin infused at 40mU/m^2·min (Caballero & Wurtman 1991; Luzi et al. 1996); 3-5 times above baseline (250-290 pmol/L) for an infusion of 20mU/m^2·min (Solini et al. 1997). Nevertheless, similar insulin infusion rates during the hyperinsulinemic euglycemic clamp do not necessarily correspond to similar conclusions with respect to the response of protein kinetics to insulin or the significance of group comparisons during the clamp.

Infusing insulin at a rate of 0.2mU/kgLBM·min (Jensen & Haymond 1991) resulted in a statistically significant decrease in Q expressed per kg LBM in LBO and lean subjects, but not UBO subjects. During the clamp plateau, Q per kg LBM was lower in the lean group compared with UBO and LBO groups. Luzi et al. (1996) studied ^{14}C-leucine kinetics, expressed per m^2, in 7 obese subjects (2 men, 5 women) and 18 lean controls (6 men, 12 women) matched for age by using 10mU/m^2·min as well as 40mU/m^2·min clamps. PIBW was the main criterion for classifying obesity. In both groups, Q and S decreased while S-B increased significantly in response to the two insulin infusions. Although O decreased significantly in both groups in response to the higher insulin infusion, O declined only in the lean group in response to the lower infusion. During the lower clamp, Q, O, and S were higher among the obese, but S-B was lower among the obese; during the higher insulin clamp, only O and S-B remained altered in the obese. This suggests that high insulin concentrations may be able to overcome abnormal rates of S and Q in the obese. Importantly, the study also shows that there is a diminished anabolic response to insulin in obesity.

Other studies, however, failed to detect differences either in the response to hyperinsulinemia or in the rates of leucine kinetics during the clamp plateau. Caballero & Wurtman (1991) reported only ^{13}C-leucine Q and O expressed per kg FFM when comparing 6 obese, but otherwise healthy women with 6 lean women. While Q dropped significantly in the obese and lean groups in response to both 10mU/m^2·min and 40mU/m^2·min insulin infusions, O stayed the same. Moreover, during the clamp plateau there were no differences in the values of the two kinetic variables between the groups. Similarly, Solini et al. (1997), by comparing 11 healthy obese to 9 lean female subjects matched for age, showed that during the 20mU/m^2·min clamp, there were no differences

in ^{14}C-leucine kinetics between the two groups. Moreover, hyperinsulinemia did not result in any statistically significant differences between baseline and the clamp in either group.

6.2.4. Hyperinsulinemic Euglycemic Clamp: Plasma Amino Acids

Luzi et al. (1996) showed that the effects of hyperaminoacidemia itself on protein kinetics are not different between obese and nonobese controls by performing a clamp where the basal levels of insulin, glucagon, and glucose were kept constant, but plasma AA concentrations were 2-3 times higher than baseline levels. Hyperinsulinemic euglycemic clamps, however, are accompanied by a decrease in plasma AAs. Jensen & Haymond (1991) demonstrated that leucine concentrations decreased in response to insulin in LBO and lean controls. Similarly, Caballero & Wurtman (1991) showed that the drop in leucine, isoleucine, and valine in response to two levels of hyperinsulinemia was statistically significant, although it is unclear whether or not the groups responded differently. Luzi et al. (1996) also showed that total AAs and BCAAs, including leucine, declined during both hyperinsulinemic clamps, in both groups, but the change was smaller in the obese group. On the other hand, Solini et al. (1997) showed that while the decrease in leucine was statistically significant in response to the clamp in both groups, total AAs did not change. Together, these results indicate that even in obesity, the equilibrium established during the clamp may not extend to AAs and this may be a confounding factor when assessing kinetics of protein metabolism.

6.2.5. Use of the Hyperinsulinemic Euglycemic Isoaminoacidemic Clamp

Chevalier et al. (2005b) studied insulin sensitivity of protein metabolism in obesity by performing a hyperinsulinemic (40mU/m^2·min; 570-760 pmol/L) euglycemic isoaminoacidemic clamp, where AAs were kept at basal levels; only the results expressed per kg of FFM will be discussed. In response to hyperinsulinemia, Q and S increased in each group and were similar between groups during the clamp. S-B also increased in both groups in response to insulin infusion, but remained lower and negative among the obese during the clamp, indicating insulin resistance of protein anabolism in obesity. O increased while B decreased comparably in both groups in response to hyperinsulinemia

and both O and B remained higher in obese subjects during the clamp. When these results are compared to the hyperinsulinemic euglycemic clamps that used the same insulin infusion rate, 40mU/m^2·min, (Caballero & Wurtman 1991; Luzi et al. 1996) one notes that B decreases consistently. On the other hand, O either increases, drops, or stays the same in the three studies. Moreover, while S decreases in the study by Luzi et al. (1996), it increases in the study by Chevalier et al. (2005b). Thus, basal levels of AAs in the presence of hyperinsulinemia stimulate protein synthesis, even among obese subjects.

6.2.6. Hyperinsulinemic Clamps: Concurrent Assessment of Insulin Sensitivity of Glucose Metabolism

Interestingly, regardless of the outcomes with respect to insulin sensitivity of protein metabolism and whether AAs were infused concurrently, all studies that simultaneously studied insulin sensitivity of glucose and protein metabolism (Caballero & Wurtman 1991; Chevalier et al. 2005b; Luzi et al. 1996; Solini et al. 1997) were able to demonstrate blunted rates of clamp glucose disposal and/or glucose infusion rates among the obese. This may further indicate that even though the technique for measuring insulin sensitivity of glucose is well established and appropriate, the methodology for measuring insulin sensitivity of protein may not always have been optimal in the studies performed so far.

6.2.7. Obstacles in Studying Kinetics of Protein Metabolism in Obesity

In addition to the type of clamp performed, other factors can affect kinetics of protein metabolism. The criteria for obesity and normal weight is not always consistent among studies, with some using PIBW and others using different cut-off points for BMI. The average BMIs for the obese and lean groups are within the current definition of obesity and normal weight for all studies except in Solini et al. (1997), where the average BMI for the obese group is 29. In fact, the latter study used a BMI>27 for the definition of obesity. Moreover, obesity is not a homogeneous disorder, since abdominal obesity shows a strong association with cardiovascular risk factors (Janssen et al. 2004; Shen et al. 2006; Zhu et al. 2005) and has adverse effects on the response of B to insulin (Jensen & Haymond 1991). Solini et al. (1997) also found negative correlations among the obese

between visceral fat and clamp B and S. With the exception of Jensen & Haymond (1991), none of the above studies make a clear and direct distinction between abdominal and lower body obesity. Indeed, Caballero & Wurtman (1991) considered the study subjects to be lower body obese, which potentially partly explains the lack of group differences. Thus, when studying kinetics of protein metabolism, both overall and abdominal obesity should be considered.

The rates of insulin infusion and the insulin levels reached during the clamp may also partly explain the different results, with Luzi et al. (1996) showing that higher insulin levels may overcome differences in certain leucine kinetic variables, as described above. Solini et al. (1997) used this argument to explain why they did not observe group differences at an insulin infusion of $20mU/m^2 \cdot min$, while Luzi et al. (1996) found differences at $10mU/m^2 \cdot min$. It nevertheless falls apart because Luzi et al. (1996) showed abnormal leucine kinetics at a higher insulin infusion, $40mU/m^2 \cdot min$.

The best way of expressing leucine kinetics for comparison is still debatable: μmol/min, divided by kg BW, divided by m^2, divided by kg FFM, or using indices of body composition as covariates, since results can differ depending on how protein kinetic variables are adjusted (Chevalier et al. 2005b; Luzi et al. 1996). A regression-based approach, using FFM as the independent variable, is the most appropriate method for adjusting leucine kinetics for body composition (Ravussin et al. 1989; Short et al. 2004b). Since FFM correlates with the dependent variable, it is a predicting factor, but if the regression line of the linear relationship does not go through the origin, it is inappropriate to divide the dependent variable by FFM (Allison et al. 1995) and FFM should be used as a covariate in the statistical analysis. None of the research involving protein metabolism in obesity to date applied these regression-based methods, raising the possibility that even when leucine kinetics were divided by kg FFM, it may have been inappropriate to do so.

The current definition of FFM is the sum of all bodily components, except "non-essential lipids"; the latter are mainly triglycerides (Wang et al. 1992). Thus, FFM consists mostly of water, proteins, minerals, glycogen, and "essential lipids" (Wang et al. 1992). The components of FFM and LBM are not systematic in the literature, especially before Wang et al. (1992) proposed the previously mentioned definition of FFM and that FFM and LBM should have the same meaning. Different definitions of FFM or LBM

may affect the definition of body fat, which is the difference between body weight and FFM or LBM, as well as the interpretation of results. The definition of FFM in the reference methods for the bioelectric impedance analysis (BIA) equations used in my protocol largely agrees with this definition of FFM (Heymsfield et al. 2005; Wang et al. 1992) (see Methods).

Caloric and protein intakes prior to the study day can affect the results of metabolic studies, particularly when nitrogen equilibrium is not present (Garlick et al. 1980; Motil et al. 1981; Pacy et al. 1994). Only some of the above studies specified some form of controlled protein and/or caloric intake (associated with weight stability) before the study day (Caballero & Wurtman 1991; Chevalier et al. 2005b; Jensen & Haymond 1991; Luzi et al. 1996; Nair et al. 1983); the controlled diet lasted at least 7 days, except in Caballero & Wurtman (1991) and Nair et al. (1983), where diet control was shorter. Particularly, the two studies that did not find defective protein metabolism in obesity in response to insulin and during the clamp plateau either did not control the subjects' dietary intake before the study (Solini et al. 1997) or the dietary intake was only controlled for 3 days prior to the study (Caballero & Wurtman 1991).

Lastly, sample sizes are often small in studies of protein metabolism in obesity, ranging from 5 to 11 obese subjects (Nair et al. 1983; Solini et al. 1997). This reduces the power of the study and each group needs to be fairly homogeneous in terms of variables that affect leucine kinetics if researchers are to detect obesity effects. Chevalier et al. (2005a) have also demonstrated sex differences in protein metabolism, implying that in studies involving both sexes, failure to adjust for sex may have lead to incorrect conclusions. For example, Nair et al. (1983) compared obese women to lean men in their study, thereby creating ambiguity over whether the differences observed were the result of obesity, sex, or both.

6.3. Type 2 Diabetes Mellitus

An effect of T2DM on kinetics of protein metabolism is controversial as a result of conflicting reports, depending on disease severity, prior dietary control, characteristics of study populations, and data analysis.

6.3.1. Postabsorptive State: Plasma Amino Acids

About 20 years ago Vannini et al. (1982) showed that improving diabetes control decreased fasting BCAA levels in T2DM. Nevertheless, Umpleby et al. (1990) showed that T2DM with supra-optimal fasting glucose levels had similar levels of fasting leucine to controls and that these levels did not change in T2DM as the day progressed. Luzi et al. (1993) also found similar levels of leucine, BCAAs, and total AAs in controls and T2DM with fasting hyperglycemia. Similarly, Pijl et al. (1994) and Halvatsiotis et al. (2002b) found that the postabsorptive concentrations of each BCAA were not different between control and T2DM subjects in whom euglycemia was achieved by overnight insulin infusion. As well, during both poor and improved glycemic control, the concentration of each of the BCAAs was not significantly different between T2DM and controls in a study by Denne et al. (1995). During poor and optimal fasting glycemic control, plasma concentrations of each of the BCAAs were not different between overweight/obese T2DM and lean or obesity-matched controls (Halvatsiotis et al. 2002a). Welle et al. (1990) also found that leucine concentrations were not altered in T2DM, regardless of diabetes treatment prior to the study day.

6.3.2. Studies Involving ^{15}N-glycine Tracer

All studies using ^{15}N-glycine methodology found abnormal protein metabolism in T2DM, regardless of the level of diabetes control. ^{15}N-glycine was ingested over a 60h period and oral antihyperglycemic agents were discontinued before or on the first day of admission, which coincided with the first day of the diet (Gougeon et al. 1998; Gougeon et al. 1994; Gougeon et al. 1997; Gougeon et al. 2000). A 6-8 day weight-maintaining diet was administered in all 4 studies, with some studies giving the same amount of protein to all participants (Gougeon et al. 1994; Gougeon et al. 1997), while other studies gave 1.5g of protein per day per kg of the body weight the person would have at a BMI of 25 (Gougeon et al. 1998; Gougeon et al. 2000). Furthermore, all study groups contained men and women and the T2DM did not have diabetic complications or other serious illnesses.

In the earliest study (Gougeon et al. 1994), 7 hyperglycemic obese T2DM were compared to obese controls, and Q, S, B were higher in the diabetic subjects, although it

is unclear if they were normalized for LBM. The same results were obtained for these 3 variables, expressed per kg FFM, by Gougeon et al. (1998) when 9 hyperglycemic obese T2DM and 7 healthy obese controls matched for age were studied. S-B per kg FFM was also smaller among diabetic subjects than controls. When glycemia was improved through insulin injections, only S and B were still higher in T2DM compared to controls. The effect of the insulin treatment was only seen as a statistically significant augmentation in S-B. Gougeon et al. (2000) showed, once again, that Q and B were greater and S-B was smaller in 13 hyperglycemic obese T2DM compared to 10 healthy obese controls. These variables were adjusted for FFM, sex, and age. S, however, was not different between groups after adjusting for the latter 3 covariates. When glycemia was improved through the use of oral antihyperglycemic agents, namely metformin and gliclazide, S became greater and B remained higher in diabetic subjects than controls. A treatment effect was only observed in S-B, which increased with improved diabetes control. Lastly, Gougeon et al. (1997) studied the effects of normalizing glucose levels in 7 hyperglycemic obese T2DM using exogenous insulin. When divided by kg BW or kg LBM, all glycine kinetic variables improved; Q, S, and B lowered while S-B augmented. These results jointly indicate that protein kinetics are impaired in obese T2DM and while improved glycemic control through the use of insulin or oral antihyperglycemic agents is associated with improvements in all or some of the kinetic variables, they do not all seem to return to the levels found in obese controls.

6.3.3. Studies Involving ^{13}C- or ^{14}C-leucine Tracer

6.3.3.1. Postabsorptive State: Leucine Carbon Flux

Using ^{13}C- or ^{14}C-leucine as tracers, only 2 studies have found altered postabsorptive protein kinetics in T2DM (Halvatsiotis et al. 2002a; Umpleby et al. 1990). Staten et al. (1986) were the first to study protein metabolism in T2DM using ^{13}C-leucine; specifically they used L-[1-^{13}C,^{15}N]leucine. The two groups were 5 obese women with T2DM, some of which had diabetic complications, studied during hyperglycemia and improved glycemia, and 5 healthy obese women of comparable age. For 1 week prior to each study day, the T2DM group received insulin injections, either at the typical dosage or at dosages to achieve better control. Leucine carbon Q and O were measured, but

there were no differences between the two groups at each level of glycemic control and there was no therapy effect. The authors suggested that there is enough insulin in T2DM to normalize protein kinetics, even during poor glycemic control. Nevertheless, the fact that the T2DM group received insulin prior to the 2 study days raises the possibility that protein kinetics were modified favorably by exogenous insulin. Subsequently, Welle & Nair (1990) measured B using ^{13}C-leucine in 5 obese T2DM women and 10 obese non-diabetic women, only half of whom had Percent Body Fat (PBF) above 35%. T2DM subjects were studied in the hyperglycemic state, after having ceased to take diabetes medication for 3 weeks; in an improved, better controlled state after 2 weeks of glyburide; and in the euglycemic state after 2 weeks of insulin injections. B per kg BW and B per kg FFM were similar between T2DM and each control group and treatments had no effect. Insulin concentrations were comparable between groups and were not altered by treatment in T2DM. Interestingly, B expressed per kg FFM was greater among the controls with more than 35% body fat, compared to "leaner" controls, which is consistent with abnormal protein kinetics in obesity. Hence, the authors concluded: "obesity per se (without diabetes) increases proteolysis". That same year, however, Umpleby et al. (1990) used ^{14}C-leucine to show that even though when expressed per kg BW, Q and S were normal, O was higher in T2DM subjects than controls. The groups consisted of 5 hyperglycemic men and women with T2DM and 5 men and women of comparable age serving as controls; the participants had either a normal BMI or were overweight. Nevertheless, some T2DM subjects had co-morbidities, T2DM subjects were on a hypocaloric diet, and it is not clear if and when diabetes medications were stopped.

More recent research of protein metabolism in the postabsorptive state using ^{13}C-leucine and ^{14}C-leucine tracers consists of larger sample sizes. Luzi et al. (1993) compared 6 subjects with T2DM (5 men, 1 woman) to 7 controls (5 men, 2 women) of similar age. Adiposity, as assessed by PIBW, was normal and similar in both groups. Controls were free of any illness and T2DM subjects did not have any other serious health problem. T2DM subjects stopped diabetic medications 1 week prior to the study, at which time they were hyperglycemic. When ^{14}C-leucine Q, S, O, and S-B were measured and expressed per m^2, there were no differences between the two groups. The

authors, nevertheless, did not rule out insulin resistance of protein metabolism: "because the fasting plasma insulin concentration was significantly increased in T2DM *vs.* control subjects, one could argue that a subtle defect exists in the ability of insulin to inhibit endogenous Leu flux".

Halvatsiotis et al. (2002b) further explored the concept of "relative" insulin resistance of protein. The study design involved 7 T2DM (5 men, 2 women) and 8 controls (5 men, 3 women) of comparable age, sex, BMI, and WHR. ^{13}C-leucine Q expressed per kg BW was measured and found not to be different at baseline. In the T2DM group, oral antihyperglycemic agents were stopped 2 or more weeks before the study day, but insulin was administered the night before the study to ensure euglycemia in the morning of the study. Moreover, fasting insulin levels were higher among T2DM subjects than controls. Thus, the authors argued that properly managed T2DM patients "appropriately adapt" to chronic high insulin levels and maintain rates of breakdown comparable to controls.

Halvatsiotis et al. (2002a) used L-[1-^{13}C,^{15}N]leucine to study carbon Q, O, and S expressed per kg FFM. Three study groups, each with 4 men and 4 women, were matched for age: T2DM subjects, controls of similar BMI and body composition, and lean controls. While the first two groups had average BMIs of 30, the lean controls had BMIs between 19 and 25. T2DM subjects were studied in the hyperglycemic state, after being off their usual diabetic treatment during 2 weeks, and in the euglycemic state, after intensive insulin therapy for 11 days and intravenous insulin during the night before the study day. Controlled T2DM had higher fasting insulin than uncontrolled T2DM, overweight/obese controls, and lean controls. The only difference in carbon leucine kinetics was found between uncontrolled T2DM and lean controls, the former having greater values in Q. This could be easily explained, however, as an obesity and not a diabetes effect. Nevertheless, the fact that among T2DM subjects, a chronic increase in insulin had no effect on carbon leucine kinetics may be indicative of insulin resistance to protein metabolism (Halvatsiotis et al. 2002a; Halvatsiotis et al. 2002b; Luzi et al. 1993).

6.3.3.2. Postabsorptive State: Leucine Nitrogen Flux and Leucine Transamination

An additional benefit of using the tracer L-[1-^{13}C,^{15}N]leucine is that leucine nitrogen flux can be studied. Two studies, already previously discussed, have used this tracer to study protein metabolism in the context of T2DM (Halvatsiotis et al. 2002a; Staten et al. 1986). The latter study found no differences in leucine nitrogen flux between the diabetic and control groups, but the sample size was fairly small (5 in each group) and insulin was administered in T2DM during the poor management phase as well as during the improved management phase. On the other hand, Halvatsiotis et al. (2002a) showed that leucine nitrogen flux was greater in hyperglycemic T2DM than euglycemic T2DM subjects. Leucine nitrogen and carbon flux are associated with reversible transamination of leucine to α-ketoisocaproate (α-KIC) (Devlin 2002; Halvatsiotis et al. 2002a) and both the rate of production of α-KIC from leucine as well as the rate of production of leucine from α-KIC were found to be elevated in hyperglycemic *vs.* euglycemic T2DM subjects (Halvatsiotis et al. 2002a). Since leucine O was not affected by the level of T2DM control, the authors proposed that the high rates of leucine transamination in hyperglycemic T2DM subjects are necessary to maintain a steady supply of AAs for gluconeogenesis.

6.3.3.3. Hyperinsulinemic Euglycemic Clamp: Leucine Carbon Kinetics

Only 2 research teams to date have published data on insulin sensitivity of protein metabolism, combining the insulin clamp with ^{13}C- or ^{14}C-leucine methodology (Halvatsiotis et al. 2002b; Luzi et al. 1993). The study designs have already been briefly described above. Halvatsiotis et al. (2002b) performed a hyperinsulinemic (0.5mU/kg/min) euglycemic clamp on T2DM subjects and controls, where clamp insulin concentrations were 2-6 times above baseline (220 pmol/L). During the clamp, insulin levels were not different between the two groups. In response to acute hyperinsulinemia, Q expressed per kg BW dropped significantly in each group, but clamp values were not different between groups. Similarly, in Luzi et al. (1993) insulin infusions at 40mU/m^2·min resulted in clamp insulin values that were 2-4 times greater than baseline (about 450 pmol/L), and insulin levels were comparable during the clamp. Q, S, and O decreased and S-B increased significantly, when divided by m^2, in each group as a result

of the clamp. There were no differences between study groups in the changes in kinetics and during the clamp. Thus, it was concluded that when insulin is augmented in the short-term, as during a clamp, changes in T2DM's protein kinetics are comparable to controls (Halvatsiotis et al. 2002b), but when T2DM subjects have significantly higher insulin levels for long periods of time, there is an adaptation in T2DM such that the protein kinetics in the two groups are comparable (Halvatsiotis et al. 2002b; Luzi et al. 1993).

6.3.3.4. Hyperinsulinemic Euglycemic Clamp: Plasma Amino Acids

In both of the above clamps, however, leucine and total BCAAs dropped in T2DM and control groups. While total AAs also declined in both groups in Luzi et al. (1993), total AAs decreased only among controls in Halvatsiotis et al. (2002b). However, the decline in AA concentrations in both studies was such that during the clamp, AA levels were comparable between groups. Pijl et al. (1994) compared 6 obese T2DM women to 6 obese controls using hyperinsulinemic euglycemic clamps. Two clamps were performed, one with insulin infusion at 1mU/kg·min and the other at 10mU/kg·min resulting in 2-7 times and 36-133 times higher insulin levels, respectively. Each BCAA was reduced in each group with the clamps, but less so in T2DM subjects for isoleucine in both clamps. Much like it was discussed in obesity, these alterations in AA levels during the hyperinsulinemic clamp may confound results.

6.3.3.5. Hyperinsulinemic Clamps: Effect of Amino Acid Infusion

To partially investigate the effect of AAs on protein kinetics in T2DM, Luzi et al. (1993) also performed a hyperinsulinemic (40mU/m^2·min; about 450 pmol/L) euglycemic hyperaminoacidemic (2 times basal levels) clamp using 10% Travasol. Similar to the hyperinsulinemic euglycemic clamp, no differences from controls were noted. Expressed per m^2, B decreased while S, O, and S-B increased significantly and to the same extent in each group. Although O, S, and S-B were not compared between the 2 clamp protocols, B was found to be lower during the hyperinsulinemic euglycemic hyperaminoacidemic clamp than during the hyperinsulinemic euglycemic clamp in controls and T2DM. Thus, AAs play a role in modulating protein kinetics in hyperinsulinemic conditions and their

role is not affected by T2DM (Luzi et al. 1993). It remains to be seen, however, what the insulin sensitivity of protein metabolism would have been if AAs had been kept at basal levels.

6.3.3.6. Hyperinsulinemic Clamps: Concurrent Assessment of Insulin Sensitivity of Glucose Metabolism

Despite conflicting results regarding insulin sensitivity of protein metabolism in T2DM, glucose metabolism has consistently been shown to be altered in T2DM, as indicated by lower clamp glucose disposal rates (Halvatsiotis et al. 2002b; Pijl et al. 1994) and glucose infusion rates compared with controls (Halvatsiotis et al. 2002b; Luzi et al. 1993).

6.3.4. Studies Involving Phenylalanine Tracers

Biolo et al. (1992) compared 8 non-obese T2DM (4 men, 4 women) on diet therapy to 6 controls (3 men, 3 women), after matching for age and BMI. Although fasting glucose was greater in T2DM, insulin was not different between the groups. Breakdown in the postabsorptive state, as calculated using L-[2,6-^{3}H]phenylalanine, was divided by kg of BW; this value was not altered in T2DM. Most recently, Halvatsiotis et al. (2002a) showed similar results in the postabsorptive state using L-[^{15}N]phenylalanine. Three groups were analyzed; overweight/obese T2DM (before and after treatment), controls of comparable weight, and lean controls, as previously described. Neither phenylalanine Q nor S nor the rate of phenylalanine hydroxylation to tyrosine, all divided by kg FFM, were different among any of the groups. An additional analysis of tyrosine Q, using tyrosine tracer methodology, also showed no differences.

Nevertheless, it has been shown that there are abnormalities in B, using L-[ring-$^{2}H_5$]phenylalanine as tracer, in a slightly different population (Denne et al. 1995). Six men and women with T2DM whose BMI ranged from overweight to morbidly obese, but who were otherwise healthy, were compared to 10 healthy male and female controls. The groups were not different with respect to age, weight, PBF, and BMI. T2DM subjects were studied after 2 weeks without treatment and again after 3-4 weeks of insulin injections, which caused fasting glucose to improve. B was calculated at baseline and

during a hyperinsulinemic (>10000 pmol/L) euglycemic clamp and it was found to be greater among T2DM participants at baseline and during the clamp. By expressing B per kg LBM, there was a group difference at baseline, but not during the clamp. The clamp effect (a decline) in B was significant in each group. Treatment had absolutely no effect on these findings. The hyperinsulinemic studies in this paper have a major drawback; not all participants were infused with insulin at the same rate nor was euglycemia always maintained. Despite this, it was shown that glucose disposal with the hyperinsulinemic clamp was lower in T2DM than controls, whether treated or not.

6.3.5. Obstacles in Studying Kinetics of Protein Metabolism in Type 2 Diabetes Mellitus

Numerous factors may explain the conflicting results among studies with leucine and phenylalanine tracers in T2DM. Many of the obstacles in protein metabolism studies in obesity also apply to T2DM. Specifically, AA levels are not controlled for during the clamp, sample sizes tend to be small, studies contain a mixture of men and women, and body composition variables such as BMI are not always matched to controls (Halvatsiotis et al. 2002b). In fact, some studies do not use BMI; they use PIBW as a measure of obesity (Luzi et al. 1993; Staten et al. 1986). To complicate matters further, there is no consensus regarding how to adjust leucine and phenylalanine kinetics for body composition and some T2DM subjects have co-morbidities (Staten et al. 1986; Umpleby et al. 1990). Caloric and protein intakes prior to the study days are also inconsistent among studies and even between diabetic subjects and controls in the same study. Some studies simply did not control the diet before the study days (Halvatsiotis et al. 2002b), while others required all subjects to consume an isoenergetic diet (Biolo et al. 1992; Denne et al. 1995; Halvatsiotis et al. 2002a; Luzi et al. 1993; Staten et al. 1986) for variable amounts of time before the study, from 3-90 days. Welle & Nair (1990) required T2DM subjects to be on an isoenergetic diet for 7 days, but the controls only had to consume it for 3 days. Protein intake is also not always consistent within a study as a percentage of total calories. Moreover, participants are not always admitted during these days of diet consumption, which raises issues of dietary adherence and questions the effect of different activity levels among the participants. Denne et al. (1995) have also

raised the question of whether or not the level of fasting glycemia has an effect on research findings in T2DM. Since hyperglycemia may be associated with hyperinsulinemia in the fasting state of T2DM subjects and since hyperinsulinemia has been linked to "relative" postabsorptive insulin resistance of protein, this may be another factor worth looking into.

Finally, there is the issue of variability of T2DM duration and whether this may account for the variation in leucine kinetics. As an example, T2DM subjects in Welle & Nair (1990) were all middle-aged and had diabetes for 1-15 years. While "Subject 1" had T2DM for 15 years, her fasting glucose was not the highest once she stopped her medication. This is compounded by the fact that when patients are officially diagnosed with T2DM, they have had the disease for some time (American Diabetes Association 2006a); the length of time between pathogenesis and diagnosis is unknown. Diabetes management is affected by many variables, genetic and environmental, and perhaps protein metabolism in T2DM is more sensitive to these factors than carbohydrate metabolism. It has also been suggested that variability in leucine kinetics in T2DM is more substantial than in controls, even among subjects with similar body composition (Welle & Nair 1990). Thus, study designs should not only attempt to match T2DM subjects as closely as possible to controls, but intra-variability of anthropometric and disease characteristics should also be kept at a minimum in each group.

