PROTEIN AND GLUCOSE METABOLISM IN RESPONSE TO HYPERINSULINEMIA WITH AND WITHOUT HYPERAMINOACIDEMIA IN MEN WITH TYPE 2 DIABETES MELLITUS

Maya Said Bassil

School of Dietetics and Human Nutrition

McGill University, Montréal

Québec, Canada

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ABSTRACT

In poorly-controlled type 2 diabetes mellitus (T2DM), 24h whole-body protein turnover is elevated and net protein balance diminished compared to nondiabetic weight-matched controls. These alterations were partly attributed to insulin resistance of protein, particularly in hyperglycemic men, determined using hyperinsulinemic, euglycemic and isoaminoacidemic clamps. However the effect of intense glycemic control on this resistance is not known. Moreover, the effect of postprandial-level hyperaminoacidemia with concurrent hyperinsulinemia and hyperglycemia on glucose and protein metabolism in T2DM is not clearly established. Thus the objectives of this thesis are to determine in men with T2DM, at the whole-body and cellular levels whether i) hyperglycemia at 8.0 vs. 5.5 mmol/L worsens insulin sensitivity of protein, ii) protein metabolism is further impaired when insulin and amino acids are clamped at peak postprandial concentrations, iii) postprandial amino acids aggravate the insulin resistance of glucose metabolism, and iv) insulin sensitivity of glucose and protein is improved with intense control of T2DM. For objectives i) and ii), eight overweight or obese men with T2DM were studied with a hyperinsulinemic, hyperglycemic (8 mmol/L) clamp, first with plasma amino acids at postabsorptive (Hyper-2) then at postprandial concentrations (Hyper-3). Whole-body protein kinetics were assessed using ¹³C-leucine tracer method. Hyper-2 results were compared to those of diabetic men whose plasma glucose was lowered to 5.5 mmol/L and fasting aminoacidemia maintained during the hyperinsulinemic clamp (Hyper-1). For

objective iii), the same protocol was used with whole-body glucose turnover assessed using ³H-glucose. Vastus lateralis biopsies were obtained at baseline and during each step of the clamp to determine the phosphorylation states of Akt, mTOR, ribosomal protein (rp) S6, and insulin receptor substrate (IRS)-1. For objective iv), protein kinetics and glucose turnover in T2DM men were measured in 7 normoglycemic (A1C 6.1±0.2%) during Hyper-1 and Hyper-3 and 8 hyperglycemic (A1C 7.1±0.2 % p<0.01) during Hyper-2 and Hyper-3. Vastus lateralis biopsies were obtained at baseline and both steps of the clamp to determine the phosphorylation states of relevant proteins in the protein synthesis pathways. Results: 1) Hyper-2 is associated with a faster turnover rate but, similar net protein anabolism as Hyper-1; 2) Hyper-3 increases net protein synthesis to levels not different from healthy lean controls; 3) hyperaminoacidemia does not worsen insulin resistance of glucose metabolism; and 4) intense glycemic control does not improve insulin sensitivity of protein metabolism. The presence of insulin resistance of protein in T2DM men has important implications for dietary protein recommendations as it suggests their protein requirements may be greater than current DRIs. Further investigation is warranted to address this issue in women and whether other dietary interventions known to improve insulin resistance of glucose metabolism in T2DM would modulate that of protein metabolism

RÉSUMÉ

Dans le diabète de type 2 (DT2) mal contrôlé, le flux global des protéines est élèvé et la synthèse nette diminuée comparés à des sujets témoins en santé. Ces altérations sont, en partie, attribuées à l'insulino-résistance des protéines, démontrée surtout chez les hommes hyperglycémiques, par l'usage de traceurs durant un test de verrouillage (clamp) hyperinsulinémique, euglycémique et isoaminoacidémique. Néanmoins, l'effet d'un contrôle intensif de la glycémie sur cette résistance n'est pas connu. De même, l'effet de l'hyperaminoacidémie postprandiale en présence d'hyperglycémie durant le clamp hyperinsulinémique sur le métabolisme du glucose et des protéines dans le DT2 n'est pas bien établi. Par conséquent, les objectifs de cette thèse sont de déterminer chez des hommes avec DT2, si, aux niveaux global et cellulaire i) une glycémie 8 versus 5.5 mmol/L aggrave l'insulino-résistance des protéines, ii) le métabolisme des protéines est davantage perturbé quand l'insuline et les acides aminés sont maintenus à des concentrations postprandiales, iii) l'hyperaminoacidémie augmente la résistance à l'insuline du glucose, iv) la sensibilité à l'insuline du glucose et des protéines est améliorée par contrôle intensif de la glycémie. Pour les objectifs i) et ii), huit hommes obèses avec diabète de type 2 ont été étudiés durant un clamp hyperinsulinémique, hyperglycémique (8 mmol/L), avec acides aminés plasmatiques maintenus à leurs concentrations postabsorptives (Hyper-2) puis postprandiales (Hyper-3). Les cinétiques globales mesurées en utilisant la 13C-leucine durant Hyper-2 ont été comparés à celles d'un groupe d'hommes

étudié préalablement chez lesquels l'hyperglycémie avait été diminuée rapidement à 5.5 mmol/L au début du clamp hyperinsulinémique et l'aminoacidémie maintenue aux niveaux de base (Hyper-1). Pour l'objectif iii), le même protocol a été utilisé pour évaluer le métabolisme global du glucose avec le 3H-glucose. En plus, trois biopsies du vastus lateralis ont été prises, soit à jeun et aux deux étapes du clamp, pour déterminer l'état de phosphorylation des protéines Akt, mTOR, ribosomal protein (rp) S6, et insulin receptor substrate (IRS)-1. Pour l'objectif iv), les cinétiques des protéines et du glucose ont été mesurées chez 7 hommes normoglycémiques (A1C : 6.1 ± 0.2) durant Hyper-1 et Hyper-3 et comparées à celles de 8 hommes hyperglycémiques (A1C : 7.1±0.2 % p<0.01) durant Hyper-2 et Hyper-3. Des biopsies du vastus lateralis ont été obtenues à jeun et durant les deux étapes du clamp pour déterminer l'état de phosphorylation des protéines pertinentes dans la voie métabolique de la synthèse protéique. Les résultats indiquent que 1) Hyper-2 est associé à un flux global des protéines plus élevé mais une synthèse nette semblable à Hyper-1; 2) Hyper-3 augmente la synthèse nette à des niveaux comparables à ceux d'hommes maigres en santé; 3) l'hyperaminoacidémie n'aggrave pas la résistance à l'insuline du glucose; 4) un contrôle glycémique plus intense n'améliore pas la sensibilité à l'insuline des protéines. La présence d'une résistance à l'insuline du métabolisme des protéines a des implications importantes vis-à-vis les recommandations alimentaires en protéine dans le DT2 car elle suggère des besoins accrus. Des études s'avèrent nécessaires pour déterminer si cela s'applique aux femmes et si des régimes alimentaires avec compositions énergétiques et nutritionnelles différentes pourraient améliorer la résistance à l'insuline des protéines dans le DT2.

CONTRIBUTION OF AUTHORS

The candidate contributed to the development of research questions and study design. She interviewed, screened, and recruited subjects for the studies and helped in conducting clamp experiments and analyzing plasma and urine samples to determine substrate and hormone concentrations as well as specific activity and isotopic enrichment. The candidate entered and verified data, conducted all statistical analyses, and wrote all manuscripts. Because the studies were conducted in the Crabtree laboratory, where 4 other researchers carry their research protocols, the candidate was exposed and involved in the recruitment, implementation and data analysis of parallel studies that led to co-authorship in abstract publication at the American Diabetes Association 71st Scientific Sessions 2011

Dr. Réjeanne Gougeon, the candidate's supervisor is the principal investigator (PI) on the operating grant secured from Canadian Institutes of Health Research (CIHR) which funded work for this thesis. Dr. Gougeon contributed to the development of the concepts and designs of the studies and supervised statistical analysis and interpretation of data for all manuscripts. She also helped in writing and edited the manuscripts presented in this thesis.

Dr. Errol Marliss, the candidate's committee member and co – investigator on the operating grant secured from CIHR was involved in training and supervising the candidate's clinical work. Dr. Marliss also helped with study design, interpretation of data as well as editing all manuscripts. Dr. José Morais, co – investigator on the operating grant secured from CIHR, was the physician responsible to obtain muscle biopsies on the clamp day. Dr. Morais also helped in editing the manuscripts.

Dr. Stéphanie Chevalier helped in conducting the clamp experiments, interpreting the data as well as editing all manuscripts.

Dr. Sergio Burgos was involved in optimizing the western blot technique to identify and quantify signaling molecules of the protein synthesis pathway from muscle biopsies. Dr. Burgos also helped in editing manuscript 2.

Dr. Hope Weiler, the candidate's committee member, guided the candidate towards achieving PhD program objectives. Dr. Weiler also helped in editing this thesis.

Research publications in refereed scientific journals

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LIST OF ABBREVIATIONS

A1C	hemoglobin glycation
ADA	American Diabetes Association
AI	Adequate Intake
ANOVA	analysis of variance
ATP	Adenosine triphosphate
AUC	area under the curve
В	rate of breakdown
BCAA	branched-chain amino acids
BIA	bioelectrical impedance analysis
BMI	body mass index
CDA	Canadian Diabetes Association
СНО	carbohydrates
CIHR	Canadian Institutes of Health Research
СТ	x-ray computed tomography
DRI	dietary reference intakes
DXA	dual energy x-ray absorptiometry
EGP	endogenous glucose production
eIF	eukaryotic initiation factor
F/U	follow-up
FFA	free fatty acids
FFM	fat-free mass

FOXO	forkhead box O3
FPG	fasting plasma glucose
GCMS	gas chromatography mass spectrometry
GDP	Guanosine diphosphate
GLP-1	Glucagon like peptide-1
GLY	glycogenolysis
GNG	gluconeogenesis
GTP	Guanosine triphosphate
HOMA	homeostatic model assessment
HP	high protein
HPLC	high pressure liquid chromatography
Hyper-1	hyperinsulinemic euglycemic isoaminoacidemic
Hyper-2	hyperinsulinemic hyperglycemic isoaminoacidemic
Hyper-3	hyperinsulinemic hyperglycemic hyperaminoacidemic
HyperAA	hyperinsulinemic hyperaminoacidemic
IAA	indispensable amino acids
IFG	impaired fasting glucose
IGF-1	insulin like growth factor-1
IGT	impaired glucose tolerance
IRMS	isotope ratio mass spectrometry
IRS	insulin receptor substrate
IsoAA	hyperinsulinemic isoaminoacidemic
KIC	α-ketoisocaproic acid
LBM	lean body mass

LC	low CHO
LSD	least significant difference
MCR	metabolic clearance rate
mg	milligram
ml	milliliter
mmol	millimole
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTORC1	mammalian target of rapamycin complex 1
mU	milliunit
0	rate of oxidation
OGTT	oral glucose tolerance test
PDK-1	3¢-phosphoinositide- dependent kinase-1
PI3K	phosphatidylinositol 3-kinase
PIP3	phosphatidylinositol 3,4,5-trisphosphate
pmol	picomole
Q	flux
R _a	rate of appearance
Rag	Ras related small GTP-binding proteins
R _d	rate of disappearance
REE	resting energy expenditure
Rheb	Ras homolog enriched in brain
RIA	radioimmunoassay
rp	ribosomal protein

RQ	respiratory quotient
S	rate of synthesis
S6K	S6 kinase
SD	standard deviation
Ser	Serine
SMBG	self monitored blood glucose readings
SPSS	Statistical Package for the Social Sciences®
T2DM	type 2 diabetes mellitus
TAA	total amino acids
Thr	threonine
TSC	tuberous sclerosis complex 2
TZD	thiazolidinediones
Ub	Ubiquitin
UrN	urinary nitrogen
WB	whole-body
WC	waist circumference
Wt	weight
μg	microgram
μmol	micromole

CHAPTER 1. INTRODUCTION

1.1. Background and rationale

The prevalence of type 2 diabetes mellitus (T2DM) has significantly increased over the past 3 decades with an estimate of 257 million people affected worldwide (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008). This number is expected to double between the years 2000 and 2030 (Hossain *et al.*, 2007). One line of defence in the management of T2DM and prevention of its complications is nutrition. While carbohydrate (CHO) and fat intake recommendations in T2DM are based on solid evidence, those for protein intake are not firmly defined (Bantle *et al.*, 2008, Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008). This is partly due to the controversial reports on protein metabolism in T2DM (Denne *et al.*, 1995, Halvatsiotis *et al.*, 2002b, Luzi *et al.*, 1993, Pereira *et al.*, 2008).

The rationale for a potential impaired protein metabolism in T2DM that might indicate higher dietary requirements compared with those of healthy people lies in the pathophysiological abnormalities of the disease, particularly insulin resistance. Insulin is known to modulate the metabolism of not only CHO and fat but also of protein. Insulin stimulates protein synthesis and suppresses breakdown *in vitro* (Jefferson *et al.*, 1972, Nakano and Hara, 1979, Tessari, 1994, Tischler *et al.*, 1982) and *in vivo* (Bennet *et al.*, 1990, Chevalier *et al.*, 2004, Hillier *et al.*, 1998). Therefore, resistance to the action of insulin is expected to impair protein metabolism as it does for glucose and fat metabolism in T2DM (Ganong, 2001).

The design used for *in vivo* determination of insulin sensitivity of protein metabolism in humans is a modification of the hyperinsulinemic euglycemic clamp. This technique is the gold standard for assessing insulin sensitivity of glucose metabolism, during which insulin is raised above basal levels and glucose is infused exogenously to maintain normal fasting concentrations or euglycemia (DeFronzo et al., 1979). Hyperinsulinemia is known to suppress proteolysis causing a decrease in amino acid concentrations below fasting levels (Fukagawa et al., 1986) that was shown to prevent detecting any action of insulin on protein synthesis (Fukagawa et al., 1989). Therefore, in a similar fashion to glucose in the conventional clamp, amino acids are prevented from dropping by infusing an amino acid solution to maintain concentrations at fasting levels or isoaminoacidemia. This hyperinsulinemic euglycemic isoaminoacidemic clamp is coupled with an isotopic dilution technique in order to assess protein metabolism in response to insulin (Chevalier *et al.*, 2004). The isotopes commonly used are amino acid tracers (¹³C-leucine, ¹⁴C-leucine, or L-ring ²H₅-phenylalanine), whose kinetics are determined during the clamp and thus serve as surrogates for wholebody protein kinetics. Kinetics include protein flux or turnover rate (Q), rates of protein synthesis (S), breakdown (B), oxidation (O) and net balance or anabolism (S-B). Using the hyperinsulinemic euglycemic isoaminoacidemic clamp, Pereira et al. (2008) have found blunted S and S-B in overweight or obese men with T2DM compared to sex- and body mass index- (BMI) matched controls. This might partly explain previous data from the same group on 24h integrated fed fasted state protein metabolism in men and women with poorly controlled T2DM (Gougeon et al., 1994). These had ~80% lower 24h whole body S-B compared to

obese controls. This is also consistent with recent findings from longitudinal studies in an elderly diabetic people who had accelerated loss of muscle mass over 6 years (Park *et al.*, 2009) and increased risk of sarcopenia (Kim *et al.*, 2010). In contrast, others have reported normal postinsulin protein kinetics in T2DM (Denne *et al.*, 1995, Halvatsiotis *et al.*, 2002b, Luzi *et al.*, 1993) but the studies did not adjust for sex, body composition, prior dietary intake and glycemic control, used different tracer methods, or lacked concomitant infusion of amino acids during the hyperinsulinemic clamp, to maintain circulating concentrations at fasting levels.

Of note is that during hyperinsulinemic, euglycemic clamps performed in poorly controlled T2DM (Denne *et al.*, 1995, Halvatsiotis *et al.*, 2002b, Luzi *et al.*, 1993, Pereira *et al.*, 2008), glycemia is lowered from hyperglycemic concentrations to euglycemia (5.0-5.5 mmol/L) in order to reproduce comparable conditions to healthy controls. However, this might create an acute normalization of glycemia that may mask impairment associated with hyperglycemia. Indeed, hyperglycemia, per se, impairs insulin sensitivity of glucose metabolism (Rossetti, 1995). Moreover, healthy subjects had altered protein metabolism during hyperinsulinemic hyperglycemic isoaminoacidemic clamp characterized by higher Q (17%), S (24%) and B (1.5 fold) (Flakoll *et al.*, 1993) compared to euglycemic clamps. However, no data exist in T2DM and thus it remains to be determined whether hyperglycemia aggravates insulin resistance of protein metabolism in T2DM.

The maximal protein anabolism occurs in the fed state when insulin, glucose and amino acids are elevated and free fatty acids are suppressed. This is

because amino acids, especially the branched amino acid leucine, have both independent (Castellino et al., 1987, Tessari et al., 1987) and synergistic effects with insulin (Adegoke et al., 2009, Castellino et al., 1987, Tessari et al., 1987) on protein synthesis. Since kinetic methods are most reliable during sustained steadystates, a fed state is simulated by clamping insulin, glucose and amino acids at postprandial levels. This hyperinsulinemic peak hyperglycemic hyperaminoacidemic clamp was associated with a substantial rise in Q, S, O and S-B (Adegoke et al., 2009, Castellino et al., 1987, Tessari et al., 1987) compared with either hyperinsulinemic isoaminoacidemic clamps or hyperaminoacidemia with insulin at baseline levels. In T2DM, insulin resistance of protein anabolism, if present, would be predicted to be maximal in the fed state. However, the literature lacks a well-designed study on the combined effect of postprandial insulin and amino acids on whole-body protein metabolism. One study by Luzi et al. (1993) found a normal whole-body protein anabolic response to clamping insulin and amino acids at postprandial concentrations in T2DM. The study, however, had a small sample size (n=6), did not account for possible sex difference, and glucose was maintained at euglycemia (5.5 mmol/L), which did not mimic fed state concentrations. Similarly, another study showed that hyperglycemic T2DM men have unaltered protein kinetics after ingesting boluses of carbohydrates and hydrolyzed protein (Manders et al., 2008). However, the total, taken in repeated boluses, was extremely large (268 g CHO and 134 g protein) and perhaps played a role in the metabolic outcomes.

At the cellular level, both insulin and leucine stimulate muscle protein synthesis through mechanisms that converge at the level of mammalian target of rapamycin-complex1 (mTORC1) (Anthony *et al.*, 2001, Drummond and Rasmussen, 2008, Kim *et al.*, 2002, Kimball *et al.*, 1999). mTORC1 activation causes a series of downstream phosphorylation of eukaryotic initiation factors (eIF) and ribosomal proteins that enhance messenger RNA (mRNA) translation. In T2DM, very few human studies have sought possible defects in the protein synthesis pathway that can reconcile the data on whole-body insulin resistance of protein metabolism. Therefore, further investigation is needed in that population to assess the effect of hyperinsulinemia \pm hyperaminoacidemia on total and phosphorylated signalling proteins of the protein synthesis pathway in skeletal muscle.

The controversy around establishing protein recommendations for T2DM is further reinforced by reports that argue against increasing dietary protein intake as this might impair glucose metabolism. In cross sectional (Linn *et al.*, 2000, Skilton *et al.*, 2008, Wolever *et al.*, 1997) and longitudinal (Liese *et al.*, 2009, Schulze *et al.*, 2003, Song *et al.*, 2004) studies, high protein consumption was associated with increased risk of insulin resistance and T2DM. Furthermore, amino acids blunt glucose uptake *in vitro* (Traxinger and Marshall, 1989, Tremblay *et al.*, 2005a, Tremblay and Marette, 2001), and in healthy humans at the whole body (Adegoke *et al.*, 2009, Krebs *et al.*, 2007, Krebs *et al.*, 2002, Pisters *et al.*, 1991, Tremblay *et al.*, 2005b) and tissue levels (Pisters *et al.*, 1991, Schwenk and Haymond, 1987). Hyperinsulinemic euglycemic clamps with amino acids infused to maintain basal (Flakoll *et al.*, 1992, Pisters *et al.*, 1991) or twice basal concentrations of total amino acids (Flakoll *et al.*, 1992, Krebs *et al.*, 2007, Krebs *et*

design to clamps without amino acid infusion and showed decreased whole-body glucose uptake in healthy men. At the cellular level the mechanism was attributed to amino acid- (specifically leucine) induced phosphorylation of mTORC1 and its target ribosomal protein S6K1. Activation of S6K1 causes serine phosphorylation of the insulin receptor substrate 1 (IRS-1) in muscle cells, which decreases its association with PI3 kinase (Tremblay et al., 2007a, Tremblay et al., 2005b). This, in turn, decreases the signaling downstream of the insulin sensitive phosphorylation cascade which results in diminished GLUT-4 translocation to the cell membrane and decreased glucose uptake. This effect was referred to as causing peripheral insulin resistance in healthy subjects (Patti et al., 1998). Findings from the above studies have been extrapolated to insulin resistant states, including T2DM. Thus it was proposed that postprandial aminoacidemia can aggravate the insulin resistance of glucose metabolism in T2DM. However, there is no study that examined these speculations in T2DM either at the whole body or cellular level.

Ameliorating glycemic control in T2DM with medications or lifestyle changes (diet and exercise) is associated with improved insulin resistance of glucose metabolism. This occurs at the hepatic level with improved suppression of endogenous glucose production in response to hyperinsulinemia (Kirwan *et al.*, 2009, Miyazaki *et al.*, 2001). Prolonged normoglycemia also increases insulin-induced peripheral glucose uptake (Ciaraldi *et al.*, 2002, Kirwan *et al.*, 2009, Pratipanawatr *et al.*, 2002). As for protein metabolism, improving glycemia with oral antidiabetic agents (sulfonylurea and metformin) (Gougeon *et al.*, 2000) or normalizing it with insulin (Gougeon *et al.*, 1998) for 7 d improved whole-body

24h protein kinetics (lower Q, S and B) in treated vs. untreated T2DM. As a consequence, net anabolism (S-B) increased to levels not different from obese controls. It remains to be determined whether sustained good diabetes control (A1C < 7%, FPG < 7 mmol/L) (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008) would improve insulin resistance of protein metabolism.

1.2. Statement of purpose

The 2 main hypotheses of the current study are that: 1) hyperglycemia in men with poorly controlled T2DM inhibits insulin stimulation (with circulating amino acids at baseline and elevated at postprandial levels) of protein synthesis, and 2) euglycemia (using intense treatment with oral antidiabetic agents) corrects the blunted insulin-stimulated net protein synthesis seen in hyperglycemic T2DM men.

Accordingly the objectives of this thesis research are:

- To use the hyperinsulinemic hyperglycemic isoaminoacidemic clamp and the L-[1-¹³C] leucine tracer method in overweight or obese men with poorly-controlled T2DM to determine if there is a worsening effect of clamping glucose at 8.0 vs.
 5.5 mmol/L on insulin sensitivity of protein;
- To use the hyperinsulinemic hyperglycemic hyperaminoacidemic clamp and L-[1-¹³C] leucine tracer method in overweight or obese men with poorly-controlled T2DM to determine whether protein metabolism is further impaired when insulin and amino acids are clamped at peak postprandial concentrations;

- To use the hyperinsulinemic hyperaminoacidemic clamp and D-[3-³H]glucose and compare it to hyperinsulinemic isoaminoacidemic clamp to determine whether postprandial amino acids worsen the already severe insulin resistance of glucose metabolism;
- 4) To use the hyperinsulinemic euglycemic isoaminoacidemic and hyperinsulinemic hyperglycemic hyperoaminoacidemic clamps with tracers of glucose and leucine in overweight or obese men with well-controlled T2DM to determine whether insulin sensitivity of glucose and protein is improved versus poorly controlled T2DM subjects;
- 5) To quantify the cellular levels of phosphorylation and expression of proteins involved in the protein synthesis pathways in skeletal muscle of overweight or obese T2DM men in poor and improved glycemic control at baseline and during hyperinsulinemic clamps at 2 levels of glycemia (euglycemia and hyperglycemia) and aminoacidemia (isoaminoacidemia and hyperaminoacidemia), to potentially define mechanisms of whole body outcomes.

CHAPTER 2. REVIEW OF THE LITERATURE

2.1. Type 2 diabetes mellitus

Type 2 diabetes mellitus (T2DM) is a chronic disease with hyperglycemia as the main feature, caused by a combination of insulin resistance and decreased insulin secretion (DeFronzo et al., 1992). Depending on the severity of either of these defects, T2DM can be viewed as a spectrum of heterogeneous disorders ranging from severe insulin resistance with relatively preserved insulin secretion on one end to highly defective insulin secretion with relatively less insulin resistance on the other end (Kahn, 2003). It is the most common type of diabetes, and is highly associated with adiposity, especially central adiposity, as well as multiple metabolic abnormalities. These, together with sustained hyperglycemia, result in macrovascular and microvascular complications leading to angiopathy, neuropathy, retinopathy and nephropathy (Health et al., 2009). This establishes T2DM as the leading cause of new cases of blindness and kidney failure. People with T2DM also have higher risk of dyslipidemia, hypertension and atherosclerosis and their main cause of death is stroke or heart disease (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008).

The prevalence of T2DM in the USA has tripled over the past 30 years (Prevention, 2008), partly due to the concurrent prevalence of obesity increasing in epidemic proportions. The incidence is also expected to double between 2000 and 2030 (Hossain *et al.*, 2007). The most recent statistics in Europe show a prevalence of 6% among middle-aged and older adults. In the USA, T2DM affects 8% (23.6 million) of the population with an estimated 24% of undiagnosed

cases (Wild *et al.*, 2004). In Canada, more than 3 million Canadians (9% of the population) have diabetes and this number is expected to reach 3.7 million by 2020 (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008). Prevalence of T2DM increases with age and is higher among specific ethnicities, namely people of African, Aboriginal, Hispanic, Asian and South Asian descent (Haffner, 1998).

2.1.1. Etiology of type 2 diabetes mellitus

Similar to other chronic diseases, T2DM etiology has been traced back to both genetic predisposition and environmental factors. Indeed several modifiable and non-modifiable risk factors for the development of T2DM are established but others are still not known.

Genes of T2DM susceptibility in humans have been identified (Lindgren and McCarthy, 2008). These might explain the increased prevalence of the disease or even the exaggerated responses to environmental risk factors in some ethnicities (Abate and Chandalia, 2003). Among the latter factors, the principal one is the adoption of what is commonly referred to as the "western lifestyle". High intakes of fat (Hu *et al.*, 2001, Meyer *et al.*, 2001) and refined low fibre carbohydrates (Hu *et al.*, 2001, Liu *et al.*, 2000) have been linked to increased risk of insulin resistance and T2DM. This is probably associated with hypercaloric intake and obesity (Abate and Chandalia, 2003). Exercise, on the other hand, is inversely related to the incidence of T2DM independently of its effect on body weight (Manson *et al.*, 1991).

2.1.2. Pathophysiology of type 2 diabetes mellitus

Two main defects exist in T2DM, insulin resistance and attenuated insulin secretion (Haffner, 1998) and there is an ongoing debate on which occurs first.

In order to better define insulin resistance, its actions need to be described. Insulin acts first by binding to its transmembrane receptor that is found on the cell membranes of many tissues including fats, muscles and liver. This triggers a series of intracellular phosphorylation cascades that ultimately modulate glucose, lipid and protein metabolism. In skeletal muscle and adipose tissue, known as "insulin sensitive tissues", insulin increases glucose uptake and suppresses glucose and fatty acid oxidation. It also enhances glycogen synthesis and lipogenesis while suppressing lipolysis and gluconeogenesis (Shepherd and Kahn, 1999). As for protein metabolism, insulin stimulates protein synthesis and suppresses breakdown (Kumar and O'Rahilly, 2005).

Insulin resistance occurs "whenever normal concentrations...produce less than a normal biologic response" (Kahn, 1978). As a consequence, more insulin is needed to normalize glycemia and the other aforementioned metabolic pathways, leading to hyperinsulinemia, a common feature of T2DM especially in the first phases of the disease. Many researchers believe that insulin resistance is the initial defect commonly associated with obesity that might lead to T2DM. Indeed, not all insulin resistant obese people develop T2DM because of their pancreatic ability to sustain the hyperinsulinemic state and overcome the reduced insulin efficiency (Kahn *et al.*, 1993, Perley and Kipnis, 1966, Polonsky *et al.*, 1988). T2DM develops when hyperinsulinemia is not sufficient to produce a normal effect, which results in impaired macronutrient metabolism causing hyperglycemia and concurrent metabolic abnormalities. Even if the original problem is insulin resistance, much debate exists about which organ is first affected (Petersen and Shulman, 2006), either adipose tissue (Lewis *et al.*, 2002), skeletal muscles (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 (Civitarese and Ravussin, 2008, Petersen *et al.*, 2004), glycogen synthase (Vaag *et al.*, 1992b) and endoplasmic reticulum stress (Boden, 2009) have been proposed to be the initial triggers. Insulin sensitivity is not altered in some T2DM patients, particularly among the non-obese (Banerji and Lebovitz, 1989, Bonora *et al.*, 1998, García-Estévez *et al.*, 2002, Gerich, 2000) and therefore the metabolic defect lies in secretion of insulin from pancreatic β cells.

2.1.3. Screening and monitoring of type 2 diabetes mellitus

The Canadian Diabetes Association (CDA) (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008) recommends screening for T2DM every 3 years for individuals \geq 40 years of age as the risk increases. As depicted in figure 2.1, diagnosis is made when plasma glucose is \geq 7 mmol/L in the fasting state, or \geq 11.1 mmol/L 2 hours after a 75 g glucose challenge also know as an oral glucose tolerance test, (OGTT). When fasting plasma glucose (FPG) is above the normal value of 5.6 mmol/L but <11.1 mmol/L, individuals are classified as either "at risk" or with "pre-diabetes" depending on the OGTT results (Figure 2.1).

After T2DM diagnosis has been confirmed, monitoring blood glucose is essential in attempting to achieve good control and thus preventing hyperglycemia and associated diabetes complications (Ohkubo et al., 1995, Patel et al., 2008). Pre- and post-meal self monitored blood glucose readings (SMBG) taken more than once during the day and for several days, are needed to assess control over an extended period of time. The recommended SMBG targets are 4.0-7.0 mmol/L pre-meal and 5.0-10.0 mmol/L 2 hours post-meal (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008). A more practical one-time test that has been commonly used by clinicians for the past 3 decades is glycated hemoglobin (A1C) expressed as percentage of total hemoglobin (Jeha and Haymond, 2007). A1C is a glycoprotein formed by the condensation of glucose with the β -chains of hemoglobin (Bunn *et al.*, 1976) and thus a higher percentage indicates a long-term poor control reflecting the average blood glucose in the preceding 3 months. CDA recommends 1 to 2 A1C tests per year for T2DM with a target lower than 7%.



Figure 2.1. Screening for T2DM in adults (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008)

Reproduced from (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008)

2hPG = 2-hour plasma glucose, FPG = fasting plasma glucose, IFG = impaired fasting glucose, IGT = impaired glucose tolerance, OGTT = oral glucose tolerance test, PG = plasma glucose

*If, despite a normal fasting value, an OGTT is subsequently performed and the 2hPG value is 7.8–11.0 mmol/L, a diagnosis of isolated IGT is made. †Prediabetes = isolated IFG, isolated IGT, IFG and IGT ‡A confirmatory laboratory glucose test (either a FPG, a casual PG or a 2hPG in a 75-g OGTT) must be done on another day in all cases in the absence of unequivocal hyperglycemia accompanied by acute metabolic decompensation.

2.1.4. Management of type 2 diabetes mellitus

2.1.4.1. Pharmacotherapy

There is much debate as to whether to initiate drug therapy for T2DM with insulin or metformin (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008). The latter is the only medication in the class of "biguanides" and is more commonly used as the first monotherapy. It lowers blood glucose by both decreasing hepatic glucose output and enhancing peripheral insulin sensitivity (DeFronzo et al., 1991). With the progression of the disease, other classes of oral antihyperglycemic drugs are added. Sulfonylureas are insulin secretagogues that act on the pancreatic β -cells to secrete more insulin and thus increase the risk of hypoglycemia (Stolar et al., 2008). Another class of controversial drugs known as thiazolidinediones (TZD) is also used in combination with other oral agents. These are potent "insulin sensitizers" that improve glycemia by increasing insulin sensitivity but are associated with a number of drawbacks. TZD complete action may take up to 3 months and these usually cause water retention and higher fat mass, which increases body weight and may aggravate congestive heart failure (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008). Moreover, a body of evidence has linked the usage of the TZD rosiglitazone with increased risk of cardiovascular events (Graham et al., 2010, Nissen and Wolski, 2007), which has led to its removal from European and North American markets (Rosen, 2010).

Alpha-glucosidase inhibitors are drugs that attenuate the digestive enzyme alpha-glucosidase that breaks down complex CHO. As a result, monosaccharides
are released and absorbed more slowly, which gives time for the pancreas to secrete insulin to lower blood glucose. DPP-inhibitors and GLP-1 receptor agonists are new classes of antidiabetic medications recently introduced on the market. These are highly efficacious in controlling glycemia but long-term safety is still under investigation (Barnett, 2006, Pratley *et al.*, 2006, Richter *et al.*, 2008). GLP-1 receptor agonists mimic the action of the incretin GLP-1 by binding to its receptor on pancreatic β -cells and increasing glucose-stimulated insulin secretion. DPP-inhibitors act by inhibiting the amino acid DPP that degrades GLP-1, and thus prolonging its stimulatory effect on insulin secretion.

If not used initially after diagnosis, insulin therapy is usually started when a combination of the maximal doses of the above oral antidiabetic drugs is not sufficient to achieve normoglycemia. This signifies a decrease in pancreatic β -cell function and compromised insulin secretion. Sources of exogenous insulin include animals (porcine or bovine) and bacteria/yeast that produce human insulin through genetic engineering. More recently, another approved class of molecules are insulin analogs which are produced by recombinant DNA technology. These modified structures of the human insulin molecule, with altered are physicochemical, biological, and pharmacodynamic properties (Vajo et al., 2001). Accordingly, different insulin types exist on the market and range in ascending order of onset, peak and duration from rapid acting, short-acting, intermediateacting to long acting insulin. Short and rapid acting insulins have higher peaks and shorter duration which make them ideal to mimic postprandial insulin. This is why these are given before each meal and the dosage is adjusted according to the amount of ingested CHO. Intermediate and long acting insulin peaks are lower

but they have longer half lives and thus are used one or twice per day to restore basal insulin. Mixtures of rapid and long acting insulin with different ratios of both are also available.

2.1.4.2. Diet

In conjunction with medications, dietary management of T2DM and coexistent morbidities is essential to ensure adequate nutritional intake, improved glycemic control, blood pressure and lipid profile as well as achieving weight loss and maintenance when needed (Bantle *et al.*, 2008, Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008). Diet recommendations have evolved from being more generalized and requiring unrealistic strict dietary and weight loss goals to being more individualized and practical to apply (Franz *et al.*, 2003). Initially, simple CHO intake was forbidden, weight loss of 18-23 kg to achieve ideal body weight was required and dependence on medications was common. Nowadays, dietary recommendations are closer to those for the general population. All food groups ought to be consumed in moderation, a small percentage weight loss may be beneficial and nutrition therapy should be tailored to the individual's needs with medications used at later stages of the disease (Franz *et al.*, 2003).

2.1.4.2.1. Carbohydrates and fat

The CDA (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008) and American Diabetes Association (ADA) (Bantle *et al.*, 2008) guidelines for CHO and fat intake for people with T2DM as well as the dietary reference intake (DRI) for healthy adults are presented in Table 2.1. Both guidelines are comparable to the DRI but CDA guidelines are more defined and specific than those of ADA. Their recommendations include greater consumption of low glycemic index foods, and higher fibre intake than for healthy persons, and unlike ADA, establish limits for simple CHO, total and unsaturated fat intake. On the other hand, only ADA sets a threshold for dietary cholesterol.

Recommendations	CDA	ADA	DRI*
CHO Quantity	45-60% (% energy, accepted range)	130g/d (minimum requirement)	RDA: 130g/d
СНО Туре	High-fibre, low glycemic index	CHO from fruits, vegetables, whole grains, legumes, and low-fat milk. Low glycemic index and load (modest additional benefit)	Limit added sugars to no more than 25% energy
Dietary Fibre	25-50g/d	14 g/1,000 kcal intake	AI: 38g/d (men), 25g/d (women)
Sucrose	Up to 10% of energy intake	NS (avoid excess energy intake)	NS
Fructose	Up to 60g/d	NS	NS
Sugar alcohols	Up to 10g/d	Daily levels established by FDA	NS
Sweeteners	Those approved by Health Canada	Daily levels established by FDA	NS
Total fat	<35% of energy	NS	NS. AMDR: 20- 35% of energy
Saturated fat	<7% of energy	<7% of energy	Minimize with nutritionally adequate diet
Polyunsaturated far	t <10% of energy	NS	NS
Omega-3 fat	Include fish and plants rich in omega-3	Two or more servings of fish per week	AI for ALA: 1.6g/d (men) 1.1g/d (women) (10% from EPA and DHA)

Table 2.1. CDA and ADA carbohydrate and fat recommendations for themanagement of T2DM versus the DRI

Recommendations	CDA	ADA	DRI*
Monounsaturated fat	Consume more and instead of saturated fat	NS	NS
Trans fat	Kept to a minimum	Should be minimized	Minimize with nutritionally adequate diet
Cholesterol	NS	<200 mg/day	NS

*DRI: dietary reference intakes for adults (19-50 years of age) (Institute of Medicine of the National Academies *et al.*, 2005); CDA: Canadian diabetes association; ADA: American diabetes association; CHO: carbohydrates; RDA: recommended daily allowances; NS: not specified; FDA: food and drug administration; ALA: α -linolenic acid; AI: adequate intake; AMDR: Acceptable Macronutrient Distribution Ranges; ALA: α -linoleic acid; EPA: eicosapentanoic acid; DHA: docosahexanoic acid

2.1.4.2.2. Protein

Protein recommendations in T2DM are still not precisely determined and both the CDA and ADA have declared that there is no solid evidence showing that the usual intake of 20% of energy intake is insufficient. This uncertainty is in part due to the controversial reports on whether protein metabolism is impaired in T2DM (discussed in a following section).