7. RATIONALE AND OBJECTIVES

There are insufficient and conflicting results with respect to whole-body protein kinetics in overweight and moderately obese subjects with T2DM in the postabsorptive and insulin-stimulated states. Insulin decreases the plasma concentration of AAs, thereby complicating the interpretation of kinetics of protein metabolism during the hyperinsulinemic euglycemic clamp. Furthermore, previous studies rarely took into account the effects of sex and regional adiposity on protein kinetics. The energy and protein intakes before the clamp study day have not always been adequately controlled. Hence, in the current study subjects were matched for BMI, waist circumference, WHR, and age; only men were studied. A weight-maintaining, protein controlled diet was consumed throughout the period of admission and T2DM stopped oral antihyperglycemic

agents and other medications that may affect glucose or protein metabolism before admission. The objectives on the last study day were:

1) To combine the hyperinsulinemic euglycemic isoaminoacidemic clamp with L-[1-^{13}C]leucine methodology to determine if there is an effect of T2DM on insulin sensitivity of protein in overweight and obese subjects.

2) To use D-[3-^{3}H]glucose and indirect calorimetry during the hyperinsulinemic euglycemic isoaminoacidemic clamp to investigate the effect of T2DM on insulin sensitivity of glucose and substrate utilization.

8. HYPOTHESES

It was hypothesized that:

1) Basal levels of leucine Q and B are higher while net balance (S-B) is more negative in T2DM than controls.

2) Insulin sensitivity of protein is reduced in T2DM *vs.* control men, particularly that in response to hyperinsulinemia, the increase in Q, S, and S-B and the decrease in B are smaller in T2DM. This results in lower Q, S, and S-B and higher B among T2DM during the clamp in comparison to controls. O is greater among T2DM subjects at baseline, in response to the clamp, and during the clamp.

3) In T2DM, insulin-mediated total glucose disposal, oxidative glucose disposal, and non-oxidative glucose disposal are lower than in controls.

4) Lipid and protein oxidation are higher in T2DM than controls.

9. METHODOLOGY

9.1. Subject Recruitment

Ethics approval for the study was obtained from the McGill University Health Centre's (MUHC) Royal Victoria Hospital (RVH) Research Ethics Board and renewed annually (**Appendix 1**). Subjects were recruited from the Metabolic Day Centre (MDC) in the RVH, through posters at the RVH and Montreal General Hospital (MGH), by contacting patients who had already participated in studies or who had previously expressed interest in studies at the Clinical Investigation Unit (CIU) of the RVH, and through newspaper advertisements. T2DM subjects were more difficult to recruit than controls and this is partly explained by the prevalence of T2DM. According to Statistics Canada (Health Indicators 2004 Jun), the 2003 prevalence of diabetes in the three health regions that include or surround the city of Montréal were 4.7% (Région de Montréal-Centre), 5.9% (Région de Laval), and 5.1% (Région de la Montérégie). The same data source indicates that the prevalence of diabetes in the province of Québec and in Canada is 4.6%. The diabetes mellitus prevalence calculation by Statistics Canada, however, includes adolescents and does not differentiate between type 1 and 2 diabetes mellitus. Taken together, the prevalence of T2DM in adults and thereby the number of potential T2DM subjects for the current study is expected to be lower than the Statistics Canada estimates. From a public health perspective, the low prevalence of T2DM is positive, but this creates a recruitment challenge.

Patients with endocrinological disorders, mainly diabetes mellitus, are referred to the MDC, where they have appointments with physicians, nurses, and dietitians. The study was introduced to physicians through written notices and personally (myself and my supervisor) and permission was obtained to approach their patients. The study was also explained to nurses, dietitians, and administrative assistants. After receiving approval from the MUHC Office of the Director of Professional Services to review patients' charts in the clinic, I looked through the charts of patients to identify potential study candidates. A tag was placed on the medical chart of each potential candidate to let health care professionals know that I was interested in approaching the patient. This was not a very fruitful recruitment strategy for T2DM subjects. Oftentimes, the MDC was extremely busy and patients either came late for appointments or were in a rush to leave

the clinic; there was limited time and space to explain the study. Many patients worked or had responsibilities which prevented them from being admitted to the hospital for a week while others simply did not want to stay in a hospital for prolonged periods of time, especially during the outbreak of *Clostridium difficile* in Québec hospitals. An advertisement of the study was also posted on the Canadian Diabetes Association (CDA) website (www.diabetes.ca) in an attempt to recruit more T2DM subjects. Although e-mails were received from interested people, they were all Ontario residents and therefore ineligible (see below).

A better recruitment strategy for T2DM subjects was the use of visually appealing posters at RVH and MGH in English and French (**Appendix 2**). The MUHC Communications Office allows posters to be placed in designated areas for limited periods of time, after which they must be replaced. Since people do not simply go to hospitals for health reasons, but also to visit family and friends and for work, posters are able to capture a larger group of potential subjects. A second successful recruitment strategy for T2DM subjects was contacting, by phone or in person, people who previously either participated in CIU studies or expressed interest in CIU studies but were ineligible. Recruitment of controls involved mainly French and English community newspaper advertisements.

9.2. Subject Eligibility

To be eligible, subjects could not be simultaneously enrolled in another study and had to be covered by the Québec medical insurance program, Medicare. Inclusion criteria included stable weight for six months with protein intake assessed as being above the Recommended Dietary Allowance (RDA) of 0.8g/kg BW/day for adults (56g/day for men) (Institute of Medicine of the National Academies et al. 2005; Shils et al. 1999), contributing close to 15% of energy requirements; the average protein intake in the Western world is greater than the RDA (Eeley et al. 1996; Smit et al. 1999). Recent inconsistent and/or fad diets were probed if present. Weight loss improves insulin resistance of glucose in the liver as well as muscle (Williams et al. 2003; Williams et al. 2000) and alters protein metabolism, although in the latter case there is also a dependence on the level of protein intake (Garlick et al. 1980). Even though eligible subjects were

placed on an isoenergetic, protein controlled diet for a few days before the clamp study, recent unstable weight or inadequate protein intake could still affect the metabolic parameters assessed on the clamp study day.

In simple terms, controls were healthy and T2DM subjects did not have any major health problem other than T2DM itself. Certain lifestyle behaviours and all serious medical conditions were exclusion criteria. The former included smoking and excessive alcohol consumption. Alcoholism leads to alterations in carbohydrate, protein, and fat metabolism (Preedy et al. 2001; Preedy et al. 1999), and smoking affects carbohydrate metabolism (Jensen et al. 1995). Serious medical disorders include cancer, infectious diseases, autoimmune disease, and cardiovascular disease (angina, stroke, myocardial infarction) and medication used to treat them. Laboratory results such as serum creatinine above 120μM and hemoglobin below 130g/L also prevented inclusion of participants in the study. These disorders or results are associated with metabolic or hemologic disturbances (Kotler 2000; Rule et al. 2006) and/or they are not compatible with donating substantial volumes of blood (clamp study day). Potential subjects taking other medications, such as diuretics, β-blockers, bronchodilators, nonsteroidal anti-inflammatory drugs, antiarrhythmics, and oral steroids were excluded from the study to prevent confounding alterations in carbohydrate and protein metabolism (Harper et al. 1995; Kaplan 1992; Löfberg et al. 2002; Short et al. 2004a). The presence of diabetes complications, namely nephropathy, neuropathy and retinopathy, among T2DM were grounds for exclusion. Since the goal of my protocol was to study T2DM in the hyperglycemic state, a short term increase in plasma glucose may have exacerbated these conditions. However, these criteria further restricted the eligible T2DM population. For T2DM, optimal or severely poor diabetes control, as assessed by fasting and postprandial glucose and A1C (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003), were also exclusion criteria. Conditions such as dyslipidemia and controlled hypertension were not exclusion criteria.

Since age, sex, and level of obesity can confound insulin resistance, inclusion criteria took these variables into account. In adults, aging is accompanied by decreases in LBM concurrently with increases in fat mass (Cohn et al. 1980; Forbes 1987). Moreover, after adjusting for LBM, insulin resistance of glucose is reported to increase with age in

men (Franssila-Kallunki et al. 1992; Fukagawa et al. 1988). In terms of protein metabolism, using a regression-based approach Short et al. (2004b) found that leucine B, S, and O decreased with age in the fasting state after adjusting for FFM. In contrast, postabsorptively, Chevalier et al. (2006) and Fukagawa et al. (1989) documented similar FFM-adjusted leucine kinetics between young and elderly subjects and Balagopal et al. (1997) found no differences in leucine kinetics between a middle-aged (52 years) and elderly group (77 years), although some differences existed between middle-aged or elderly and the young group. During the hyperinsulinemic euglycemic isoaminoacidemic clamp, the elderly also show a smaller leucine net balance (S-B) (Chevalier et al. 2006). Adiposity, overall and abdominal, appears to be the important determinant of insulin resistance of glucose and protein, and not age (Boden et al. 1993; Chevalier et al. 2006; Kohrt et al. 1993). Since no differences in postabsorptive protein metabolism were found between middle-aged and elderly persons (Balagopal et al. 1997) and in an attempt to maximize recruitment, the age range for inclusion was set from 18 to 70 years of age. A study goal was to match groups for age.

Women have greater PBF and smaller LBM than men (Cohn et al. 1980; Forbes 1987). Moreover, the effects of sex on protein metabolism have been documented by Chevalier et al. (2005a) and include a blunted net balance in women during the hyperinsulinemic euglycemic isoaminoacidemic clamp. Short et al. (2004b), however, failed to find sex differences after adjusting for FFM. In the latter study, it is not clear at which stage of the menstrual cycle women were studied, which has been shown to alter protein metabolism (Tipton 2001). Thus, to avoid the confounding effect of sex, only men were studied in the current experimental protocol.

Obese individuals, as assessed by BMI, are at increased risk for insulin resistance of glucose and protein metabolism, as previously discussed. It was therefore important to match both study groups for BMI. Regional obesity, as assessed by waist circumference (mainly) and WHR, increases the risk of insulin resistance of glucose and protein (Jensen & Haymond 1991; Kohrt et al. 1993), and hence, groups were also matched for abdominal obesity.

Determining eligibility of interested subjects involved various steps. First, a pre-screening questionnaire (**Appendix 3**) was done with potential subjects to see if they fit

the above criteria. After I met with the research nurse and the study physician to review the filled out pre-screening questionnaire, a decision was made on whether or not to invite the potential subject for a screening visit. The screening visit took place in the CIU at 8 am, after an overnight fast; T2DM subjects did not take any medication that morning. The consent form was explained and questions regarding the study were answered; consent forms were available in English and French, although only English versions are provided in **Appendix 4 and 5**. Once the consent form was signed by the participant, weight and height were taken followed by REE (Deltatrac, SensorMedics, Yorba Linda, CA) and BIA (RJL-101A Systems, Detroit, MI) measurements. Blood and urine samples were collected for standard blood and urine tests. This was followed by a chest X-ray and an electrocardiogram (ECG) to assess pulmonary and cardiac functions respectively. A detailed medical history was also obtained through an interview with the research nurse. Based on the results from blood tests, urine tests, chest X-ray, ECG, and a physical exam, the study physician decided whether or not the potential subject was fit physiologically to do the study.

Control subjects who were selected to participate in the study underwent a standard Oral Glucose Tolerance Test (OGTT). Briefly, subjects came to the CIU in the morning after an overnight fast and 75g of glucose were ingested (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003). Blood samples were taken at -15min, 0min, and every half an hour afterwards and plasma glucose concentrations were measured using G7M Micro-Stat (Analox Instruments USA, Lunenberg, MA). If, at 2 hours, the plasma glucose was 7.8-11.0 mmol/L and the fasting glucose was less than 6.1 mmol/L, the subject was diagnosed with Impaired Glucose Tolerance (IGT) (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003). Fasting glucose levels between 6.1 and 6.9 mmol/L with a 2 hour OGTT less than 7.8 mmol/L is indicative of Impaired Fasting Glucose (IFG) (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003). When the fasting glucose is between 6.1 and 6.9 mmol/L and the 2 hour OGTT is between 7.8 and 11.0 mmol/L, then both IFG and IGT are present (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003).

9.3. Study Protocol

The total length of the study was 7 days for control subjects and 8 days for T2DM subjects. For the first 6 days (controls) or 7 days (T2DM), subjects were placed on an isoenergetic, protein controlled diet (see below). On the last study day, the clamp took place. T2DM subjects were admitted to the CIU of the RVH for all 8 days, while control subjects usually spent the first two days at home, although they had the option of being admitted to the CIU on day 1 or 2. In the period between the screening visit and the first day of the study, subjects were instructed to maintain the same eating and lifestyle habits.

Approximately one week before admission, T2DM subjects stopped taking their oral antihyperglycemic agents and only started treatment again once the study was complete. The importance of this requirement is twofold: first, since the goal of the study was to investigate T2DM metabolism in the hyperglycemic state, removal of T2DM treatment ensured that glycemic control would worsen and second, oral antihyperglycemic agents as well as insulin therapy are confounding variables because they improve glycemic control, insulin resistance of glucose, and kinetics of protein metabolism in T2DM subjects (Andrews et al. 1984; Bailey et al. 1996; Beeson et al. 2003; Carey et al. 2002; Gougeon et al. 1998; Gougeon et al. 2000; Paterson et al. 1985; Pratipanawatr et al. 2002; Roden et al. 2005; Tan et al. 2004). The exact time when oral antihyperglycemic agents were stopped depended on the level of T2DM control before admission and the oral antihyperglycemic agents being taken. Simultaneously, the time selected by the study physician for T2DM to stop their oral antihyperglycemic agents attempted to minimize excessive hyperglycemia on an outpatient basis. Oral antihyperglycemic agents like metformin and thiazolidinediones (Bailey & Turner 1996; Lebovitz 2005), which improve insulin resistance in a more direct manner, were stopped in advance of oral antihyperglycemic agents like glyburide, which stimulate insulin production by the pancreas (Lebovitz 2005). Once off oral antihyperglycemic agents, T2DM subjects were instructed to check blood glucose levels regularly and these results were monitored by the research team. Most T2DM subjects in the study were taking oral antihyperglycemic agents. One subject was only using diet to control T2DM. Another subject was on insulin and oral antihyperglycemic agents; on admission, the dosage of Humulin®-N (Eli Lilly Canada Inc, Toronto, Canada) was reduced and a sliding scale of

Humulin®-R (Eli Lilly Canada Inc, Toronto, Canada) was added to maintain hyperglycemia but prevent excessively high glucose levels. Poorly controlled T2DM induced by stopping or reducing treatment in a brief period of time is not expected to have a significant negative impact on T2DM management and health in the long term. Lipid-lowering medication was also stopped upon admission, but some anti-hypertensives that do not affect carbohydrate or protein metabolism were continued throughout the study.

During the admission period (first 6 days for controls and first 7 days for T2DM), subjects had their own room, fridges (one for food and water, the other for urine bottles), and washroom in the CIU. They also had access to shower facilities. On Day 1, between 7:45 am and 8 am the subjects voided completely and the urine was discarded. Starting at 8am on Day 1, all urine was collected in appropriate bottles on a 24 hour cycle until the clamp study day; the last urine collection was actually a 23 hour collection because the subjects had to wake up earlier on the day of the clamp protocol. Each morning, subjects were awoken at approximately 7:45 am. They voided completely and finished a 24 hour urine collection. They were weighed in light clothing or a hospital gown. The weight-maintaining, protein controlled diet started at 8 am each day and was consumed at specific times (see below). Subjects were encouraged to drink enough water to quench their thirst, but not to consume more than 2 L per day. For the most part, the consumption of water was below 2L per day, but in cases of substantial hyperglycemia in T2DM, this volume was slightly exceeded. No other food or beverages were permitted. Subjects were sometimes allowed to temporarily leave the hospital, as long as the diet and urine collections were followed as previously mentioned. Since exercise can alter protein metabolism and insulin sensitivity of glucose (Borghouts et al. 2000; Segal et al. 1991; Waterlow 1984; Wolfe et al. 1982), physical activity was kept to walks in and around the hospital.

A daily log was kept by all subjects that included information such as water consumption, bowel movements, presence of any symptoms, and blood pressure measured by the research nurse (**Appendix 6**). Blood samples were taken on Days 1, 4, 6 for T2DM subjects and Days 4 and 6 for controls; analyses of numerous variables such as lipids, electrolyte balance, and hemoglobin were done by the RVH laboratory to ensure

that physiological variables were within an acceptable range during the study. A sample of the daily urine collections was sent to the lab to ensure that variables such as creatinine and urea were within acceptable ranges and to ensure that collections were being done properly.

T2DM subjects were also provided with an Accu-Chek® Advantage glucose meter (Roche Diagnostics, Indianapolis, IN), which replaced an older model in 2005, the AccuSoft glucose meter (Roche Diagnostics, Indianapolis, IN). Appropriate glucose strips for the glucose meters (Roche Diagnostics, Indianapolis, IN) and hospital lancets were also given to the participants. They were required to test and make note of their blood glucose levels 4 times per day on Days 1-6: before the 8 am (fasting), 2 pm, 5 pm, and 8 pm meals and 3 times per day on Day 7: before the 8 am (fasting), 12 pm, and 5 pm meals. If glucose values at 8 am, 2 pm, and 8 pm (Days 1-6) or at 8am and 12 pm (Day 7) exceeded 15 mmol/L, small amounts of insulin (Humulin®-R) were given subcutaneously, based on a sliding insulin scale devised by the study physician, to prevent excessive hyperglycemia. The sliding scale for each subject for specific times during the day depended on the level and pattern of hyperglycemia throughout the day, determined by self-blood glucose monitoring. Even though rigorous insulin therapy alters carbohydrate and protein metabolism (Andrews et al. 1984; Gougeon et al. 1998; Pratipanawatr et al. 2002), the small amounts of insulin given in the study are not expected to significantly alter the kinetics of protein and glucose measured on the day of the clamp. Moreover, no insulin was administered after 5 pm on Day 7, providing a large gap of time until the clamp protocol was initiated the next morning. Lastly, hyperglycemia itself induces insulin resistance of glucose (Pratipanawatr et al. 2002) and this would therefore counteract any effectiveness of insulin in the long term.

During the admission period but before the day of the clamp study, body circumferences were measured using a precise tape measure. Waist circumference was measured according to WHO procedures, between the iliac crest and the lowest rib, with the tape parallel to the floor (World Health Organization 1995). Hip circumference, also measured according to WHO recommendations, was the greatest circumference at the level of the buttocks, with the measuring tape parallel to the floor (World Health

Organization 1995). The same person measured the circumferences of all participants to ensure consistency.

9.3.1. Diet

The caloric requirements to maintain each subject's weight throughout the study were calculated as 1.5 multiplied by REE (Gougeon et al. 2000; Harris et al. 1919). REE was measured during the screening visit using Deltatrac (SensorMedics, Yorba Linda, CA). When the diet was formulated, the percentage of calories coming from each macronutrient included: 60% from carbohydrates, 25% from fat, and 15% from protein. This macronutrient distribution is within the Acceptable Macronutrient Distribution Ranges (AMDR) (Institute of Medicine of the National Academies et al. 2005). The protein intake was set at 1.7g/kg FFM, which is equivalent to 1.23g protein/kg BW and 1.28 g protein/kg BW in the control and T2DM groups respectively. This is a generous protein intake, above the RDA, thereby ensuring nitrogen balance in the population (Institute of Medicine of the National Academies et al. 2005).

The diet consisted of the following main items: 2% milk (Québon, Natrel, Longueuil, QC), cereal (All Bran, Kellogg Canada Inc, Mississauga, ON), and Ensure (Ross, Abbott Laboratories, St. Laurent, QC). Every day, subjects received 30g of cereal and 150mL of milk for the 8 am meal. The rest of the diet consisted of cans of Ensure, a liquid formula containing necessary vitamins and minerals that is available in three flavors, namely chocolate, vanilla, and strawberry, from the RVH kitchen. In order to achieve the desired level of protein intake and macronutrient distribution, however, it was necessary to use polymers of glucose and canola oil in the daily diet of some subjects. In these situations, one third of the calories would come from canola oil (Canola Harvest®, Canbra Foods Ltd., Lethbridge, AB; 9.1 kcal/g) and the rest from a glucose polymer powder. Glucose polymers were obtained from the RVH, and once Caloreen (Nestlé, Nestlé Clinical Nutrition, North York, ON; 3.84 kcal/g) was discontinued, we switched to Polycose (Ross, Abbott Laboratories, Columbus, OH; 3.80 kcal/g). On days 1-5 (controls) and days 1-6 (T2DM), there were six meals throughout the day, every three hours, from 8am to 11pm. On day 6 (controls) and day 7 (T2DM) meal times were more frequent, at 8 am, 10 am, 12 pm, 2 pm, 4 pm, and 5 pm because of a concurrent protocol

to assess gluconeogenesis using deuterated water. Depending on each subject's caloric needs, a can or two of Ensure was given for breakfast. There was an attempt to divide the cans of Ensure evenly among the meals, depending on caloric needs. All nutritional information associated with the diet can be found in **Tables 1** and **2**.

The diet enhanced hyperglycemia in T2DM subjects, partly due to fast absorption of meals and the main source of carbohydrate was sucrose. Since the renal threshold for glucose is 10.0 mmol/L in blood drawn from a vein or 11.1 mmol/L in blood drawn from an artery (Ganong 2001), prolonged substantial hyperglycemia results in significant glycosuria in T2DM subjects. Replacement of these calories was necessary to prevent weight loss. To determine the magnitude of daily glycosuria, glucose concentrations in a sample of urine from the 24 hour urine collections were analyzed using G7M Micro-Stat (Analox Instruments USA, Lunenberg, MA) every morning. This value was multiplied by the total volume of urine in that specific 24 hour collection and by 4kcal/g glucose to yield the caloric loss. That day, half of the calories associated with glycosuria were replaced with a glucose polymer, Caloreen or Polycose depending on availability, and the other half with canola oil. These ingredients were mixed thoroughly with Ensure. This assurance of retention of the required energy from the diet slightly changed the percentage contribution of each macronutrient to the diet.

9.3.2. Clamp Study

On the last study day, the hyperinsulinemic euglycemic isoaminoacidemic clamp was performed. The hyperinsulinemic euglycemic clamp, devised by DeFronzo et al. (1979), has been subsequently altered and the methodological approach in the current study is based on Banerji & Lebovitz (1989) and Saad et al. (1994) (Chevalier et al. 2004). To concurrently assess insulin sensitivity of kinetics of protein metabolism without the confounding effect of sub-basal plasma AAs, it is necessary to perform the hyperinsulinemic euglycemic isoaminoacidemic clamp. A hindrance in this type of clamp has been the inability to keep most AAs at postabsorptive levels, but our lab has recently perfected the technique by finding an appropriate AA solution (10% TrophAmine without electrolytes; B Braun Medical Inc, Irvine, CA) and by varying its rate of infusion (Chevalier et al. 2006; Chevalier et al. 2004; Chevalier et al. 2005a;

Chevalier et al. 2005b). The tracers L-[1-^{13}C]leucine and D-[3-^{3}H]glucose were administered to assess leucine and glucose kinetics respectively, like in previous studies (Banerji & Lebovitz 1989; Chevalier et al. 2004; Matthews et al. 1980; Saad et al. 1994).

Subjects arrived at the McGill Nutrition and Food Science Centre, RVH, from the RVH CIU in the fasting state before 8 am on the day of the clamp study. A catheter was placed into the antecubital vein on one side of the body to infuse substrates, insulin, and tracers while on the other side of the body, a catheter was placed into a dorsal hand vein retrogradely to obtain blood samples (Chevalier et al. 2004; Saad et al. 1994). Blood in the vein was arterialized by keeping the sampling hand in a warming box at 65-70°C (Chevalier et al. 2004; Saad et al. 1994; Zello et al. 1990).

The time course of the experiment, the infusions involved, and the sampling frequency are depicted in **Figure 1**. At 8 am, 0.1 mg/kg of $NaH^{13}CO_2$ (MassTrace Inc., Woburn, MA) in the aqueous form was ingested and a bolus of L-[1-^{13}C]leucine (Isotech, Sigma-Aldrich, St. Louis, MO) equal to 0.5 mg/kg was administered intravenously to attain a steady state more quickly (Chevalier et al. 2004; Wolfe & Chinkes 2005). For the remainder of the experiment, L-[1-^{13}C]leucine was infused intravenously at a rate of 0.008 mg/kg·min (Chevalier et al. 2004). Furthermore, at 8 am a 22 μCi bolus injection of D-[3-^{3}H]glucose (PerkinElmer Inc., Life and Analytical Sciences, Boston, MA) was immediately followed by a 0.22 μCi/min D-[3-^{3}H]glucose infusion that lasted until the end of the study (Chevalier et al. 2004). The time at which the L-[1-^{13}C]leucine and D-[3-^{3}H]glucose constant infusions were initiated is labeled as -180 minutes (min) and this is the experimental start time. The clamp protocol begun at 0 min, when the infusion of insulin (Humulin R; Eli Lilly Canada Inc, Toronto, Canada) was initiated (Chevalier et al. 2004). The first nine minutes of the insulin infusion involved boli that diminished steadily in magnitude, followed by a constant infusion of insulin at 40mU/m^2·min that continued until the end of the clamp (Banerji & Lebovitz 1989; Chevalier et al. 2004). At 4 min, the 10% TrophAmine AA infusion was started at a rate of 0.004 mL/kg FFM·min in leaner controls, 0.0035 mL/kg FFM·min in obese controls, and 0.0035 mL/kg FFM·min in T2DM subjects. To maintain postabsorptive AA levels, BCAA plasma concentrations were determined by fluorometric assay and the AA solution infusion rate was altered accordingly if necessary. To achieve euglycemia (5.5 mmol/L) and steady

state in isotopic enrichment (specific activity), a glucose infusion, consisting of 20% dextrose (Avebe b.a., Foxhol, The Netherlands) and D-[3-^{3}H]glucose, called a "hot glucose infusion" or simply "hot ginf" due to the presence of a glucose tracer (Finegood et al. 1987) was used. Specifically, at 4 min after the start of insulin infusion, the hot ginf was started in controls. In T2DM, because they were hyperglycemic, hot ginf infusion was delayed by more than 4 minutes after the start of the insulin infusion; specifically, the hot ginf infusion was initiated when plasma glucose reached approximately 5.5 mmol/L. Plasma glucose readings (GM7 Micro-Stat; Analox Instruments USA, Lunenberg, MA) were entered into the Andres Glucose Clamp Program version 2.0-8/96, which generated the magnitude of the glucose infusion rate necessary to maintain euglycemia. During the experimental protocol, two steady states were obtained: one during the postabsorptive state and the other during the clamp. A steady state or plateau refers to an isotopic equilibrium, when the ^{13}C enrichment in plasma and CO_2 as well as the specific activity (SA) of tritiated glucose in plasma are constant, and to physiological equilibrium, when plasma hormone and substrate concentrations are not changing (El-Khoury 1999).

Syringes containing individually adjusted concentrations of L-[1-^{13}C]leucine, D-[3-^{3}H]glucose, insulin, 10% TrophAmine, and hot ginf were prepared by the study physician on the day of the clamp or the evening before. The insulin was mixed with 2 mL of heparinized blood from the subject to avoid insulin from being adsorbed by the syringe or infusion line (DeFronzo et al. 1979); surface area was calculated as in Wang et al. (1992). The syringes were placed in separate pumps (Harvard Apparatus Inc., Holliston, MA): they were set for the continuous infusion of L-[1-^{13}C]leucine and D-[3-^{3}H]glucose solutions as well as insulin (after priming) while digital pumps for 10% TrophAmine and hot ginf allowed one to specify the rates of infusion of these solutions throughout the clamp.

Blood samples were obtained at baseline (-180 min), -90 min, -60 min, every 10 minutes from -40 to 0 min, and every 5 minutes after the start of the insulin infusion. Blood samples were partitioned into different tubes for different analyses. Screwcap vials containing approximately 1.5 mL of blood were spun in a centrifuge (Micro-Centrifuge, Model 235B; Fisher, An Allied Company) for 30 seconds and the plasma was used for the fluorometric assay and to determine plasma glucose with the GM7 Micro-

Stat glucose analyzer. Breath samples, which are necessary to assess $^{13}CO_2$ enrichment, were obtained at baseline, at -60 min, every 10 minutes from -40 to 0 min, at 60 min and every 30 minutes thereafter until the clamp steady state was reached, when breath samples were collected every 10 minutes. Indirect calorimetry for 20 minutes was done 3 times, after the start of tracer infusions but before the postabsorptive steady state and during the postabsorptive and clamp steady states. Urine samples were also collected throughout the clamp procedure.

During the clamp, investigators identified the steady state for AAs and glucose based on the constancy of their respective infusion rates. Nevertheless, postabsorptive and clamp plateaus for glucose and leucine kinetics were ultimately designated based on the stability of SA and enrichment, respectively. Postabsorptive plateau values for glucose kinetics, leucine kinetics, plasma insulin, plasma glucagon, and serum FFA concentrations were the average of values obtained every 10 minutes from -40 to 0 min. The clamp plateau period lasted 30 minutes. Clamp plateau glucose kinetics, leucine kinetics, insulin, and glucagon concentrations were calculated as the average of values obtained every 10 minutes during the designated clamp steady state. Clamp FFAs were based on one time point during the clamp steady state because this was not a main endpoint and it is widely reported in the literature that plasma FFAs are quite sensitive to insulin (Kumar & O'Rahilly 2005). Measurements of insulin, glucagon, and FFA concentrations as well as plasma D-[3-^{3}H]glucose radioactivity for each time point were done in duplicate. Exceptions to the above statements are: 1) the calculation of postabsorptive plateau glucose kinetics in T2DM subjects (see below in section 9.3.4.) and 2) the number of time points used for the calculation of leucine kinetics (see below in section 9.3.3.). For the great majority of subjects, the clamp steady state for leucine kinetics coincided with the clamp steady state for glucose kinetics.

Blood collected throughout the clamp study was divided into specific tubes, depending on the endpoint. Blood was added to heparin-containing tubes and centrifugation followed; the supernatant was used for immediate determination of plasma glucose and BCAA (fluorometric assay) concentrations. A portion of this plasma underwent further preparatory steps (Slocum et al. 1991) before being stored at -70°C for subsequent analysis of AAs by High Pressure Liquid Chromatography (HPLC). For

FFAs, after blood was allowed to clot, samples were centrifuged and stored at -20°C. For hormone, glucose SA, and α-KIC measurements, tubes and their particular additives were placed on ice before adding blood; the additives were, respectively, heparin and trasylol, heparin and sodium fluoride, and heparin only. Once blood had been added, these tubes were centrifuged and the supernatants stored at -20 °C until specific assays were performed (Section 9.3.7).

9.3.2.1. Fluorometric Assay for Branched Chain Amino Acids

To maintain postabsorptive plasma AA concentrations in our experimental protocol once the insulin infusion was initiated, a quick and reliable method for quantification of plasma AAs was necessary. Based on the work by Beckett et al. (1996), who combined spectrophotometry with bacterial leucine dehydrogenase to measure plasma BCAA concentrations, our lab recently developed a similar technique using fluorometry (Chevalier et al. 2006; Chevalier et al. 2004; Chevalier et al. 2005a; Chevalier et al. 2005b). Each BCAA undergoes oxidative deamination in the presence of the enzyme *Bacillus cereus* leucine dehydrogenase and the cofactor NAD^+, thereby producing NADH and the corresponding branched chain keto acids, namely α-ketoisocaproate, α-keto-β-methylglutarate, and α-ketoisovalerate (Chevalier et al. 2004; Ohshima et al. 1978). *Bacillus cereus* leucine dehydrogenase (E.C. 1.4.1.9, specific activity of 47.0 U/mg; Calbiochem, Calbiochem-Novabiochem, La Jolla, CA), in a buffer of 25 mmol/L sodium phosphate (Fisher Chemicals, Fisher Scientific, St. Laurent, QC), was stored at -70°C until the day of the clamp study, when it was diluted in 25 mmol/L sodium phosphate buffer with 1mg/mL of bovine serum albumin (Sigma, Sigma Chemical, St Louis, MO) (Chevalier et al. 2004). β-NAD (Roche, Roche Diagnostics, Laval, QC) was dissolved in a buffer of 0.1 mol/L sodium carbonate (Fisher Chemicals, Fisher Scientific, St. Laurent, QC) (Chevalier et al. 2004). Each sample for analysis (2 mL) consisted of 25 μL of plasma, 4 mmol/L of NAD, 0.5U of leucine dehydrogenase, and buffer (0.1 mol/L potassium phosphate, 2 mmol/L EDTA, and 0.02% mercaptoethanol; Fisher Chemicals, Fisher Scientific, St. Laurent, QC) (Chevalier et al. 2004).

A spectrofluorometer (Turner model 430 with xenon lamp, Sequoia-Turner, Mountain View, CA) measured the amount of fluorescence from NADH during the first 4 minutes of the reaction, which occurred at 37°C; wavelengths for excitation and emission were 355 nm and 485 nm respectively (Chevalier et al. 2004). The same experimental procedures were followed for a sample blank, which consisted of all the components of a typical sample but did not contain any enzyme. The sample blank established background fluorescence, which was subtracted from the fluorescence obtained in enzyme-containing samples. A standard curve was created before the start of insulin infusion, using 0-250 μmol/L BCAA standards that were prepared and analyzed under the same conditions as the plasma-containing samples (Chevalier et al. 2004). The relative ratios of BCAAs (Sigma, Sigma Chemical, St Louis, MO) in the standard BCAA mixture were similar to the relative ratios normally found in plasma (Chevalier et al. 2004). The BCAA concentration in each plasma-containing sample was determined simply by interpolating NADH fluorescence from the standard curve (fluorescence *vs.* BCAA concentration), since the molar ratio of BCAA consumed to NADH formed during this biochemical reaction is one to one (Chevalier et al. 2004). The fluorometric assay is fairly accurate when compared to the lengthier HPLC technique (Chevalier et al. 2004).

In the fluorometric assay utilized, plasma BCAAs were chosen as indicators of plasma AA concentrations because BCAAs are highly sensitive to insulin. Nevertheless, the concentrations of different AAs change differently in response to the hyperinsulinemic euglycemic clamp. To maintain isoaminoacidemia, it was necessary to use an AA solution that contained relatively elevated levels of AAs that decreased in response to insulin, like BCAAs, but low levels of AAs that were expected to either augment or not vary (Chevalier et al. 2004). Different AA solutions were tested in our laboratory and 10% TrophAmine was shown to be the most appropriate solution (Chevalier et al. 2004). 10% TrophAmine consists of 18 typical AAs and taurine, but no glutamine or asparagine because they are not stable in aqueous form.

9.3.2.2. Glucose Oxidase Technique

Once the insulin infusion was initiated, plasma glucose levels were analyzed using a GM7 Micro-Stat glucose analyzer every 5 minutes. The chemical reaction underlying

this technique is catalyzed by glucose oxidase and it involves the conversion of β-D-glucose and oxygen to gluconic acid and hydrogen peroxide; the amount of oxygen consumed is indicative of amount of glucose in the sample (Analox Instruments [date unknown]). Procedures for this technique were followed according to the manufacturer's instructions (Analox Instruments [date unknown]). Briefly, calibration was done with a 150 mg/dL glucose standard solution and verified with 150, 144, and 90 mg/dL standard samples (Beckman Coulter Inc., Fullerton, CA). A maximum error of 2% was acceptable, but measurement error was kept even lower by using the 150 mg/dL glucose standard as a sample in between plasma samples and calibrating accordingly. Measurements of plasma-containing samples were made in duplicate.

9.3.3. L-[1-^{13}C]Leucine Kinetics

L-[1-^{13}C]leucine is currently the most common tracer in the study of kinetics of whole body protein metabolism (El-Khoury 1999). This tracer methodology is based on a two pool model, an AA pool and a bigger protein pool, at the level of the whole body (**Figure 2**) (Abrams & Wong 2003; O'Keefe et al. 1974; Waterlow et al. 1977). AAs, including leucine, can enter the AA pool from protein breakdown (B) and from intravenous infusion (I); they can leave the AA pool to synthesize protein (S) and to be oxidized (O); and the AA flux or turnover is known as Q. These five variables are rates, typically expressed in μmol/min, and at steady state, the equations are as follows (Abrams & Wong 2003):

$$Q = S + O = B + I$$

When L-[1-^{13}C]leucine is used as a tracer, the variables refer to the rate at which leucine is incorporated into protein (S), oxidized (O), released from protein breakdown (B), or infused (I). This stochastic model is a simplification (Cynober 1995; Wolfe & Chinkes 2005), but numerous of its underlying assumptions were previously upheld (Cynober 1995). For example, the isotope L-[1-^{13}C]leucine is indistinguishable from the much more common L-[1-^{12}C]leucine and hence, the tracer moves into the same compartments and undergoes the same biochemical reactions as the tracee (Cynober 1995). Furthermore, recycling of the leucine tracer, that is, the release of tracer once it has been

incorporated into protein is negligible when the experimental protocol lasts up to 4-8 hours (Cynober 1995; Schwenk et al. 1985).

BCAA transaminase catalyzes the reversible conversion of leucine to α-KIC; this reaction is fast and occurs within the cell (Devlin 2002; Matthews et al. 1982; Thompson et al. 1988). Intracellular leucine enrichment is lower than plasma leucine enrichment because leucine from protein breakdown "dilutes" the isotope concentration in the cell (Cynober 1995; Matthews et al. 1982). Since plasma ^{13}C-α-KIC is a better indicator of intracellular leucine enrichment than plasma ^{13}C-leucine (Matthews et al. 1982; Thompson et al. 1988) and since one is ultimately interested in the movement of leucine through the various pathways within the cell, ^{13}C-α-KIC enrichment is used in the calculations of leucine kinetics. This is the reciprocal model, as opposed to the minimal model which uses plasma leucine enrichment. The actual precursor for S is leucyl-tRNA (Bier 1989) and even though it is unclear how accurately plasma α-KIC reflects this precursor pool (Bier 1989), recent studies indicate that plasma α-KIC is still better than plasma leucine in calculations of S (Cynober 1995; El-Khoury 1999; Horber et al. 1989; Watt et al. 1991). α-KIC is converted irreversibly to isovaleryl CoA by branched chain α-keto acid dehydrogenase (BCKDH), releasing the first carbon as CO_2 (Devlin 2002). The ^{13}C enrichment of CO_2 released at this step will ultimately be used in calculating leucine O. The following steady state equations for leucine Q and O from Matthews et al. (1980) have been adapted for the reciprocal model:

$$\mathbf{Q = i\,[E_i/E_p - 1]}$$

$$\mathbf{O = V_{13CO2}\,[1/E_p - 1/E_i] \times 100}, \text{ where } \mathbf{V_{13CO2} = [V_{CO2}\,E_{CO2} / kgBW]\,[(44.6 \cdot 60)/(100 \cdot r)]}$$

Enrichment is expressed as Atom Percent Excess (APE). $\mathbf{E_i}$ is the enrichment of L-[1-^{13}C]leucine administered, as specified by the manufacturer and **i** is the rate of L-[1-^{13}C]leucine infusion in μmol/kgBW·hr. $\mathbf{E_p}$ is the enrichment of ^{13}C-α-KIC in plasma, according to the equation APE = $100(r_s-r_b)/[(r_s-r_b)+1]$, where r is the ratio ^{13}α-KIC/^{12}α-KIC, the subscript s refers either to postabsorptive or clamp steady state, and the subscript b refers to baseline (-180 min) (Wolfe & Chinkes 2005). $\mathbf{V_{13CO2}}$ is the rate of $^{13}CO_2$

production in μmol/kgBW·hr, while V_{CO2} is the rate of CO_2 production in mL/min and E_{CO2} is the ^{13}C enrichment of CO_2 exhaled. $^{13}CO_2$ enrichment is initially obtained as delta (δ) and is converted to APE (Wolfe & Chinkes 2005); the ratio $^{13}CO_2/^{12}CO_2$ is the key component of the calculation. APE for each sample is the difference between the APE value obtained for that time point and the APE value at -180 min (Wolfe & Chinkes 2005).

The above equations also include conversion factors: 100 changes APE into a fraction, 44.6 in μmol/mL converts the units of air from mL to μmol at standard temperature and pressure according to Avogadro's law, and 60 converts minutes to hours. The term **r** is the recovery factor, which is the proportion of $^{13}CO_2$ generated during oxidation that is exhaled; the remainder stays in the body's bicarbonate pool (Chevalier et al. 2004; Cynober 1995; El-Khoury 1999; Matthews et al. 1980). Based on previous bicarbonate studies in our laboratory, **r** equals 0.671 during the postabsorptive steady state and 0.799 during the clamp steady state for all subjects.

Four time points were used for baseline $^{13}CO_2$ and ^{13}C-α-KIC enrichments, while four time points were used for clamp ^{13}C-α-KIC enrichment and three time points for clamp $^{13}CO_2$ enrichment. The values of Q and O for each steady state were calculated from the averages of $^{13}CO_2$ and ^{13}C-α-KIC enrichments for each plateau. Since Q and O are calculable and I is known, S and B are determined by subtraction. In the postabsorptive steady state, B=Q because leucine is not infused and cannot be made in the body; S=Q-O. In the clamp steady state, B=Q-I and S=Q-O. Similar to glucose kinetics (see below), the sum of B and I are known as rate of appearance (R_a) while O and S are known as rate of disappearance (R_d); S is also known as non-oxidative R_d.

Indirect calorimetry (Deltatrac; SensorMedics, Yorba Linda, CA) was used to measure V_{CO2}. Breath samples were collected in an appropriate balloon and using a syringe, breath was placed in specific tubes (Chevalier et al. 2004). When Vacutainer tubes (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ) stopped being manufactured in 2005, our laboratory compared the suitability of various available substitutes and Kendall tubes (Tyco Healthcare Group LP, Mansfield, MA) were selected. E_{CO2} was determined using Micromass 903D (Vacuum Generators, Winsforce, United Kingdom), an isotope ratio mass spectrometer (Chevalier et al. 2004).

Assessment of ^{13}C-α-KIC enrichment in plasma involved a series of steps (Chevalier et al. 2005b; Mamer et al. 1988). Protein was precipitated from the plasma sample and the supernatant was collected. At a basic pH, $NaBH_4$ reduced α-KIC to α-hydroxyisocaproate. The solution was acidified, followed by extraction with ethyl acetate. Once dried, *N*-methyl-*N*-(*tert*-butyldimethylsilyl)trifluoroacetamide (Regis Technologies Inc., Morton Grove, IL) was used to obtain a t-butyldimethylsilyl derivative of α-hydroxyisocaproate, which was measured using a gas chromatograph-mass spectrometer (GCMS 5988A; Hewlett-Packard, Palo Alto, CA) (Chevalier et al. 2006; Chevalier et al. 2005b).

Adjustments to $^{13}CO_2$ enrichment were necessary during the clamp because infusions of glucose and AAs diluted background enrichment (Chevalier et al. 2004; Chevalier et al. 2005b). The exact magnitude of the adjustment was determined in a different set of studies in our laboratory; it depended on the rate of glucose and AA infusions (Fukagawa et al. 1989). The factor was 10.1% for lean, 7.0% for obese, and 7.0% for T2DM subjects.

9.3.4. Glucose Turnover Using D-[3-^{3}H]glucose

A commonly used tracer to study glucose turnover is the radioactive isotope D-[3-^{3}H]glucose. Tritium is lost in the glycolytic step where dihydroxyacetone phosphate is converted to glyceraldehyde 3-phosphate as water and in the pentose phosphate pathway, the latter being a minor contribution (Finegood et al. 1987; Horton et al. 1996; Wolfe & Chinkes 2005). Glucose turnover studies are based on a single pool model and at steady state, the rate at which glucose moves into plasma, rate of appearance (R_a), equals the rate at which it leaves plasma to enter tissues, rate of disappearance (R_d) (Wolfe & Chinkes 2005). Other main assumptions include that D-[3-^{3}H]glucose does not behave differently from the more abundant glucose isotope in the body and that recycling of D-[3-^{3}H]glucose is minor during the clamp study protocol; both of these assumptions appear to be valid (Finegood et al. 1987; Koivisto et al. 1990; Steele 1959).

Subsequently, Finegood et al. (1987) showed that during the hyperinsulinemic euglycemic clamp, in addition to the constant D-[3-^{3}H]glucose infusion, including tracer in the glucose infusion minimized alterations in SA throughout the experimental protocol,

which in turn reduced the probability of obtaining negative EGP values; this is known as the hot glucose infusion method, or "hot ginf" (Finegood et al. 1987; Finegood et al. 1988). The current experimental protocol used the hot ginf method along with the equations for R_a and R_d proposed by Finegood et al. (1987):

$$R_a(t) = I/SA_p(t) - [(pV \cdot G(t) \cdot dSA_p(t)/dt)/SA_p(t)] + [SA_g \cdot Ginf(t)/SA_p(t)] - Ginf(t)$$
$$R_d(t) = I/SA_p(t) - [(pV \cdot G(t) \cdot dSA_p(t)/dt)/SA_p(t)] + [SA_g \cdot Ginf(t)/SA_p(t)] - pV \cdot (dG(t)/dt)$$

In the previous formula, R_a represents endogenous glucose R_a, which equals EGP; glucose R_d is the rate of glucose disposal. SA is a measure of isotopic enrichment and is defined as the amount of radioactivity, in μCi, divided by the total amount of glucose in a sample (Wolfe & Chinkes 2005). $SA_p(t)$ refers to SA in the plasma sample at time t in μCi/mg, SA_g refers to SA of the exogenous glucose solution in μCi/mg, G(t) refers to the glucose concentration in plasma at time t in mg/dL, and Ginf(t) is the rate of glucose infusion in mg/min·kg. Any variables expressed as derivatives refer to changes in variables with respect to time. The product of p and V is called the "effective volume" by Finegood et al. (1987). While p represents the fast mixing fraction of body glucose and equals 0.65, V represents glucose's volume of distribution in the body in dL/kg and it is 25% of body weight (Finegood et al. 1987; Radziuk et al. 1978; Wolfe & Chinkes 2005). With "effective volume", the above equations take into account that whole body glucose turnover is not truly explained by a single pool model because body glucose consists of multiple pools with different mixing rates (Finegood et al. 1987; Steele 1959; Wolfe & Chinkes 2005).

The approach taken to prepare the hot ginf solution in the current experimental protocol relied upon the work by Finegood et al. (1988). Based on a series of assumptions and by combining the equations for R_a during the postabsorptive and clamp plateaus, a formula and value for SA_g was generated. It was assumed that R_a equaled 2mg/kg/min during the postabsorptive plateau and R_a would be completely inhibited during the clamp plateau. Based on the literature and previous experiments in our laboratory, a value for glucose infusion rate was assumed; it was also assumed that T2DM subjects would have lower glucose infusion rates than control subjects. Lastly, it

was assumed that the derivative of SA with respect to time during the postabsorptive and clamp plateaus equaled 0 (definition of an isotopic steady state). Ultimately, the goal was to maintain plasma glucose SA as close as possible (within 25%) of postabsorptive plateau glucose SA.

A smoothing program, OOPSEG, was used to determine $dSA_p(t)/dt$ and $dG(t)/dt$ (Bradley et al. 1993; Finegood et al. 1987). OOPSEG established the "error-free curve" of $SA_p(t)$ and G(t) with respect to time after finding the most probable magnitude of measurement error (Bradley et al. 1993). For control subjects, OOPSEG created this curve based on all $SA_p(t)$ and G(t) data points, but only the values corresponding to the time range of the two plateaus, as previously specified, were used in the calculations of R_a and R_d. Calculations of R_a and R_d for T2DM subjects, however, were more complex because their fasting hyperglycemia declined to euglycemia throughout the clamp protocol. First, when OOPSEG analyzed the entire data set for T2DM subjects, it created a curve for G(t) where plasma glucose declined steadily during the postabsorptive period, thereby violating the definition of a postabsorptive steady state. Second, the decrease in plasma glucose was accompanied by fluctuations in postabsorptive plasma glucose SA and SA was not always constant during the time designated for the postabsorptive plateau among the controls (-40 to 0 min). Thus, during the postabsorptive state, a period of stable plasma glucose SA was selected as the postabsorptive plateau for each T2DM subject. For most T2DM subjects, the plateau was from -30 to 0 min, although some plateaus were from -60 min to 0 min. The values for $SA_p(t)$ and G(t) for the selected period of time were run through OOPSEG, after which postabsorptive R_a and R_d were calculated. For the clamp plateau in T2DM subjects, $SA_p(t)$ and G(t) from 0 min until the end of the clamp were run through OOPSEG and the last 30 minutes of the clamp were selected as the clamp steady state. Lastly, T2DM urine samples were analyzed for glucose (GM7 Micro-Stat) throughout the clamp protocol, and adjustments to R_d were made accordingly (DeFronzo et al. 1979).

9.3.5. Bioelectric Impedance Analysis

BIA is a technique that assesses body composition, specifically Fat-Free Mass (FFM) and fat mass. The BIA methodology used in the current study (RJL-101A; RJL

Systems, Detroit, MI) involved placing four electrodes in specific locations of the hand and foot on the same side of the body (RJL Systems c2005). Since physical activity and hydration status affect BIA results (Baumgartner et al. 1998; Tagliabue et al. 1992), subjects were given some water if they felt excessively thirsty and laid flat on a bed for at least 5 minutes before BIA measurements were taken. Their legs were apart and arms away from the body (RJL Systems c2005). The appropriate skin areas were wiped with an alcohol swab and allowed to dry. Electrodes supplied by the manufacturer were split in half to provide the four electrodes necessary for each test. Each of the four electrodes was attached to an appropriate clip. Two electrodes sent 800 μA of current through the body at a frequency of 50 kHz and the other two electrodes picked up the decrease in voltage (Lukaski et al. 1985). According to the manufacturer's specifications, current-sending electrodes were positioned immediately behind the middle finger and middle toe, while the top of each receiving electrode split the head of the ulna and the medial malleolus in half (RJL Systems c2005). At least two readings of resistance and reactance were obtained between 5 and 10 minutes.

Impedance, defined as the "ability to restrain ac current" (Hecht 1996), consists of resistance and reactance. The former is the main component of impedance in the human body and both variables are measured in BIA (Baumgartner et al. 1998; Lukaski et al. 1985). The human body can be thought of as a group of resistors and capacitors that are associated with resistance and reactance respectively (Baumgartner et al. 1998; Heymsfield et al. 2005; RJL Systems c2005). BIA itself, however, is not able to delineate body composition (Heymsfield et al. 2005). Reference methods such as underwater weighing, dilution techniques, and dual-energy X-ray absorptiometry (DXA) as well as regression-based statistics have been utilized to establish FFM equations for BIA methodology (Heymsfield et al. 2005). BIA equations were devised for a given population and sex because the reference methods themselves depend upon characteristics such as age, sex, and obesity (Heymsfield et al. 2005). After a thorough review of BIA equations available in the literature, equations by Goran, Kushner, and Roubenoff for men were selected for the determination of FFM in the current experimental protocol:

Goran (Goran et al. 1995), men:

$FFM=(0.43*height^2/R)+(0.24*weight)+6.2-(0.10*SSF)+11.8$

Kushner (Kushner et al. 1990; Kushner et al. 1986), men:

$D_2O\text{-}TBW=0.396(height^2/R)+(0.143*weight)+8.399$

$FFM=D_2O\text{-}TBW/(1.04)(0.73)$

Roubenoff (Roubenoff et al. 1997), men:

$FFM=9.1536+0.4273(height^2/R)+0.1926*weight+0.0667*X_c$

FFM is in kg, height is in cm, weight is in kg, R is resistance in ohms, X_c is reactance in ohms, D_2O-TBW is total body water using deuterium (kg), and SSF represents suprailiac skinfold thickness in mm. All SSF measurements were determined using Lange skinfold calipers (Beta Technology Incorporated, Santa Cruz, CA) by the same trained technician. A skinfold in the mid-axillary line, about 2 cm above the iliac crest, was grabbed and measured in subjects in whom it was possible despite their adiposity, according to the manufacturer's instructions (Beta Technology Inc. 1985); the average of two SSF values was used in the Goran equation.

The Goran equation uses underwater weighing as its reference method and it was devised from a large population of lean and obese men and women who were younger than 50 years of age. The Goran equation can be applied to an obese population (Goran et al. 1994), but its use of SSF, a measure of subcutaneous fat, may be problematic in obesity because it appears that the ratio of subcutaneous fat to total fat is smaller in obese subjects than in lean subjects (Heymsfield et al. 2005). Moreover, the accuracy of SSF measurements may be compromised in obese individuals (Heymsfield et al. 2005). In the current experiment, the Goran equation was used for two of the younger controls (< 65 years old), one subject with a BMI of 25.4, the other with a BMI of 30.5. The decision to use the Goran equation to estimate FFM in the obese control subject is not a contradiction of my previous statements, but rather, it is based on clinical evidence of physical fitness and the fact that BIA equations specific for obese individuals resulted in an unusually low PBF for this subject.

For the most part, however, BIA equations designed for lean populations lead to an overestimation of FFM in obese subjects (Baumgartner et al. 1998; Deurenberg 1996). The Kushner equation for men was deemed the most appropriate currently available equation for our severely overweight and obese controls, except for the obese subject described above, and all T2DM subjects. The Kushner equation uses deuterated water as its reference method and it was devised from a population with a large BMI range, which included people above the age of 65. It was decided that the Kushner equation was better than the Goran equation to estimate FFM for all T2DM subjects, even though their BMI ranged from 24-35, because they all showed abdominal obesity (waist circumference above action level 1 or 2), which may affect FFM estimates in itself (Baumgartner et al. 1998; Deurenberg 1996); moreover, the population used to devise the Kushner equation actually included a small number of T2DM subjects. The Kushner equation was not used for lean controls because FFM is underestimated when obtained using total body water *vs.* underwater weighing in lean young and elderly subjects (Goran et al. 1994).

For the controls who were 65 years of age or older (two were 65, one was 68 years old), the Roubenoff equation was used. The Roubenoff equation uses DXA as the reference method and it is based on an elderly population of the Framingham Heart Study. It was deemed necessary to use this equation due to age-related changes in body composition, such as an increase in the proportion of fat mass (Cohn et al. 1980), that were not adequately addressed in the other two equations above. Lastly, the accuracy of BIA in estimating FFM on an individual basis has been criticized because its equations are population-based (Deurenberg 1996). Nevertheless, it is important to remember that the reference methods have inherent errors and the reliability of their FFM estimates also depends on the compartmental model used (Goran et al. 1994; Heymsfield et al. 2005; Wang et al. 1992).

9.3.6. Indirect Calorimetry and Nitrogen Balance

Indirect calorimetry, with a ventilated hood (Deltatrac, SensorMedics, Yorba Linda, CA), was utilized to obtain REE on the screening day and to calculate rates of glucose and fat oxidation on the day of the clamp. Procedures were followed according the manufacturer's instructions (SensorMedics 1988). On both days, at the McGill

Nutrition and Food Science Centre, data were collected for 20 minutes after subjects lay on a bed for at least 30 minutes. Subjects were asked if the temperature was appropriate and if not, blankets were given or the air conditioner was turned on. Indirect calorimetry was explained and during measurements, while lying on the bed, subjects were asked to keep their hands at the side of the body, to keep their legs apart, to breathe normally, not to move or speak, and not to fall asleep. Notes were taken of the time and nature of any deviations from these procedures and if, as a result, meaningful alterations occurred in data points for REE, VO_2 (rate at which O_2 is used up in mL/min), and VCO_2 (rate at which CO_2 is generated in mL/min), the specific measurements were excluded.

To calculate glucose and fat oxidation during baseline and clamp steady states, the following equations were used (SensorMedics 1988):

Glucose oxidation (g/24 hr) = 5.926 (VCO_2) – 4.189 (VO_2) – 2.539 (UrN)
Fat oxidation (g/24 hr) = 2.432 (VO_2) – 2.432 (VCO_2) – 1.943 (UrN)

Protein oxidation was calculated according to the following equation (SensorMedics 1988):

Protein oxidation (g/24 hr) = 6.25 (UrN)

UrN, in g/day, refers to excretion of nitrogen in urine. It is the sum of nitrogen from urea and creatinine, obtained from measurements of daily urine collections throughout admission by the RVH laboratory, as well as estimates of nitrogen from urinary uric acid (0.3 g/day) and ammonia (0.28 g/day) from previous studies in our laboratory. Changes in the urea pool, as evidenced by alterations in serum urea (Jéquier et al. 1987; Simonson et al. 1990), were also factored into the calculation of UrN according to Jéquier et al. (1987), except that estimates of the urea pool size were based on FFM in my protocol, as in previous studies by our laboratory (Chevalier et al. 2006; Chevalier et al. 2004; Chevalier et al. 2005a; Chevalier et al. 2005b). Measurements of serum urea were also done by the RVH laboratory. UrN was usually the average of three study days; days were selected based on the quality of urine collections, including the consistency of creatinine

levels. Values for glucose, fat, and protein oxidation were converted to mg/min for analysis in my protocol. The Respiratory Quotient (RQ) at each steady state is VCO_2 divided by VO_2.

Nitrogen balance was determined by subtracting the sum of 1) UrN, as calculated above, 2) fecal losses (70 mg nitrogen per g nitrogen intake) (Gougeon et al. 2000), and 3) miscellaneous losses (5 mg nitrogen per kg BW) (Gougeon et al. 2000) from nitrogen intake, determined from the study diet.