Epidemiological studies examining the dietary habits that are associated with increased risk for T2DM in adults found increased risk with higher protein intake (hazard ratio 2.15 [95% CI 1.77–2.60] highest vs. lowest quartile) (Sluijs *et al.*, 2010) especially in the form of red meat (Schulze *et al.*, 2003, Song *et al.*, 2004). Confounders such as saturated fat, nitrites and nitrates were also linked to increased risk of T2DM. Elevated circulating amino acids could also impair glucose metabolism and induce insulin resistance as it has been shown acutely in healthy subjects (Tremblay *et al.*, 2005b, Tremblay and Marette, 2001, Tremblay *et al.*, 2007b) (to be discussed in a following section).

In terms of glycemic control, high protein (HP) low CHO (LC) diets have been associated with an improved glycemic control (lower A1C, fasting and postprandial glucose concentrations) in short term intervention studies spanning 2 to 16 weeks (Boden *et al.*, 2005, Gannon and Nuttall, 2004, Gannon *et al.*, 2003, Nuttall *et al.*, 2008b) (Table 2.2). The CHO:protein ratio of these diets ranged from a strict low CHO diet containing only 21 g CHO per day (with 30% of energy from protein) to a moderately restricted diet with a 30:30 ratio. In one study (Boden *et al.*, 2005), HP-LC diet was coupled with decreased energy intake and 2% weight loss. Hence, one cannot differentiate between the effect on glycemia of the diet per se from that of the associated appetite and energy suppression and weight loss. However, Gannon and Nuttall, Gannon and Nuttall, 2004, 2006, Nuttall and Gannon, 2006, Nuttall et al., 2008b) (Table 2.2) demonstrated an improved glycemic control in T2DM men after 5 weeks of consuming a HP LC diet with 2 different percentages of CHO without energy restriction or weight change versus a standard diet. The same group also tested different macronutrient proportions to identify the best outcome with respect to glycemic control (Gannon and Nuttall, 2006). Two diets with different CHO:protein ratios; 30:20 and 30:30 for 5 weeks were equally effective compared to the standard diet with a ratio of 55:15. These diets resulted in a 38% decrease in 24h glucose, a reduction in fasting glucose to near normal and a decrease in A1C from 9.8% to 7.6%. Conversely, others (Sargrad et al., 2005, Wycherley et al., 2010) did not find any amelioration in glycemia in T2DM individuals on energy restricted HP-LC (30:40) beyond that of isoenergetic control diet (table 2.2). In one study (Wycherley et al., 2010) reduction in blood glucose was not different despite more weight loss in the HP-LC group. In another study (Sargrad et al., 2005), A1C and insulin sensitivity were improved only in the control group.

The beneficial effects of HP-LC diet can be attributed to the higher protein and/or the lower CHO content of the diet. Proteins, when consumed alone, do not increase plasma glucose concentrations as do CHO (Krezowski *et al.*, 1986), although most amino acids can be converted to glucose by gluconeogenesis. Moreover, proteins and especially certain amino acids such as arginine, lysine and leucine are known to have insulinotropic properties (Lentine and Wrone, 2004)

by directly stimulating the pancreas rather than indirectly through incretin secretion (Frid et al., 2005). Adding (Manders et al., 2005) or substituting (Gannon et al., 2003) protein for CHO in meals resulted in a higher insulin and lower glucose response in T2DM. Thus protein's ability to stimulate insulin is preserved in T2DM as opposed to that of glucose, which is commonly attenuated (van Loon *et al.*, 2003). In addition, protein's stimulation of insulin was found to be even more potentiated in T2DM (Nuttall et al., 1984) compared with healthy controls (Krezowski et al., 1986). Insulin net AUC after ingestion of 50 g protein in the form of lean beef was higher in T2DM versus healthy subjects and lowered plasma glucose below baseline (Nuttall et al., 1984). All of the above findings are in contrast with a recent study in which substituting protein for fat instead of CHO in a 741 kcal meal (CHO:protein:fat 51:15:34 vs. 51:30:19) did not affect postprandial insulin and glucose response in obese subjects with T2DM (Papakonstantinou et al., 2010). These subjects were newly diagnosed with T2DM and treated only with diet and might, therefore, have behaved similarly to healthy controls postprandially, with CHO being the main contributor to glucose and insulin response.

Longer term effects of HP-LC diets on diabetes control are not well established and thus further investigation is required. In a study by Brinkworth et al in 2004 (table 2.2) persons with T2DM were assessed 1 year after 8 weeks of energy restricted diet followed by 4 weeks of weight maintenance using HP-LC or standard diet. After these 12 weeks, subjects were asked to maintain the same weight maintenance diet they were assigned. At the end of the year, the HP-LC group had greater weight loss (-3.7 vs. -2.2 kg) but the diet had no effect on glycemic control (A1C or FPG) (Brinkworth *et al.*, 2004).

Protein recommendations for diabetic nephropathy are also not strictly defined. Most renal dietitians aim not to exceed the RDA of 0.8 g/kg/d (Bantle et al., 2008) due to concerns of accelerated progression of the disease with higher intake (Lentine and Wrone, 2004). However usual protein intake of people with T2DM is commonly up to 1.2 g//kg/d (\sim 20% of energy intake) in certain populations (Cruz and Calle-Pascual, 2004). Two recent meta-analyses (Pan et al., 2008, Robertson et al., 2007) of randomized controlled trials (RCT) have examined the effect of low protein (LP) diets on the progression of renal disease in diabetes. The first one (Pan et al., 2008) reviewed studies conducted over 6 to 24 months and comparing LP diets (0.8 g/kg/d) to usual protein intake (1-1.2 g/kg/d). Looking at the standard and weighted mean differences respectively, LP diet was associated with lower proteinuria (-0.69 mg/d) and A1C (-0.31%) but with no change in glomerular filtration rate or creatinine clearance rate. Despite the former findings, the authors concluded that LP diets do not significantly delay the progression of renal disease in diabetes. In the second meta-analysis (Robertson *et al.*, 2007), a similar conclusion was drawn from RCT of a minimum duration of 4 months with LP diet content of 0.8 g protein /kg/d vs. 1.1-1.2 g/kg/d. One study (Meloni et al., 2002) investigated the effect of a more restricted LP diet (0.6 g/kg/d) for 12 months on renal disease progression. No improvement in renal function compared with usual diet was observed, but LP diets resulted in malnutrition indicated by lower albumin and pre-albumin levels by the end of the study.

In summary, the evidence available on the adequate protein intake for best outcomes in T2DM is inconclusive. Higher protein intake might be needed to overcome the impaired protein metabolism and achieve better glycemic control. On the other hand, long term usefulness of HP diet is still not established, while prospective studies show a negative relationship between HP diet and the incidence of T2DM. Lower protein intake has a slight but protective effect on the progression of renal disease in T2DM although prolonged very low intake might lead to malnutrition.

Author	Subjects	Intervention	Diet (CHO: protein: fat ratio)	Results
(Gannon and Nuttall, 2004)	8 overwt & obese men (mean age: 63 y) with T2DM	5 week cross over design with 5 week wash out	Isoenergetic -Control diet: 55:15:30 -HP diet: 20:30:50	-No change in body wt -HP diet: 22% lower 24-h serum glucose and 36% lower A1C
(Brinkworth <i>et al</i> ., 2004)	15 obese men & 23 obese women (mean age: 62 y) with T2DM randomly assigned to control or HP diet	8 weeks of energy restricted then 4 weeks of energy balance 1 y F/U	-Control diet: 55:15:30 -HP diet: 40:30:30	-Week 64: more wt reduction with HP diet (-3.7 vs2.2 kg) -No effect on glycemic control (FPG or A1C)
(Boden <i>et al.</i> , 2005)	3 obese men & 7 obese women (mean age: 51 y) with T2DM	Usual diet for 7 d then LC diet for 2 weeks	Ad Lib -Usual diet: 40:19:31 -LC: 21g CHO:28:68 -Same g protein in both diets	LC vs. usual diet -↓ energy intake (by 1000calories) and wt loss (-1.6 kg) -↓FPG (16%) & A1C (7%) ↑insulin sensitivity (75%)
(Sargrad <i>et al.</i> , 2005)	obese men & women with T2DM (mean age: 48 y) : 6 on control and 6 on HP diet	8 weeks on control or HP diet Hyperinsulinemic clamp before and after diet	Isoenergetic -Control diet: 55:15:30 -HP diet: 40:30:30	-Same wt loss in both groups (~2.4 kg) -↓ in A1C (16%) & FPG (18%) & ↑glucose uptake 34% after control but not HP diet

Table 2.2. Studies on effect of high protein (HP) low CHO (LC) diets on glycemic control in T2DM

Author	Subjects	Intervention	Diet (CHO: protein: fat ratio)	Results
(Nuttall <i>et al</i> ., 2008b)	8 overwt & obese men (mean age: 59 y) with T2DM	5 week on HP diet after standard diet	Isoenergetic -HP diet: 30:30:40	-Fasting glucose \downarrow 40 % -24 h glucose AUC \downarrow 45 %. -A1C \downarrow 1.7%
(Wycherley <i>et al.</i> , 2010)	obese men and women (mean age: 55 y) with T2DM: 16 on control and 12 on HP diet	16 weeks on energy restricted control or HP diet	Isoenergetic -Control diet : 53:19:26 - HP diet: 43:33:22	-Same reduction in wt (~8.7 %) and in A1C (9%) and blood glucose (26%) in both groups

WC: waist circumference, wt: weight, AUC: area under the curve, F/U: follow up

2.1.4.3. Body weight and composition

People with T2DM are more likely to be overweight or obese and to have high waist circumferences (>102 cm) and waist to hip ratios (Goodpaster *et al.*, 2003, Han et al., 1998, Tsui et al., 1998). Moreover, methods of assessing body composition like bioelectrical impedance analysis (BIA) and dual-energy X-ray absorptiometry (DXA) have revealed high body fat percentage in T2DM with more fat concentrated at the abdominal region compared to healthy individuals $(172 \pm 79 \text{ vs. } 145 \pm 66 \text{ cm}^2 \text{ for men; } 162 \pm 66 \text{ vs.} 116 \pm 54 \text{ cm}^2 \text{ for women; both } p$ < 0.0001) (Goodpaster et al., 2003, Han et al., 1998, Tsui et al., 1998). Abdominal visceral fat, which is quantified by more precise techniques like Xray computed tomography (CT) scans and magnetic resonance imaging (MRI), is specifically highly correlated with glucose intolerance, insulin resistance and increased risk of T2DM (r with fasting insulin was 0.37 for men and 0.40 for women, both p < 0.0001) (Goodpaster *et al.*, 2003). Even lean individuals with increased abdominal visceral fat may have impaired glucose tolerance (Despres et al., 1989).

Ectopic fat is defined as fat deposited in tissues other than adipose tissues, such as muscles and liver. Ectopic fat in the liver and skeletal muscles is associated with insulin resistance independent of obesity (Rasouli *et al.*, 2007, Yki-Jarvinen, 2002). Moreover, intermuscular fat or fat spread between deep and visceral fascia of the muscles is higher in individuals with insulin resistance or T2DM (Goodpaster *et al.*, 2000, Hilton *et al.*, 2008, Itani *et al.*, 2002, Shulman *et al.*, 1990a).

Despite having higher muscle mass than lean controls due to higher body size and obesity, people with T2DM appear to have lower muscle performance that is thought to be associated with increased disabilities in older age. Park and colleagues (2006) found lower leg and arm muscle strength in a cross section of older men with T2DM compared with those without. Both men and women with T2DM also had lower muscle quality (p<0.001) defined as maximal strength per unit of muscle mass (Park et al., 2006). The same group reported ~30% greater decline in leg muscle strength and quality in older subjects with T2DM over 3 years compared with healthy age-matched controls (Park et al., 2007). The difference remained significant (p<0.03) even after adjusting for confounding variables. Middle-aged (58 years) obese T2DM men and women, with peripheral neuropathy, also showed lower scores on physical performance tests. Cases had lower calf muscle strength, muscle power, ratio of leg muscle power to leg muscle volume compared with healthy controls. These impairments were highly correlated with intramuscular fat infiltration that was 2 times higher than controls (Hilton et al., 2008). Nevertheless the contribution of neuropathy to muscle function cannot be excluded. Indeed, Andersen et al (2004) found lower ankle and knee strengths in adults with T2DM compared to age, sex and BMI matched controls with strength negatively correlated with the degree of neuropathy (Andersen et al., 2004).

T2DM also aggravates the loss of muscle mass associated with aging. Older adults with T2DM followed for 6 years had accelerated loss of appendicular muscle mass compared with non-diabetic controls with more pronounced loss in those whose diabetes was only newly diagnosed at the study entry (Park *et al.*, 2009). Consistent with this, the more recent Korean Sarcopenic Obesity Study showed that sarcopenia (or low skeletal muscle mass) is more prevalent among older people with T2DM (15.7 vs. 6.9%) (Kim *et al.*, 2010).

2.2. Insulin resistance of glucose metabolism in type 2 diabetes mellitus

Insulin suppresses endogenous glucose production (EGP) and stimulates glucose uptake and utilization. EGP is mainly derived from liver, with a minor contribution from the kidneys (Meyer *et al.*, 2004). On the other hand, insulin-stimulated glucose uptake occurs mostly in the periphery by the insulin sensitive tissues, namely skeletal muscle and adipose tissue. In T2DM, both pathways are resistant to the action of insulin.

The gold standard to measure whole-body insulin resistance of glucose metabolism is the hyperinsulinemic euglycemic clamp, in which insulin is infused to raise concentrations above basal levels and glucose is maintained at euglycemia or normal fasting concentrations, by infusing a dextrose solution (DeFronzo *et al.*, 1979). Glucose infusion rate reflects sensitivity, such that the lower the infusion rates the higher the insulin resistance. However, this method does not permit differentiation between hepatic and peripheral resistance. This is why tracers of glucose (¹³C, ²H or ³H glucose) are administered during the clamp to estimate EGP, which is a reflection of hepatic insulin sensitivity and the rate of glucose uptake (R_d), another measure of peripheral insulin sensitivity (Finegood *et al.*, 1987). The blood sampling and analysis are conducted after 2-3 hours of tracer infusion when the "plateau" has been reached. This is defined as a steady state of

infusion rate, substrate and hormonal concentrations as well as constant isotopic enrichment (²H or ¹³C glucose) or specific activity (³H glucose). Glucose tracers are also used in the fasting state to quantify postabsorptive EGP and R_d. The following sections review *in vivo* human studies that assessed both EGP (Tables 2.3 and 2.4) and glucose uptake (Table 2.5) in T2DM. Unless specified otherwise, in studies using hyperinsulinemic euglycemic clamps, insulin was maintained at comparable concentrations in T2DM and control groups. Also, subjects were from both sexes and controls were always matched for age, sex, and BMI.

2.2.1. Hepatic insulin resistance

Postabsorptive EGP is commonly elevated in hyperglycemic overweight or obese T2DM (Basu *et al.*, 2005, Boden *et al.*, 2001b, Campbell *et al.*, 1988, Firth *et al.*, 1987, Gastaldelli *et al.*, 2000, Jeng *et al.*, 1994, Meyer *et al.*, 2004, Staehr *et al.*, 2001, Turk *et al.*, 1995, Vaag *et al.*, 1995, Woerle *et al.*, 2006), and in those who are lean (DeFronzo *et al.*, 1985, Firth *et al.*, 1987, Gastaldelli *et al.*, 2000, Groop *et al.*, 1989a, Rooney *et al.*, 1993) compared with sex, age and BMI matched controls (table 2.3). This usually occurs despite fasting hyperinsulinemia and is thus indicative of hepatic insulin resistance. When renal and hepatic contributions to EGP were quantified, both were higher than normal (Meyer *et al.*, 2004). EGP correlates with FPG in T2DM and is believed to contribute to hyperglycemia together with peripheral insulin resistance (Bogardus *et al.*, 1984). However, others have reported unchanged (Boden *et al.*, 2001b, Jeng *et al.*, 1994) or even lower (Pigon *et al.*, 1996) fasting EGP in mildly hyperglycemic T2DM compared with healthy controls. In the 2 former studies, mean FPG of T2DM was lower than 10 mmol/L, whereas in the latter one it was 7 mmol/L and subjects were newly diagnosed and had excellent long term glycemic control (A1C=5%). Therefore, as suggested by DeFronzo (1988), there might be a threshold concentration for FPG above which elevated EGP becomes the main abnormality that causes hyperglycemia. At lower FPG concentrations, the abnormality is more at the level of insulin resistance in the periphery. FPG threshold values have been proposed for this relationship (DeFronzo, 1988, Jeng *et al.*, 1994), including 7.8 mmol/L (DeFronzo, 1988).

Postabsoprtive EGP is the sum of glycogenolysis (GLY) and gluconeogenesis (GNG). Consistent with the EGP findings in T2DM, fasting GNG was reported to be higher (Basu *et al.*, 2005, Boden *et al.*, 2001b, Gastaldelli *et al.*, 2000, Woerle *et al.*, 2006), unchanged with lower FPG concentrations (Boden *et al.*, 2001b) or lower (Turk *et al.*, 1995) vs. healthy matched controls (Table 2.2). In the latter study, T2DM subjects were hyperglycemic but their FPG was normalized to 5 mmol/L by an overnight lowdose insulin infusion, which is a potential confounder. As for GLY, it has not always matched the trend of EGP in T2DM suggesting that GNG is the main contributor to hyperglycemia. Concurrent with elevated EGP, higher (Woerle *et al.*, 2006), normal (Basu *et al.*, 2005, Gastaldelli *et al.*, 2000) or even lower rates of GLY (Boden *et al.*, 2001b) have been reported in T2DM.