9.3.7. Other Assays: Insulin, Glucagon, Free Fatty Acids, D-[3-^{3}H]glucose, and Amino Acids (HPLC)

A radioimmunoassay (RIA) utilizing [^{125}I] porcine insulin, human insulin, and antibodies for bovine insulin (Linco Research, St. Charles, MO), with charcoaling for separation of unbound hormone, was used to measure subjects' plasma insulin concentrations (Sigal et al. 1994). A Glucagon RIA Kit (Linco Research, St. Charles, MO) was utilized to establish plasma pancreatic glucagon concentrations. The last step of both assays was analysis by a gamma counter; using standard curves, the level of radioactivity in samples was interpolated to establish hormone concentrations. Serum FFA concentrations were measured using the NEFA C Assay Kit (Wako Chemicals USA Inc, Richmond, VA), the principle of which is a series of enzyme-catalyzed reactions, involving FFAs, ultimately resulting in a colored product that is quantified (Chevalier et al. 2005b; Wako Chemicals USA).

Measurements of plasma D-[3-^{3}H]glucose radioactivity were made using a radioactive assay, according to Finegood et al. (1987). Briefly, plasma proteins were precipitated, the supernatant was isolated and dried, water and scintillation cocktail (ICN Biomedical, Irvine, CA) were added, followed by analysis in a Beckman scintillation counter (Beckman Coulter LS6500 Multi-Purpose Scintillation Counter, Fullerton, CA). Ion-exchange HPLC was used to determine plasma AA concentrations (Chevalier et al. 2005b). Specifically, buffers containing lithium enabled appropriate separation of AAs; elution time ascertained the AA type and ninhydrin was used to establish AA levels in each sample (Slocum & Cummings 1991). All of the above substrate and hormone measurements, except HPLC AA measurements, were done in duplicates.

9.3.8. Statistical Analyses

Subject characteristics were compared by two-tailed unpaired t-tests. To determine the response of variables to the hyperinsulinemic euglycemic isoaminoacidemic clamp, two-way repeated measures analysis of variance (ANOVA) was utilized, with group and clamp as main effects. The two time periods were baseline and clamp steady states. When a statistical interaction was found, two-tailed paired and unpaired t-tests were done to determine which relationships were significant, as per Chevalier et al. (2005b). The variables that were analyzed using repeated measures ANOVA include: plasma concentrations of substrates and hormones, indirect calorimetry results, kinetics of glucose and protein metabolism, and plasma AA concentrations.

A linear regression approach was utilized to determine whether kinetics of protein and glucose metabolism should be adjusted for FFM (Allison et al. 1995; Ravussin & Bogardus 1989). The regression approach first establishes, in each study group, if the dependent variable correlates with FFM and if so, if the regression line of the linear relationship goes through the origin. If the correlation is significant and the line goes through the origin, then the dependent variable is divided by FFM. If there is a correlation, but the curve does not go through the origin, then FFM must be treated as a covariate in the statistical analysis, by using analysis of covariance (ANCOVA). If there is no correlation, then no adjustments should be made for FFM. Pearson correlation coefficient (r) was used to determine the relationship between glucose and leucine kinetics. When adjustments for FFM were necessary, partial correlations (partial r) were performed.

Significance was set at 0.05 and power at 80%. The sample size calculation used the change in net balance, defined as the change in leucine S-B from baseline to clamp, as the endpoint; the group difference and standard deviation were estimated from previous leucine kinetics studies in the laboratory. All statistical analyses were performed using SPSS 11.0 for Windows (SPSS Inc., Chicago, IL).

Table 1: Nutritional information for Ensure, All Bran cereal, and 2% milk

	Ensure® (1 can = 235mL)	Québon 2% milk (150mL)	Kellogg's® All Bran® (30g)
Energy (kcal)	250	77.0	78.0
Protein (g)	9.4	5.2	3.6
Fat (g)	6.7		0.9
Polyunsaturated (g)	1.4		
Linoleic acid (g)	1.3		
Linolenic acid	0.21		
Monounsaturated (g)	2.8		
Saturated (g)	0.5		0.18
Cholesterol (mg)	3.8		0
Trans fats (g)			0
Carbohydrate (g)	38		22.8
Sugars (g)			5.4
Starch (g)			7.2
Fiber (g)			10.2
Sodium (mg)	250	77	264
Potassium (mg)	375	238	348
Chloride (mg)	284		
Vitamin A	38% *		
Vitamin D_3	30% *		
Vitamin E	27% *		
Vitamin C	25% *		
Thiamine	25% *		
Riboflavin	27% *		
Niacin	26% *		
Vitamin B_6	28% *		
Folacin	27% *		
Vitamin B_{12}	25% *		
Pantothenic acid	25% *		
Calcium	27% *		
Phosphorous	25% *		
Magnesium	26% *		
Iron	27% *		
Zinc	44% *		
Iodide	25% *		
Copper (mg)	0.5		
Manganese (mg)	1.3		
Selenium (mg)	0.013		
Chromium (mg)	0.011		
Molybdenum (mg)	0.028		
Biotin (mg)	0.035		

* Expressed as percentage of Recommended Daily Intake.

Table 2: Nutritional information for Caloreen, Polycose, and canola oil

	Nestlé® Caloreen® (per 100g)	Polycose® (per 100g)	Canola oil (per 100g)
Energy (kcal)	370	380	910
Protein (g)	0.0		0
Fat (g)	0.0		101
Polyunsaturated (g)			28
Linoleic acid (g)			19.2
Linolenic acid			9.3
Monounsaturated (g)			58
Saturated (g)			8
Cholesterol (mg)		0	
Trans fats (g)			1.6
Carbohydrate (g)	96	94	0
Sodium (mg)	52	≤ 120	
Potassium (mg)	8.1	≤ 10	
Chloride (mg)		≤ 223	
Vitamin A			
Vitamin D_3			
Vitamin E			
Vitamin C			
Thiamine			
Riboflavin			
Niacin			
Vitamin B_6			
Folacin			
Vitamin B_{12}			
Pantothenic acid			
Calcium		≤ 30 mg	
Phosphorous		≤ 15 mg	
Magnesium			
Iron			
Zinc			
Iodide			
Copper (mg)			
Manganese (mg)			
Selenium (mg)			
Chromium (mg)			
Molybdenum (mg)			
Biotin (mg)			
Water (g)		6	

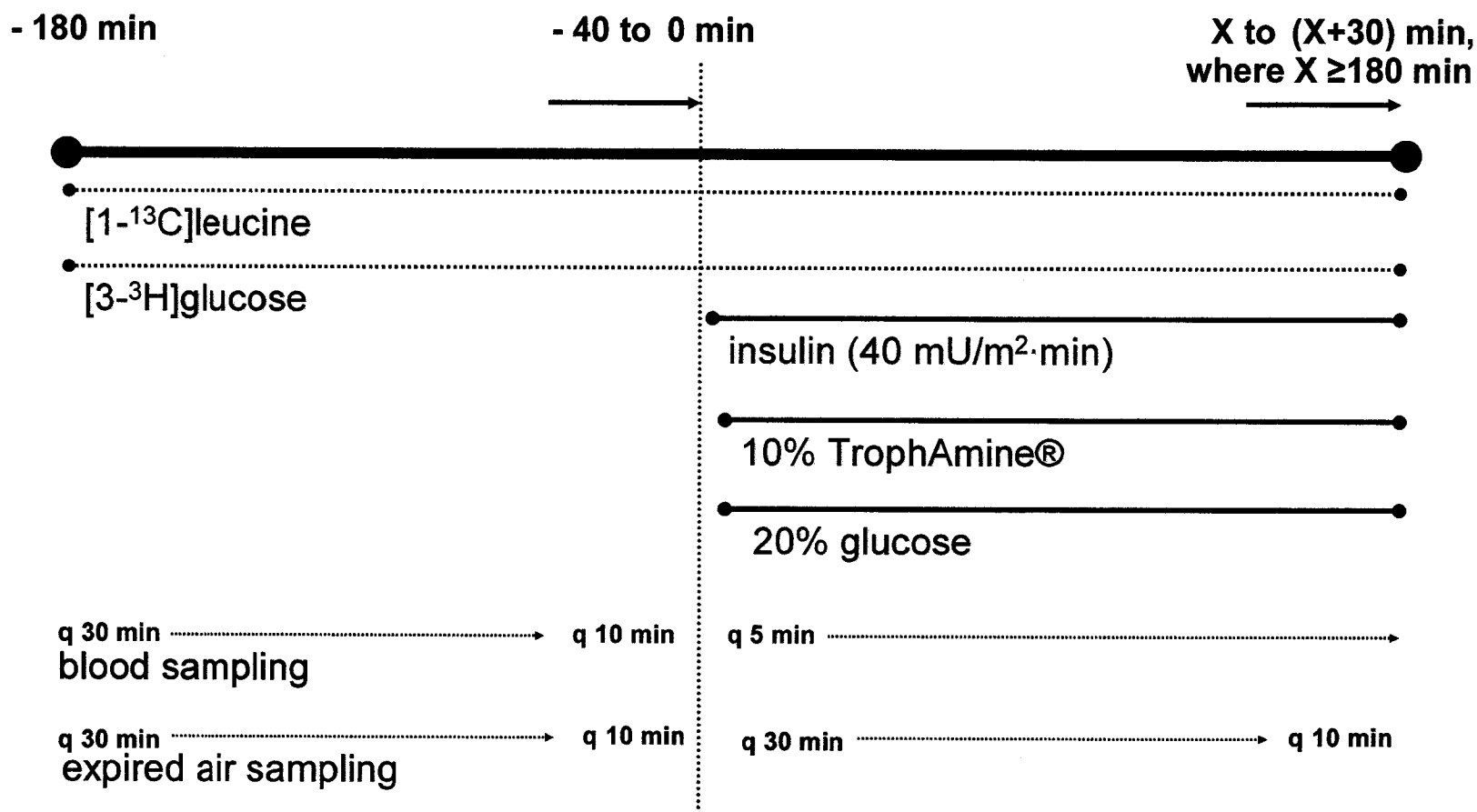

Figure 1. The hyperinsulinemic euglycemic isoaminoacidemic clamp combined with [1-^{13}C]leucine and [3-^{3}H]glucose tracer methodologies. The study protocol starts at -180 minutes (min), baseline plateau takes place from -40 to 0 min, and the clamp plateau occurs during the last 30 minutes of the protocol. The vertical line indicates insulin infusion start time (0 min). Horizontal lines represent the period of time during which a tracer, substrate, or insulin was infused. Infusion of 10% TrophAmine® started at 4 min in all subjects; 20% glucose infusion began at 4 min in control subjects, but in subjects with type 2 diabetes mellitus, there may have been a delay due to hyperglycemia. Blood and breath samples were taken periodically throughout the study, for example every (q) 10 min during the baseline plateau.

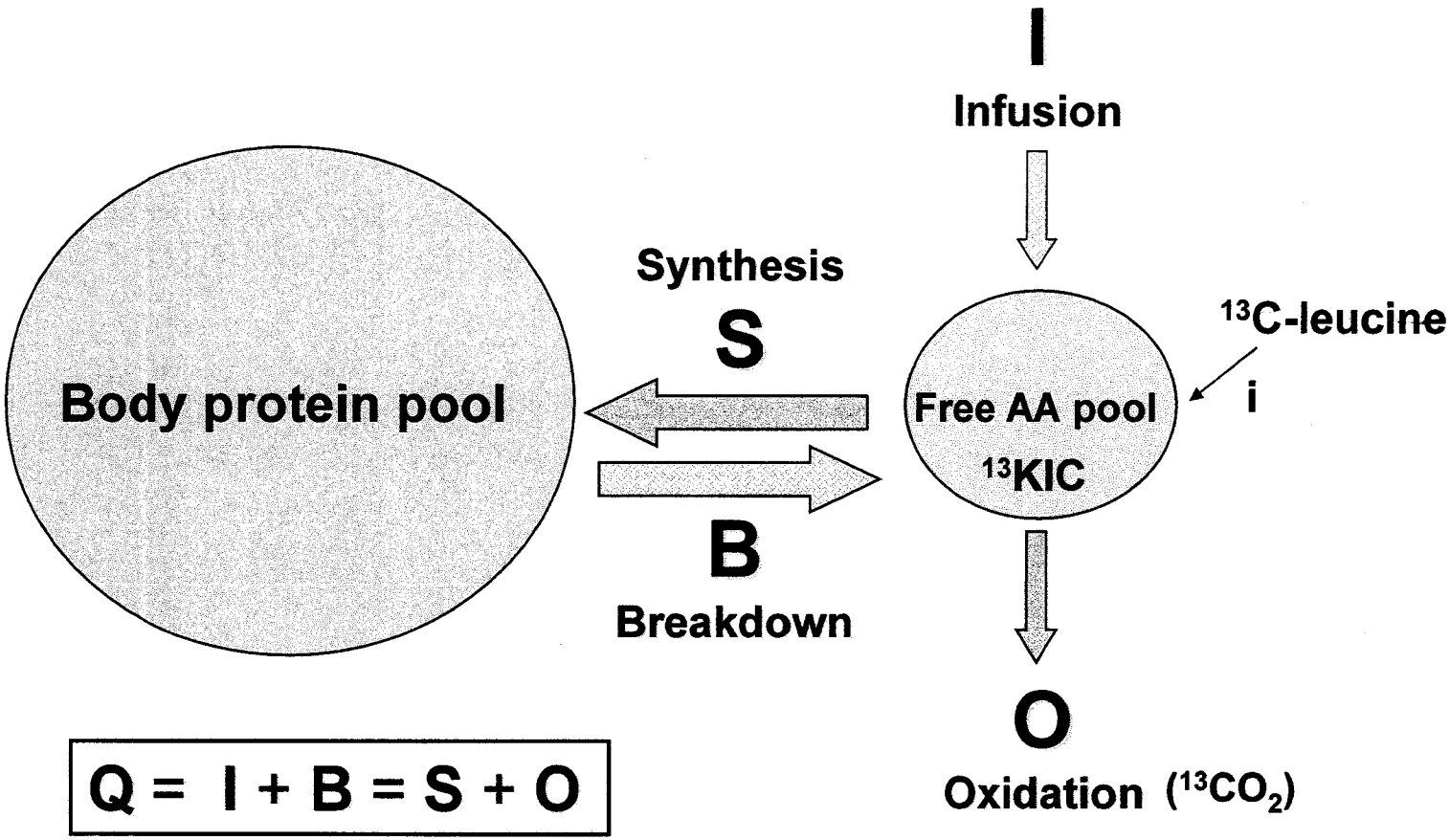

Figure 2. Whole body two-pool [1-^{13}C]leucine kinetics model (Abrams & Wong 2003; Golden et al. 1977). Amino acids enter the free amino acid (AA) pool from protein breakdown (B) or from intravenous infusion (I). Amino acids can leave the free AA pool to be incorporated into protein (S) or to be oxidized (O) to CO_2. When [1-^{13}C]leucine tracer is infused at a rate of i, it mixes with other amino acids in the free AA pool and rates of total leucine flux (Q), leucine B, leucine S, and leucine O can be determined. Leucine I is the leucine infusion rate. Calculations of Q and O require measurements of ^{13}C-α-ketoisocaproate (^{13}KIC) and $^{13}CO_2$ enrichment in plasma and breath, respectively.

10. RESULTS

10.1. Subject Characteristics

Ten men with T2DM and eleven without were recruited and studied (**Table 3**). The average age of T2DM subjects did not differ from the average age of controls (55 ± 1 *vs.* 44 ± 5 years, p=0.071). Waist circumference was also similar between T2DM and control subjects (104.2 ± 3.9 *vs.* 100.7 ± 2.4 cm, p=0.440). All control subjects were Caucasian and with the exception of two control subjects, all had waist circumferences above action level 1 (≥94 cm) or action level 2 (≥102 cm), which are cut-off points designed for Caucasian populations (Lean et al. 1995; World Health Organization 2000). While eight of the ten T2DM subjects were Caucasian, one T2DM subject was South Asian and the other was Chinese. All of the Caucasian T2DM subjects showed abdominal obesity, with a waist circumference above action level 1 or 2. Although the data on appropriate waist circumference cut-off points for specific Asian populations are scarce and incomplete (Razak et al. 2005; World Health Organization 2000), if recent cut-off values for males of 78 cm (action level 1) (Misra et al. 2006) and 80-84 cm (Ko et al. 1999) are applied to the South Asian and Chinese participants, respectively, they also show abdominal obesity. Thus, with the exception of two controls, subjects were abdominally obese. Moreover, neither WHR nor hip circumference differed between the two study groups (**Table 3**).

BMI ranged from 24 to 35 kg/m^2 in both study groups. In control subjects, the specific range was from 24.2 to 34.6 while BMI ranged from 24.4 to 34.5 in T2DM subjects. The BMIs of the two non-Caucasian T2DM subjects were 24.4 and 25.9, but the Chinese and South Asians show an increased risk of morbidities at BMIs below 25.0, even though it is still unclear exactly what the BMI cut-off points should be for Chinese and South Asian populations (Deurenberg et al. 1998; Ko et al. 1999; Misra et al. 2006; World Health Organization 2000). Hence, using BMI cut-off points for Caucasians and proposed cut-off points for Chinese and Asian Indians, which are lower than for Caucasians (Deurenberg et al. 1998; Ko et al. 1999; Misra et al. 2006; World Health Organization 2000), the great majority of the subjects in the current study were either overweight or obese. BMI did not differ between T2DM and control subjects (29.3 ± 1.2 *vs.* 29.1 ± 1.0 kg/m^2, p=0.877) and neither did weight and height (**Table 3**). Lastly, FFM

and PBF were comparable between T2DM and control subjects (64.6 ± 3.1 *vs.* 63.2 ± 2.0 kg, p=0.695; 28.0 ± 1.5 *vs.* 29.0 ± 1.2 %, p=0.590).

Blood lipid levels given in **Table 3** refer to screening day values. T2DM and control subjects showed comparable serum cholesterol (4.54 ± 0.22 *vs.* 5.29 ± 0.35 mmol/L, p=0.093), HDL cholesterol (0.98 ± 0.08 *vs.* 1.16 ± 0.08 mmol/L, p=0.122), LDL cholesterol (2.81 ± 0.21 *vs.* 3.37 ± 0.33 mmol/L, p=0.188), and triglycerides (1.78 ± 0.37 *vs.* 1.67 ± 0.20 mmol/L, p=0.803). All these averages are within the normal ranges specified by the RVH clinical laboratory (**Table 3**), although individually, the values of some of the variables were higher than optimal. Elevated lipid levels in controls or T2DM subjects and lipid-lowering medication in T2DM subjects were not exclusion criteria, although the latter were stopped before admission. The lipid results for T2DM subjects, however, must be interpreted with some caution because some T2DM subjects were taking lipid-lowering medication; on the day of the screening, they were merely asked not to take any of their medication in the morning.

A1C values, which are indicative of glycemic control in the previous 2 to 3 months (American Diabetes Association 2006b), were higher among T2DM subjects than controls (6.9 ± 0.2 vs. 5.4 ± 0.1 %, p<0.001). Screening day A1C values were used because T2DM medication was not necessarily stopped at the same time before admission in all subjects and the effects of T2DM medication would not have worn off by Day 1. None of the control subjects exceeded the normal A1C range specified by the RVH clinical laboratory (4.7-6.0%), while eight of the ten T2DM subjects exceeded this range. Half of the T2DM subjects had A1C values above 7.0%, the A1C threshold for optimal T2DM management (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003). Thus, although most T2DM subjects had higher than normal A1C values, half could be classified as being poorly controlled based on A1C results, but all were unequivocally hyperglycemic when studied.

FPG is reported for the day of the clamp (**Table 3**), after the weight-maintaining, protein controlled diet had been consumed and the confounding effects of T2DM and other medications on metabolism did not exist. Fasting glucose was higher among T2DM than control subjects (10.4 ± 0.5 *vs.* 5.2 ± 0.1 mmol/L, p<0.001). All T2DM subjects had fasting glucose concentrations that exceeded 7.0 mmol/L, which is higher than the

threshold for fasting glucose in optimally controlled T2DM (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003). None of the control subjects had IFG. Lastly, OGTTs were done in control subjects (**Table 3**), 2 of whom had IGT with 2h OGTT plasma glucose concentrations between 7.8 and 11.0 mmol/L (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003); these control subjects behaved like the other controls with respect to the main endpoints.

The average daily weight-maintaining diet, in kcal or in kcal/kg FFM, was comparable in T2DM and control groups (2970 ± 112 *vs.* 2874 ± 99 kcal, p=0.527; 46.2 ± 1.0 *vs.* 45.5 ± 0.7 kcal/kg FFM · day, p=0.559). Protein intake did not differ between T2DM and control subjects (114 ± 4 *vs.* 110 ± 4 g/day, p=0.471) and by design, protein intake per kg FFM was set at approximately 1.7g/kg FFM (1.78 ± 0.03 *vs.* 1.73 ± 0.03 g/kg FFM · day, p=0.313) and 15% of daily energy requirements (15.4 ± 0.1 *vs.* 15.2 ± 0.1 %, p=0.442). Replenishing of the diet was done in T2DM subjects, as necessary, based on urinary glucose analyses performed every morning in our laboratory (data not shown). Both T2DM and control subjects were in nitrogen balance on the day of the clamp (0 ± 0.38 vs. 0.20 ± 0.33 g/day, p=0.697).

Additional clinical features of T2DM subjects are shown in **Table 4**. The duration of T2DM ranged from 1 to 13 years, with the average duration being 7 ± 1 years. Nine of the T2DM subjects were taking at least one oral antihyperglycemic agent and most T2DM subjects were taking metformin and glyburide before the start of the study protocol. One subject (#2 in **Table 4**) who was diagnosed with T2DM three years before the study was not taking any medication for T2DM. On the day of the clamp, this subject had the lowest FPG of all T2DM subjects. Another subject (#8 in **Table 4**) who had T2DM for 12 years was taking metformin and two different types of insulin. It was verified that subject #8 was indeed diagnosed with T2DM and not type 1 diabetes mellitus; the fairly recent addition of insulin to his T2DM treatment regimen further substantiated this. In subsequent analyses of plasma insulin levels, steps were taken to remove insulin antibodies in this subject's plasma.

Adherence to the study protocol by all subjects was ensured through various approaches. First, the study procedures were explained to each participant at least twice, on different days. Second, during admission participants were reminded in person or by

phone of their meal times, especially at the beginning of the study week; they were asked randomly if procedures had been followed in a specific manner; they kept in constant contact with various members of the research team, including myself, a nurse, and the study physician; they were required to keep a log (see Methods) and to tell a member of the research team if and for how long they were planning to leave RVH; they were expected to eat the meals in their room. Third, specific variables indicated whether or not the study protocol had been followed. For instance, substantial changes in weight would indicate that the food is not being consumed in its entirety and substantial fluctuations in urinary creatinine, which is an indicator of muscle mass (Gropper et al. 2005), or urinary electrolytes may signify improper 24 hour urine collections. All participants adhered satisfactorily to the study protocol.

10.2. Substrate and Hormone Concentrations

Insulin concentrations did not differ between groups either at baseline or during the clamp and increased to the same extent as a result of insulin infusion (clamp effect, ANOVA, $p<0.001$) (**Table 5**). The insulin levels obtained during the clamp for T2DM subjects and controls were 656 ± 22 and 647 ± 22 pmol/L, respectively. In the literature, a different statistical test, namely an unpaired t-test, is often used to compare baseline insulin levels between groups. When this is done in my protocol, T2DM subjects show elevated baseline insulin concentrations (121 ± 15 *vs.* 75 ± 7 pmol/L, $p=0.018$). These differences are likely explained by the fact that although multiple comparisons are made in two-way ANOVA, α still equals 0.05, thereby minimizing Type I error, the probability of rejecting the null hypothesis when it is indeed true (Moore et al. 2003; Munro 2001). Hence, the focus will be on the ANOVA results.

Plasma glucagon, which antagonizes various aspects of insulin action, was not controlled for in the current experiment. Glucagon showed a different pattern from insulin (**Table 5**). At baseline glucagon did not differ between T2DM and control groups (unpaired t-test; 27 ± 4 *vs.* 23 ± 2 pmol/L, $p=0.398$), but while glucagon concentrations did not change in the T2DM group in response to the clamp, they decreased significantly in the control group. During the clamp, T2DM subjects had a greater concentration of glucagon than control subjects (unpaired t-test; 28 ± 4 *vs.* 16 ± 2 pmol/L, $p=0.008$).

Furthermore, the Glucagon to Insulin ratio (G/I) reflects the net hepatic effect of these two hormones (Ganong 2001). At baseline, G/I was lower in T2DM subjects than controls, but the reverse was true during the clamp (baseline: 0.23 ± 0.02 *vs.* 0.33 ± 0.04, p=0.038; clamp: 0.04 ± 0.00 *vs.* 0.03 ± 0.00, p=0.004). Even though G/I decreased significantly in both groups in response to the clamp, the magnitude of the decline was smaller in T2DM subjects.

At baseline, plasma glucose was significantly higher in T2DM subjects than in controls (9.11 ± 0.55 vs. 5.02 ± 0.08 mmol/L, p<0.001). While glucose declined significantly from baseline to the clamp plateau in T2DM subjects, it was raised significantly in controls (**Table 5**) such that clamp plasma glucose did not differ between T2DM and control groups (5.53 ± 0.03 vs. 5.51 ± 0.02 mmol/L, p=0.542) as per protocol. BCAA concentrations, as measured using HPLC, did not differ between T2DM subjects and controls during baseline or the clamp (**Table 5**). Postabsorptive BCAA levels were maintained during the clamp (T2DM: 458 ± 17 to 448 ± 20 μmol/L; Controls: 433 ± 13 to 429 ± 13 μmol/L; clamp effect, p=0.208; group x clamp interaction, p=0.525). Plasma insulin, glucose, and BCAA concentrations are shown in **Figure 4**. It indicates that "clamping" of insulin, glucose, and BCAAs was achieved. Serum FFAs did not differ between groups but decreased significantly in T2DM by 73% and in control subjects by 80% in response to the clamp (T2DM: 610 ± 42 to 166 ± 21 μmol/L; Controls: 525 ± 65 to 106 ± 6 μmol/L; clamp effect, p<0.001; group x clamp interaction, p=0.755) (**Table 5**).

Isoaminoacidemia refers to all plasma AAs and not merely BCAAs. My protocol used BCAA concentrations as a guide during the experiment in making alterations to AA infusion rates to try to achieve constant plasma concentrations of all AAs, as has been obtained in previous studies from this lab (Chevalier et al. 2006; Chevalier et al. 2004). The concentrations of individual plasma AAs, at baseline and during the clamp, measured using HPLC are given in **Table 6**. While leucine concentrations did not alter in response to the clamp, the concentrations of the other two BCAAs, valine and isoleucine, decreased and increased respectively (clamp effect, p<0.05). Among individual BCAAs, there were no group differences at baseline, during the clamp, or in response to the clamp. Threonine concentrations dropped in both groups, but methionine and lysine levels increased in T2DM and control subjects as a result of the clamp protocol. In response to

the clamp, phenylalanine concentrations increased only in controls while histidine levels increased significantly in both groups, but more so in the controls. Tryptophan concentrations did not change due to the clamp, but were higher in T2DM subjects at baseline and during the clamp (group effect, $p<0.001$). Total essential AAs, however, were kept at postabsorptive levels during the clamp; there were no group differences in concentrations during the two plateaus or in response to hyperinsulinemia.

Among the non-essential AAs, ornithine concentrations were higher among T2DM subjects than controls at baseline and during the clamp, but postabsorptive concentrations were maintained during the clamp. Only clamp effects ($p<0.05$) were observed in glutamine (decrease), glycine (increase), tyrosine (decrease), citrulline (decrease), and arginine (increase) concentrations. Glutamate concentrations decreased in T2DM subjects due to hyperinsulinemia, but they did not alter in control subjects; there were no group differences at baseline or during the clamp. Both serine and asparagine concentrations decreased significantly in both groups as a result of the clamp, but the magnitude of the changes was greater among T2DM subjects. There were no differences between groups in the concentrations of these two AAs at baseline and during the clamp. Both alanine and taurine concentrations were not altered by the clamp and did not show group differences. Total non-essential AAs, however, decreased in response to the clamp in T2DM and control groups (clamp effect, $p<0.001$). The sum of all measured plasma AAs showed a statistical interaction with a p value of 0.048: although total plasma AAs did not differ between the two groups at baseline or during the clamp plateaus, the T2DM showed a small but statistically significant decline in plasma AAs from baseline to the clamp (**Table 6**).

Taken together, these results indicate that the experimental protocol was able to maintain postabsorptive concentrations of insulin-sensitive AAs like leucine and BCAAs (as a sum) as well as total essential plasma AA concentrations, but that at the individual level, other AA concentrations fluctuated. Of the statistically significant changes in AA concentrations from baseline to the clamp, the highest alterations were a decrease of 28% in asparagine in T2DM and a 28% increase in arginine among control groups. Total non-essential AAs decreased significantly and comparably in T2DM and control groups, but

only by 8% and 5%, respectively. Similarly, the decrease in total AAs among T2DM subjects was only 5%.