In healthy subjects, physiologic hyperinsulinemia like that attained during a mixed meal suppresses EGP to levels not different from zero. This occurs directly by inhibiting the hepatic gluconeogenic and glycogenolytic enzymes and indirectly by lowering FFA and glucagon concentrations (Lewis et al., 1996). Hyperinsulinemic euglycemic clamps have been used to assess EGP in response to hyperinsulinemia in T2DM subjects compared with matched controls (Table 2). EGP has been consistently higher and not fully suppressed as in controls at postprandial insulin (infusion rate: 40 mU/m².min or 1 mU/kg.min, serum concentrations: 450-810 pmol/L) (Bogardus et al., 1984, Campbell et al., 1988, DeFronzo et al., 1985, Firth et al., 1987, Groop et al., 1989a, Turk et al., 1995) or lower concentrations (infusion rate: 4-20 mU/m².min or 0.4-0.5 mU/kg.min, serum concentrations: 20-300 pmo/L) (Basu et al., 2005, Campbell et al., 1988, Del Prato et al., 1993, Firth et al., 1987, Groop et al., 1989a, Rooney et al., 1993, Staehr et al., 2001, Turk et al., 1995), illustrating hepatic insulin resistance. Conversely, higher supraphysiological insulin concentrations (infusion rate: 100-400 mU/m².min or 2-10 mU/kg.min, serum concentrations: 1248-13,758 pmol/L) completely suppressed EGP, similarly to healthy controls (Bogardus *et al.*, 1984, Campbell et al., 1988, Firth et al., 1987, Groop et al., 1989a, Rooney et al., 1993, Staehr et al., 2001, Turk et al., 1995). Normal suppression of EGP in T2DM was also reported in 4 studies with lower physiological hyperinsulinemia (Pigon et al., 1996, Staehr et al., 2001, Turk et al., 1995, Vaag et al., 1995). In three of these studies (Staehr et al., 2001, Turk et al., 1995, Vaag et al., 1995), insulin was infused overnight to normalize glycemia before the clamp, which might have also normalized the hepatic metabolic abnormalities. Similarly, impaired fasting EGP was not evident in newly diagnosed T2DM subjects (Pigon et al., 1996) with mild hyperglycemia (FPG, 7 mmol/L), and with excellent glycemic control (A1C, 5%).

Subjects might not have yet developed the hepatic defect associated with poorer glycemic control and more advanced stages of the disease. Postprandial EGP was also quantified using a meal study, during which both insulin and glucose were elevated compared with controls. EGP was higher than normal after the intake of 75 g oral glucose (Meyer *et al.*, 2004) or a mixed meal composed of 50% CHO, 30% fat, and 20% protein (Woerle *et al.*, 2006). Del Prato et al. (1993) reproduced the same results using hyperinsulinemic, hyperglycemic (15 mmol/L) clamps simulating peak meal insulin and glucose concentrations in T2DM. GNG and GLY assessed concurrently with EGP were higher compared with controls both during hyperinsulinemic euglycemic clamps (Basu *et al.*, 2005) and mixed meal studies (Woerle *et al.*, 2006).

 Table 2.3. Studies on postabsorptive endogenous glucose production (EGP) in T2DM*

Author	Subjects	T2DM FPG	Outcomes	Methods	Main findings in T2DM
Basu et al. 2005	10 lean vs. 10 obese vs. 11 obese T2DM (age=59 y)	10 mmol/L	16h postabsorptive EGP, GNG and GLY	[³ H]-glucose & deuterated water	Higher EGP (22%) and GNG (31%) in T2DM vs. lean and obese Higher GLY (31%) in T2DM vs. lean but not obese
Boden et al. 2001	13 hyperglycemic T2DM obese vs. 14 matched mildly hyperglycemic T2DM vs. 7 controls (age=59 y)	10 mmol/L vs. 7.1 mmol/L	16h postabsorptive EGP, GNG and GLY	[3- ³ H]-glucose and deuterated water	EGP (28%) and GNG (17.5%) higher in T2DM (FPG>10 mmol/L) vs. controls but not T2DM (FPG<10 mmol/L) GLY in all T2DM < controls (p<0.03)
Bogardus et al. 1984	15 obese T2DM (age=32 y) and 19 BMI matched but younger controls (age=25 y)	13.7 mmol/L, range of 7.5- 17.2 mmol/L	Postabsorptive EGP	[3- ³ H]-glucose	EGP positively correlated with FPG in T2DM but not in controls (r=0.9)
Campbell et al. 1988	14 overwt T2DM (age=54 y) vs. 14 controls	11 mmol/L	10-12 h postabsorptive EGP	[³ H]-glucose	Higher EGP (31%)
DeFronzo et al. 1985	9 normal wt T2DM (age=52 y) vs. 10 controls	8.8 mmol/L	Postabsorptive EGP	[³ H]-glucose	EGP slightly higher (2.50 vs. 2.17 mg/kg.min)
Firth et al. 1987	14 obese T2DM (age=56 y)vs. 10 obese controls 6 lean T2DM (age=61 y) vs. 5 lean controls	Obese:10.4 mmol/L Lean: 11.2 mmol/L	Postabsorptive EGP	[³ H]-glucose	EGP 50% higher in obese T2DM vs. obese controls. EGP tended to be higher (p =0.13) in lean T2DM vs. lean controls

Author	Subjects	T2DM FPG	Outcomes	Methods	Main findings in T2DM
Gastaldelli et al. 2000	28 obese T2DM (age=58 y)vs. 12 obese controls 9 lean T2DM (age=59 y) vs. 6 lean controls	Obese: 8.2 mmol/L Lean: 8.5 mmol/L	15h postabsorptive EGG and GNG	6,6-[² H]glucose and deuterated water	EGP higher in T2DM lean (35%) and obese (16%) vs. controls. GNG flux higher in T2DM obese (24%) and lean (2x) vs. controls GLY in T2DM not different vs. lean controls
Groop et al. 1989	9 lean T2DM (age=61 y) vs. 8 controls	9.5 mmol/L	15h postabsorptive	[³ H]-glucose	EGP 17% higher (83 vs. 71 mg/m ² .min)
Jeng et al. 1994	T2DM BMI \leq 30 (age=35-70 y) 3 groups of 11 each: DM1 DM2, and DM3 vs. 18 controls	DM1, FPG <10 mmol/L DM2, FPG=10-14 mmol/L;DM3, FPG >14 mmol/L	EGP quantified from 9 to 10 a.m	[³ H]-glucose infused for 12-h period, from 22h00 to 10h00	No difference between EGP of DM1 and controls DM-2 (21%) and DM-3 (29%) had higher EGP vs. controls (p<0.01)
Meyer et al. 2004	10 obese T2DM (age=52 y) &10 controls	9.9 mmol/L	12h postabsorptive total, renal and hepatic EGP	[³ H]-glucose. renal vein catheterization and p- aminohippuric acid clearance technique	Higher total (76%), hepatic (38%) and renal (4x) EGP
Pigon et al. 1996	7 mildly hyperglycemic lean T2DM (age=46 y) vs. 10controls	7 mmol/L, normal long term control (A1C=5%)	14h postabsorptive EGP	[6- ³ H]-glucose	EGP 10%lower

Author	Subjects	T2DM FPG	Outcomes	Methods	Main findings in T2DM
Rooney et al. 1993	8 lean T2DM (age=54 y) vs. 8 controls	10.7 mmol/L	Postabsorptive EGP and hepatic glucose (G)/glucose 6 phosphate (G6-P) cycling	[2- ³ H] - and [6- ³ H]glucose	EGP (38%) and G/G6-P (63%) cycle higher
Staehr et al. 2001	10 overwt T2DM (age=54 y) vs. 10 controls	9.3 mmol/L	16h postabsorptive EGP (FPG normalized to 5 mmol/L by a overnight low-dose insulin)	[3- ³ H]-glucose	EGP 13% higher
Turk et al. 1995	7 overweight T2DM (age=53 y) and 7 controls	normalized to 5 mmol/L overnight by a low-dose insulin infusion	16h postabsorptive EGP and GNG	[6- ³ H]-glucose and ¹⁴ CO ₂	EGP 20% higher GNG (% incorporation of $^{14}CO_2$ in glucose) lower in T2DM (p<0.05)
Vaag et al. 1992a	12 obese T2DM (age=56 y) vs.12 lean controls	10.7 mmol/L	12h postabsorptive EGP	[3- ³ H]-glucose	Higher EGP (32%)
Vaag et al. 1995	10 overwt T2DM (age=62 y) vs. 10 controls	10.9 mmol/L	12h postabsorptive (i.v. insulin infusion to normalize glycemia inT2DM)	[3- ³ H]-glucose	EGP 26% higher

Author	Subjects	T2DM FPG	Outcomes	Methods	Main findings in T2DM
Woerle et al, 2005	26 obese T2DM (age=53 y) vs. 15 controls	12 mmol/L, A1 _C = 8.6%	Baseline EGP, GNG and GLY	[3- ³ H]glucose and [¹⁴ C]bicarbonate and oral [6,6- dideutero] glucose	Higher fasting EGP (30%), GNG (46%) and GLY (27%)

*Unless specified otherwise, subjects in all studies were from both sexes and controls were age, sex and BMI matched with T2DM subjects Mean values are shown

FPG: fasting plasma glucose (1 mmol/L=18.163 mg/dl), GNG: gluconeogenesis, GLY: glycogenolysis

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Author	Subjects	Outcomes	Methods	Insulin (infusion rate and/or concentrations)	Main findings
Basu et al. 2005	10 lean vs. 10 obese nondiabetic vs. 11 obese T2DM (age=59 y)	EGP, GNG and GLY	Clamp and [³ H]- glucose & deuterated water	140 pmol/L	Higher EGP (5x), GNG (3x) and GLY (2x) in T2DM > obese > lean
Bogardus et al. 1984	15 obese T2DM (age=32 y) and 19 younger (25 y) controls	EGP	Clamp (2 steps) and [3- ³ H]-glucose	1^{st} step insulin: 40 mU/ m ² .min (135 μ U/mL) 2^{nd} step insulin: 400 mU/ m ² .min (1738 μ U/mL)	EGP completely suppressed only with high insulin
Campbell et al. 1988	14 T2DM (age=54 y) vs. 14 controls	EGP	Clamp (sequential infusion of insulin at 3 consecutive rates) and [³ H]-glucose	0.4, 1.0, and 10 mU/kg.min (48, 104, and 2,011 μU/mL,)	Higher EGP in T2DM in the 2 first steps (20-40%) but was suppressed totally in the 3 rd step
DeFronzo et al. 1985	9 T2DM (age=52 y) vs. 10 controls	EGP	Clamp and [³ H]- glucose	40 mU/m ² .min (108 µU/mL)	EGP slightly greater (0.35 vs. 0.16 mg/kg.min)

Table 2.4. Studies on post-insulin EGP in T2DM*

Author	Subjects	Outcomes	Methods	Insulin (infusion rate and/or concentrations)	Main findings
Del Prato et al. 1993	7 lean T2DM (age=56 y) vs. 7 controls	EGP	Clamp (T2DM&Controls) and [³ H]glucose Additional Hyperinsulinemic hyperglycemic (15 mmol/L) clamp for T2DM	20 mU/m ² .min(~260 pmol/L)	EGP higher than controls in both clamps
Firth et al. 1987	14 obese T2DM (age=56 y) vs. 10 obese controls 6 lean T2DM (age=61 y) vs. 5 lean controls	EGP	Clamp (sequential infusion of insulin at 3 consecutive rates)and [³ H]- glucose	0.4, 1.0, and 10.0 mU/kg.min (51, 106, & 2,293 μU/ml)	EGP higher in obese (p<0.001) and lean (p<0.03) T2DM vs. controls in first 2 steps. EGP suppressed similarly in 3 rd step
Groop et al. 1989	9 lean T2DM (age=61 y) vs. 8 controls	EGP	Clamp (5 steps on 2 days) and [³ H]-glucose	Day1: 4, 20, and 100 mU/m ² .min (22, 37, and 208 µU/mL); Day2: 10 and 40 mU/m ² .min (10 and 75 µU/ml)	↑EGP in T2DM during all clamp steps (p<0.05-0.01) except the highest insulin EGP was fully suppressed

Author	Subjects	Outcomes	Methods	Insulin (infusion rate and/or concentrations)	Main findings
Meyer et al. 2004	10 obese T2DM (age=52 y) and 10 controls	EGP	75 g oral glucose using [³ H]-glucose infusion and [6,6 ² H2]-glucose enriched in oral glucose	Peak insulin: 166 (T2DM) < 325 pmol/L (controls)	AUC: total (2x), renal (2x) and hepatic EGP (3x) were higher in T2DM
Rooney et al. 1993	8 hyperglycemic lean T2DM (age=54 y) vs. 8 matched controls (M&F)	EGP and hepatic glucose/ glucose 6 phosphate cycling	Clamp (2-step insulin infusion) and [2- ³ H] and [6- ³ H]glucose	0.4 and 2.0 mU/kg/min	0.4mU: higher EGP in T2DM (10.1 vs 5.2 μmol/kg.min) but same G/G6-P cycle 2mU insulin: EGP suppressed same in both but higher G/G6-P cycle (4x)
Pigon et al. 1996	7 lean T2DM (age=46 y) vs. 10 controls	EGP	Clamp (2 steps_[6- ³ H]-glucose	0.25 then 1.0 mU/kg.min (169 then 520 pmol/L)	-EGP did not differ at 2 levels of insulin
Staehr et al. 2001	10 overweight T2DM (age=54 y) vs. 10 controls	EGP	Clamp (2 steps) and [3- ³ H]-glucose	20 or 40 mU/ m ² .min	EGP higher (45%) in T2DM at 20 but not at 40 mU/ m ² .min

Author	Subjects	Outcomes	Methods	Insulin (infusion rate and/or concentrations)	Main findings
Turk et al. 1995	7 overweight T2DM (age=53 y) vs. 7 controls	EGP and GNG	Clamp and [6- ³ H]- glucose and ¹⁴ CO ₂ FPG of T2DM normalized to 5 mmol/L by an overnight low-dose insulin infusion	0.5 or 1 mU/kg.min (300 or 600 pmol/L)	 -No difference in EGP between groups in both clamps - Higher GNG (4-6x) in T2DM
Vaag et al. 1995	10 overweight T2DM (age=62 y) vs. 10 controls	EGP	Clamp: 120 to 150 min after the withdrawal of a prior i.v. insulin infusion to normalize glycemia in T2DM	40 mU/m ² .min (80 μU/mL)	No difference in EGP between groups
Woerle et al, 2005	26 obese T2DM (age=53 y) vs. controls	EGP, GNG and GLY	Mixed meal and [3- ³ H]glucose, [¹⁴ C]bicarbonate and oral [6,6-dideutero] glucose	Peak insulin: 179 (T2DM) < 290 pmol (controls)	-EGP and GLY decreased from baseline but remained 2x higher in T2DM -Higher GNG in T2DM

*Unless specified otherwise, Clamp stands for hyperinsulinemic euglycemic clamp. Subjects in all studies were from both sexes and controls were age, sex and BMI matched with T2DM subjects. Mean values are shown.FPG: fasting plasma glucose, GNG: gluconeogenesis, GLY: glycogenolysis, insulin: 1mU/L=6 pmol/L, AUC: area under the curve

2.2.2. Peripheral insulin resistance

Glucose transport proteins GLUT1 to 4 have been identified on cell membranes with some only expressed in specific tissues. GLUT1 through 3 are sensitive to increasing concentrations of plasma glucose above normal fasting values (such as in fasting T2DM or postprandially). GLUT4, however, is an insulin sensitive glucose transporter expressed only in the periphery, namely in skeletal muscle and fat tissues and contributes to most of the glucose uptake by these tissues. In response to hyperinsulinemia, GLUT4 is translocated from vesicles inside the cell to the cell membrane. It is estimated that 90% of the infused glucose during hyperinsulinemic euglycemic clamp is disposed of by peripheral tissues in the healthy individual (DeFronzo *et al.*, 1985).

In hyperglycemic T2DM, fasting whole-body rate of glucose disposal (R_d) is usually elevated compared with matched controls (Campbell *et al.*, 1988, Ciaraldi *et al.*, 2005, DeFronzo *et al.*, 1985, Firth *et al.*, 1987, Groop *et al.*, 1989a, Meyer *et al.*, 2004, Thorburn *et al.*, 1990, Vaag *et al.*, 1995) (Table 2.5). This is due to concurrent hyperglycemia and so most of the glucose uptake is non-insulin mediated. Studies in which T2DM was well controlled (Pigon *et al.*, 1996) or glycemia was normalized by overnight insulin infusion (Staehr *et al.*, 2001), postabsorptive R_d was lower than that of controls despite hyperinsulinemia (Table 2.5). In one study, recently diagnosed lean or obese T2DM with mean FPG <15 mmol/L had fasting R_d not different from lean or obese controls (Hother-Nielsen and Beck-Nielsen, 1991). When peripheral skeletal muscle glucose R_d was

estimated from leg glucose uptake, it was found to be the same (Kelley *et al.*, 1992, Williams *et al.*, 2001) higher (DeFronzo *et al.*, 1985) or lower (Ciaraldi *et al.*, 2005) compared with sex-, age- and BMI-matched healthy controls.

During hyperinsulinemic euglycemic clamps (Table 2.5), whole-body glucose R_d has been consistently reported to be lower in T2DM vs. controls at low (Campbell et al., 1988, Del Prato et al., 1993, Firth et al., 1987, Pigon et al., 1996, Staehr et al., 2001), peak postprandial-like (Bonadonna et al., 1993, Campbell et al., 1988, Ciaraldi et al., 2005, DeFronzo et al., 1985, Firth et al., 1987, Groop et al., 1989a, Pigon et al., 1996, Staehr et al., 2001, Vaag et al., 1995) and supraphysiological insulin concentrations (Campbell et al., 1988, Firth et al., 1987, Groop et al., 1989a, Hother-Nielsen and Beck-Nielsen, 1991). Only when insulin was clamped at concentrations three times higher (1000 vs. 309 pmol/L) than those of controls during an euglycemic clamp (Thorburn et al., 1990), did T2DM subjects have similar whole-body glucose R_d. This is another manifestation of insulin resistance of glucose in T2DM since higher hormone concentrations were needed to induce a normal "biological response". Only one study (Groop et al., 1989a), surprisingly reported at 20 mU insulin/m².min higher glucose R_d in 9 lean hyperglycemic T2DM compared with 8 weight-matched nondiabetic controls.

Since most of the infused glucose is disposed of by peripheral tissues during hyperinsulinemic euglycemic clamps, these are thus the main organs responsible for the blunted whole-body glucose uptake in T2DM. This was demonstrated in studies where leg and forearm glucose uptake was directly assessed using arteriovenous catheterization and glucose tracers or dynamic positron emission tomography (PET) imaging techniques (Table 2.5). Leg (DeFronzo *et al.*, 1985, Williams and Kelley, 2000) and forearm (Bonadonna *et al.*, 1996, Bonadonna *et al.*, 1993) glucose uptake was lower in T2DM lean and obese subjects during euglycemic clamps with postprandial (500-600 pmol/L) insulin concentrations. Kelley et al. (1993) found similar leg glucose uptake in lean T2DM as in controls, which contradicted their previous results of lower leg R_d in obese T2DM (Kelley *et al.*, 1992), suggesting an interaction with body composition.

When glucose is transported inside the cell, it undergoes oxidative or nonoxidative disposal. The latter can be either glycogen synthesis or non-oxidative glycolysis (Del Prato *et al.*, 1993, Kelley *et al.*, 1993, Vaag *et al.*, 1995, Young *et al.*, 1988) during which glucose is converted to pyruvate and then lactate without being oxidized in the Krebs cycle. During hyperinsulinemic clamps, glycogen synthesis is by far the larger contributor to overall non-oxidative glucose disposal (Pratipanawatr *et al.*, 2002, Shulman *et al.*, 1990b). In the fasting state, nonoxidative disposal was elevated (Groop *et al.*, 1989b, Thorburn *et al.*, 1990) and oxidative disposal higher (Golay *et al.*, 1988), similar (Groop *et al.*, 1989b), or lower (Thorburn *et al.*, 1990) in T2DM compared with controls. During the hyperinsulinemic euglycemic clamp, oxidative glucose disposal is lower (Butler *et al.*, 1990, Del Prato *et al.*, 1993, Golay *et al.*, 1988, Vaag *et al.*, 1995) or similar (Yokoyama *et al.*, 2006), whereas non-oxidative glucose disposal is lower (Butler *et al.*, 1990, Golay *et al.*, 1988, Vaag *et al.*, 1995), as is glycogen synthesis (Del Prato *et al.*, 1993, Vaag *et al.*, 1995), yet non-oxidative glycolysis is higher in T2DM vs. healthy controls (Del Prato *et al.*, 1993, Vaag *et al.*, 1995). While both oxidative (~25%) and non-oxidative glucose disposal (~31%) were lower in T2DM at insulin infusion rates of 40 and 100 mU/m²·min, no differences were found at 20 mU/m²·min (Groop *et al.*, 1989b). Kelley et al. (1992) measured vastus lateralis leg muscle pyruvate dehydrogenase and glycogen synthase activities, enzymes implicated in the glucose oxidation and glycogen synthesis pathways respectively. Both were lower in T2DM subjects vs. controls at baseline (~63%) and during the clamp (~77%), consistent with the lower glucose oxidation and glycogen synthesis data.

Of note is that during the above hyperinsulinemic euglycemic clamps, plasma glucose of T2DM subjects is lowered from hyperglycemic concentrations to euglycemia (5-5.5 mmol/L) to reproduce conditions comparable to controls. However, in clamps where glycemia in T2DM was increased (Thorburn *et al.*, 1990) or maintained at fasting hyperglycemic (isoglycemic) concentrations (Vaag *et al.*, 1992a) vs. euglycemia in healthy controls, glucose R_d was not different between groups. Similar findings were obtained in meal studies during which postprandial glucose concentrations were higher than controls (Meyer *et al.*, 2004, Woerle *et al.*, 2006). 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 R_d is corrected for glycemia, metabolic clearance rate of glucose (R_d divided by glucose concentrations) was

lower in T2DM (Meyer *et al.*, 2004, Woerle *et al.*, 2006). Consistent with this, when both T2DM and control subjects were studied using hyperinsulinemic hyperglycemic clamps of comparable insulin and glucose concentrations (approximately 10 mmol/L), T2DM subjects had lower glucose R_d (Shulman *et al.*, 1990b).

Author	Subjects	FPG of T2DM	Outcomes	Methods	Main findings in T2DM
Bonadonna et al. 1993	6 lean T2DM (age=49 y) vs. 5 controls	10.9 mmol/L	Clamp R _d and forearm glucose uptake	 Overnight intravenous infusion of insulin to normalize glycemia Clamp insulin 240 pmol/min.m² (500 pmol/L) Catheterization balance technique using radioactive 3- O-methyl-D-glucose 	Lower whole body R _d (67%) and forearm glucose uptake (84%)
Bonadonna et al. 1996	5 lean T2DM and 7 controls		Rate of transmembrane influx and intracellular phosphorylation of D-Glucose in the forearm muscle during clamp	-Clamp insulin (500 pmol/L) -brachial artery and deep forearm vein catheterization -experiment repeated with hyperinsulinemic hyperglycemic clamp in T2DM only to mimic controls R _d	 -During euglycemic clamp lower rate of influx (41%) and phosphorylation (86%) into forearm tissues - During hyperglycemic clamp, rate of influx similar to controls (during euglycemic clamp) but 60% lower phosphorylation
Butler et al. 1990	7 obese T2DM vs. 7 controls		Clamp glucose oxidation (low and high insulin)	-Substrate oxidation -Clamp insulin low (~270 pmol/L) high (~ 17 μmol/L)	-Glucose oxidation and storage lower at both concentrations -Intracellularly derived glucose oxidation similar to controls

Table 2.5. Studies on postabsorptive and post-insulin glucose uptake in T2DM*

Author	Subjects	FPG of T2DM	Outcomes	Methods	Main findings in T2DM
Campbell et al. 1988	14 overweight T2DM (age=54 y) vs. 14 controls	10.9 mmol/L	R _d , postabsorptive and during clamp with sequential infusion of insulin	-[³ H]-glucose -Clamp insulin: 0.4, 1.0, and 10 mU/kg.min (48, 104, and 2,011µU/mL)	-Postabsorptive $R_d > \text{controls (31\%)}$ -Clamp $R_d < \text{controls at all levels of}$ insulin (23-40%)
Ciaraldi et al. 2005	14 obese T2DM (age=52 y) vs. 16 controls	12.8 mmol/L	-R _d postabsorptive and during clamp -Basal muscle glucose leg uptake -GLUT1 and 4 quantitation and gene expression	-[3- ³ H]-glucose -Clamp insulin: 720 pmol/mL.min -Arteriovenous catheterization limb balance -Muscle biopsies (vastus lateralis)	-Postabsorptive $R_d > \text{controls} (23\%)$ -clamp R_d in < controls (~45%) -Basal leg glucose uptake in T2DM 40%< controls - lower GLUT1 but same GLUT 4
Del Prato et al. 1993	7 lean T2DM (age=56 y) vs. 7 controls	10 mmol/L	Clamp R _d	Insulin: 20 mU/m ² .min (~260 pmol/L)	- R _d 54% lower
DeFronzo et al. 1985	9 normal weight T2DM (age=52 y) vs. 10 controls	158 mg/dl (8.8 mmol/L)	-R _d postabsorptive and during clamp -Basal and clamp glucose leg muscle uptake	[³ H]-glucose Insulin; 40mU/m ² .min (108 μU/mL) Arteriovenous catheterization	 - R_d slightly higher at baseline and 38% lower during clamp -leg glucose uptake higher at baseline but 45%lower during clamp
Golay et al. 1988	16 lean T2DM (age=54 y) vs. 11 controls	8.4 mmol/L	Substrate oxidation, postabsorptive and during clamp	Indirect calorimetry Clamp insulin: 40 mU/m ² min (100 mU/L)	-Glucose oxidation 42% lower at baseline and 34% lower during clamp -50% lower Clamp rate of non- oxidative glucose disposal

Author	Subjects	FPG of T2DM	Outcomes	Methods	Main findings in T2DM
Groop et al. 1989	9 lean T2DM (age=61 y) vs. 8 controls	172 mg/dl (9.6 mmol/L)	R _d postabsorptive and during clamp (3 rates of insulin infusion on 2 days)	- [3 H]-glucose - Clamp insulin Day1: 4, 20, and 100 mU/m ² .min (10± 1, 75±5, and 208± 16 µU/mL) Day2: 10 and 40 mU/m ² . min (22±2, 37±4 µU/mL)	- Postabsorptive $R_d > controls$ (17%) -No difference in R_d at 2 lower insulin conc. -At the two highest insulin infusion steps R_d lower by 30% -At 20 mU/m ² .min: R_d in T2DM > controls
Firth et al. 1987	14 obese T2DM (age=56 y) vs. 10 controls 6 lean T2DM (age=61 y) vs. 5 lean controls	Obese: 188 mg/dl (10.4 mmol/L)Lean: 202 mg/dl (11.2 mmol/L)	R _d postabsorptive and during clamp (sequential infusion of insulin)	Insulin: 0.4, 1.0, and 10.0 mU/kg.min (51, 106, & 2,293 µU/ml)	- Postabsorptive R_d in T2DM lean (1.5x) and obese (50%) > controls - Clamp R_d in T2DM lean (p<0.02) and obese (p<0.001) < controls (all insulin infusion rate)
Hother- Nielsen & Beck- Nielsen.1991	12 obese T2DM vs. 10 lean T2DM vs. matched controls	Lean and obese <15 mmol/L	R_d postabsorptive and during clamp	Supraphysiological insulin concentrations	-Baseline R _d not different between groups (both lean & obese) -Clamp R _d lower in T2DM

Author	Subjects	FPG of T2DM	Outcomes	Methods	Main findings in T2DM
Kelley et al. 1992	8 moderately obese T2DM men vs. 8 controls		Postabsorptive and clamp leg glucose uptake, oxidation and storage glycogen synthase and pyruvate dehydrogenase activities	-Overnight insulin to normalize glucose in T2DM. -Leg arteriovenous balance study to determine [3- ³ H] glucose uptake and O ₂ and CO ₂ Clamp insulin: 40 mU/m ² min (500-600 pM) Muscle biopsies at baseline & clamp	 Lower clamp leg glucose uptake (40%), oxidation (53%), and storage (77%) lower basal and clamp pyruvate dehydrogenase (63%) and glycogen synthase (81%) activities Clamp nonoxidative glycolysis similar in both groups
Kelley et al 1993	10 lean T2DM and 10 controls		Same as above study	Same as above study	-Similar Leg glucose uptake, oxidation, and storage lower in both groups -Similar glycogen synthase activity -Lower non oxidized glycolysis
Meyer et al. 2004	10 obese T2DM (age=52 y) and controls	9.9 mmol/L	-Fasting and Postprandial R _d -Renal and forearm glucose turnover	75 g oral glucose using [³ H]- glucose infusion and [6,6 ² H2]- glucose enriched in oral glucose renal and forearm vein catheterization and p- aminohippuric acid clearance technique	-Fasting R_d 76% higher in T2DM -Over the 4-½ hour postprandial period, R_d 18%> in T2DM bur MCR 43% lower Fasting and postprandial renal glucose uptake was higher in T2DM (2x) but muscle glucose uptake was same
Author	Subjects	FPG of T2DM	Outcomes	Methods	Main findings in T2DM
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Pigon et al. 1996	7 mildly lean T2DM (age=46 y) vs. 10 controls	7 mmol/L	Clamp R _d (2 step clamp)	[6^{-3} H]-glucose Insulin: 0.25 then 1.0 mU/kg BW.min (169 then 520 μ U/mL)	- R _d 25-30% lower in T2DM
Thorburn et al. 1990	8 lean T2DM (age=59 y) vs. 8 controls	9.9 mmol/L	R _d postabsorptive and during clamp. (3 types) Glycogen synthase activity at baseline and during clamps	[³ H]-Glucose - Controls: insulin (150 pmol/ml.min) and glucose at 5 mmol/L -T2DM: 2 clamps G: insulin at 150 pmol/ml.min (309 pmol/L) and glucose at 15-30 mmol/L I: insulin at 450 pmol/ml.min (1000 pmol/L) and glucose at 5m muscle biopsies	 Postabsorptive R_d > controls R_d same as controls during G and I Glucose Oxidationlower by 26- 28% at baseline and during clamps Basal non-Oxidative glucose 3x higher at basal and 63% higher during clamps Glycogen synthase 60% lower at baseline and 41% lower during G clamp, but same as controls during I clamp
Vaag et al. 1992a	12 obese T2DM (age=56 y) vs. 12 lean controls	10.7 mmol/L	R _d , postabsorptive and during isoglycemic clamp	- [3- ³ H]-glucose Insulin: 40 mU. m ² . min	-Fasting and clamp R_d , glucose oxidation and storage similar in both groups

Author	Subjects	FPG of T2DM	Outcomes	Methods	Main findings in T2DM
Vaag et al. 1995	10 overweight T2DM (age=62 y) vs. 10 controls	10.9 mmol/L	R _d , postabsorptive and during clamp	Insulin: 40mU/m ² .min (80µU/mL) i.v. insulin infusion to normalize glycemia in T2DM before clamp	-Fasting R _d 26% higher at baseline -Clamp R _d 2x lower -Lower baseline and clamp glucose oxidation and storage but non oxidative glycolysis suppressed less
Williams et al. 2001	13 obese T2DM (age=50 y) vs. 14 lean vs. 15 obese controls	9.5 mmol/L	Transport of and phosphorylation of glucose across leg, postabsorptve and during clamp (3 insulin infusions)	-Dynamic positron emission tomography (PET) imaging of 2-deoxy-2[1 ⁸ F]fluoro-Dglucose Arteriovenous leg glucose uptake of [2- ³ H] glucose Insulin 0, 40, or 120 mU/m ² . min (92, 587, or 2097 pmol/L)	-Same basal leg glucose uptake across groups. During clamp -Low leg glucose transport (25- 36%) - Low leg glucose phosphorylation
Woerle et al, 2005	26 obese T2DM (age=53 y) and 15 controls	12 mmol/L	Postmeal R _d	[3- ³ H]glucose and indirect calorimetry	-Whole body R _d and glucose storage was same in 2 groups but MCR was lower in T2DM (p<0.001) -Glucose oxidation (p<0.004) was lower but non-oxidative glycolysis was higher in T2DM

*Unless specified otherwise, Clamp stands for hyperinsulinemic euglycemic clamp. Subjects in all studies were from both sexes and controls were age, sex and BMI matched with T2DM subjects. Mean values are shown. FPG: fasting plasma glucose, MCR: metabolic clearance rate, R_d : Glucose disposal, Insulin: 1mU/L=6 pmol/L, FPG: 1 mmol/L=18.016 mg/dl, BW: body weight.

2.2.2.1. Molecular mechanisms of peripheral insulin resistance

At the molecular level, insulin-mediated glucose disposal in the periphery (adipose tissue and skeletal muscle) is initiated by the binding of insulin to its transmembrane receptor. This causes tyrosine autophosphorylation of the receptor β -subunit on the interior surface and activation of its intrinsic tyrosine kinase. The latter phosphorylates several intracellular proteins, including the insulin receptor substrates (IRS) that provide docking sites for p85, the regulatory subunit of type I phosphatidylinositol 3-kinase (PI3K). Activation of the catalytic p110 subunit of PI3K generates the lipid byproduct phosphatidylinositol 3,4,5-trisphosphate (PIP3). Activated PIP3 induces a phosphorylation cascade involving stimulation of a 3-phosphoinositide- dependent kinase-1 (PDK-1). Two potential PI3K/PDK-1 downstream targets are the protein kinase B/Akt (PKB) family of proteins and the atypical protein kinase C family members. These get phosphorylated on Thr410 and Thr308, respectively. Full activation of Akt further requires phosphorylation on Ser473 by the putative PDK-2. Akt substrate TBC1D4 is especially associated with insulin-induced GLUT4 translocation (Hoehn et al., 2008). It is important to note that there exist Akt-independent mechanisms through which insulin increases GLUT4 translocation and mediates glucose transport (Farese *et al.*, 2007). Further investigation of the exact pathways is still required (Elmendorf, 2002, Gonzalez and McGraw, 2006).

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 and Mari, 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 and Kolterman, 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 and Kolterman, 1981, Olefsky et al., 1982). 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). The different metabolic abnormalities (hyperinsulinemia, hyperglycemia and nutrition overload, dyslipidemia, inflammation etc.) observed in T2DM induce defects at different levels of the insulin signal transduction pathway (Hoehn et al., 2008, Martyn et al., 2008, Muoio and Newgard, 2008a, Muoio and Newgard, 2008b, Petersen and Shulman, 2006). A large body of evidence has focused on IRS proteins as the major defect in the cascade. IRS serine/threonine phosphorylation, tyrosine de-phosphorylation and degradation have been thought to cause its deactivation and subsequent decreased PI3K associated activity (Hoehn *et al.*, 2008). This ultimately leads to diminished translocation of GLUT4 to the cell membrane of skeletal muscle and adipose tissue in response to insulin (Kahn, 1996, Kotani *et al.*, 2004). However, IRS-1 independent defects in the insulin transduction pathway contributing to insulin resistance have also been identified (Hoehn *et al.*, 2008).

2.2.3. Effect of glucose, fatty acids and amino acids on glucose disposal

2.2.3.1. Effect of glucose and fatty acids

Hyperglycemia contributes itself to insulin resistance. Using animal models of T2DM, chronic hyperglycemia was shown to blunt glucose disposal rate and this was attributed to a defect in intracellular glucose phosphorylation resulting in decreased glucose transport. Such "glucotoxicity" also impedes insulin-mediated glucose transport by altering the function of the glucose transporter, GLUT4 (Rossetti 1995). Acute normalization of glycemia by insulin infusion improves hepatic insulin resistance by decreasing fasting and postinsulin EGP in some studies (Boden *et al.*, 2001b, Jeng *et al.*, 1994, Vaag *et al.*, 1995) but not others (Staehr *et al.*, 2001, Turk *et al.*, 1995). Peripheral insulin resistance does not appear, however, to be improved by short term normalization of control.

Chronic normoglycemia, however, achieved with oral antidiabetic agents (metformin, TZD or sulfonylurea) (Ciaraldi *et al.*, 2002, Pratipanawatr *et al.*,

2002, Vestergaard *et al.*, 1995) or lifestyle modification (diet and exercise) (Kirwan *et al.*, 2009) has been associated with improved insulin stimulated glucose R_d and better suppression of EGP.

Fatty acids are usually elevated in T2DM in the postabsorptive state (Del Prato et al., 1993, Meyer et al., 2002, Nurjhan et al., 1992, Rooney et al., 1993), and in response to hyperinsulinemia (Bavenholm et al., 2003, Del Prato et al., 1993, Golay et al., 1988, Groop et al., 1989a, Groop et al., 1991, Rooney et al., 1993). This can be interpreted as index of insulin resistance of lipid metabolism, as insulin fails to fully suppress lipolysis (Kumar and O'Rahilly, 2005, Lewis et al., 2002). It was suggested by Randle et al. (1963) 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 and Mandarino, 2000, 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., 1989b). Nevertheless, the glucose fatty acid cycle cannot totally describe the abnormalities associated with FFA-induced insulin resistance (Boden, 1997, Boden and Chen, 1995, Boden et al., 1991, Kelley and Mandarino, 2000). 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 and Shulman, 2002, Lewis et al., 2002).