10.3. Adjusting Variables of Glucose, Protein, and Fat Metabolism for Body Composition

To determine whether glucose, protein, and fat metabolism variables should be adjusted for FFM, a linear regression approach was utilized. Baseline and clamp glucose R_a, R_d, oxidative R_d, and non-oxidative R_d did not correlate with FFM in each group and thus, none of these variables were adjusted for FFM (data not shown). Glucose infusion rate as well as baseline and clamp fat oxidation did not correlate with FFM and therefore one did not have to adjust for FFM. Glucose infusion rates were, however, also divided by FFM to facilitate comparisons with previous studies.

Baseline and clamp leucine Q, B, and S correlated with FFM in each group separately and the regression line of the linear relationship went through the origin (data not shown). Leucine O at baseline did not correlate with FFM among T2DM subjects, although among the controls, O correlated with FFM and the regression line of the linear relationship went through the origin. Clamp leucine O did not correlate with FFM either in controls or T2DM subjects. Both baseline and clamp leucine O correlated with FFM and the regression line of the linear relationship went through zero when the analysis was done with all participants. Based on these results and since O is related algebraically to other leucine kinetic variables that were divided by FFM, it was most appropriate to divide leucine O by FFM. A similar situation arose with baseline and clamp S-B. At baseline, S-B only correlated with FFM in the control group and when all participants were analyzed together; in both instances, the linear relationship went through the origin. During the clamp, S-B did not correlate with FFM in the control group, T2DM group, or when all participants were analyzed together. Nevertheless, since S and B were separately normalized for FFM, it was physiologically meaningful to divide baseline and clamp S-B by FFM. In correlations involving baseline and clamp leucine kinetics, FFM was used as a covariate.

AA infusion rate did not correlate with FFM in each group. Nevertheless, AA infusion rates were expressed per min and per kg FFM · min and leucine infusion rates

were expressed per kg FFM · min to facilitate comparison with other leucine kinetic variables. None of ΔQ, ΔS, ΔB, ΔO, and Δ(S-B) correlated with FFM in each study group, but in the ensuing analysis these variables were either divided by FFM and in correlations, FFM was used as a covariate; for a given variable, Δ refers to the difference between clamp and baseline values. With the exception of the partial correlation between glucose infusion rate and ΔQ, the significance of the correlations involving Δ's in leucine kinetics was not altered by adjusting for FFM (data not shown). The rationale is that FFM is a confounder of these variables in the baseline and clamp states and it is unlikely, from a physiological and statistical perspective that this confounding effect is completely removed by subtracting baseline from clamp values.

In the case of protein oxidation, there was a correlation with FFM among controls and this relationship went through zero. Among T2DM subjects, significance of the correlation was borderline ($p=0.05$), but the line did not go through zero. The approach in this situation was to use one way ANCOVA with FFM as a covariate. A group comparison of protein oxidation (mg/min) was also done without any adjustments (unpaired t-test) to facilitate comparisons with glucose and fat oxidation, both of which are expressed as mg/min; this did not alter the significance of the results.

10.4. Indirect Calorimetry Results

VO_2, VCO_2, and RQ increased to a similar extent in both groups in response to the clamp (**Table 7**). There were no group differences in these three variables. Fat oxidation also decreased comparably due to hyperinsulinemia, and the groups did not differ at baseline or during the clamp. The two study groups showed similar protein oxidation, regardless of whether an unpaired t-test or one-way ANCOVA is used.

10.5. Kinetics of Glucose Metabolism

The results for glucose metabolism endpoints are found in **Table 8**. Glucose infusion rates were significantly and dramatically lower in T2DM subjects than in controls (185 ± 25 *vs.* 481 ± 48 mg/min, $p<0.001$). Likewise, total glucose R_d during the clamp was lower in T2DM subjects (232 ± 19 *vs.* 486 ± 48 mg/min, $p<0.001$). At baseline, however, total glucose R_d was greater among T2DM subjects than controls (202

± 11 *vs.* 149 ± 7 mg/min, p=0.001). Moreover, while total glucose R_d increased in controls in response to the clamp (p<0.001), there was no change in T2DM subjects. Non-oxidative glucose R_d showed a similar pattern to total glucose R_d (**Table 8**). Non-oxidative glucose R_d was greater in T2DM than controls at baseline (143 ± 20 *vs.* 73 ± 14 mg/min, p=0.008), but it was lower in T2DM than controls during the clamp (100 ± 15 *vs.* 324 ± 40 mg/min, p<0.001). Controls showed an increase in non-oxidative glucose R_d in response to hyperinsulinemia (p<0.001), but T2DM subjects showed no alterations. On the other hand, oxidative glucose R_d increased significantly in response to hyperinsulinemia (clamp effect, p<0.05) and there were no group differences at baseline, during the clamp, or in response to the clamp.

When oxidative and non-oxidative glucose R_d were expressed as percentages of total glucose R_d, the following results were found. Percent oxidative R_d was greater among T2DM than controls during the clamp (57.2 ± 5.1 *vs.* 34.6 ± 3.3, p=0.001) and the reverse was true for percent non-oxidative R_d during the clamp (42.8 ± 5.1 *vs.* 65.4 ± 3.3, p=0.001). There was no difference between T2DM and control subjects for both variables at baseline. In response to the clamp, percent non-oxidative R_d decreased in T2DM subjects and increased in controls, while percent oxidative R_d increased in T2DM subjects and decreased in controls.

Endogenous glucose R_a (EGP) was greater among T2DM subjects at baseline and during the clamp, although it was suppressed in both groups by hyperinsulinemia (clamp effect, p<0.05; group x time interaction, p=0.837) (**Table 8**). **Figure 5** shows and compares total glucose R_d and endogenous glucose R_a expressed per kg BW. It is important to note, however, that using the linear regression approach with baseline and clamp R_a and R_d as endpoints and BW as the independent variable, no correlations were found (data not shown). Ultimately, the point of **Figure 5** is to facilitate comparison of the current research with previous results in the literature.

10.6. Kinetics of Protein Metabolism

All results for leucine kinetics are shown in **Table 9**. The rate of AA infusion, expressed per ml/min, mg/min, and mg/kg FFM · min did not differ between groups. The rate of leucine flux (Q) showed a group x clamp interaction (p=0.030); there were no

group differences in Q at baseline and during the clamp, but while Q increased significantly in response to hyperinsulinemia among control subjects (2.64 ± 0.08 to 2.86 ± 0.09 μmol/kg FFM · min, $p<0.001$), the changes observed in T2DM subjects (2.62 ± 0.08 to 2.70 ± 0.08 μmol/kg FFM · min, $p=0.097$) were not significant. The rate at which leucine is incorporated into protein (S) showed a group x clamp interaction ($p=0.004$). Although there were no group differences in S at baseline and during the clamp, the clamp caused an increase in S among the controls (2.09 ± 0.06 to 2.21 ± 0.08 μmol/kg FFM · min, $p=0.005$), but no alterations were observed in the T2DM group (2.07 ± 0.06 to 2.03 ± 0.06, $p=0.316$). The rate of leucine release due to protein breakdown (B) did not differ between T2DM and control groups at baseline and during the clamp and B was diminished due to hyperinsulinemia in both study groups (clamp effect, $p<0.001$). The rate of leucine oxidation (O) and net balance (S-B) increased as a result of the clamp in both groups (clamp effect, $p<0.05$), but there were no group differences for both variables. When O, S, and B were expressed as percents of Q, only clamp effects were observed ($p<0.05$) (**Table 9**); percent O increased, while both percent S and percent B decreased in response to the clamp.

Figure 6 provides a schematic representation of the statistical interaction observed for Q and S. The inability of hyperinsulinemia to stimulate protein synthesis in the T2DM group is especially obvious in panel B of **Figure 6**. When leucine kinetics were expressed as percentage change, the message resonates with the results obtained for repeated measures ANOVA (**Figure 7**). The percentage change in leucine flux and the rate at which leucine is used for protein synthesis was lower in T2DM than controls (Q: 3.3 ± 1.7 *vs.* 8.6 ± 1.7 %, $p<0.05$; S: -1.7 ± 1.7 *vs.* 5.6 ± 1.5 %, $p<0.05$).

10.7. Correlations

An important component of this study was also to investigate the relationship between glucose and protein metabolism. First, it is important to determine if AA and glucose infusion rates correlate with their respective kinetics. AA infusion rates correlated significantly ($p<0.05$) with clamp leucine Q (partial $r=0.505$), clamp leucine S (partial $r=0.453$), clamp leucine S-B (partial $r=0.796$), ΔQ (partial $r=0.550$), ΔS (partial $r=0.561$), and ΔS-B (partial $r=0.845$) (**Table 10**). The glucose infusion rate showed a

robust and significant correlation with clamp total glucose R_d ($r=0.981$, $p<0.001$) and clamp non-oxidative glucose R_d ($r=0.928$, $p<0.001$); the correlation with clamp oxidative glucose R_d was weaker ($r=0.607$, $p=0.004$) (**Table 11**). Taken together, these data indicate that while the glucose infusion rate correlated with all clamp glucose R_d kinetics, AA infusion rates correlated with most, but not all, clamp leucine kinetic variables and changes in leucine kinetics. The partial correlations between AA infusion rate and clamp net balance (S-B) and change in net balance (Δ S-B; measure of the protein anabolic response to insulin) were especially robust, explaining 63% and 71% of the variance, respectively. As shown in **Figure 8**, there were significant correlations between AA and glucose infusion rates, expressed in mg/min ($r=0.509$, $p=0.018$). AA infusion rate correlated with total glucose disposal during the clamp (**Table 11**) and glucose infusion rate correlated with clamp S-B (partial $r=0.585$, $p=0.007$), Δ Q (partial $r=0.533$, $p=0.015$), Δ S (partial $r=0.653$, $p=0.002$), and Δ S-B (partial $r=0.514$, $p=0.02$) (**Table 10**).

Both clamp total glucose R_d and clamp non-oxidative glucose R_d correlated significantly with clamp S-B (partial $r=0.588$ and partial $r=0.558$, respectively), Δ Q (partial $r=0.447$ and partial $r=0.500$, respectively), and Δ S (partial $r=0.589$ and partial $r=0.624$, respectively) (**Table 10**). Interestingly, clamp total glucose R_d also correlated with Δ S-B (partial $r=0.501$, $p=0.024$) and clamp non-oxidative glucose R_d correlated with clamp leucine S (partial $r=0.456$, $p=0.043$) (**Table 10**). Clamp oxidative glucose R_d only correlated with Δ S-B (partial $r=0.491$, $p=0.028$) (**Table 10**). Overall, when statistically significant correlations exist, leucine kinetics, including leucine S as well as S-B and their respective deltas correlate with total clamp glucose R_d and non-oxidative glucose R_d.

The control group in my protocol consists of overweight and obese individuals, and hence, these participants are not completely healthy. To assess the full impact of T2DM, in the presence of excess fat, the T2DM group was compared to a group of healthy lean men previously studied in our laboratory (Chevalier et al. 2004). These subjects also underwent a hyperinsulinemic euglycemic isoaminoacidemic clamp. The percentage change in leucine S and leucine Q was significantly smaller among subjects with T2DM than lean controls (S: -1.7 ± 1.7 *vs.* 21.6 ± 3.6 %; Q: 3.3 ± 1.7 *vs.* 19.2 ± 2.2 %, $p<0.001$ for both) (**Figure 9**).

Table 3. Comparison of age, anthropometrics, body composition, serum lipids, glycemic control, admission diet, and nitrogen balance between controls and subjects with type 2 diabetes mellitus (T2DM) [1]

	T2DM (n=10)	Controls (n=11)	P value [2]
Age (years)	55 ± 1	44 ± 5	0.071
Weight (kg)	90.5 ± 5.5	89.3 ± 3.4	0.857
Height (cm)	175.1 ± 2.2	175.3 ±1.7	0.941
BMI (kg/m^2)	29.3 ± 1.2	29.1 ± 1.0	0.877
Waist circumference (cm)	104.2 ± 3.9	100.7 ± 2.4	0.440
Hip circumference (cm)	105.2 ± 2.9	105.5 ± 1.8	0.938
Waist to Hip ratio	0.99 ± 0.01	0.95 ± 0.01	0.087
FFM (kg)	64.6 ± 3.1	63.2 ± 2.0	0.695
Percent body fat (%)	28.0 ± 1.5	29.0 ± 1.2	0.590
Serum Cholesterol (mmol/L) [3,6]	4.54 ± 0.22	5.29 ± 0.35	0.093
HDL (mmol/L) [3,7]	0.98 ± 0.08	1.16 ± 0.08	0.122
LDL (mmol/L) [3,8]	2.81 ± 0.21 [3]	3.37 ± 0.33	0.188
Triglycerides (mmol/L) [3,9]	1.78 ± 0.37	1.67 ± 0.20	0.803
A1C (%) [3,10]	6.9 ± 0.2	5.4 ± 0.1	<0.001
FPG (mmol/L) [5]	10.4 ± 0.5	5.2 ± 0.1	<0.001
2h OGTT (mmol/L)	NA	6.4 ± 0.6	NA
Energy intake			
kcal/day	2970 ± 112	2874 ± 99	0.527
kcal/kg FFM · day	46.2 ± 1.0	45.5 ± 0.7	0.559
Protein intake			
g/day	114 ± 4	110 ± 4	0.471
g/kg FFM · day	1.78 ± 0.03	1.73 ± 0.03	0.313
% of daily energy	15.4 ± 0.1	15.2 ± 0.1	0.442
Nitrogen balance (g/day) [5]	0 ± 0.38	0.20 ± 0.33	0.697

[1]Data presented as mean ± SEM. BMI, Body Mass Index; FFM, Fat Free Mass; HDL, High Density Lipoprotein; LDL, Low Density Lipoprotein; A1C = glycosylated hemoglobin; FPG, Fasting Plasma Glucose; OGTT, Oral Glucose Tolerance Test; NA, Not Applicable.
[2]Unpaired t-test.
[3]Screening day.
[4]n=9.
[5]Clamp day.
[6]Normal range (RVH clinical laboratory) = 3.6-6.2 mmol/L.
[7]Normal range (RVH clinical laboratory) = 0.9-1.4 mmol/L.
[8]Normal range (RVH clinical laboratory) = 1.6-4.1 mmol/L.
[9]Normal range (RVH clinical laboratory) = .0-2.3 mmol/L.
[10]Normal range (RVH clinical laboratory) = 4.7-6.0%.

Table 4. Type 2 diabetes mellitus (T2DM) duration and medication in subjects with the disease

Subject #	T2DM duration (years)	T2DM medication before admission
1	3	metformin
2	3	none
3	1	glyburide
4	9	glyburide, metformin
5	2	glyburide, metformin
6	9	metformin
7	13	glyburide, metformin
8	12	metformin, insulin (NPH 20/80, aspart)
9	4.5	glyburide, metformin
10	11	glyburide, metformin

Table 5. Concentrations of substrates and hormones in plasma or serum during baseline and hyperinsulinemic euglycemic isoaminoacidemic clamp plateaus among subjects with type 2 diabetes mellitus (T2DM) and controls [1]

	T2DM (n=10)	Controls (n=11)	P value for group x clamp interaction [2]
Plasma insulin (pmol/L) [3]			
Baseline	121 ± 15	75 ± 7	0.167
Clamp	656 ± 22	647 ± 22	
Plasma glucagon (pmol/L)			
Baseline	27 ± 4	23 ± 2 [4]	0.001
Clamp	28 ± 4 [5]	16 ± 2	
Plasma glucagon/insulin			
Baseline	0.23 ± 0.02 [4, 5]	0.33 ± 0.04 [4]	0.017
Clamp	0.04 ± 0.00 [5]	0.03 ± 0.00	
Plasma glucose (mmol/L)			
Baseline	9.11 ± 0.55 [4, 5]	5.02 ± 0.08 [4]	<0.001
Clamp	5.53 ± 0.03	5.51 ± 0.02	
Plasma BCAAs (μmol/L) [6]			
Baseline	458 ± 17	433 ± 13	0.525
Clamp	448 ± 20	429 ± 13	
Serum FFAs (μmol/L) [3]			
Baseline	610 ± 42	525 ± 65	0.755
Clamp	166 ± 21	106 ± 6	

[1]Data presented as mean ± SEM. BCAAs, Branched Chain Amino Acids; FFAs, Free Fatty Acids.
[2]Statistical interaction from repeated measures ANOVA.
[3]Clamp effect, repeated measures ANOVA, $p<0.05$.
[4]Paired t-test in a given group, $p<0.05$.
[5]Unpaired t-test at a given time (baseline or clamp), $p<0.05$.
[6]BCAAs measured using HPLC.

Table 6. Amino acid concentrations (μmol/L) at baseline and during the clamp among type 2 diabetes mellitus (T2DM) and control subjects [1]

	T2DM (n=10)	Controls (n=11)	P value for group x clamp interaction [2]
Valine [3]			
Baseline	241 ± 9	230 ± 6	0.420
Clamp	225 ± 9	217 ± 5	
Isoleucine [3]			
Baseline	67 ± 3	61 ± 3	0.201
Clamp	72 ± 4	63 ± 3	
Leucine			
Baseline	150 ± 6	142 ± 5	0.223
Clamp	151 ± 8	150 ± 5	
Tryptophan [4, 5]			
Baseline	58 ± 2	43 ± 3	0.674
Clamp	62 ± 2	46 ± 3	
Threonine [3]			
Baseline	102 ± 7	113 ± 6	0.934
Clamp	90 ± 6	102 ± 4	
Phenylalanine			
Baseline	63 ± 4	54 ± 2 [6]	0.024
Clamp	62 ± 3	58 ± 3	
Methionine [3]			
Baseline	26 ± 1	24 ± 1	0.259
Clamp	27 ± 2	26 ± 1	
Lysine [3]			
Baseline	204 ± 10	190 ± 9	0.451
Clamp	212 ± 14	204 ± 10	
Histidine			
Baseline	84 ± 7 [6]	82 ± 4 [6]	0.008
Clamp	91 ± 6	100 ± 4	
Essential AAs (sum) [5]			
Baseline	1006 ± 28	931 ± 26	0.174
Clamp	1003 ± 40	963 ± 26	
Taurine			
Baseline	35 ± 2	37 ± 2	0.383
Clamp	33 ± 2	36 ± 2	
Ornithine [4]			
Baseline	84 ± 9	61 ± 4	0.355
Clamp	81 ± 10	61 ± 4	
Glutamate			
Baseline	104 ± 10 [6]	80 ± 10	0.020
Clamp	93 ± 9	83 ± 10	

Table 6. Amino acid concentrations (μmol/L) at baseline and during the clamp among type 2 diabetes mellitus (T2DM) and control subjects [1] (*cont*)

	T2DM (n=10)	Controls (n=11)	P value for group x clamp interaction [2]
Serine			
Baseline	102 ± 7 [6]	94 ± 6 [6]	0.015
Clamp	83 ± 5	88 ± 6	
Alanine			
Baseline	308 ± 15	310 ± 18	0.687
Clamp	302 ± 12	297 ± 14	
Glutamine [3]			
Baseline	534 ± 22	489 ± 15	0.184
Clamp	452 ± 22	429 ± 13	
Glycine [3]			
Baseline	172 ± 11	208 ± 19	0.186
Clamp	187 ± 12	231 ± 20	
Tyrosine [3]			
Baseline	62 ± 4	62 ± 4	0.209
Clamp	48 ± 3	45 ± 4	
Citrulline [3]			
Baseline	41 ± 1	38 ± 3	0.063
Clamp	35 ± 2	29 ± 3	
Asparagine			
Baseline	43 ± 2 [6]	38 ± 2 [6]	0.013
Clamp	31 ± 2	30 ± 2	
Arginine [3]			
Baseline	86 ± 5	74 ± 5	0.228
Clamp	102 ± 6	95 ± 4	
Non-essential AAs (sum) [3]			
Baseline	1571 ± 45	1492 ± 36	0.144
Clamp	1447 ± 51	1423 ± 38	
Total AAs [5]			
Baseline	2594 ± 69 [6]	2410 ± 59	0.048
Clamp	2455 ± 86	2388 ± 65	

[1]Data presented as mean ± SEM. AAs, Amino Acids. Amino acid concentrations determined by HPLC.
[2]Statistical interaction from repeated measures ANOVA.
[3]Clamp effect, repeated measures ANOVA, $p<0.05$.
[4]Group effect, repeated measures ANOVA, $p<0.05$.
[5]T2DM, n=9; Controls, n=10.
[6]Paired t-test in a given group, $p<0.05$.

Table 7. Results for indirect calorimetry measurements at baseline and clamp plateaus and protein oxidation in subjects with type 2 diabetes mellitus (T2DM) and controls [1]

	T2DM (n=10)	Controls (n=11)	P value for group x clamp interaction [2]
VO_2 (mL/min) [3]			
Baseline	270 ± 11	262 ± 9	0.123
Clamp	272 ± 12	271 ± 8	
VCO_2 (mL/min) [3]			
Baseline	213 ± 10	211 ± 8	0.155
Clamp	232 ± 9	238 ± 9	
RQ [3]			
Baseline	0.79 ± 0.02	0.80 ± 0.01	0.766
Clamp	0.85 ± 0.02	0.88 ± 0.01	
Fat oxidation (mg/min) [3]			
Baseline	75 ± 7	66 ± 6	0.777
Clamp	46 ± 9	35 ± 6	
Protein oxidation (mg/min)			
One way ANCOVA (NS)	71 ± 2 [4]	69 ± 2 [4]	NA
Unpaired t-test (NS)	72 ± 3	68 ± 3	

[1]Data presented as mean ± SEM. VO_2, rate at which oxygen is used up; VCO_2, rate at which CO_2 is generated; RQ, Respiratory Quotient; NS, Not Significant; NA, Not Applicable.
[2]Statistical interaction from repeated measures ANOVA.
[3]Clamp effect, repeated measures ANOVA, $p<0.05$.
[4]Means adjusted for fat free mass.

Table 8. Kinetics of glucose metabolism during baseline and clamp plateaus and clamp glucose infusion rate in subjects with type 2 diabetes mellitus (T2DM) and controls [1]

	T2DM (n=10)	Controls (n=11)	P value for group x clamp interaction [2]
Clamp glucose infusion			
mg/min	185 ± 25 [3]	481 ± 48	NA
mg/kg FFM · min	2.94 ± 0.39 [3]	7.61 ± 0.68	
Total glucose R_d (mg/min)			
Baseline	202 ± 11 [3]	149 ± 7 [4]	<0.001
Clamp	232 ± 19 [3]	486 ± 48	
Oxidative glucose R_d (mg/min) [5]			
Baseline	60 ± 19	76 ± 13	0.507
Clamp	132 ± 15	162 ± 17	
Non-oxidative glucose R_d (mg/min)			
Baseline	143 ± 20 [3]	73 ± 14 [4]	<0.001
Clamp	100 ± 15 [3]	324 ± 40	
Oxidative R_d as % of total Rd			
Baseline	29.0 ± 9.6 [4]	51.5 ± 8.6 [4]	0.002
Clamp	57.2 ± 5.1 [3]	34.6 ± 3.3	
Non-oxidative R_d as % of total Rd			
Baseline	71.0 ± 9.6 [4]	48.5 ± 8.6 [4]	0.002
Clamp	42.8 ± 5.1 [3]	65.4 ± 3.3	
Endogenous Glucose R_a (mg/min) [5,6]			
Baseline	188 ± 12	149 ± 6	0.837
Clamp	45 ± 12	3 ± 10	

[1]Data presented as mean ± SEM. FFM, Fat Free Mass; R_d, rate of disposal; R_a, rate of appearance. [2]Statistical interaction from repeated measures ANOVA. [3]Unpaired t-test at a given time (baseline or clamp), $p<0.05$. [4]Paired t-test in a given group, $p<0.05$. [5]Clamp effect, repeated measures ANOVA, $p<0.05$. [6]Group effect, repeated measures ANOVA, $p<0.05$.

Table 9. Kinetics of protein metabolism at baseline and clamp plateaus and clamp amino acid infusion rate in type 2 diabetes mellitus (T2DM) and control subjects [1]

	T2DM (n=10)	Controls (n=11)	P value for group x clamp interaction [2]
Clamp AA infusion			
ml/min	0.41 ± 0.03	0.43 ± 0.02	NA
mg/min	41.0 ± 2.5	43.4 ± 2.2	NA
mg/kg FFM · min	0.65 ± 0.05	0.69 ± 0.04	NA
Leucine infusion (I)			
(μmol/kg FFM · min)	0.69 ± 0.05	0.74 ± 0.05	NA
Leucine Flux (Q)			
(μmol/kg FFM · min)			
Baseline	2.62 ± 0.08	2.64 ± 0.08 [3]	0.030
Clamp	2.70 ± 0.08	2.86 ± 0.09	
Leucine Oxidation (O) [4]			
(μmol/kg FFM · min)			
Baseline	0.56 ± 0.04	0.55 ± 0.03	0.749
Clamp	0.68 ± 0.03	0.65 ± 0.02	
Leucine incorporation into protein (S)			
(μmol/kg FFM · min)			
Baseline	2.07 ± 0.06	2.09 ± 0.06 [3]	0.004
Clamp	2.03 ± 0.06	2.21 ± 0.08	
Leucine release from protein breakdown (B) [4]			
(μmol/kg FFM · min)			
Baseline	2.62 ± 0.08	2.64 ± 0.08	0.145
Clamp	2.01 ± 0.06	2.12 ± 0.08	
Leucine Net Balance (S-B) [4]			
(μmol/kg FFM · min)			
Baseline	-0.56 ± 0.04	-0.55 ± 0.03	0.383
Clamp	0.01 ± 0.04	0.09 ± 0.04	
Leucine O as a % of Leucine Q [4]			
Baseline	21.1 ± 1.1	20.6 ± 0.9	0.171
Clamp	25.0 ± 0.8	22.8 ± 0.6	
Leucine S as a % of Leucine Q [4]			
Baseline	78.9 ± 1.1	79.4 ± 0.9	0.171
Clamp	75.0 ± 0.8	77.2 ± 0.6	
Leucine B as a % of Leucine Q [4]			
Baseline	100	100	0.827
Clamp	74.5 ± 1.6	74.1 ± 1.4	

[1]Data presented as mean ± SEM. NA, Not Applicable; AA, Amino Acid. [2]Statistical interaction from repeated measures ANOVA. [3]Paired t-test in a given group, $p<0.05$. [4]Clamp effect, repeated measures ANOVA, $p<0.05$.

Table 10. Correlations between amino acid infusion rate, glucose infusion rate, glucose kinetics, and leucine kinetics [1]

	Amino acid infusion rate (mg/min)	Glucose infusion rate (mg/min)	Clamp total glucose R_d (mg/min)	Clamp oxidative glucose R_d (mg/min)	Clamp non-oxidative glucose R_d (mg/min)
Clamp leu Q (μmol/min)	0.505 [2]	0.364	0.314	0.101	0.330
Clamp leu O (μmol/min)	0.406	-0.063	-0.147	0.155	-0.227
Clamp leu S (μmol/min)	0.453 [2]	0.442	0.411	0.067	0.456 [2]
Clamp leu B (μmol/min)	0.064	0.156	0.124	-0.107	0.183
Clamp leu S-B (μmol/min)	0.796 [2]	0.585 [2]	0.588 [2]	0.357	0.558 [2]
Δ Leu Q (μmol/min)	0.550 [2]	0.533 [2]	0.447 [2]	0.061	0.500 [2]
Δ Leu O (μmol/min)	0.084	-0.114	-0.171	-0.198	-0.129
Δ Leu S (μmol/min)	0.561 [2]	0.653 [2]	0.589 [2]	0.177	0.624 [2]
Δ Leu B (μmol/min)	-0.322	0.123	0.072	-0.339	0.206
Δ Leu S-B (μmol/min)	0.845 [2]	0.514 [2]	0.501 [2]	0.491 [2]	0.409

[1]Partial correlations, adjusted for fat free mass. Correlations include all participants. Δ = Clamp value – Baseline value. Leu, leucine. Refer to Tables 8 and 9 for other abbreviations.
[2] $p<0.05$.

Table 11. Correlations between amino acid infusion rate, glucose infusion rate, and kinetics of glucose metabolism during the hyperinsulinemic euglycemic isoaminoacidemic clamp [1]

	Amino acid infusion rate (mg/min)	Glucose infusion rate (mg/min)
Glucose infusion rate (mg/min)	0.509 [2]	1
Clamp total glucose R_d (mg/min)	0.458 [2]	0.981 [2]
Clamp oxidative glucose R_d (mg/min)	0.431	0.607 [2]
Clamp non-oxidative glucose R_d (mg/min)	0.381	0.928 [2]

[1]Pearson correlation coefficients. Correlations include all participants. R_d, rate of disposal.
[2] $p<0.05$.

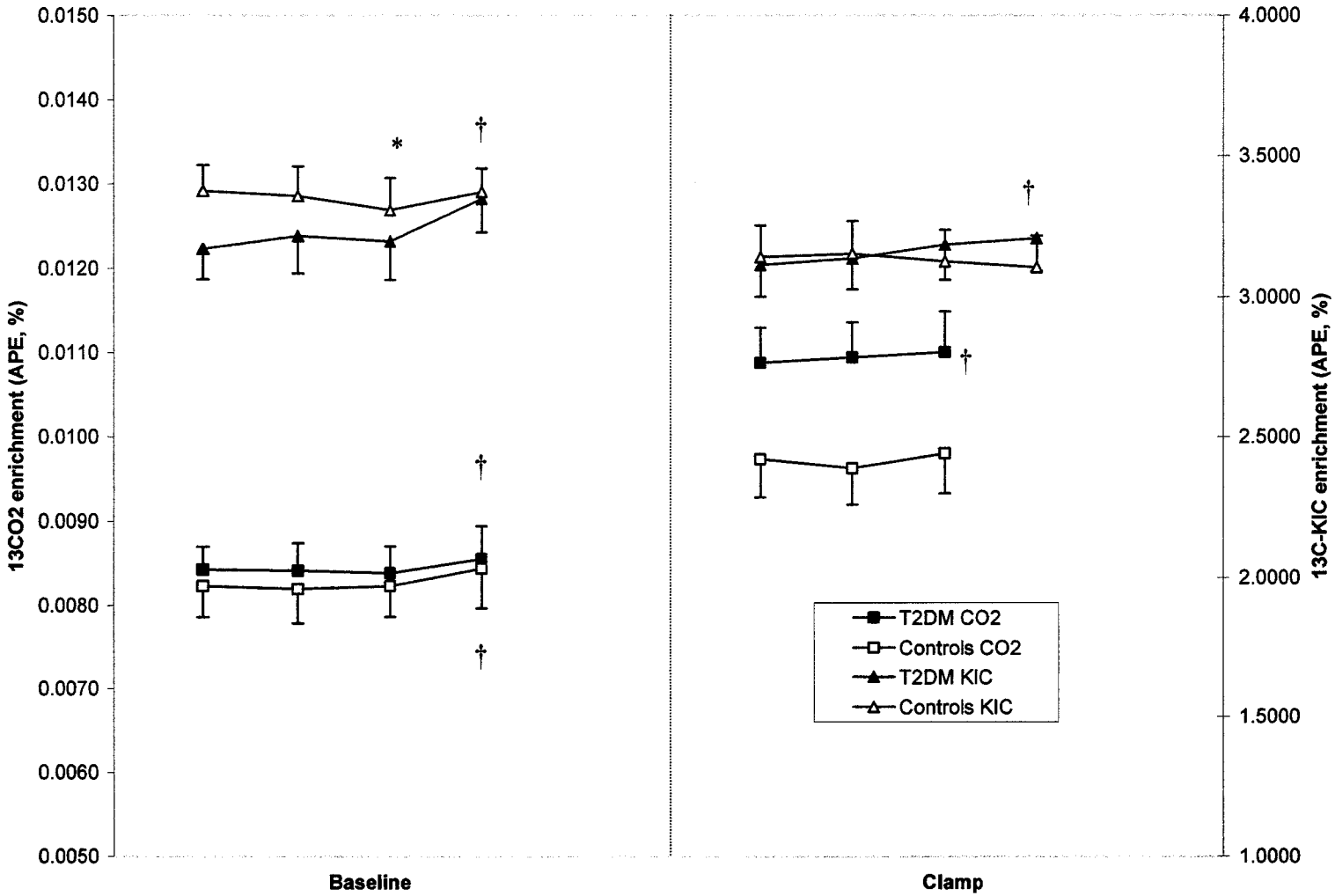

Figure 3. ^{13}C-α-ketoisocaproate (^{13}C-KIC) and $^{13}CO_2$ enrichment in plasma and breath samples, respectively, during baseline and clamp plateaus. The middle vertical line indicates the start of insulin infusion; the horizontal line is not to scale. At certain times, the number of measurements in the control or type 2 diabetes mellitus (T2DM) group was not complete; *, n=10 and †, n=9. Error bars represent SEM.

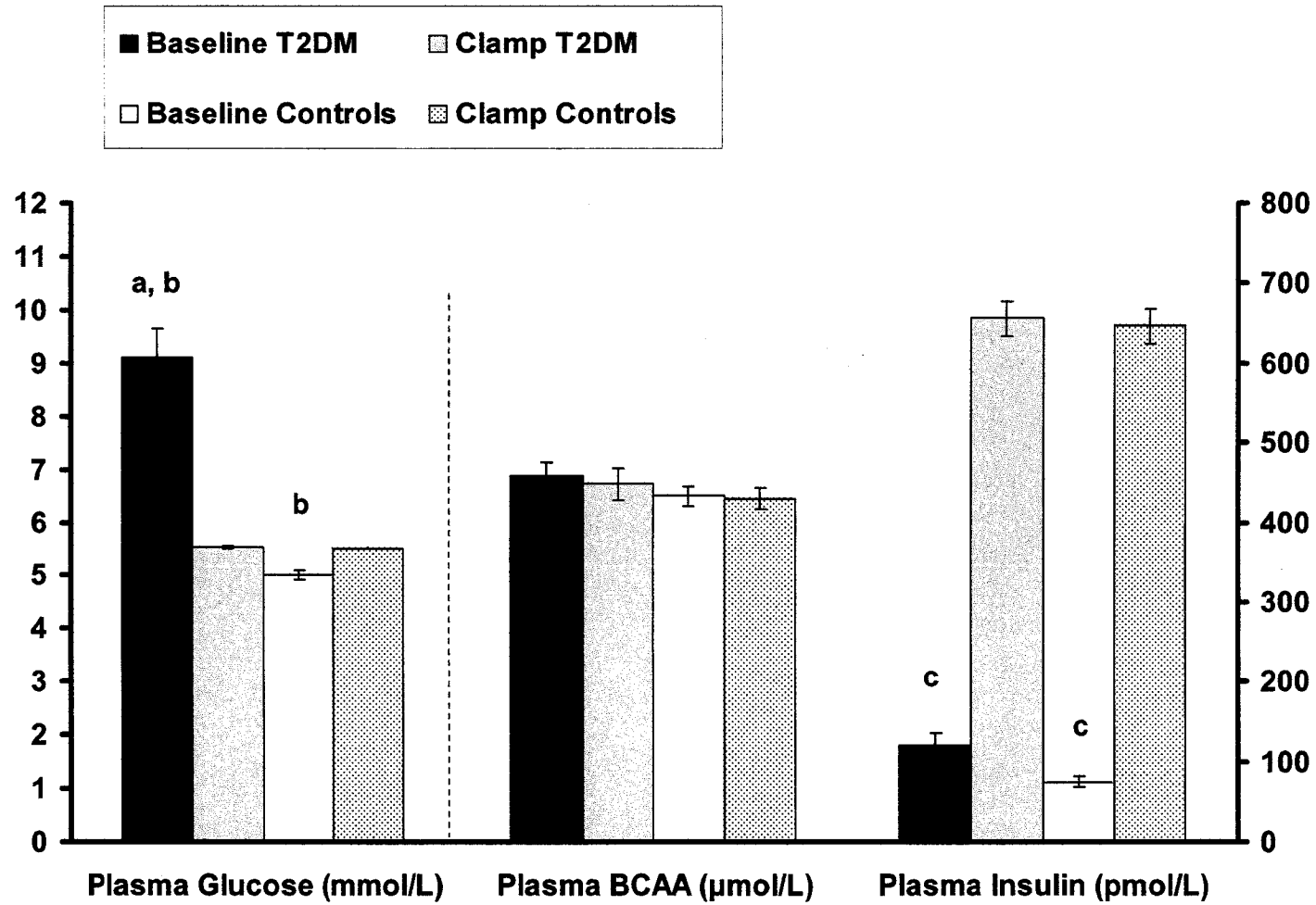

Figure 4. Effect of clamp protocol on plasma glucose, branched chain amino acid (BCAA), and insulin concentrations of type 2 diabetes mellitus (T2DM) and control groups. **a**, group difference, baseline (unpaired t-test, $p<0.05$); **b**, difference between baseline and clamp in a group (paired t-test, $p<0.05$); **c**, clamp effect (repeated measures ANOVA, $p<0.05$). During the clamp plateau, plasma glucose, BCAA, and insulin concentrations did not differ between groups. Error bars refer to SEM.

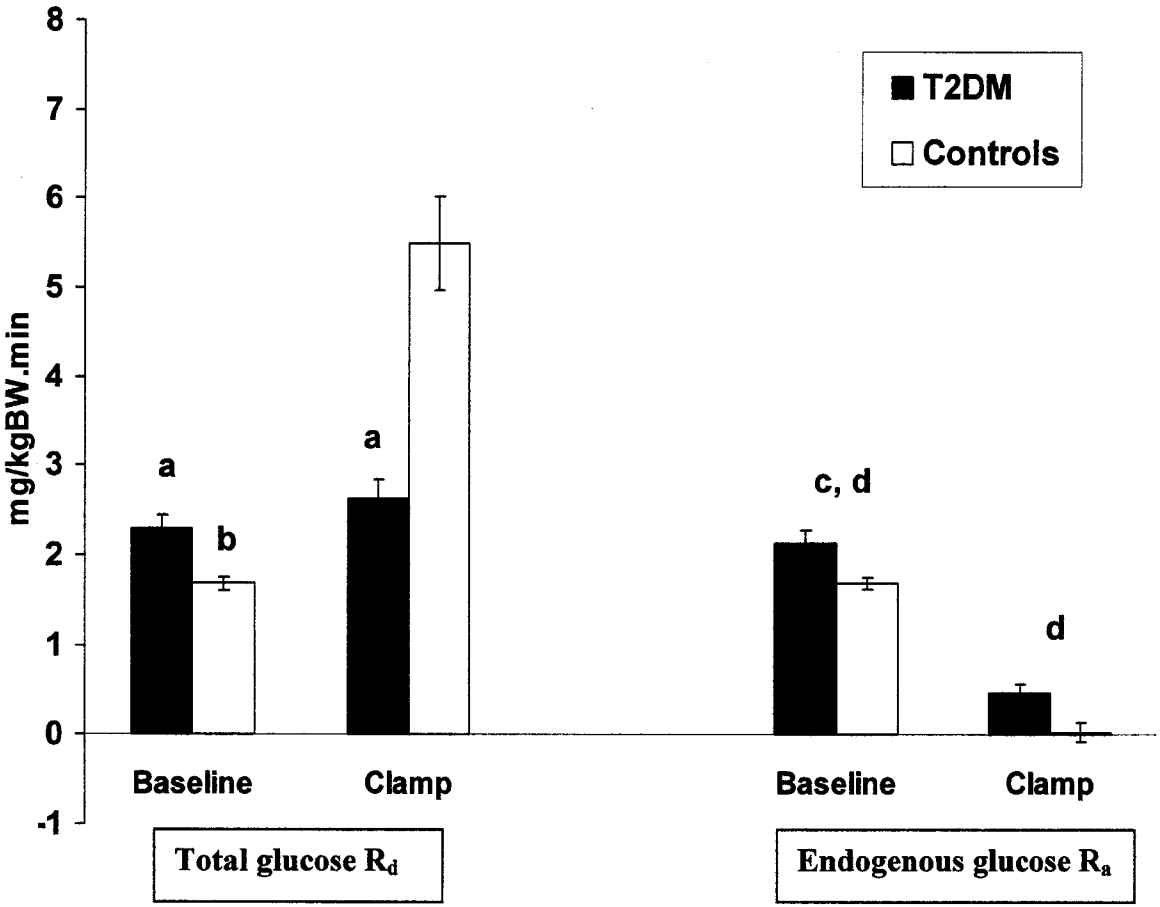

Figure 5. Baseline and clamp plateaus total glucose disposal (R_d) and endogenous glucose appearance (R_a) in type 2 diabetes mellitus (T2DM) and control groups. **a**, group difference at baseline or during the clamp (unpaired t-test, $p<0.01$); **b**, difference between baseline and clamp in a group (paired t-test, $p<0.001$); **c**, clamp effect (repeated measures ANOVA, $p<0.001$); **d**, group effect (repeated measures ANOVA, $p=0.001$). Error bars refer to SEM.

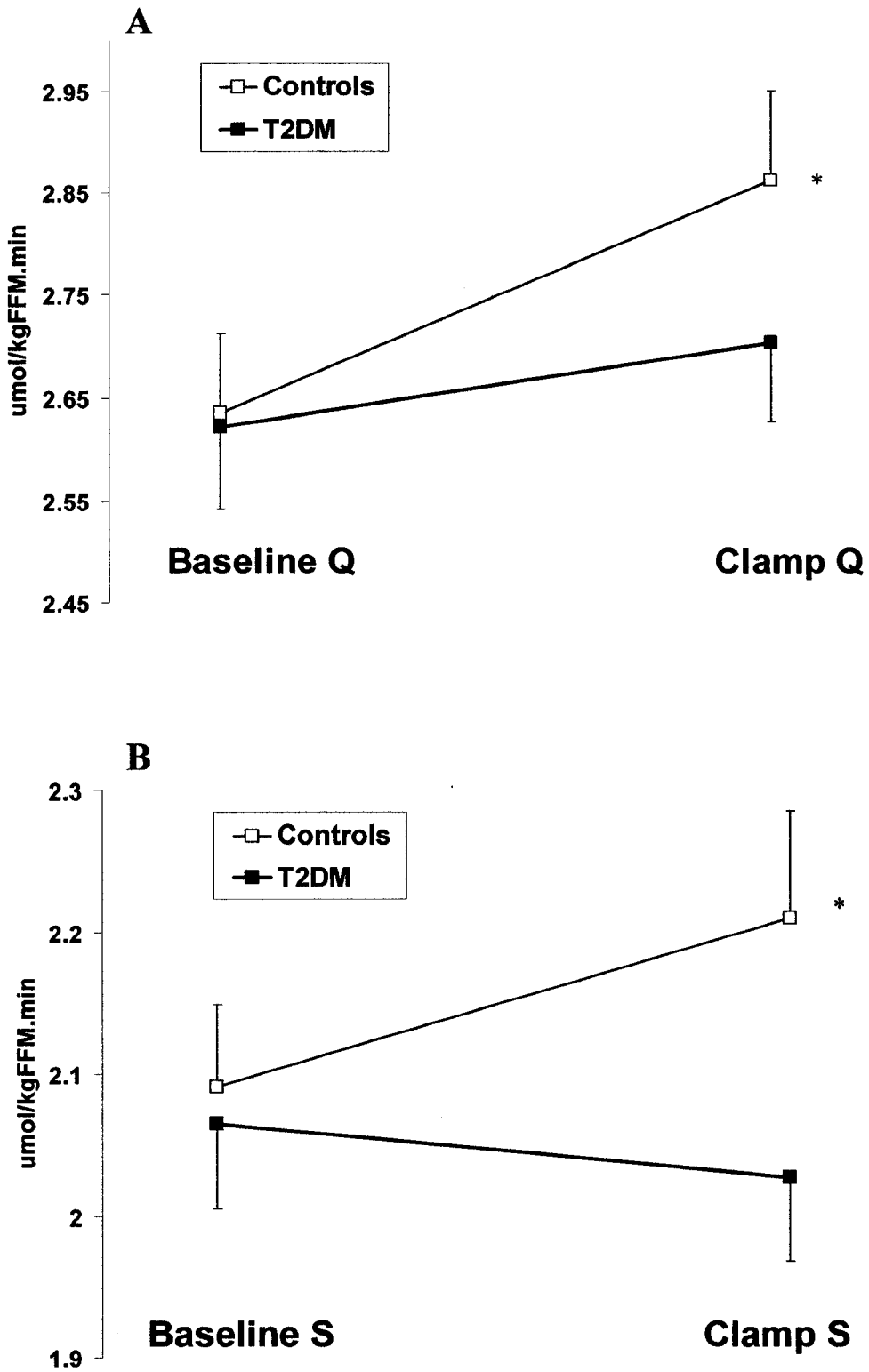

Figure 6. (A) Effect of clamp on total leucine flux (Q) in subjects with type 2 diabetes mellitus (T2DM) and controls. (B) Effect of clamp on rate at which leucine is incorporated into protein (S). *, difference between baseline and clamp plateaus in a group (paired t-test, $p<0.05$). Error bars refer to SEM.

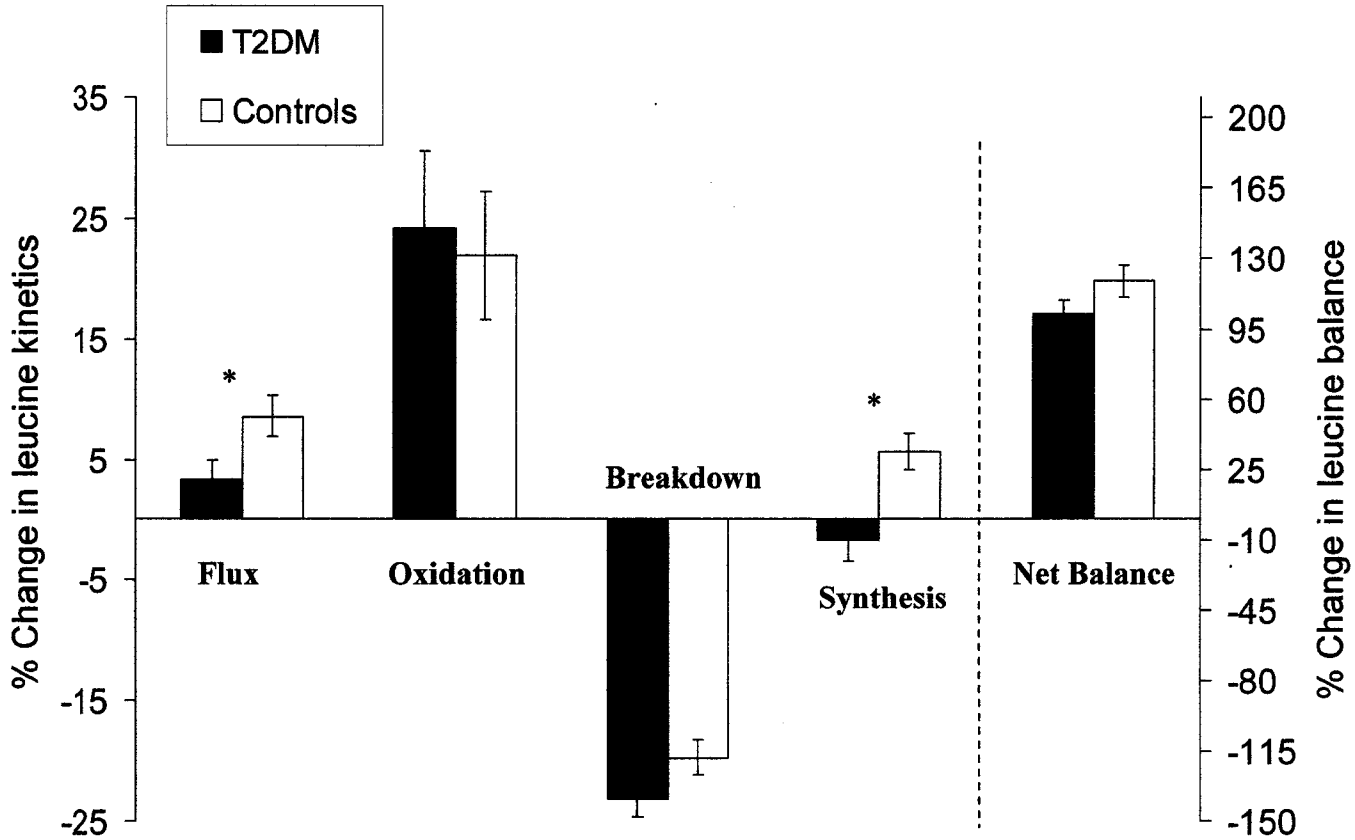

Figure 7. Percentage (%) change in leucine kinetics and leucine balance, from baseline to clamp plateau, in subjects with type 2 diabetes mellitus (T2DM) and controls. *, group difference (unpaired t-test, $p<0.05$). % Change in leucine kinetics = 100 (Clamp value – Baseline value)/Baseline value. % Change in leucine balance = 100 x [Clamp net balance – Baseline net balance] / (|Baseline net balance|), where S and B represent synthesis and breakdown, respectively, and Net Balance = S-B. Error bars refer to SEM.

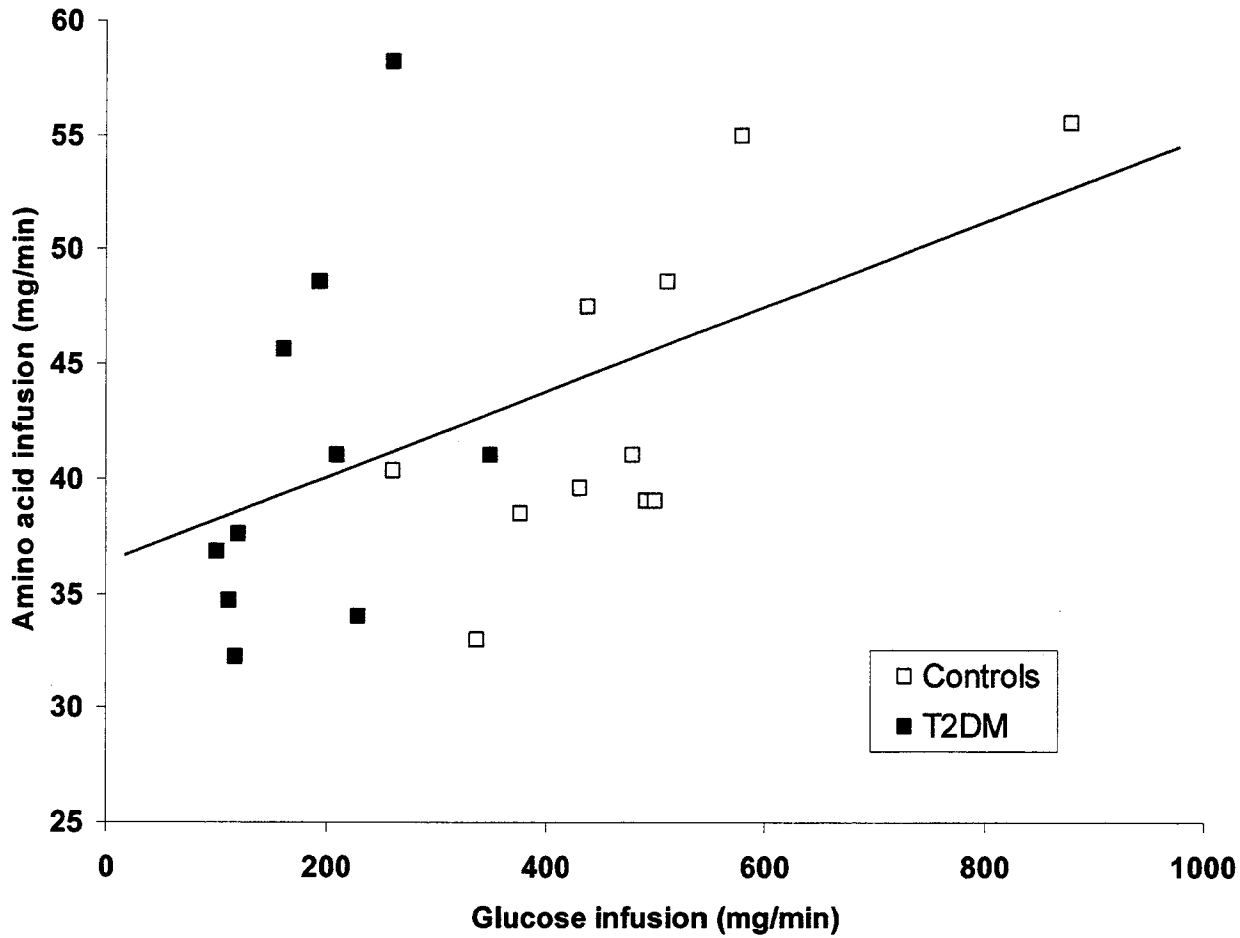

Figure 8. Correlation between amino acid and glucose infusion rates. Pearson correlation coefficient; r=0.509, p=0.018. T2DM represents subjects with type 2 diabetes mellitus.

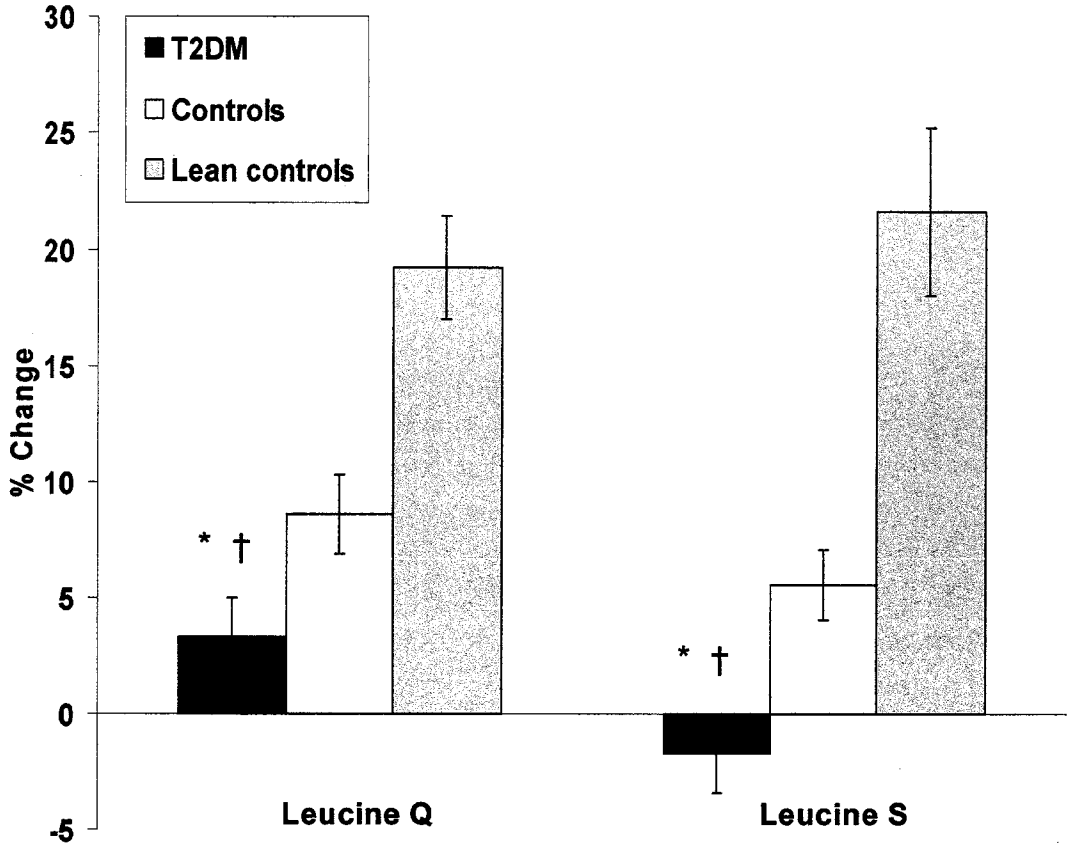

Figure 9. Comparison of the percentage change in the rate of leucine flux (Q) and the rate at which leucine is incorporated into protein (S) in subjects with type 2 diabetes mellitus (T2DM), controls matched for age and obesity (Controls), and healthy, young, lean controls previously studied in our laboratory using the hyperinsulinemic euglycemic isoaminoacidemic clamp (Chevalier et al. 2004). Percentage change = 100 (Clamp value – Baseline value)/Baseline value. *, $p<0.05$, T2DM *vs.* controls; †, $p<0.001$, T2DM *vs.* lean controls. Error bars represent SEM.

11. DISCUSSION

The main findings of this study are that when hyperglycemic T2DM men are matched for age, body composition, and abdominal obesity to non-diabetic male controls and both groups undergo a hyperinsulinemic euglycemic isoaminoacidemic clamp, T2DM participants show 1) blunted insulin-stimulated increase in whole body protein synthesis and protein turnover, as assessed using L-[1-^{13}C]leucine, 2) diminished insulin-mediated total and non-oxidative glucose disposal, and 3) similar rates of protein oxidation as well as clamp glucose and fat oxidation. Furthermore, insulin sensitivity of glucose and protein metabolism are positively associated, as determined by bivariate and partial correlations.