Moreover, triglycerides are elevated in T2DM muscle (22% higher vs. healthy controls) (Goodpaster and Kelley, 2002, Kelley, 2002, Kelley and Mandarino, 2000), possibly due to a diminished ability of the mitochondria to oxidize fats (Goodpaster and Kelley, 2002, Petersen and 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 (r = 0.57; p< 0.001) and during insulin-stimulated states (r = 0.61; p< 0.001) (Basu *et al.*, 2005). Based on positive correlations between EGP and lipid oxidation, Groop et al. (1989b) 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)

2.2.3.2. Effect of amino acids

In cross sectional (Linn *et al.*, 2000, Skilton *et al.*, 2008, Wolever *et al.*, 1997) and longitudinal (Liese *et al.*, 2009, Schulze *et al.*, 2003, Song *et al.*, 2004) studies, high protein consumption was associated with increased risk of insulin resistance and T2DM. This was partly attributed to amino acid induced blunting of glucose uptake that was demonstrated *in vitro* (Traxinger and Marshall, 1989, Tremblay *et al.*, 2005a, Tremblay and Marette, 2001), and in healthy humans at

the whole body (Adegoke et al., 2009, Krebs et al., 2007, Krebs et al., 2002, Pisters et al., 1991, Tremblay et al., 2005b) and tissue levels (forearm muscle) (Pisters et al., 1991, Schwenk and Haymond, 1987). Hyperinsulinemic euglycemic clamps were performed in healthy individuals, during which insulin was raised to peak postprandial levels (~400 pmol/L) and were compared in a cross over design to hyperinsulinemic euglycemic clamps with amino acids infused to raise the concentrations by 2.1 fold. (Flakoll et al., 1992, Krebs et al., 2007, Krebs et al., 2002, Tremblay et al., 2005b). This resulted in a 25-33% decrease in whole-body clamp glucose R_d during the latter clamp (Krebs et al., 2007, Krebs et al., 2002, Tremblay et al., 2005b) and was confirmed by a lower uptake at the periphery with 33% less forearm glucose uptake (Flakoll et al., 1992). The same results were reported with supraphysiological clamp insulin concentrations (10 mU/kg.min) (Flakoll et al., 1992) or when amino acids were infused to maintain fasting concentrations (vs. no infusion) (Pisters *et al.*, 1991). Furthermore Adegoke et al. (2009) observed that raising glucose and amino acids to postprandial levels during hyperinsulinemic clamps in healthy men ("fed clamp") resulted in a 40% lower glucose metabolic clearance rate (glucose R_d divided by glycemia) vs. hyperinsulinemic euglycemic isoaminoacidemic clamps.

At the cellular level, the proposed mechanism was not due to substrate competition for oxidation between amino acids and glucose at the level of pyruvate dehydrogenase, but rather to blunted insulin signaling and glucose transport (Krebs *et al.*, 2002). Indeed, amino acid (leucine in specific) induced phophorylation of mTORC1 (Krebs *et al.*, 2007) and its substrate ribosomal

protein S6K-1 (Um *et al.*, 2006) causes a negative feedback phosphorylation of certain serine residues on IRS-1 (Tremblay *et al.*, 2007a, Tremblay *et al.*, 2005b). This, in turn, reduces IRS-1 associated PI3-kinase activity (Patti *et al.*, 1998, Tremblay and Marette, 2001), which further deactivates downstream components of the phosphorylation cascade. As a result GLUT4 translocation to the cell membrane is diminished and glucose uptake is decreased. Some of the common IRS-1 serine residues identified in humans, whose inhibitory phosphorylation was associated with mTOR/S6K1 activation, are Ser-636/639, Ser-312 and Ser-1101. Findings from the above studies have been extrapolated to insulin resistant states, including T2DM. Thus it was proposed that postprandial aminoacidemia can aggravate the insulin resistance of glucose metabolism in T2DM. However, there is no study that examined these speculations in T2DM either at the whole body or cellular level.

2.3. Insulin resistance of protein metabolism in type 2 diabetes

2.3.1. Effect of insulin on protein metabolism

In addition to its well-established role in regulating glucose and fat metabolism (Ganong 2001), insulin is also known to modulate protein metabolism. In a myriad of *in vitro* studies, insulin stimulated protein synthesis and suppressed protein breakdown in skeletal and smooth muscle as well as other body organs (Jefferson *et al.*, 1972, Nakano and Hara, 1979, Tessari, 1994, Tischler *et al.*, 1982). In situations of insulin deficiency, like in type 1 diabetes mellitus (T1DM), subjects had increased protein turnover (elevated breakdown,

synthesis and oxidation) compared to controls, in the fasting state (Nair *et al.*, 1987, Nair *et al.*, 1983, Robert *et al.*, 1985, Umpleby *et al.*, 1986) and failed to suppress nitrogen excretion to the same extent as controls following a protein free diet (Lariviere *et al.*, 1992). These abnormalities in protein metabolism in T1DM were restored to normal with insulin therapy (Lariviere *et al.*, 1992, Nair *et al.*, 1987, Umpleby *et al.*, 1986).

In order to examine the effect of insulin on protein metabolism in vivo, hyperinsulinemic euglycemic clamps and amino acid tracers are used to assess whole body or regional protein kinetics in healthy individuals. Using this technique, insulin suppressed protein breakdown with no effect on synthesis (Castellino et al., 1987, Flakoll et al., 1989, Frexes-Steed et al., 1990, Tessari et al., 1986). Only one study using the same protocol reported a stimulatory effect of insulin on protein synthesis in skeletal muscles (Biolo et al., 1995) and others even found a decrease in synthesis vs. baseline (Frexes-Steed *et al.*, 1990). These were attributed to the concurrent decrease in the concentration of free amino acids below fasting levels (Fukagawa et al., 1986) due to suppression of protein breakdown by insulin and thus setting a non-physiological limit of amino acid availability for synthesis (Tessari et al., 1986). Therefore, to identify a potential independent effect of insulin in modulating amino acid incorporation into proteins and prevent the confounding drop in amino acids, these should be administered concomitantly to maintain baseline concentrations (isoaminoacedemia). Indeed, a hyperinsulinemic euglycemic isoaminoacidemic clamp enhanced whole-body

(Chevalier *et al.*, 2004, Chevalier *et al.*, 2005a) and skeletal muscle (Bennet *et al.*, 1990, Hillier *et al.*, 2000) protein synthesis.

2.3.2. Independent and added effect of amino acids on protein metabolism

Following a meal, one cannot distinguish between the effects of insulin on protein metabolism from that of the concomitant rise in amino acids. *In vitro* (Buse and Reid, 1975) and *in vivo* (Anthony *et al.*, 1999) studies have identified an independent effect of the BCAA leucine on increasing protein synthesis with and without elevated insulin. The "pancreatic" clamp technique was also used in which somatostatin was infused to maintain insulin at postabsorptive levels and amino acids were increased. Protein Q, S, O and S-B increased while B decreased (Castellino *et al.*, 1987, Tessari *et al.*, 1987). Clamping insulin, glucose and amino acids at peak postprandial concentrations in healthy subjects (simulating the fed state) caused a substantial rise in Q, S, O and S-B and drop in B (Adegoke *et al.*, 2009, Castellino *et al.*, 1987, Tessari *et al.*, 1987) compared to either hyperinsulinemic isoaminoacidemic clamps or hyperaminoacidemia with insulin at baseline postabsorptive levels. This illustrates the synergistic stimulatory effect of insulin and amino acids on protein anabolism

2.3.3. Protein metabolism in type 2 diabetes

T2DM was recognized as "*diabetes mellitus, lipidus et proteinus!*" (Marliss and Gougeon, 2002) since the added effect of insulin resistance and eventual relative or absolute insulin deficiency are anticipated to further impair protein metabolism beyond that due to obesity (Chevalier *et al.*, 2006b, Chevalier

et al., 2005b). Protein metabolism in T2DM has recently gained interest among investigators who assessed whole body and regional protein metabolism using isotopic tracers under different physiological conditions. Amino acid tracers (13 C or 14 C leucine, L-ring 2 H₅-phenylalanine) are infused in the fasting state and during the period of the clamp and kinetics of the corresponding amino acid are markers of whole body or regional Q, B, S and S-B that are calculated at steady state (Bier, 1992). Altered protein metabolism in T2DM, if present, raises concerns about protein restrictions that accompany prescribed energy-restricted diets (such as a vegan diet) to improve glycemic control (Barnard *et al.*, 2006) or to mitigate against renal damage (Locatelli *et al.*, 1991). The following section and Table 2.6 include studies on whole body and muscle protein metabolism in T2DM. Controls in all studies were healthy and matched for sex, age and body weight with the T2DM subjects (mean age rage, 36-68 y).

2.3.3.1. Integrated fed-fasted state studies

Whole body protein kinetics in the integrated fed-fasted state were quantified using the 60h oral ¹⁵N glycine method (Table 2.6). Obese T2DM hyperglycemic men and women (oral antihyperglycemic agents discontinued) were compared to non-diabetic controls matched for BMI (Gougeon *et al.*, 1994). Following an isoenergetic diet, the T2DM hyperglycemic subjects had 31% accelerated N flux (higher Q, S and B) and lower net balance (S-B) (-0.3 vs. 2 gN/d) (Table 2.6). Moreover, unlike the controls, T2DM subjects failed to achieve N equilibrium during 4 weeks of a very low energy diet (N losses were ~3 fold more). This suggests that in T2DM, the protein requirement to maintain N equilibrium might rise more steeply than in nondiabetic persons as energy intake decreases below needs. Improving glycemia with antidiabetic agents (gliclazide \pm metformin) or normalizing it with insulin (150 \pm 13 U/d) for 7 d improved ¹⁵N-glycine kinetics (lower Q, S and B) in treated vs. untreated T2DM (Table 2.6). As a consequence, net anabolism (S-B) increased to levels not different from obese controls, albeit at a persistent higher flux (higher S and B). This was true even after adjusting for body weight (Gougeon *et al.*, 1997a), FFM (Gougeon *et al.*, 1997b), age and sex (Gougeon *et al.*, 2000). Therefore, there is a clear worsening effect of hyperglycemia on 24h whole body protein metabolism, which is improved with glycemic control, but at the expense of higher N flux.

2.3.3.2. Postabsorptive protein turnover

Stable isotope¹³C-leucine or radioactive ¹⁴C-leucine were used to quantify fasting whole body protein metabolism in T2DM after attaining a plasma steady state ¹³C enrichment or ¹⁴C specific activity (Table 2.6). No difference in protein kinetics was found between T2DM women vs. controls (Staten *et al.*, 1986) even after adjusting for body weight (BW) and fat free mass (FFM) (Pereira *et al.*, 2008, Welle and Nair, 1990). Similar results were obtained in men with T2DM (Pereira *et al.*, 2008) and in a heterogeneous sample of T2DM men and women, where Q, S and O expressed per kg BW (Halvatsiotis *et al.*, 2002a, Umpleby *et al.*, 1990) or per m² (Luzi *et al.*, 1993) were not different than controls. Likewise, in studies using labeled ²H₅-phenylalanine to assess postabsorptive whole-body (Biolo *et al.*, 1992) or vastus lateralis muscle (Halvatsiotis *et al.*, 2002b) protein metabolism (Table 2.6), none of the kinetics was altered in a T2DM sample of obese men and women vs. controls. Reasons for failing to detect postabsorptive effects of diabetes include that net protein catabolism is at its maximum physiological rate, or because hyperglycemia was previously corrected by insulin infusion (Halvatsiotis *et al.*, 2002a, Halvatsiotis *et al.*, 2002b, Staten *et al.*, 1986)

2.3.3.3. Protein turnover in response to hyperinsulinemia

Amino acid tracers were also used to quantify protein metabolism in T2DM during a hyperinsulinemic euglycemic clamp (Table 2.6). No difference in changes from baseline kinetics were observed in T2DM men and women vs. controls whether insulin was clamped at 220 pmol/L (Halvatsiotis *et al.*, 2002a), 450 pmol/L (Luzi *et al.*, 1993) or at supra-physiological levels that exceeded 1000 pmol/L (Denne *et al.*, 1995). In all the latter studies, there was an insulin-induced drop in blood amino acid concentrations that might have masked a potential resistance to the action of insulin especially at the level of protein synthesis. Furthermore, other limitations include small sample size and heterogeneous samples (men and women), lack of adjustment for confounders (like FFM and BW), differing degrees of glycemic control, previous dietary protein and total energy intake, and different analytical methods used to determine protein kinetics.

When amino acid levels were maintained at postabsorptive concentrations by a continuous infusion of an amino acid solution during hyperinsulinemic (525605 pmol/L) euglycemic (5.5mmol/L) clamp, T2DM hyperglycemic overweight or obese subjects responded differently according to sex. There was a 69% lesser increase from baseline in Q and no increase in S in male T2DM vs. controls. As a result net anabolism was not different from zero indicating an insulin resistance of protein metabolism. In women there was no worsening effect of T2DM on protein kinetics compared to obese controls. The latter, however, showed marked insulin resistance of protein metabolism vs. lean women (Pereira *et al.*, 2008).

Raising amino acids to postprandial concentrations (2 times over baseline) during a hyperinsulinemic euglycemic clamp improved protein kinetics to levels not different from controls (Luzi *et al.*, 1993). Nevertheless, the sample was small and had a mixture of men and women. Also glycemia was maintained at normal fasting concentrations (5.5 mmol/L) which might mask any impairment associated with hyperglycemia, commonly found in T2DM. Similarly Manders et al. (2008) reported normal postprandial muscle protein synthesis in hyperglycemic T2DM men after the consumption of CHO+protein hydrosylate boluses. However, the total, taken in repeated boluses, was extremely large (268 g CHO and 134 g protein) and it is therefore possible that this played a role in the metabolic outcomes. Further studies are needed to determine whether the protein anabolic response to typical postprandial conditions (hyperinsulinemia, hyperglycemia and hyperaminoacidemia) is altered in men with T2DM.

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Studies	Reference	Subjects	Gender	Methods	Treatment	Protein Turnover Outcome
24 h whole- body protein turnover	Gougeon et al. 1994	7 obese hyperglycemic (age=36 y) T2DM and 7 controls	1 man and 6 women	¹⁵ N glycine kinetics	OHA discontinued.	Protein Q (31%), S (16%)and B (21%) higher, and S-B lower in T2DM
	Gougeon et al. 1997a	7 obese T2DM (age=46 y; FPG=10.6 mmol/L)	3 men and 4 women	¹⁵ N glycine kinetics per BW or LBM	Insulin injections to normalize glycemia	Q (17%), S (18%) & B (24%) decreased and S-B (3.6x) increased in treated vs. untreated T2DM
	Gougeon et al. 1997b	9 obese T2DM and 7 controls (age=48 y FPG= 14.1 mmol/L)	3 men and 6 women	¹⁵ N glycine kinetics per kg FFM	Insulin injections to improve glycemia	S and B higher (30%) but S-B improved to obese levels in treated vs. untreated T2DM

Studies	Reference	Subjects	Gender	Methods	Treatment	Protein Turnover Outcome
	Gougeon et al. 2000	13 obese T2DM (age=51 y; FPG= 11.5 mmol/L) and 7 controls	6 men and 7 women	¹⁵ N glycine kinetics adjusted for FFM, age & sex	Oral antidiabetic agents to improve glycemia	Q (20%), S (22%) & B (24%) higher and S-B (45%) lower in untreated T2DM vs. controls S-B not different from controls in treated T2DM
Post- absorptive protein turnover	Staten et al, 1986	5 obese T2DM and 5 controls	women	L-[1- ¹³ C, ¹⁵ N] leucine kinetics	Insulin 2 d prior to study at typical dosage or to achieve better control	No differences in WB protein kinetics. No effect of insulin therapy
	Welle & Nair, 1990	5 obese hyperglycemic T2DM and 10 controls	women	¹³ C-leucine kinetics per BW and FFM	OHA discontinued 3 wks prior to study	WB B not different
	Umpleby et al, 1990	5 hyperglycemic T2DM (age=50 y), and 5 controls	men and women	¹⁴ C-leucine kinetics per BW	Not clear	WB Q and S normal but O higher in T2DM
	Biolo et al, 1992	8 non-obese T2DM (age=55 y) and 6 controls	men and women	L-[2,6- ³ H] phenylalanine kinetics per BW	Diet therapy	WB B not different

Studies	Reference	Subjects	Gender	Methods	Treatment	Protein Turnover Outcome
	Luzi et al, 1993	6 T2DM (age=64 y), and 5 controls	men and women	¹⁴ C-leucine kinetics per m ²	OHA discontinued 1 wk prior to study	WB Q, S, O and S-B not different among groups
	Denne et al, 1995	6 overweight or obese T2DM (age=41 y) and 10 controls	5 women 1 men	L-[ring- ² H ₅] phenylalanine kinetics per LBM	Treatment discontinued 2weeks prior to study and then insulin for 3-4 weeks to improve fasting glucose	T2DM: WB B 36% greater, leg B 2x lower. No treatment effect
	Halvatsiot is et al, 2002a	5 T2DM, and 8 controls	men and women	¹³ C-leucine kinetics per BW	OHA discontinued 2 or more wks prior to study, but insulin the night before to ensure euglycemia	WB Q not different
	Halvatsiot is et al, 2002b	8 hyperglycemic T2DM (age=56 y), 8 controls of similar BMI and 8 lean controls	4 men and 4 women	L-[1- ¹³ C, ¹⁵ N] leucine kinetics per FFM	OHA discontinued 2 or more wks prior to study, then intensive insulin for 11 d and the night before to ensure euglycemia	25% greater WB Q in untreated T2DM vs. lean but not obese controls No difference in muscle (vastus lateralis) protein synthesis

Studies	Reference	Subjects	Gender	Methods	Treatment	Protein Turnover Outcome
	Pereira et al, 2008	17 hyperglycemic T2DM (age=53 y) and 23 controls	10 men and 7 women	¹³ C leucine kinetics per FFM adjusted for sex	Diabetes medications discontinued 1 week pre-study	No difference in any WB kinetic variable
protein turnover in response to hyperinsulin emia and euglycemia	Halvatsiot is et al, 2002a	5 T2DM, and 8 controls	men and women	¹³ C-leucine kinetics per BW	Same as above. Clamp insulin=220 pmol/L	WB Q not different
	Luzi et al, 1993	6 T2DM (age=64 y), and 5 controls	5 men and 1 women	¹⁴ C-leucine kinetics per m ²	Same as above. Clamp insulin=450 pmol/L	No difference in WB changes from baseline or clamp kinetics
	Denne et al, 1995	6 overweight or obese T2DM (age=41 y) and 10 controls	1 men and 5 women	L-[ring- ² H ₅] phenylalanine kinetics per LBM	Same as above. insulin>1000 pmol/L	No difference in changes from baseline (decline in B) or clamp kinetics
		controls				No suppression vs. 42% suppression of leg B in controls

Studies	Reference	Subjects	Gender	Methods	Treatment	Protein Turnover Outcome
protein turnover in response to hyperinsulin emia euglycemia with added amino acids	Luzi et al. 1993	6 T2DM (age=64 y), and 5 controls	5 men and 1women	¹⁴ C-leucine kinetics per m ²	Same as above. Clamp insulin=450 pmol/L Clamp AA (10% Travasol) 2X basal levels	No difference in WB changes from baseline and from hyperinsulinemic euglycemic clamp kinetics
	Pereira et al. 2008	17 hyperglycemic T2DM (age=53 y) and 23 controls	10 men and 7 women	¹³ C leucine kinetics per FFM, adjusted for sex	Same as above. Clamp insulin=525-605 pmol/L Clamp AA (10% Trophamine) at basal levels	Compared to controls: M: smaller increase from baseline in WB Q (69%) smaller and no increase in WB S (vs. 5% increase in controls). No difference between T2DM F and controls

Studies	Reference	Subjects	Gender	Methods	Treatment	Protein Turnover Outcome
	Manders et al. 2008	10 hyperglycemic T2DM (age=68 y) and 10 controls	men	L-[ring- ² H ₅] phenylalanine and L-[ring- ² H ₂] tyrosine kinetics	Diabetes medications continued through the study. CHO boluses administered during study (total of 268 g).	No differences in WB kinetics and muscle protein synthesis
	Manders et al. 2008	Same as above	Same as above	Same as above	Same as above. CHO+protein hydrosylate boluses administered during study (total of 268 g CHO and 134 g protein).	No differences in WB kinetics and muscle protein synthesis

WB: whole-body

2.4. Cellular mechanisms of protein metabolism

2.4.1. Protein synthesis

The translational efficiency or mRNA translation initiation appears to be the main step of the protein synthesis pathway at the cellular level that is acutely regulated by hormonal and substrate availability. It involves a complex multistep activation of initiation factors (eIFs) (Yoshizawa et al., 1997). Insulin and insulin like growth factor-1 (IGF-1) stimulate protein synthesis by activating Akt through PI3-kinase. This leads to Serine²⁴⁴⁸ phosphorylation of mammalian target of rapamycin complex 1 (mTORC1) and its activation. This takes place through different steps; activated Akt increases the phosphorylation of the tuberous sclerosis complex 2 (TSC) which deactivates the TSC1/2, converting Rheb (Ras homolog enriched in brain) from GDP to GTP bound form (Inoki et al., 2003). The active, GTP-bound form of Rheb directly interacts with mTORC1 to stimulate its activity (Sancak et al., 2007). Akt activation by growth factors can activate mTORC1 in a TSC1/2-independent manner by promoting the phosphorylation and dissociation of PRAS40 from mTORC1 (Sancak et al., 2007). One downstream target of mTORC1 is the phosphorylation of 4E-BP1, which causes dissociation of the complex 4E-BP1-eIF4E. The freed eIF4E binds to eIF4G to form the eIF4F complex, which then binds to mRNA to proceed with translation in the ribosome subunit (Kimball et al., 2002, Kimball et al., 1997, Kimball et al., 1994). Another downstream signaling target of mTOR is the phosphorylation of the ribosomal protein S6 protein kinase (S6K1), which gets

activated and leads to hyperphosphorylation of another ribosomal protein S6, which enhances the translation of a particular class of mRNA. For the mRNA to proceed with translation at the ribosomal subunit, it requires activation of eIF2B, a rate limiting step for protein synthesis (Anthony *et al.*, 2001, Yoshizawa *et al.*, 1997).

Amino acids, especially the essential BCAA leucine, were also shown to independently activate mTOR and promote protein synthesis in skeletal muscles (Drummond and Rasmussen, 2008). The exact mechanisms are still not clear but appear to involve Rag proteins, a family of guanosine triphosphatases that interact with mTORC1 and promote its intracellular perinuclear localization, which favours its activation by Rheb (Sancak et al., 2008). However, mTOR dependent pathway only partially explains the cellular mechanisms involved in leucine mediated increase in skeletal muscle protein synthesis (Kimball et al., 2002). Leucine also stimulates protein synthesis by activating eIF2B (Kimball et al., 1998). The presence of both insulin and amino acids appear to have a synergistic effect on protein synthesis with mTOR being the convergence point of action. In vitro and animal studies have shown the enhanced phosphorylation of 4E-BP1 and S6K1 by insulin and amino acids vs. either one of the two studied alone (Anthony et al., 2001, Baum et al., 2005, Kimball et al., 2002). Also recently, one human in vivo study showed that insulin induced reductions in amino acid concentrations blunted the activation of mTOR/S6K1 signalling in skeletal muscles in healthy and T2DM subjects, emphasizing the need to maintain the availability of amino acids in order to observe an insulin stimulation of protein synthesis (Drummond and Rasmussen, 2008). Similarly, Adegoke et al. (2009) reported marked increased in phosphorylation of Akt, mTOR, S6K1, S6 and 4E-BP1 in healthy men during a clamp simulating the fed state (hyperinsulinemic, hyperglycemic, hyperaminoacidemic).

In T2DM, there was a blunted insulin mediated protein synthesis in skeletal muscles of rats characterized by lower dissociation of 4E-BP1.eIF4E and activation of S6K1 and S6 vs. controls (Anthony *et al.*, 2002). Orally administered leucine restored protein synthesis to normal control levels without increasing 4E-BP1/S6K1 phosphorylation, suggesting an insulin-independent protein synthesis pathway (Anthony *et al.*, 2002). One human study found decreased total and mTOR and eIF2 α phosphorylation in T2DM subjects vs. controls (Drummond and Rasmussen, 2008). However no study has extensively quantified the total and phosphorylated forms of the different intermediates of the protein synthesis pathways in poorly vs. well controlled T2DM with hyperinsulinemia \pm hyperaminoacidemia.

2.5. Protein breakdown

In skeletal muscles, the majority of protein degradation occurs through the activation of the ubiquitin (Ub)-proteasome pathway which is ATP dependent and involves ubiquitin ligases (E3s) that catalyse the ubiquitination of specific proteins triggering their degradation by the 26S proteasome (Goldberg, 2003, Lorite *et al.*, 2001, Mitch and Goldberg, 1996) (Taillandier *et al.*, 1996). This Ub-proteasome system is activated in various catabolic conditions ranging from fasting to surgery and cancer cachexia (Baracos, 2000, Bossola *et al.*, 2001,

Bossola et al., 2003, Hasselgren and Fischer, 2001, Hasselgren et al., 2002, Lecker et al., 1999). Under these conditions, the Ub-proteasome components including Ub conjugating enzyme $E2_{14k}$ that catalyzes the first irreversible reaction in the pathway (Adegoke et al., 2002) (Olasunkami et al, 2002), the E3s Atrogin-1/MAFbx (Gomes et al., 2001) and MURF-1 and proteasome (Baracos et al., 1995) were highly expressed. Insulin and IGF-1 suppress the Ub-proteasome system in cultured myocytes by mTOR mediated blocking of nuclear translocation of FOXO, a transcription factor known to increase the expression of MAFbx and MURF-1 (Latres et al., 2005). Insulin also lowered levels of Ub conjugating enzyme $E2_{14k}$, in the skeletal muscle of fed vs. fasting rats (Wing and Banville, 1994). Moreover, raising the amino acids to postprandial levels during hyperinsulinemic hyperglycemic clamp in men further suppressed proteolysis manifested by a 20% decrease in ubiquitinated proteins compared to the effect of insulin alone (Adegoke et al., 2009). However, no studies have been conducted in humans with poorly or well controlled T2DM to identify any resistance to the action of insulin (with or without hyperaminoacidemia) to suppress muscle breakdown at the cellular levels concurrent with the whole body data.

Bridge 1

Impaired protein anabolism was reported in the 24h integrated fed-fasted state (Gougeon et al., 1994) and in response to insulin in poorly controlled T2DM. The latter was demonstrated in overweight or obese hyperglycemic men compared with matched controls during hyperinsulinemic euglycemic isoaminoacidemic clamp (Pereira et al., 2008). However, it is not clear whether sustained hyperglycemia during the clamp would worsen the insulin resistance of protein metabolism. Furthermore, little is known about the anabolic response to raising both insulin and amino acids to postprandial concentrations in T2DM. Therefore, in the current manuscript, protein kinetics using ¹³C-leucine were measured during hyperinsulinemic hyperglycemic (8 mmol/L) isoaminoacidemic clamp in men with poor glycemic control and compared to kinetics of matched hyperglycemic men who underwent the same protocol but with glycemia lowered to 5.5 mmol/L during the clamp. Kinetics of protein metabolism were also quantified during hyperinsulinemic hyperglycemic hyperaminoacidemic clamp to determine whether the anabolic response to postprandial insulin and amino acids is also impaired. The results of this study have implications with regard to setting protein recommendations in T2DM that are still not firmly established (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008)

CHAPTER 3. MANUSCRIPT 1

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Postprandial hyperaminoacidaemia overcomes insulin resistance of protein anabolism in men with type 2 diabetes

M. Bassil, E.B. Marliss, J.A. Morais, S. Pereira, S. Chevalier and R. Gougeon

McGill University Nutrition and Food Science Centre, Montreal, QC, Canada, H3A 1A1.

Corresponding author: Réjeanne Gougeon

McGill Nutrition and Food Science Centre MUHC/Royal Victoria Hospital 687 Pine Avenue West, H6.61 Montreal, QC H3A 1A1 Canada Tel: (514) 843-1665 Fax: (514) 843-1706 E-mail: rejeanne.gougeon@muhc.mcgill.ca

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3.1. ABSTRACT

Aims/hypothesis Although protein is usually ignored when considering insulin resistance, we have shown resistance of protein concurrent with glucose metabolism in men with type 2 diabetes during a hyperinsulinaemic clamp at euglycaemia and fasting aminoacidaemia. We hypothesized that this resistance is even worse during conditions that simulate the postprandial state, when anabolism should be maximal.

Methods Eight overweight and obese men with type 2 diabetes underwent a hyperinsulinaemic-hyperglycaemic (8 mmol/l) clamp, first with plasma amino acids at postabsorptive (Hyper-2) then at postprandial concentrations (Hyper-3). Whole-body protein kinetics were assessed using L-1[¹³C]-leucine. Hyper-2 results were compared to those of diabetic men whose plasma glucose was lowered to 5.5 mmol/l and fasting aminoacidaemia maintained during the hyperinsulinaemic clamp (Hyper-1)

Results In Hyper-2 vs. Hyper-1 clamps, leucine flux $(2.99\pm0.16 \text{ vs.} 2.62\pm0.06 \text{ }\mu\text{mol} \text{ kg} \text{ [fat-free mass (FFM)^{-1}] min^{-1}}\text{)}$, rates of synthesis $(2.31\pm0.15 \text{ vs.} 1.98\pm0.06)$ and breakdown $(2.38\pm0.16 \text{ vs.} 2.00\pm0.07)$ were higher (p<0.05), but leucine oxidation and net balance did not differ. In Hyper-3 vs. Hyper-2 clamps, leucine flux and synthesis and oxidation rates increased markedly as did net balance $(0.84\pm0.09 \text{ vs.} -0.07\pm0.04 \text{ }\mu\text{mol} \text{ [kg FFM]^{-1} min^{-1}}, p<0.0001)$.

Conclusions/Interpretations In type 2 diabetic men, insulin resistance of protein metabolism is of the same magnitude at 8 vs. 5.5 mmol/l, but turnover rates are higher with hyperglycemia. Contrary to our hypothesis, sustained

postprandial-level hyperaminoacidaemia stimulated positive net protein balance comparable to that previously found in lean nondiabetic men. This was sufficient to overcome the insulin resistance of protein anabolism.

Keywords Leucine kinetics. Hyperinsulinaemic clamp. Protein metabolism. Insulin resistance. Type 2 diabetes. Fed state. Hyperaminoacidaemia.

Abbreviations

BCAA	branched-chain amino acids
FFM	fat-free mass
IAA	indispensible amino acids
KIC	α-ketoisocaproic acid
LSD	least significant difference
R _a	rate of appearance
Rag	Ras related small GTP-binding proteins
R _d	rate of disappearance
REE	resting energy expenditure
Rheb	Ras homolog enriched in brain
SPSS	Statistical Package for the Social Sciences [®]
TAA	total amino acids

3.2. Introduction

Abnormal protein metabolism is not widely recognized as part of the consequences of insulin resistance. Despite there being few clinical signs of abnormal protein metabolism in type 2 diabetes, we have demonstrated the presence of insulin resistance of protein metabolism in men (Pereira *et al.*, 2008). Furthermore, a recent study of older men and women followed for six years showed that those with type 2 diabetes lost more appendicular lean body mass, and this was even more pronounced when diabetes was diagnosed only at study

entry (Park *et al.*, 2009). Type 2 diabetes was also associated with increased risk of sarcopenia in the Korean Sarcopenic Obesity Study (Kim *et al.*, 2010).

Insulin stimulates protein synthesis and suppresses catabolism in vitro (Jefferson et al., 1972, Nakano and Hara, 1979, Tessari, 1994, Tischler et al., 1982) and in vivo (Adegoke et al., 2009, Bennet et al., 1990, Hillier et al., 1998). Amino acids, especially the branched-chain amino acids (BCAA), have effects on protein synthesis that are both independent of (Castellino et al., 1987, Tessari et al., 1987) and synergistic with insulin (Adegoke et al., 2009, Castellino et al., 1987, Tessari et al., 1987). In order to dissect the effects of insulin from those of amino acids on whole body protein metabolism, hyperinsulinaemic-euglycaemic clamps and isotopic tracer techniques have been used. Amino acids are infused to maintain fasting serum concentrations (isoaminoacidaemia) which otherwise fall due to insulin suppression of proteolysis (Castellino *et al.*, 1987, Nygren and Nair, 2003). Using this "Hyper-1 clamp", we have found blunted whole body anabolic responses to insulin in overweight and obese men with poorly controlled type 2 diabetes. There was a significant association between insulin resistance of glucose and of protein metabolism (Pereira et al., 2008). Women with type 2 diabetes did not show added diabetes effects compared to overweight and obese controls (Pereira et al., 2008), in whom we had reported insulin resistance of protein anabolism vs. lean women and men (Chevalier et al., 2005b). This justifies why men only were studied in the present protocol. Of note is that during conventional hyperinsulinaemic clamps in type 2 diabetes (Denne et al., 1995, Halvatsiotis et al., 2002b, Luzi et al., 1993, Pereira et al., 2008), plasma glucose is decreased from hyperglycaemia and maintained at euglycaemia (5.5 mmol/l) in order to reproduce comparable conditions as in lean and obese controls. This could create an acute normalization that may mask impairment associated with hyperglycaemia. For this reason, in the present study, plasma glucose was maintained at 8 mmol/l during a two-step hyperinsulinaemic clamp (Hyper2/hyper 3).

Under normal physiological conditions, the greatest protein anabolism occurs in the fed state, during which concentrations of insulin, glucose and amino acids are elevated and those of NEFA are low. Thus, insulin resistance of protein anabolism, if present, would be predicted to be maximal in this state. However, kinetic methods are most reliable during sustained steady-states. We therefore developed the hyperinsulinaemic- hyperglycaemic-hyperaminoacidaemic (Hyper-3) technique with all concentrations clamped at peak values reached postprandially, thereby simulating a fed steady-state (Adegoke *et al.*, 2009). This caused a marked increase in whole-body net protein accretion in healthy lean men during Hyper-3, compared to Hyper-1. There was an additive effect of raised amino acids to that of insulin (Adegoke et al., 2009). Normal whole-body protein anabolic response to clamping insulin and amino acids at postprandial concentrations in type 2 diabetes was reported by Luzi et al. (Luzi et al., 1993). The study, however, included both sexes, and glucose was maintained at euglycaemia (5.5 mmol/l) which did not mimic fed-state concentrations. Similarly, another study showed that hyperglycaemic diabetic men have unaltered protein kinetics after ingesting boluses of carbohydrates and hydrolysed protein (Manders *et al.*, 2008). The total, taken in repeated boluses, being extremely large (268 g carbohydrates and 134 g protein) may, however, indicate that in type 2 diabetes, the protein anabolic capacity is maintained with generous protein intakes.

The present study was designed to investigate the effect of hyperglycaemia on whole-body protein kinetics in type 2 diabetes during isoaminoacidaemia (Hyper-2 vs. Hyper-1) and to quantify the effect of hyperaminoacidaemia during Hyper-3. Results have been presented in part in abstract form (Bassil, 2009), and results of the Hyper-1 group are from 8 subjects selected from a previous study (Pereira *et al.*, 2008) to match for body composition.

3.3. Methods

Sixteen white overweight and obese men (8 per protocol) aged <65 years, with conventionally controlled type 2 diabetes were studied (Table 3.1). Individuals were screened with fasting blood and urine samples, electrocardiograms and chest X-rays and a complete physical examination. Participants signed the study consent form approved by the institutional research ethics board. Exclusion criteria included smoking, unstable weight for the previous 6 months, insulin therapy, abnormal dietary habits assessed by 24 h dietary recall, significant hepatic, hematologic, renal, pulmonary, or cardiovascular dysfunction, and medications known to affect metabolism.

Individuals were admitted for 4 to 6 days to the MUHC/Royal Victoria Hospital Clinical Investigation Unit. All except one subject were treated with oral

antihyperglycaemic drugs: metformin, 13; sulfonylureas, 11; and repaglinide and a thiazolidinedione, 1 each. Eight individuals were treated with statins and nine with antihypertensive agents. All medications except antihypertensives were held on the clamp day until the end of the experiment. Participants consumed a formula-based (Ensure® and Glucerna®, Ross Laboratories, Montreal, QC, Canada) isoenergetic, protein controlled diet divided in 5 equal meals from 08:00 to 20:00 hours for 6 to 8 days. It provided 1.7 g protein [kg FFM]⁻¹ d⁻¹ (15-16 % of energy), 59-60% of energy from carbohydrate and 25% from fat. Weight was maintained by calculating total energy expenditure based on resting energy expenditure (REE) (Deltatrac, SensorMedics, Yorba Linda, CA, USA), multiplied by 1.5-1.6. An energy supplement [two-thirds glucose polymer (Polycose®; Abbott laboratories, St-Laurent, QC, Canada) and one third vegetable oil) was given to correct for energy loss in the preceding day's measured glycosuria. Subjects' habitual daily physical activity assessed using the MONICA Optional Study of Physical Activity (MOSPA) questionnaire (1997) reflected a sedentary lifestyle. Prior to clamp studies, activity was limited for 6 to 8 days to short nonbrisk walks. Circumferences (waist, hip, chest, calf and thigh) were measured according to World Health Organization 1995 criteria and body composition was determined by bioimpedance analysis for all individuals (RJL-101A Systems, Detroit, MI) and by dual energy x-Ray Absorptiometry (DXA, Lunar Prodigy Advance, GE Healthcare, Madison, WI) for Hyper-2/ Hyper-3 subjects in whom FFM measured by both methods did not differ significantly. Capillary glucose>15 mmol/l, measured before meals (Accuchek III, Boehringer Mannheim) was treated with small doses of subcutaneous insulin except during the 15 h prior to the clamp.