Postabsorptive leucine, BCAA, and total AA concentrations were comparable in T2DM and control subjects in the current protocol, consistent with most studies (Denne et al. 1995; Halvatsiotis et al. 2002a; Halvatsiotis et al. 2002b; Luzi et al. 1993; Pijl et al. 1994; Umpleby et al. 1990; Welle & Nair 1990), but not Vannini et al. (1982). A BCAA dehydrogenase assay was combined with fluorometry to permit quick adjustments to the AA infusion rate throughout the insulin clamp. Total BCAAs and total essential AAs were comparable to baseline. Among the BCAAs, leucine remained constant, while isoleucine increased in both groups and valine decreased in both groups. Total non-essential AAs decreased in response to hyperinsulinemia, but this is likely not to have affected the results for protein kinetics because essential AAs play the principal role in stimulating protein synthesis. First, protein synthesis has been shown to increase in skeletal muscle of healthy elderly when plasma essential AAs are doubled (Volpi et al. 2003); the effects of essential AAs only and both essential and non-essential AAs are similar. Second, protein synthesis increases at the whole body level in healthy volunteers when total plasma BCAA concentrations are quadrupled (Louard et al. 1990). Third, translation initiation is augmented in the skeletal muscle of healthy subjects when plasma leucine concentrations are tripled (Greiwe et al. 2001). By similar reasoning, it is unlikely that the small decline in total AAs in T2DM would have affected the results, since essential AAs were maintained at postabsorptive levels in the T2DM group during the clamp. The decrease in total AAs among T2DM subjects was due to an 8% decline in non-essential AAs; in controls, non-essential AAs only decreased by 5%. Moreover, the

change in total AAs in T2DM subjects was 5%, which is a very small physiological change. Among lean healthy men studied with the hyperinsulinemic euglycemic isoaminoacidemic clamp in our laboratory, the change in plasma AAs from baseline to the clamp, although not significant, was also a decrease of 5% (Chevalier et al. 2004). Under those circumstances, we were clearly able to show an insulin-stimulated increase in whole-body protein synthesis.

In my protocol, group differences were found for tryptophan and ornithine, which were higher among T2DM subjects at baseline and during the clamp. Although it is not immediately clear why tryptophan concentrations were augmented in T2DM subjects, greater ornithine concentrations could be associated with elevated urea synthesis, the latter being previously documented in hyperglycemic T2DM subjects (Almdal et al. 1994; Devlin 2002). Furthermore, a group effect for ornithine was previously found in obese subjects at baseline and during the hyperinsulinemic euglycemic isoaminoacidemic clamp (Chevalier et al. 2005b). The largest fluctuation in individual AAs in response to hyperinsulinemia was 28% for asparagine and arginine, two non-essential AAs.

Clearly, the changes in AAs observed in my protocol are minimal, especially when compared to the changes in plasma AAs in previous studies that have attempted to maintain postabsorptive AA concentrations during the hyperinsulinemic euglycemic clamp (Castellino et al. 1987; Flakoll et al. 1989; Frexes-Steed et al. 1990; Heslin et al. 1992; Russell-Jones et al. 1994), in which some AA concentrations increased by at least 100%. Indeed, our research group has also been able to maintain isoaminoacidemia during the hyperinsulinemic euglycemic clamp in healthy young and obese populations (Chevalier et al. 2004; Chevalier et al. 2005b). Moreover, after a thorough literature search, nobody has ever shown any physiological or biochemical effects of such small changes in AA concentrations. Lastly, in my protocol, an isocaloric diet with generous protein content and comparable protein composition was consumed for 7 days (T2DM) or 6 days (controls) before measurements of insulin sensitivity of protein and glucose metabolism were made on the clamp study day.

It is important to maintain plasma AA concentrations at postabsorptive levels during the clamp because *in vivo* human studies have indicated that AAs and insulin have independent effects on protein metabolism. At the level of the whole body, the effect of

insulin on kinetics of protein metabolism depends on plasma AA concentrations (Castellino et al. 1987; Tessari et al. 1987). In studies that have previously attempted to maintain isoaminoacidemia during a hyperinsulinemic euglycemic clamp in healthy subjects, with varying degrees of success, physiological hyperinsulinemia does not change Q (Castellino et al. 1987; Flakoll et al. 1989; Frexes-Steed et al. 1990; Russell-Jones et al. 1994), B declines (Castellino et al. 1987; Flakoll et al. 1989; Frexes-Steed et al. 1990; Heslin et al. 1992; Russell-Jones et al. 1994), S does not change (Castellino et al. 1987; Flakoll et al. 1989; Frexes-Steed et al. 1990; Heslin et al. 1992; Russell-Jones et al. 1994), O increases (Castellino et al. 1987; Heslin et al. 1992; Russell-Jones et al. 1994) or does not change (Flakoll et al. 1989; Frexes-Steed et al. 1990), and S-B increases (Castellino et al. 1987; Heslin et al. 1992). The results for a hyperinsulinemic euglycemic isoaminoacidemic clamp in healthy young men (Chevalier et al. 2004), where postabsorptive AA concentrations were maintained, were mostly similar, as insulin decreased B, increased Q and S-B, had no effect on O, but in contrast, insulin increased S. For the majority of studies, the direction of the effect of AAs on kinetics of protein metabolism is as follows: Q, O, S, and S-B augment (Castellino et al. 1987; Tessari et al. 1987) and B declines (Castellino et al. 1987; Flakoll et al. 1989; Tessari et al. 1987). In Flakoll et al. (1989), however, the AA-induced increase in O and S did not reach statistical significance.

The response of S to insulin deserves to be explored further because of the conflicting *in vivo* results in the literature. In skeletal muscle, only insulin diminishes B, while both insulin and AAs stimulate S; in splanchnic tissues, only AAs increase S and suppress B (Nygren & Nair 2003). During fasting, skeletal muscle accounts for 27-34% of whole body synthesis (Nair et al. 1988; Tessari et al. 1996), which is greater than for splanchnic tissues (Tessari et al. 1996); in the fed state, most protein synthesis occurs in skeletal muscle (Rennie et al. 1982). At a given insulin concentration, fluctuations in AAs will likely have a more important impact on whole body S than whole body B because protein synthesis in skeletal muscle, a large contributor to whole body S, is also affected by plasma AA concentrations (Nygren & Nair 2003). At the level of the whole body, B has been consistently shown to decrease during a hyperinsulinemic euglycemic clamp, regardless of whether AAs declined or were maintained at postabsorptive levels

(Castellino et al. 1987; Chevalier et al. 2004; Flakoll et al. 1989; Fukagawa et al. 1985; Meek et al. 1998; Petrides et al. 1994; Tessari et al. 1987; Tessari et al. 1986). On the other hand, while S decreases during a hyperinsulinemic euglycemic clamp (Castellino et al. 1987; Flakoll et al. 1989; Meek et al. 1998; Petrides et al. 1994; Tessari et al. 1987; Tessari et al. 1986), it increases during the hyperinsulinemic euglycemic isoaminoacidemic clamp (Chevalier et al. 2004). Hence, the direction of the response of S to insulin is dependent upon substrate availability. Lastly, different approaches to adjusting for body composition, especially in studies including both sexes, are likely to further occult the true effect of insulin on protein synthesis (Chevalier et al. 2005a; Chevalier et al. 2005b).

Molecular studies corroborate the findings of Chevalier et al. (2004) and the results for our control group, which indicate that insulin stimulates protein synthesis. Insulin augments the initiation of messenger RNA translation and the synthesis of ribosomes (Bolster et al. 2004; Harris et al. 2006; Wullschleger et al. 2006) via stimulation of mammalian target of rapamycin (mTOR), which is downstream of phosphatidylinositol 3-kinase (PI3K); among other actions, mTOR phosphorylates eukaryotic initiation factor 4E binding protein 1 (eIF4E-BP1) and 70 kDa ribosomal protein S6 kinase ($p70^{S6k}$) (Wullschleger et al. 2006). Once eIF4E-BP1 is phosphorylated, it liberates eIF4E (Wullschleger et al. 2006), a factor necessary for the initiation of translation (Shah et al. 2000). Phosphorylation of the kinase $p70^{S6k}$ speeds up translation initiation (Greiwe et al. 2001). Insulin has been shown to stimulate phosphorylation of eIF4E-BP1 and $p70^{S6k}$ in cell lines as well as in rodent skeletal muscle (Anthony et al. 2002; Patti et al. 1998). In human skeletal muscle, the hyperinsulinemic euglycemic clamp is associated with an increase in the phosphorylation of $p70^{S6k}$ (Greiwe et al. 2001; Hillier et al. 2000), although there was no alteration in the amount of phosphorylated eIF4E-BP1 (Hillier et al. 2000). Nevertheless, plasma AAs diminished during the clamp, and AAs, especially BCAAs and leucine itself, have been shown to augment the levels of phosphorylated eIF4E-BP1 and $p70^{S6k}$ in cell lines as well as rat and human skeletal muscle (Anthony et al. 2002; Greiwe et al. 2001; Patti et al. 1998; Wullschleger et al. 2006). Since there is an overlap between AA and insulin pathways, which occurs at mTOR (Wullschleger et al. 2006), the decline in AAs may have affected

the results with respect to eIF4E-BP1 phosphorylation (Hillier et al. 2000). Furthermore, leucine has been shown to stimulate translation initiation through the phosphorylation of eIF4G, a pathway that is separate from the insulin pathway (Kimball et al. 2006; Wullschleger et al. 2006). The stimulation of eIF4E-BP1 and $p70^{S6k}$ phosphorylation (Greiwe et al. 2001; Patti et al. 1998) and skeletal muscle protein synthesis (Anthony et al. 2002) is greater when AAs and insulin are both present. When plasma AAs decrease, as in the hyperinsulinemic euglycemic clamp, protein synthesis is not necessarily expected to increase because of a diminished availability of substrates and due to diminished stimulation of the mTOR and eIF4G pathways. Insulin also controls transcription of over 700 genes in human skeletal muscle during a hyperinsulinemic euglycemic clamp (Rome et al. 2003).

In the current protocol, baseline leucine S did not differ in T2DM *vs.* control subjects, which agrees with previous studies (Halvatsiotis et al. 2002a; Luzi et al. 1993; Umpleby et al. 1990). Studies involving phenylalanine tracers also found no group differences in T2DM *vs.* control subjects in the postabsorptive state (Halvatsiotis et al. 2002a). In the current protocol, in response to hyperinsulinemia, S did not change in T2DM subjects, while it increased in control subjects. This differs from the results obtained by Luzi et al. (1993), where S diminished comparably in T2DM and control groups in response to a hyperinsulinemic euglycemic clamp; S increased comparably in both groups in response to a hyperinsulinemic euglycemic hyperaminoacidemic clamp. The discrepancy in results is likely explained by our ability to maintain isoaminoacidemia, the fact our study only consisted of men, and the fact protein kinetics were adjusted for FFM based on a regression method. However, similar to both clamps in Luzi et al. (1993), we found no differences in S during the clamp plateau. Thus, the differences observed in response to hyperinsulinemia were not great enough to result in statistically significant differences during the clamp plateau.

In the current experiment, no group differences were found in leucine O at baseline, which is consistent with previous studies (Halvatsiotis et al. 2002a; Luzi et al. 1993; Staten et al. 1986). Umpleby et al. (1990), however, showed that basal O was greater among T2DM *vs.* control subjects, but T2DM subjects were not on an isocaloric diet. In response to the clamp in my protocol, O increased comparably in both groups.

This contrasts with the response to a hyperinsulinemic euglycemic clamp performed by Luzi et al. (1993) where O diminished to the same extent in both groups. The drop in O may be explained by the lowering of plasma AAs and BCAAs in that study; indeed, a hyperinsulinemic euglycemic hyperaminoacidemic clamp was associated with an increase in O, both in T2DM and controls (Luzi et al. 1993). During our clamp, there were no group differences in O, which agrees with Luzi et al. (1993).

Skeletal muscle has a maximal capability to synthesize protein and clearly, if the body cannot dispose of leucine through protein synthesis, it will have to be oxidized (Rennie et al. 2006; Rennie et al. 2002). The first step in leucine oxidation is the conversion of leucine to α-KIC by BCAA transaminase, followed by the irreversible decarboxylation of α-KIC in a reaction that is catalyzed by BCKDH (Aftring et al. 1988; Devlin 2002; Nakai et al. 2000). BCKDH activity and regulation is tissue-dependent (Frick et al. 1989; Miller et al. 1988) and in rat skeletal muscle, leucine stimulates BCKDH activity (Aftring et al. 1988). Even though isoaminoacidemia was maintained in the current protocol, including constant concentrations of leucine, protein synthesis may have reached a maximum by the end of the clamp protocol, and consequently, additional leucine was oxidized (Bohé et al. 2001; Rennie et al. 2006; Rennie et al. 2002). Due to insulin's diminished ability to stimulate S in T2DM subjects, more AAs were diverted to oxidation in the presence of comparable rates of AA infusion, but statistical significance for group differences in O was not achieved. The role of insulin in AA oxidation is somewhat unclear in *in vivo* studies, with some indicating no effect (Chevalier et al. 2004; Flakoll et al. 1989; Frexes-Steed et al. 1990) while others point to stimulation (Castellino et al. 1987; Heslin et al. 1992), although it appears to stimulate it in rodent adipose cells (Frick & Goodman 1989). Hence, it is possible, although not definitive, that the combination of high physiological insulin and postabsorptive AA concentrations could have stimulated leucine oxidation in the current protocol. In previous protocols using the hyperinsulinemic euglycemic isoaminoacidemic clamp by our research group, leucine O either increased in response to the clamp (Chevalier et al. 2006; Chevalier et al. 2005a; Chevalier et al. 2005b) or it was not significant (Chevalier et al. 2004).

Total flux, Q, which at steady state equals the sum of S and O, only increased in the controls in response to hyperinsulinemia, although there were no differences between

T2DM and control subjects during the clamp. This diminished increase among T2DM subjects was due to the inability of insulin to significantly increase S; this is analogous to the situation in insulin-mediated glucose disposal, where non-oxidative glucose disposal is the pathway that is most severely impaired in T2DM (Shulman et al. 1990). Luzi et al. (1993) showed that Q diminished in response to a hyperinsulinemic euglycemic clamp, but that no group differences were found during the clamp steady state. Nevertheless, the concomitant drop in AAs decrease S and O and served as a confounding variable.

Rates of leucine B did not differ in T2DM *vs.* control subjects at baseline, there was no difference in response to hyperinsulinemia, and they did not differ during the clamp. This agrees with the great majority of previous studies (Biolo et al. 1992; Halvatsiotis et al. 2002a; Halvatsiotis et al. 2002b; Luzi et al. 1993; Staten et al. 1986; Umpleby et al. 1990; Welle & Nair 1990), except Denne et al. (1995) who showed that postabsorptive B was greater among T2DM subjects and that clamp B was only greater among T2DM subjects when not adjusted for body composition. It is also important to reiterate that the subjects in the current protocol were matched for abdominal obesity, which is reported to be associated with a smaller decrease in protein breakdown in response to the hyperinsulinemic euglycemic clamp (Jensen & Haymond 1991). Rates of net balance (S-B) at baseline, the increase in response to the clamp, and S-B during the clamp have also been found to be similar between T2DM subjects and controls (Luzi et al. 1993), as in the current study.

In my protocol, postabsorptive EGP was elevated in T2DM subjects, which is consistent with numerous studies (Basu et al. 2005; Campbell et al. 1988; DeFronzo et al. 1985; Firth et al. 1987; Gastaldelli et al. 2000; Groop et al. 1989; Meyer et al. 2004; Rooney et al. 1993; Vaag et al. 1992a). A positive correlation has been shown to exist between FPG and postabsorptive EGP, although FPG thresholds for this linear relationship have been proposed, including 7.8 mmol/L (DeFronzo 1988). The FPG of T2DM subjects in my protocol (10.4 ± 0.5 mmol/L) was above 7.8 mmol/L, substantiating the fact that poor glycemic control in T2DM is associated with augmented rates of EGP.

Elevated postabsorptive EGP observed in T2DM is a result of augmented postabsorptive GNG (Basu et al. 2005; Boden et al. 2001b; Gastaldelli et al. 2000), GNG

from alanine (Consoli et al. 1990), and GNG from lactate (Consoli et al. 1990), while glycogenolysis is either lower or similar in T2DM *vs.* controls (Basu et al. 2005; Boden et al. 2001b; Gastaldelli et al. 2000). Alanine production is increased in T2DM (Consoli et al. 1990) and Zawadzki et al. (1988) found that the Cori cycle is augmented in obese T2DM subjects. Lactate levels were not measured in my protocol and no group differences were found for postabsorptive plasma alanine concentrations. This does not necessarily mean that GNG from alanine was not elevated in my protocol because rates of flux can be increased in the absence of elevated concentrations if both production and tissue uptake are increased to the same extent (Halvatsiotis et al. 2002a).

Although hyperinsulinemia diminished EGP in both T2DM and control subjects to the same extent, EGP remained higher among T2DM subjects during the hyperinsulinemic euglycemic isoaminoacidemic clamp, thus proving insulin resistance with respect to EGP. A greater EGP in T2DM *vs.* control subjects was also found in hyperinsulinemic euglycemic clamps that attained high physiological insulin concentrations, which are comparable to the approximately 600 pmol/L clamp insulin concentrations in the current protocol (Abate et al. 1996; Campbell et al. 1988; DeFronzo et al. 1985; Groop et al. 1989), with the exception of Pigòn et al. (1996). In the study by Pigon et al. (1996), however, most T2DM subjects were on diet therapy and had fasting glycemia in the optimal range. Indeed, FPG thresholds of 11 to 12 mmol/L have been proposed for augmented clamp EGP (DeFronzo 1988), although increased EGP was found in T2DM subjects at slightly lower FPG in my study.

In the present experiment, postabsorptive total glucose disposal was greater in T2DM subjects than in controls, which is consistent with several studies (Baron et al. 1985; Campbell et al. 1988; Ciaraldi et al. 2005; Groop et al. 1989; Thorburn et al. 1990). The underlying mechanism is increased insulin-independent glucose disposal through the mass action of plasma hyperglycemia in T2DM (Baron et al. 1985; Del Prato et al. 1997). Baron et al. (1985) found that the fraction of total postabsorptive glucose disposal that is due to insulin-independent glucose disposal was comparable between T2DM and control subjects (70-75%). In the context of elevated fasting insulin concentrations among T2DM, insulin-mediated glucose disposal could also be increased postabsorptively. Although not significant, the slightly higher basal insulin concentrations in T2DM

subjects may also explain the elevated glucose disposal. The CNS does not contribute to the observed increase in glucose disposal (Del Prato et al. 1997). Although Baron et al. (1988) showed that the main location for augmented glucose disposal when FPG increased was skeletal muscle in healthy subjects, the contribution of skeletal muscle to postabsorptive glucose disposal is less clear in hyperglycemic T2DM subjects. Postabsorptive glucose disposal in skeletal muscle of hyperglycemic T2DM has been found to be either increased (DeFronzo et al. 1985), the same (Williams et al. 2001), or even diminished (Ciaraldi et al. 2005) compared to controls.

Recently, evidence for glucose resistance with respect to glucose disposal in T2DM has been accumulating (Del Prato et al. 1997; Nielsen et al. 1998). It is defined as diminished glucose-induced glucose disposal and it may be the result of the adverse effects of long-term hyperglycemia, known as glucose toxicity (Del Prato et al. 1997). Importantly, even though oxidative and non-oxidative glucose disposal are affected (Del Prato et al. 1997), it appears that glucose resistance is selective, involving particularly oxidative glucose disposal. Indeed, this agrees with the results in my protocol, where the increase in total postabsorptive glucose disposal is not distributed evenly between oxidative and non-oxidative glucose disposal; postabsorptive non-oxidative glucose disposal is greater among T2DM subjects, but there is no difference in postabsorptive oxidative glucose disposal between T2DM subjects and controls. The results for postabsorptive oxidative glucose disposal agree with those of Groop et al. (1989), but Golay et al. (1988) found oxidative disposal to be elevated in T2DM and Thorburn et al. (1990) found it to be diminished in T2DM subjects. On the other hand, the results for postabsorptive non-oxidative glucose disposal are consistent with those of Groop et al. (1989) and Thorburn et al. (1990).

In my protocol, total glucose disposal only increased in controls in response to hyperinsulinemia, such that total glucose disposal was lower in T2DM *vs.* controls during the clamp. In hyperinsulinemic euglycemic clamps that achieved a similar level of hyperinsulinemia, insulin-mediated glucose disposal has also consistently been shown to be diminished in T2DM (Abate et al. 1996; Bavenholm et al. 2003; Bonadonna et al. 1993; Campbell et al. 1988; DeFronzo et al. 1985; Firth et al. 1987; Golay et al. 1988; Groop et al. 1989; Kelley et al. 1992; Pigon et al. 1996; Turk et al. 1995; Vaag et al.

1995). Most of the insulin-mediated glucose disposal in T2DM and control subjects occurs in skeletal muscle (DeFronzo et al. 1985). Diminished insulin-stimulated glucose disposal has been reported in T2DM skeletal muscle (Bonadonna et al. 1993; DeFronzo et al. 1985; Kelley et al. 1992).

In the current study, oxidative glucose disposal increased comparably in response to hyperinsulinemia, such that the study groups did not differ during the clamp. At comparable hyperinsulinemia, other researchers found oxidative glucose disposal to be lower among T2DM subjects during the hyperinsulinemic euglycemic clamp (Bavenholm et al. 2003; Golay et al. 1988; Groop et al. 1989; Vaag et al. 1995), but at slightly higher insulin concentrations (900 pmol/L), there were no differences between groups (Yokoyama et al. 2006). In the current experiment, there was a tendency for clamp glucose oxidation to be greater in controls, but statistical significance was not reached.

In response to hyperinsulinemia, non-oxidative glucose disposal only increased among control subjects and was diminished in T2DM *vs.* control subjects during the clamp. In euglycemic clamps of comparable hyperinsulinemia, other researchers have also shown that non-oxidative glucose disposal is impaired in T2DM (Golay et al. 1988; Groop et al. 1989; Vaag et al. 1995). The main component of non-oxidative glucose disposal is glycogen synthesis (Pratipanawatr et al. 2002; Shulman et al. 1990) and glycogen synthase activity is impaired in T2DM skeletal muscle during the clamp (Vaag et al. 1995). Consistent with numerous studies (Golay et al. 1988; Pratipanawatr et al. 2002; Shulman et al. 1990), we were also able to show that the main abnormality in insulin-mediated glucose disposal in poorly controlled T2DM is through the non-oxidative pathway; furthermore, only 42.8 ± 5.1 % of the total clamp disposal in T2DM subjects was through this pathway, which was significantly smaller than in control subjects (65.4 ± 3.3 %, $p < 0.05$). Since the RQ was less than 1 during both steady states, net lipogenesis is believed not to have occurred (Jéquier et al. 1987).

The main mechanism responsible for diminished insulin-stimulated glucose disposal and non-oxidative glucose disposal in T2DM is blunted glucose transport in skeletal muscle (Bonadonna et al. 1993; Cline et al. 1999; Williams et al. 2001). Movement of GLUT4 to the cell membrane from storage sites in the cytoplasm requires stimulation of the PI3K pathway (Ganong 2001; Kahn 1996; Kahn et al. 2005; Kido et al.

2001). Although various pathways emanate from insulin receptor activation, it is the PI3K pathway that appears to be blunted in T2DM, leading to the concept of "selective insulin resistance" when referring to the metabolic disorder (Groop et al. 2005). Impaired insulin-mediated glucose disposal during the clamp in skeletal muscle of T2DM subjects may also involve a decreased rate of conversion of glucose to glucose 6-phosphate (Bonadonna et al. 1996; Williams et al. 2001), diminished pyruvate dehydrogenase activity (Kelley et al. 1992), and decreased glycogen synthase activity (Kelley et al. 1992; Pratipanawatr et al. 2002; Vaag et al. 1995). In any given patient with T2DM, blunted total, oxidative, and non-oxidative glucose disposal is the sum of abnormalities in glucose uptake, enzymatic processes, and substrate feedback mechanisms within the cell.

In the current study, FFA concentrations and fat oxidation did not differ between groups at baseline or during the clamp, but decreased comparably in response to hyperinsulinemia. Indeed, insulin's ability to diminish FFAs and lipid oxidation in T2DM and control subjects is widely documented (Bavenholm et al. 2003; Golay et al. 1988; Groop et al. 1989). Although numerous other studies are consistent with my results for postabsorptive plasma FFA concentrations (Basu et al. 2005; Bavenholm et al. 2003; Groop et al. 1989; Iozzo et al. 2003; Thorburn et al. 1990; Vaag et al. 1992a), other researchers have found them to be elevated among T2DM subjects (Del Prato et al. 1993; Meyer et al. 2002; Nurjhan et al. 1992; Rooney et al. 1993). The K_m for the suppression of plasma FFA concentrations during the hyperinsulinemic euglycemic clamp is comparable between T2DM and control subjects (Groop et al. 1989; Groop et al. 1991b) and the value for K_m is not very much higher than basal insulin concentrations, the difference being approximately 20-60 pmol/L (Groop et al. 1989). Thus, even small increases in plasma insulin, which are commonly observed in T2DM subjects (including my protocol, although there were no group differences), can cause a huge decrease in plasma FFAs. This may explain discrepancies in the literature and the reason why no differences in postabsorptive FFA concentrations were observed in my study. In terms of lipid oxidation in the fasting state, various other studies agree with my results (Bavenholm et al. 2003; Golay et al. 1988; Groop et al. 1989; Groop et al. 1991b; Thorburn et al. 1990; Vaag et al. 1992a).

A number of studies have shown that plasma FFAs during a hyperinsulinemic euglycemic clamp with insulin concentrations comparable to the current study are greater in T2DM subjects than controls (Bavenholm et al. 2003; Golay et al. 1988; Groop et al. 1989). At first glance, this appears to be at odds with my results. Nevertheless, abdominal obesity is not only linked to diminished insulin sensitivity of glucose, but also to a blunted effect of insulin on plasma FFA concentrations (Pedersen et al. 1993). While the T2DM and control subjects in my study were matched for abdominal obesity, the studies by Groop et al. (1989) and Golay et al. (1988) did not measure and compare indices of abdominal obesity. On the other hand, T2DM subjects in Bavenholm et al. (2003) showed higher visceral fat and WHR than controls. In this case, it is unclear whether the elevated FFAs concentrations among T2DM subjects during the clamp were due to T2DM itself or the abdominal obesity characteristic of T2DM. In my protocol, where subjects were matched for abdominal obesity, no differences in clamp FFA concentrations were observed.

Similar to the results in the current study, Golay et al. (1988) and Groop et al. (1989) also found no differences in lipid oxidation during a clamp of comparable hyperinsulinemia. Bavenholm et al. (2003), on the other hand, showed higher lipid oxidation rates among T2DM subjects during a clamp with similar insulin concentrations to mine. The higher visceral fat among T2DM subjects in this study may explain the higher T2DM clamp lipid oxidation, since there is a positive correlation between abdominal obesity and clamp lipid oxidation (Pedersen et al. 1993). Lastly, the significance of group differences in plasma FFAs does not necessarily parallel that of lipid oxidation because in addition to being affected by FFA concentrations, lipid oxidation is also determined by other metabolic aspects, including insulin action in adipose tissue and oxidation efficiency (Devlin 2002; Groop et al. 1991a; Kelley & Mandarino 2000a; Kumar & O'Rahilly 2005; Lewis et al. 2002; Petersen & Shulman 2006).

Parallelism between insulin sensitivity of glucose and protein metabolism was observed. There was an interest in determining the correlation between AA and glucose infusion rates because this would allow the study of insulin sensitivity of glucose and protein without the need for tracers. The correlation was significant, but only somewhat

robust (r=0.509). Significant positive partial correlations were also found between glucose infusion rates and clamp net balance (S-B), the change in S, the change in Q, and the change in net balance (measure of the protein anabolic response to insulin). Clamp non-oxidative glucose disposal also showed partial correlations with clamp net balance (S-B), clamp S, the change in Q, and the change in S. Oxidation of glucose, fat, and protein did not show significant group differences, indicating that the main pathological abnormalities in this T2DM population do no lie in oxidation.

One can postulate that abnormalities in the PI3K pathway in T2DM lead not only to insulin resistance of glucose, but also to a diminished ability of insulin to stimulate protein synthesis. Indeed, there were positive correlations between clamp non-oxidative glucose disposal, a severely blunted insulin-mediated glucose disposal pathway in T2DM (Shulman et al. 1990), and the change in S and clamp S, after adjusting for FFM; the change in S explained about 40% of the variance in clamp non-oxidative glucose disposal. Co-localization of such abnormalities could occur in skeletal muscle, which is important for both glucose disposal and protein synthesis (Bonadonna et al. 1993; DeFronzo et al. 1985; Kelley et al. 1992; Nair et al. 1988; Rennie et al. 1982; Tessari et al. 1986). Indeed, transcription of genes that are components of the insulin pathway, such as the gene for the PI3K subunit p85α, is blunted in T2DM skeletal muscle in response to the hyperinsulinemic euglycemic clamp (Ducluzeau et al. 2001). Moreover, a generous protein diet may be helpful in reducing insulin resistance, since both insulin and AAs stimulate mTOR. AAs in the culture medium and an AA infusion in healthy humans, however, induce insulin resistance of glucose at the cellular and whole body levels respectively (Krebs et al. 2003; Patti et al. 1998; Pisters et al. 1991; Tremblay et al. 2001; Wullschleger et al. 2006), at least partly through downregulation of the PI3K pathway via $p70^{S6k}$ (Tremblay & Marette 2001; Wullschleger et al. 2006). Nevertheless, diets with generous protein intakes (30% of energy) decreased blood glucose in T2DM subjects (Gannon et al. 2003), potentially by reducing EGP (Tappy et al. 1994).

It is noteworthy that the glucose infusion rate, an indication of insulin sensitivity in the periphery, was lower among controls in my study (7.61 ± 0.68 mg/kg FFM·min) than the glucose infusion rate for healthy lean young male controls (9.75 ± 0.63 mg/kg FFM·min) in an analogous study previously done in our laboratory using the

hyperinsulinemic euglycemic isoaminoacidemic clamp (Chevalier et al. 2004). The controls in the current experiment included overweight and obese subjects, thus explaining this group's insulin resistance of glucose metabolism. Furthermore, insulin resistance of protein metabolism has been documented in obesity (Chevalier et al. 2005b) and protein metabolism in my control group is not completely normal. Indeed, the magnitude of the blunted response of leucine S and Q to hyperinsulinemia in hyperglycemic T2DM subjects with excess fat becomes more impressive when T2DM subjects are compared to the group of healthy lean men previously studied in our laboratory (Chevalier et al. 2004) (Figure 9).

The 2002 American Diabetes Association (ADA) recommendations state that a protein intake close to what is observed at the population level, about 15-20% of energy, is appropriate for T2DM patients without diabetic nephropathy (American Diabetes Association 2002; Franz et al. 2002). The ADA also indicates that the level of protein intake may need to be increased from the RDA (0.8g protein/kg BW/day) up to 1.0g protein/kg BW/day in poorly controlled T2DM (American Diabetes Association 2002; Franz et al. 2002); this recommendation is largely based on ^{15}N-glycine studies of Gougeon et al. (1994, 1997, 1998, 2000) in T2DM subjects. The upper limit placed on daily protein intake for T2DM (1.0 g/kg BW) is mainly based on data from type 1 diabetes mellitus patients (American Diabetes Association 2002; Franz et al. 2002). Recently, however, Wrone et al. (2003) showed that a protein intake greater than 19% of energy only augments the risk of microalbuminuria in type 1 and type 2 diabetes mellitus if hypertension is also present. The findings of my study support abnormal protein metabolism in poorly controlled T2DM and further highlight the need for more research on the effects of elevated protein intakes on glycemic management in T2DM patients without diabetic nephropathy.

An estimate of the additional protein synthesized per minute in controls is obtained when the group difference in the change in leucine S (μmol/min) is converted to g of leucine per minute and then to g of protein per minute (leucine constitutes approximately 8% of total body protein). Approximately 1 g of additional protein is synthesized in control *vs.* T2DM subjects per hour and over a 9 hour period, corresponding to 3 meals, 9 g of additional protein are synthesized in controls. Hence, as

a rough estimate, hyperglycemic T2DM subjects would require approximately an additional 0.1 g of protein/kg BW/day or 0.9 g protein/kg BW/day to maintain the same rate of whole body protein synthesis as controls. This estimate from my protocol agrees with ADA guidelines for protein intake in patients with poorly controlled T2DM but no nephropathy. The elderly with T2DM may represent an especially vulnerable population because the insulin resistance of protein associated with aging (Chevalier et al. 2006) may be compounded by the reduced ability of insulin to stimulate protein synthesis in T2DM.

While glucagon was not different between groups at baseline, it diminished only in the control group in response to hyperinsulinemia such that T2DM subjects showed a higher glucagon concentration during the clamp. The glucagon to insulin ratios were lower in T2DM at baseline, but higher during the clamp. Studies show that glucagon concentrations at baseline or during the hyperinsulinemic euglycemic clamp (Basu et al. 2005; Golay et al. 1988; Halvatsiotis et al. 2002b; Luzi et al. 1993; Turk et al. 1995) and the hyperinsulinemic euglycemic hyperaminoacidemic clamp (Luzi et al. 1993) did not differ between T2DM and control subjects, but Staehr et al. (2001) found greater glucagon concentrations in T2DM subjects, postabsorptively and during the clamp, even though insulin was infused the night before the clamp protocol. The mechanism for the inability of hyperinsulinemia to lower glucagon levels in T2DM subjects could be blunted insulin signaling in the pancreatic α cell, where glucagon is made (Xu et al. 2006). Glucagon, which acts at the level of the liver, contributes to increased EGP in T2DM (Baron et al. 1987; Consoli 1992; Del Prato et al. 2004; Gastaldelli et al. 2000) and may be involved in the elevated EGP levels among T2DM during the clamp.

In protocols using high levels of AAs, glucagon, insulin, and growth hormone, it has been suggested that glucagon diminishes protein synthesis in healthy humans (Charlton et al. 1996). Nevertheless, these results must be interpreted with caution because 1) The glucagon concentrations used were 4 times higher than baseline and to put this into perspective, glucagon concentrations only doubled when healthy subjects were given a 400 kcal meal consisting mostly of protein (60%), the latter being a potent stimulus of glucagon release (Blom et al. 2006) and 2) Glucagon's actions depend on the relative concentrations of insulin (Lewis et al. 1997). Hence, the implications for our study are unclear. Nygren & Nair (2003) recently showed that when a hyperinsulinemic

euglycemic clamp is performed in isoaminoacidemic conditions (total AAs), insulin infusion does not alter glucagon concentrations; these experimental conditions are not associated with decreases in protein synthesis, either in skeletal muscle or in the splanchnic region. Taken together, a fundamental metabolic abnormality in T2DM, and not the lack of alteration in glucagon concentrations likely accounts for the diminished ability of insulin to stimulate protein synthesis.

This study has several limitations. First, although T2DM was poorly managed in the diabetic subjects according to the CDA Clinical Practice Guidelines (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee 2003), the subjects were not excessively hyperglycemic. Improved glycemic control in T2DM results in increased insulin-stimulated total glucose disposal and non-oxidative glucose disposal (Pratipanawatr et al. 2002). It is possible that the T2DM subjects were not hyperglycemic enough to detect group differences in S and Q during the clamp. Second, T2DM subjects showed a large range in disease duration and it has been demonstrated that as time passes after diagnosis, T2DM management worsens (Harris et al. 2005; U.K. Prospective Diabetes Study Group 1995). It is possible that one of the factors affecting insulin sensitivity is also duration of T2DM; if so, this would increase variability in the insulin sensitivity of kinetics of protein metabolism. Third, unlike the rest of the study participants, two T2DM subjects were not Caucasian. Interestingly, they showed the second and third highest AA infusion rates (in mg/min) of all the T2DM subjects and this would have added variability to protein metabolism variables. Indeed, Welle & Nair (1990) have suggested that variability of protein kinetics is greater among T2DM subjects than controls. Fourth, even though subjects were matched for age, body composition, and abdominal obesity, it is possible that the sample size was too small. These limitations do not diminish the importance of the main findings in my protocol, which indicate that the responses of protein synthesis and turnover to insulin are diminished in T2DM, beyond the effects of body composition.

12. CONCLUSION

The response of whole body leucine S and Q to insulin is blunted in men with moderately poorly controlled T2DM compared to non-diabetic men of similar obesity and even more markedly when compared to healthy lean men. The T2DM and non-diabetic study groups were well matched for age, FFM, and abdominal obesity. Although insulin sensitivity of glucose correlates with that of protein metabolism, insulin resistance of glucose still appears to be more prominent in T2DM subjects. The study utilized a hyperinsulinemic euglycemic isoaminoacidemic clamp to eliminate the confounding effect of a decline in plasma AAs when studying insulin sensitivity of protein metabolism. After a thorough literature search, it appears to be the first time ^{13}C-leucine methodology has been combined with a hyperinsulinemic euglycemic isoaminoacidemic clamp to study insulin sensitivity of protein metabolism in T2DM. In light of the current findings, more research is necessary to determine the acute and chronic effects of different levels of protein intake and glycemic control on insulin sensitivity in T2DM. Such information will be critically important in determining what the correct protein intake should be for persons with T2DM and how it will need to be tailored to promoting metabolic control in each person.

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Appendices

Centre universitaire de santé McGill
McGill University Health Centre

Institut de Recherche de l'Hôpital Royal Victoria
Research Institute of the Royal Victoria Hospital

April 20, 1999

Dr. R. Gougeon
Nutrition & Food Science Centre
H6.90

RE: RESEARCH ETHICS BOARD REVIEW
REB PROTOCOL NO. 99-720

Dear Dr. Gougeon:

Thank you for submitting your study entitled, "Gluconeogenesis and insulin sensitivity of protein in type 2 diabetes mellitus" for review by the Research Ethics Board of the Royal Victoria Hospital.

The protocol and consent form were reviewed by the Medicine-A Subcommittee at their meeting held on April 6, 1999, and the following comments were made:

1. The committee found these consent forms, too long and too technical. It recommends that the consent forms be written using the usual format, using headings to the paragraphs and for easy layperson terms consumption (grade 8 education level) For your convenience, enclosed is a guidelines for consent forms.
2. Technical words should be described in lay terms with the technical term in brackets after each description.
3. Clarify the amount of days regarding each admission.
4. Potential infection after the use of IV is mentioned in page 3 section 10 of the protocol, therefore, this should be added as a potential risk in the RISK section.
5. Paragraph #2, to be rewritten in a more user friendly form with shorter sentences.
6. It is recommended to include in the consent form "Women of childbearing potential who are not using barrier methods of contraception (condom, IUD, diaphragm etc.) will be excluded from the study. Should you nevertheless become pregnant in the course of being a subject in this study you should notify your physician and principal investigator immediately"
7. In paragraph # 4 add in brackets (not radioactive) after a stable isotope.
8. A statement regarding the patients rights should be included in the consent form: "If you have questions concerning your rights as a research subject and wish to discuss them with someone not associated with the study, you may contact the hospital ombudsman at 842-1231, local 5655".

Type 2 Diabetes Study

The McGill Nutrition & Food Science Centre is seeking participants for a research study looking at how diabetes affects blood glucose levels and the state of protein in the body. (This is not a trial for a new medication)

www.diabetes.org.uk

If you:

- **Have type 2 diabetes**
- **Are younger than 65 yrs of age**
- **Have no other major health problems**
- **Are willing to stay at the Clinical Investigation Unit of the Royal Victoria Hospital for 8 days,**

You are invited to call Connie or Sandra at 843-1665 between 9am and 5pm.

Benefits of participating in the study include knowing your energy requirements, knowing your percent body fat, getting dietary and lifestyle counseling, and knowing the results of your blood and urine tests. You will be compensated for your participation!

Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665 Connie or Sandra 843-1665

Une étude sur le diabète de type 2

Le Centre de nutrition et des sciences de l'alimentation de l'université McGill recherche des personnes pour une étude des effets du diabète sur le glucose sanguin et le métabolisme des protéines dans le corps. (Cette étude n'implique pas de nouveaux médicaments)

www.diabetes.org.uk

Si vous:

- **Souffrez du diabète de type 2**
- **Avez moins de 65 ans**
- **N'avez aucun autre problème de santé majeur**
- **Êtes prêt à passer 8 jours à l'Unité de recherche clinique de l'hôpital Royal Victoria,**

On vous invite à appeler Connie ou Sandra au 843-1665 entre 9h00 et 17h00.

En participant à cette étude vous connaîtrez vos besoins en énergie, aurez accès à une consultation en alimentation et en gestion de votre diabète et aux résultats de vos examens de sang et d'urine. Une compensation vous sera versée pour votre participation!

Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665 Connie ou Sandra 843-1665

Questionnaire for potential subjects

Date:_____

Name:_____ **Phone #:**_____

Age:_____ **Weight:**_____ **Height:**_____ **BMI:**_____

1. Currently in another study? ☐ Yes - End date?
☐No

2. Stable weight for 6 months? ☐Yes
☐No
☐Don't know

3. Allergies? ☐Yes
☐No

4. Smoker? ☐Yes
☐No

5. Vegetarian (red meat, eggs, cheese, milk)? ☐Yes
☐No - How much red meat?

6. Drink alcohol (wine, beer)? ☐Yes – Less than 3 drinks/day?
☐No

7. Medicare card/Hospital card? ☐Yes
☐No

8. Type 2 DM? ☐ Yes - For how many years?
- Medication (dosage, frequency)?
☐No

9. Glycemic control:

	Date & Value	Date & Value	Date & Value	Date & Value	Date & Value	Date & Value	Date & Value
Hemo A1C							
BS Monitoring							

10. ♀ Post-menopausal? ☐**Yes - Last period?**
☐**No - Regular periods?**

11. Surgery/Hospitalization? ☐**Yes – Why? When?**
☐**No**

12. Other Medical Conditions & Medications

MEDICAL CONDITION	HOW MANY YRS?	CURRENT MEDICATIONS (DOSAGE, FREQUENCY)
Diabetes complications		
Heart/High blood pressure		
Thyroid		
Anemia/Fe deficiency		
Kidney		
Liver		
Respiratory		
Cancer		
Cholesterol/Lipids		
Psychiatric		
Other:		

13. Herbs & Vitamins? ☐ **Yes – Which ones?**
☐**No**

14. Activities/Exercise:

Treating Physician: _____

CONSENT FORM

Royal Victoria Hospital
Clinical Investigation Unit

GLUCONEOGENESIS AND INSULIN SENSITIVITY OF PROTEIN IN TYPE 2 DIABETES MELLITUS- DIABETIC SUBJECT

I have been invited by Dr. Réjeanne Gougeon, to take part in a study of the impact of my Type 2 diabetes on the effectiveness of insulin on my body's protein metabolism. I will be studied during a weight-maintaining diet with my blood glucoses moderately high. The purpose of the study is to better understand the body's protein metabolism, so as to be able to prescribe the best diets and treatments for each patient. I will be admitted for eight (8) days. The study will include:

1) *Admission*: My admission to the Clinical Investigation Unit of the Royal Victoria Hospital will take place after I have a physical examination, standard blood and urine tests, chest X-ray and electrocardiogram. I will also have my body fat and lean tissues measured by a safe, painless, very low electric current (bioelectrical impedance analysis) and my resting metabolic rate measured by breathing under a plastic canopy for 20 minutes (indirect calorimetry). For this study at high blood glucose levels, I will stop my diabetic medications for at least three (3) days prior to my admission.

2) *Special Diet*: For breakfast I will have cereal and milk, but the rest of my food will be a liquid formula diet that contains all the necessary nutrients that I must consume completely. My blood glucose will be tested four (4) to six (6) times daily. My blood glucose will never be allowed to rise to levels constituting a risk to my health. If required to keep sugars controlled, insulin injections may be given, but only for a short period. I will test my blood glucose and I will call the nurse if I feel uncomfortable or my blood glucose level surpasses 18 mmol/L. There is a risk of dehydration if I do not drink an adequate amount of water during this period to compensate for the formation of urine.

3) On day 7, I will participate in a test to measure gluconeogenesis rates (the body's production of sugar). For this I will eat the same diet until 6pm at which point I will begin a fasting period. In the evening I will drink a 150 ml (5 ounces) glass of water with deuterium, as part of the water itself. A urine sample and 9 ml of venous blood will be collected at 4 pm and again at 8 am and 11 am the next day. Deuterated water is another stable isotope (not radioactive). Deuterated water has caused slight, tolerable and short-lived dizziness in some people.

4) *Insulin Study*: On day 8, insulin sensitivity determination and protein metabolism studies

will be performed at the Crabtree Nutrition Lab in the Hospital. This will last approximately 8 hours. On that morning, while I am fasting and resting comfortably in a bed, I will receive two intravenous catheters, one in my arm and the other in my hand. The second one will be used for repeated, painless blood samples to be taken. This catheter will be in a vein on the back of my hand, which will be placed in a warming box at 65°C to make the blood in the vein similar to that of an artery. This is because the blood vessels dilate, and is not a painful or uncomfortable procedure. The other catheter will be attached to a plastic tube for infusion of saline, radioactive tritiated glucose, insulin, ^{13}C-leucine (another stable isotope), glucose and a mixture of amino acids. The amount of radioactive material introduced will be about 150 μCi (5.55 MBq). It may be up to 250 μCi (9.25MBq) if the study lasts longer. It represents a radiation exposure similar to that received from a standard X-ray of the chest. The dose is minimal and disappears totally from the body (mainly in urine) within 2 ½ weeks of administration. I will receive an initial dose of tritiated glucose, sodium bicarbonate and ^{13}C-leucine followed by a continuous infusion. Blood samples of 3-9 ml (2 tsps) each will be taken starting after 2 hours, and every 10 minutes for 60 minutes. A mixture of amino acids (protein) and dextrose (sugar) will be infused together with the insulin to keep my blood amino acid and blood sugar levels constant. Blood samples of 1 mL will be taken every 5 minutes after the insulin has started in order to verify my blood glucose and amino acid levels and 3-9mL every 30 minutes for 3 hours and 10 minutes during the last half-hour. Several times during the day, I shall be asked to breathe under the plastic canopy for 20 minutes to measure my metabolic rate. Samples of my breath will be taken, about 20 times throughout the study by simply blowing air into a special bag.

5) During my stay in hospital, I may participate in individual sessions for training in behaviour modification. This will be for my benefit, to learn the principles of bringing about changes in my lifestyle. During the whole period of the study, I agree to stay on the ward unless given a special pass to leave for short periods.

6) All my urine will be collected in special containers provided to me. These urine collections must be carefully and completely done. I will be weighed each morning in nightclothes, after urinating. My blood pressure and heart rate will be checked regularly by the nurses. I will use a single dipstick to check my urine for glucose and ketones every morning. I will be taught the methods for measuring my own blood glucose using reagent strips and a reflectometer (e.g. Accuchek® meter). This involves a finger prick using a lancet, which causes mild discomfort. This is a very standard procedure used by most people with diabetes.

7) *Measurements of body composition*: At each study, body composition assessment will be done using heavy water dilution technique, the very low electric current (bioelectrical impedance analysis), and circumference measurements of my arm, chest, waist, hip and thighs with a measuring tape.

8) I may undergo total body magnetic resonance imaging (MRI) at the Montreal Neurological Institute. It will require that I lie on a stretcher for approximately 20 minutes while I move

through the scanner. It is possible that I find it uncomfortable to remain lying on my back, face up for this period of time. If I am claustrophobic or have any implanted device containing metal, I do not have to undergo the MRI which measures my lean and fat tissues.

9) *Risks and Benefits*: The risks involved in consuming the diets, in blood sampling and having moderately high blood glucoses for a short period are considered to be minimal. I may experience some changes in my bowel movement habits with the diet, but special attention is paid to minimise them. There may be slight pain or discomfort while doing blood tests with a slight risk for bruising and infection. The amount of blood drawn over the entire study will not exceed that in an ordinary blood donation. There is a risk of low blood sugar with therapy but my blood glucose will be monitored to reduce the risk to a minimum. Because of tritiated glucose, I will not plan to start a pregnancy for 2 months following the study.

My participation in this study is voluntary. Although the oral agent and the insulin therapy, and contact with the medical, dietetic and nursing experts may benefit me, the other procedures are not expected to provide any direct benefit to me. It is hoped that the information obtained will lead to the advancement of scientific knowledge. The data obtained will be treated confidentially and it will not be possible to identify me personally in any publication of the results. Any questions I may have about the diet and the results of the study will be answered by contacting Dr. Réjeanne Gougeon at (514) 843-1665. If I have any questions concerning my rights as a research subject and wish to discuss them with someone not associated with the study, I may contact the hospital ombudsman at (514) 934-1934, local 35655.

10) Women of childbearing potential who are not using barrier methods of contraception (condom, IUD, diaphragm etc.) will be excluded from the study. Should I nevertheless become pregnant in the course of being a subject in this study I will notify my physician and Dr. Réjeanne Gougeon immediately.

11) The funds that I will receive because of volunteering for this research are to defray expenses as a compensation for loss and/or inconveniences that are related to my participation. Receipt of funds is not the reason I volunteered.

I _____ consent to be a subject in this project.

I am free to withdraw from the study at any time, without prejudice. Early termination of this project, for any reason, will not compromise my medical care.

I have had the time to read this consent form carefully and the opportunity to ask questions about this research project and have them answered to my satisfaction.

Dated at Montreal, this _____ day of _____ 200__.

SUBJECT _____
(signature)

INVESTIGATOR _____

(signature)

CONSENT FORM

Royal Victoria Hospital

Clinical Investigation Unit

GLUCONEOGENESIS AND INSULIN SENSITIVITY OF PROTEIN IN TYPE 2 DIABETES MELLITUS: OBESE NONDIABETIC SUBJECTS

I have been invited by Dr. Réjeanne Gougeon, to take part in a study of the impact of Type 2 diabetes on the effectiveness of insulin on my body's protein metabolism as an obese control subject without diabetes and with normal blood sugar levels. I will be studied during a weight-maintaining diet. The purpose of the study is to better understand the body's protein metabolism, so as to be able to prescribe the best diets and treatments for each patient. I will be admitted for seven (7) days. The study will include:

1) *Admission*: My admission to the Clinical Investigation Unit of the Royal Victoria Hospital will take place after I have a physical examination, standard blood and urine tests, chest X-ray and electrocardiogram. I will have also my body fat and lean tissues measured by a safe, painless, very low electric current (bioelectrical impedance analysis) and my resting metabolic rate measured by breathing under a plastic canopy for 20 minutes (indirect calorimetry). I will also have a glucose tolerance test for which I will be given 75g (1/3 cup) of glucose and a standard small plastic tube (intravenous catheter) will be inserted into a vein in my arm for painless blood sampling at 0, 30, 60, 90, 120 and 180 minutes after drinking the glucose solution.

2) *Special Diet*: For breakfast I will have cereal and milk, but the rest of my food will be a liquid formula diet that contains all the necessary nutrients and that I must consume completely.

3) *Protein Study*: From days 3 to 5 of the study, I shall take part in a test that will last 60 hours, to measure protein metabolism. Starting at 8:00 am I shall be given an amino acid dissolved in 5 mL (one teaspoon) of water. Amino acids are the "building blocks" of protein in food and in the body. This amino acid, called ^{15}N-glycine, is in a special form (an isotope) and is without risk since it is found in low concentrations in foods, and is widely used in tests such as this one. It is not radioactive. Every 3 hours for the next 60 hours, including overnight, when a member of the research team will awaken me. I will consume this solution of ^{15}N-glycine and will urinate into appropriate containers. A blood test will also be done at the beginning and at the end of each ^{15}N-glycine test.

4) On day 6, I will participate in a test to measure gluconeogenesis rates (the body's production of sugar). For this I will eat the same diet until 6 pm at which point I will begin a fasting period. At 11 pm, I will drink a 150 mL (5 ounces) glass of water with deuterium, as part of the water itself. A urine sample and 9 mL of venous blood will be collected at 4 pm and again at 8 am and 11 am the next day.
Deuterated water is another stable isotope (not radioactive). Deuterated water has caused slight, tolerable and short-lived dizziness in some people.

5) *Insulin Study*: On day 7, insulin sensitivity determination and protein metabolism studies will be performed at the Crabtree Nutrition Lab in the Hospital. This will last approximately 8 hours. On that morning, while I am fasting and resting comfortably in a bed, I will receive two intravenous catheters, one in my arm and the other in my hand. The second one will be used for repeated, painless blood samples to be taken. This catheter will be in a vein on the back of my hand, which will be placed in a warming box at 65°C to make the blood in the vein similar to that of an artery. This is because the blood vessels dilate, and is not a painful or uncomfortable procedure. The other catheter will be attached to a plastic tube for infusion of saline, radioactive tritiated glucose, insulin, ^{13}C-leucine (another stable isotope), glucose and a mixture of amino acids. The amount of radioactive material introduced will be about 150 μCi (5.55 MBq). It may be up to 250 μCi (9.25MBq) if I am young, muscular and very sensitive to the effects of insulin. It represents a radiation exposure similar to that received from a standard X-ray of the chest. The dose is minimal and disappears totally from the body (mainly in urine) within 2 ½ weeks of administration. I will receive an initial dose of tritiated glucose, sodium bicarbonate and ^{13}C-leucine followed by a continuous infusion. Blood samples of 3-9 mL (2 tsps) each will be taken starting after 2 hours, every 10 minutes for 60 minutes. A mixture of amino acids (protein) and dextrose (sugar) will be infused together with the insulin to keep my blood amino acid and blood sugar levels constant. Blood samples of 1 mL will be taken every 5 minutes after the insulin has started to verify my blood glucose and amino acid levels and 3-9 mL every 30 minutes for 3 hours and every 10 minutes during the last half-hour. After 2 ½ hours and every hour thereafter, I shall be asked to breathe under the plastic canopy for 20 minutes to measure my metabolic rate. Samples of my breath will be taken, about 20 times throughout the study by simply blowing air into a special bag.

6) During my stay in hospital, I may participate in individual or group sessions for training in behaviour modification. This will be for my benefit, to learn the principles of bringing about changes in my lifestyle. During the whole period of the study, I agree to stay on the ward unless given a special pass to leave for short periods.

7) All my urine will be collected in special containers provided to me. These urine collections must be carefully and completely done. I will be weighed each morning in nightclothes, after urinating. My blood pressure and heart rate will be checked regularly by the nurses. I will be taught the methods for measuring my own blood glucose using reagent strips and a reflectometer (e.g. Accuchek® meter). This involves a finger prick using a lancet,

which causes mild discomfort. This is a very standard procedure used by most people with diabetes. I will make such measurements up to 6 times on days 4 and 5. This involves a finger prick using a lancet, which cases mild discomfort. This is a very standard procedure used by most people with diabetes.

8) *Measurements of body composition*: Body composition assessment will be done using heavy water dilution technique, the very low electric current (bioelectrical impedance analysis), and circumference measurements of my arm, chest, waist, hip and thighs with a measuring tape.

9) I may undergo total body magnetic resonance imaging (MRI) at the Montreal Neurological Hospital. It will require that I lie on a stretcher for approximately 20 minutes while I move through the scanner. It is possible that I find it uncomfortable to remain elongated for this period of time of that I become anxious once in the scanner or when asked to hold my breath. If I am claustrophobic or have any implanted device containing metal, I do not have to undergo the MRI, which measures my lean and fat tissues.

10) *Risks and Benefits*: The risks involved in consuming the diets and in blood sampling are considered to be minimal. I may experience some changes in my bowel movement habits with the diet, but special attention will be paid to minimise them. There may be slight pain or discomfort while doing blood tests with a slight risk for bruising and infection. The amount of blood drawn over the entire study will not exceed that in an ordinary blood donation. Because of the tritiated glucose, I will not plan to start a pregnancy for 2 months following the study.

My participation in this study is voluntary. Although the contact with the medical, dietetic and nursing experts may benefit me, the other procedures are not expected to provide any direct benefit to me. It is hoped that the information obtained will lead to the advancement of scientific knowledge. The data obtained will be treated confidentially and it will not be possible to identify me personally in any publication of the results. Any questions I may have about the diet and the results of the study will be answered by contacting Dr. Réjeanne Gougeon at 845-1665. If I have any questions concerning my rights as a research subject and wish to discuss them with someone not associated with the study, I may contact the hospital ombudsman at 842-1231, local 35655.

11) Women of childbearing potential who are not using barrier methods of contraception (condom, IUD, diaphragm etc.) will be excluded from the study. Should I nevertheless become pregnant in the course of being a subject in this study I will notify my physician and Dr. Réjeanne Gougeon immediately.

12) The funds that I will receive because of volunteering for this research are to defray expenses as a compensation for loss and/or inconveniences that are related to my participation. Receipt of funds is not the reason I volunteered.

I _____ consent to be a subject in this project.

I am free to withdraw from the study at any time, without prejudice. Early termination of this project, for any reason, will not compromise my medical care.

I have had the time to read this consent form carefully and the opportunity to ask questions about this research and have them answered to my satisfaction.

Dated at Montreal, this _____ day of _____ 200__.

SUBJECT _____
(signature)

INVESTIGATOR _____

(signature)

Name: _____ RVH No.: _____

Date	Day of the week	Day of CIU diet	Weight (kg)	Temp. (°C)	Water consumed (1 bottle = 500mL)	Time of last "morning" urine sample	Bowel move-ments	Physical activity	Blood pressure & Pulse	Comments