On the clamp day (Figure 3.1) at 08:00 hours, catheters were inserted in an antecubital vein for infusions and in the opposite hand for blood sampling. The hand was kept in a heated box at 65-70°C to arterialise venous blood (Zello et al., 1990). Then, a bolus of 0.1 mg/kg of oral NaH¹³CO₂ (MassTrace Inc., Woburn, MA) and of 0.5 mg/kg of intravenous L-1-[¹³C]leucine (Isotech, Sigma-Aldrich, St Louis, MO) was given, followed by a constant infusion rate of 0.008 mg kg⁻¹ min⁻¹ for leucine kinetic determination. A primed infusion of biosynthetic regular human insulin (Humulin R; Eli Lilly Canada Inc, Toronto, Canada) was given concurrently at 1.1 mU (kg FFM)⁻¹ min⁻¹. Low ¹³C-enriched 20% glucose in water (Avebe b.a., Foxhol, The Netherlands) and an amino acid solution (TrophAmine[®] 10% without electrolytes, B. Braun Medical Inc., Irvine, CA, USA) (Table 3.2) were infused at variable rates to maintain constant concentrations based on plasma measurements of glucose and BCAA as indicator of total amino acids (TAA) at 5-minute intervals. Glucose was clamped at 8 mmol/l in Hyper-2 and at 5.5 mmol/l in Hyper-1. Total BCAA were maintained at individual subjects' fasting concentrations in both Hyper-2 and Hyper-1.

As depicted in Figure 3.1, 3 h after the start of insulin infusion, Hyper-2 was followed by Hyper-3 by increasing BCAA to 750 μ mol/l. Insulin, glucose and BCAA targets for Hyper-3 were obtained from peak postprandial concentrations reached during a meal test (2981 kJ, 30 g protein) in lean, healthy subjects (data not shown). The L-[1-¹³C]-leucine infusion rate was increased by

50% to prevent dilution of isotopic enrichment by exogenous leucine, and the insulin infusion was decreased by 17% to correct for the stimulation of endogenous insulin secretion by increasing amino acid concentrations (Nuttall and Gannon, 1991). The Hyper-3 clamp lasted for 3 h after which all infusions were stopped. In the Hyper-1 study, the 3 h hyperinsulinaemic period followed 3 h of postabsorptive state with only tracer infusions (data not presented).

Steady states of glucose and amino acid concentrations and infusion rates were achieved within 120 min and maintained until 180 min. Kinetics were calculated during the last 30 min ("plateau") of each phase of the clamps. Indirect calorimetry was performed for 20 min at baseline and during plateaus. Arterialised blood samples were collected for substrates, hormones and isotopic enrichment, at baseline and every hour until 50 min prior to end of the clamps, and thereafter at 10 min intervals. Simultaneously, expired air samples were collected in evacuated tubes for analysis of ¹³CO₂ enrichment (Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ).

L-[1-¹³C]-leucine kinetics were calculated according to (Matthews *et al.*, 1980b), using plasma [1-¹³C]- α -ketoisocaproic acid (KIC) enrichment (reciprocal model) (Matthews *et al.*, 1982), providing leucine total rate of appearance (R_a flux), endogenous R_a (protein breakdown), oxidation and non-oxidative endogenous rate of disappearance (R_d protein synthesis). The ¹³C enrichments of expired CO₂ and VCO₂ from indirect calorimetry were used in calculating leucine oxidation. The recovery factor, the proportion of ¹³CO₂ generated during oxidation that is exhaled (Cynober, 1995, Matthews *et al.*, 1980b) was 0.799

during Hyper-1 and Hyper-2 steady states and 0.824 for Hyper-3, based on previous bicarbonate studies done in our laboratory. In the calculation of leucine oxidation rates, correction was made to ${}^{13}CO_2$ enrichment because low [1- ${}^{13}C$] glucose solutions dilute the natural enrichment, as previously described (Chevalier *et al.*, 2004). A factor of 7.0% was used as determined by additional clamp studies done in obese control subjects with or without type 2 diabetes during which ${}^{13}C$ leucine was omitted.

Assays Enrichment of plasma [13 C]- α -KIC was determined by GC-MS (5988A; Hewlett-Packard, Palo Alto, CA) after derivatization with N-methyl-N-(tertbutyldimethylsilyl)trifluoroacetamide (Regis Technologies Inc., Morton Grove, IL) as detailed previously (Chevalier *et al.*, 2005a). Expired ${}^{13}CO_2$ enrichment was measured by isotope ratio mass spectrometry (Vacuum Generators, Winsforce, United Kingdom), plasma glucose concentration by glucose oxidase (GM7 Micro-Stat; Analox Instruments USA, Lunenberg, MA) and total BCAA during clamp, by an enzymatic, fluorometric assay (FP-6200, Jasco Corporation, Tokyo, Japan). BCAA are oxidatively deaminated to their corresponding ketoacid, by leucine dehydrogenase in the presence of NAD^+ . NADH is generated stochiometrically; its fluorescence is measured at 4 min of the reaction, at 37°C, 355 nm excitation and 485 nm emission wavelengths (Chevalier et al., 2006a). Serum insulin, C-peptide and glucagon were determined by radioimmunoassay (Millipore, Billerica, MA). Serum NEFA concentrations were measured by a colorimetric assay (NEFA C, Wako Chemicals USA Inc, Richmond, VA) and reverse-phase HPLC was used to determine individual
plasma amino acid concentrations after pre-column derivatization with ophtalaldehyde (Chevalier *et al.*, 2005b).

Statistical analyses Results are presented as means \pm SEM. Repeated measures ANOVA was used to assess clamp effects on kinetics, hormones and substrates within-subject (baseline vs. Hyper-1, and baseline vs. Hyper-2 vs. Hyper-3, with LSD post-hoc test) and between-subject responses to the clamp (Hyper-1 vs. Hyper-2). Data comparisons between Hyper-1 and Hyper-2 clamp periods were made using independent t-tests. Pearson's coefficient was used for simple correlations between parameters, and stepwise multiple regression to estimate the magnitude of variation that is explained by each parameter. HOMA was non-normally distributed and hence log-transformed, and presented as lnHOMA-IR. Based on a paired design (Lachin, 1981) and SD from our previous results (Adegoke *et al.*, 2009), a sample size of 8 subjects was needed to detect a 20% difference in protein synthesis rates between Hyper-2 and Hyper-3 clamps, (one-tailed $\alpha = 0.05$, $\beta = 0.10$). Analyses were performed using SPSS 17.0 for Windows (SPSS, Chicago, IL).

3.4. Results

Age, height and HbA_{1C} were matched between participant groups. Mean weight, BMI, % body fat and waist circumferences were non-significantly different (Table 3.1). Percent body fat and waist circumferences of all subjects were above healthy reference values (World Health Organization, 2000).

Baseline and clamp substrate, respiratory gas exchange and hormone concentrations are presented in Table 3.3. By design, participants were

hyperglycaemic at baseline; glucose was decreased to 5.5 mmol/l during Hyper-1, and maintained at 8 mmol/l during Hyper-2 and Hyper-3. Compared with baseline, hyperinsulinaemia did not affect REE or respiratory quotient (RQ) during Hyper-2 but increased RQ in Hyper-1. Raising amino acids significantly increased REE without affecting RQ during Hyper-3. Fasting concentrations of TAA and indispensable amino acids (IAA) and BCAA were maintained during Hyper-1 and Hyper-2, but leucine concentrations during Hyper-2 increased slightly. Subsequently during Hyper-3, TAA were significantly increased by 32%, IAA by 53%, BCAA by 64% and leucine by 100%. Subjects were hyperinsulinaemic at baseline, and insulin infusion markedly increased serum concentrations to postprandial targets during Hyper-1 and Hyper-2, without change in C-peptide in the latter. During Hyper-3, insulin concentrations achieved in Hyper-2 were maintained by the 17% decrease in infusion rate, and C-peptide increased slightly (12%) but significantly compared to baseline and Hyper-2 (p=0.049). Compared to baseline, glucagon decreased slightly during Hyper-2 but not Hyper-1, while raising amino acids caused a 44% increase during Hyper-3 (p < 0.005). Serum NEFA were significantly suppressed by insulin during all three clamps to concentrations not different among protocols.

Leucine kinetics are presented in Figure 3.2. The effect of hyperglycaemia on leucine kinetics was tested by comparing Hyper-2 (8 mmol/l) to Hyper-1 (5.5 mmol/l) (Figure 3.2a). Leucine flux (total R_a), nonoxidative R_d (protein synthesis) and endogenous R_a (breakdown) were significantly higher in Hyper-2, but leucine

oxidation and net balance (R_d minus endogenous R_a) were not different. In neither clamp was the net balance anabolic.

During Hyper-3, amino acids were infused at a three- to fourfold increased rate to reach and maintain postprandial concentrations (Figure 3.2b). Hyperaminoacidaemia caused significantly higher leucine flux and oxidation than during Hyper-2, synthesis increased by 17% (p<0.05) and breakdown decreased by 20% (p<0.005), which resulted in a marked increase in net balance from -5 ± 3 µmol/min to 60 ± 5 µmol/min (p<0.0001)

During Hyper-2, controlling for FFM, leucine flux correlated positively with markers of whole-body adiposity: weight (r=0.953, p=0.001), BMI (r=0.853, p=0.015), fat mass (r=0.953, p=0.001) and abdominal adiposity, inferred by waist circumference (r=0.889, p=0.007). However, stepwise regression analysis showed that weight was the only independent variable that predicted 97% of the variance in flux such that when they were controlled for weight, correlations between flux and body composition were lost. However, controlling for body weight, net leucine balance was found to be negatively correlated with waist circumference (r= -0.879, p=0.021). During Hyper-3, net leucine balance correlated negatively with log_e HOMA-IR (r = -0.745, p=0.034)

3.5. Discussion

This study showed a marked protein anabolic response to clamping insulin and amino acids at postprandial levels while maintaining the elevated fasting glucose at 8 mmol/l in type 2 diabetic men. This occurred despite evidence of insulin resistance of protein metabolism. Indeed the anabolic response to hyperinsulinaemia (Fig 3.2a) was blunted to the same extent as when plasma glucose had been clamped at 5.5 mmol/l and amino acids at postabsorptive levels. It occurred at the "cost" of accelerated protein turnover rates with glycemia at 8 mmol/L (Pereira *et al.*, 2008). The clinically pertinent implication is that physiological postprandial hyperaminoacidaemia can overcome such insulin resistance of protein anabolism in obese type 2 diabetic persons at a commonly observed level of hyperglycemia.

The Hyper-3 phase of the clamp was designed to simulate a fed steadystate, with target hyperglycaemia, hyperinsulinaemia and hyperaminoacidaemia at physiological postprandial concentrations of lean healthy subjects. This allowed for testing of one variable, increased amino acid provision, vs. Hyper-2. It required that insulin be maintained at the same concentrations, and this was achieved by lowering its infusion rate to compensate for the anticipated increase in endogenous insulin secretion due to hyperaminoacidaemia (Manders *et al.*, 2005, Nuttall and Gannon, 1991). This was confirmed by the slightly higher Cpeptide concentrations. Thus, the unchanged peripheral serum insulin was the consequence of decreased exogenous and increased endogenous contributions. A decrease in hepatic insulin extraction as recently reported by Lan-Pidnainy and Wolever cannot be excluded (Lan-Pidhainy and Wolever) . Glucagon was also increased, though in the presence of 3.5-fold higher insulin, the glucagon to insulin ratio was minimally altered (0.07 ± 0.01 vs. 0.05 ± 0.01 in Hyper-2). The substantial increase in net protein balance with hyperaminoacidaemia was the result of both stimulated synthesis and suppressed breakdown (Figure 3.2b). Leucine oxidation rate increased concurrently, consistent with greater protein contribution to energy expenditure compared to Hyper-2. If we assume that the thermic effect of the 25 g of protein administered is 105 kJ over 6h [~25% of energy content of the protein (Gougeon, 2001)], this matches the REE mean difference of 108 kJ over 6 h between Hyper-2 and Hyper-3.

The marked increase in net balance in Hyper-3 prompted us to compare these leucine kinetic responses to those of 8 healthy men (Figure 3.3) from the group previously studied (Adegoke *et al.*, 2009). They had lower BMI of $21.5 \pm$ 0.6 kg/m², age 29 \pm 3 yrs, and FFM 59 \pm 2 kg (all p<0.05 vs. the type 2 diabetic men in the present study). Their hyperglycaemia (7.87 \pm 0.05 mmol/l) and hyperaminoacidaemia (BCAA 709 \pm 24, leucine 272 \pm 11, TAA 3408 \pm 241, IAA $1630 \pm 101 \,\mu\text{mol/l}$ did not differ from the participants with type 2 diabetes in the present study. However, hyperinsulinaemia was even greater ($788 \pm 83 \text{ pmol/l}$) due to a larger C-peptide response. Notwithstanding, none of the kinetic variables differed between groups, indicating the potency of amino acids themselves in the anabolic response. Interestingly, in lean nondiabetic subjects, the anabolic response was increased from a positive net leucine balance of 0.25 in Hyper-1 (Chevalier et al., 2004) to 0.99 µmol leucine (kg FFM)⁻¹ min⁻¹ in Hyper-3 (Adegoke *et al.*, 2009), whereas in type 2 diabetes it reached the same levels in Hyper-3, but from zero net balance in Hyper-1/Hyper-2. This underscores the effectiveness of hyperaminoacidaemia in overcoming their insulin resistance of protein metabolism.

The "normalized" protein kinetic response during Hyper-3 is consistent with results of Luzi et al (Luzi et al., 1993) and of Manders et al (Manders et al., 2008), albeit with different subject characteristics. The latter reported that the muscle protein synthetic response in men with longstanding type 2 diabetes was not impaired after ingestion of carbohydrate and protein boluses. Our total amount of infused amino acids (25 g) was much less than the ingested protein (134 g) in (Manders et al., 2008). We clamped BCAA at 770 µmol/l and most other amino acids at peak concentrations attained after mixed meal ingestion with 30 g protein in lean, healthy subjects. However, we have reported lower peak postprandial amino acid concentrations (BCAA= 650 pmol/l) in poorly controlled type 2 diabetes after consuming a similar meal (Mourad *et al.*, 2009). Thus, it remains to be determined at what levels and durations of hyperaminoacidaemia protein kinetics become normalized in type 2 diabetes, as there could be a shift in "doseresponse" to the right, requiring larger amounts of exogenous protein than recommended requirements. Finally, despite normal mean anabolic response, net leucine balance during Hyper-3 was negatively correlated with an index of whole body insulin resistance of glucose metabolism, lnHOMA, supporting the notion that insulin resistance might still affect postprandial protein anabolism. Therefore, greater magnitudes of postprandial hyperaminoacidaemia in type 2 diabetes might increase protein synthesis and net protein anabolism to levels that could mask a persistent underlying insulin resistance of protein metabolism. Indeed, in elderly, muscle protein synthesis in response to hyperaminoacidemia and glucose-induced hyperinsulinaemia was found to be impaired (Volpi *et al.*, 2000), but was improved with an increased supply of amino acids (Volpi *et al.*, 1998). Amino acids, especially leucine, were shown to stimulate protein synthesis with and without elevated insulin (Anthony *et al.*, 1999, Buse and Reid, 1975, Castellino *et al.*, 1987, Tessari *et al.*, 1987). Leucine activates the mammalian target of rapamycin (mTORC1) signalling (Kim *et al.*, 2002, Kimball *et al.*, 1999) by a yet unclearly defined mechanism, but may involve Ras-related small GTP-binding proteins (RAG) proteins that promote its intracellular perinuclear localization, favouring its activation (Sancak *et al.*, 2008).

Our objective for the Hyper-2 clamp was to test the effect of insulin on protein metabolism in type 2 diabetes while maintaining "usual" fasting hyperglycaemia and amino acid concentrations. Clamping glycaemia at 8.0 vs. 5.5 mmol/l was associated with significantly higher leucine flux, synthesis and breakdown rates, but those of leucine infusion, oxidation and net balance were not different (Figure 3.2a). Such an effect has been reported in normal subjects, even at pharmacologic hyperinsulinaemia (Flakoll *et al.*, 1993). Negative postabsorptive net balance (Pereira *et al.*, 2008) was brought only to equilibrium in both Hyper-1 and Hyper-2, in contrast to a positive balance in nondiabetic subjects (Chevalier *et al.*, 2004, Flakoll *et al.*, 1993, Pereira *et al.*, 2008). Thus, in Hyper-2, the metabolic abnormality responsible for the hyperglycaemia was associated with blunted anabolism, with even greater "inefficiency" in response to insulin, namely greater rates of turnover. This is consistent with our previous reports of accelerated integrated 24h protein turnover rate in hyperglycaemic type 2 diabetes (Gougeon *et al.*, 1997a, Gougeon *et al.*, 1994, Gougeon *et al.*, 1997b, Gougeon *et al.*, 2000). A factor in the greater leucine turnover in Hyper-2 might be the non-significant higher body weight and adiposity of Hyper-2 subjects, suggested by the strong correlation between these variables and leucine flux, and by weight being an independent determinant of turnover rate. Moreover, waist circumference (controlled for weight) was significantly and negatively correlated with net balance as previously demonstrated in obesity by us and others (Chevalier *et al.*, 2005b, Luzi *et al.*, 1996, Pereira *et al.*, 2008).

Some limitations of our protocol need to be considered. First, the simulated fed steady-state does not mimic the temporal non-steady state changes in substrate and hormone concentrations that occur during meal absorption. Second, Hyper-2 preceded Hyper-3 and thus the time of exposure to exogenous insulin and amino acids may have influenced the response to the second phase. Third, the amino acid composition of TrophAmine[®] (table 3.2) is different from that of usual food proteins, being particularly enriched in indispensable amino acids, especially leucine. Thus, it remains to be shown whether similar findings, or magnitude of effects, would be seen with oral feeding.

This study has shown that, in overweight and obese men with type 2 diabetes, protein turnover is at an accelerated rate in hyperglycaemic clamps while insulin resistance of protein metabolism is of the same magnitude as found with euglycaemic clamps. Conversely, the whole body protein anabolic response to postprandial hyperaminoacidaemia appears to be normal. Therefore, the

postprandial protein anabolic capacity is preserved in type 2 diabetes in these conditions. Assuming constant kinetics, the mean net anabolism of 61 µmol/min in Hyper-3 would give an estimated 10 g protein over 3 hours, corresponding to the "meal" with 25 g of amino acids contained in TrophAmine. This would restore one fourth of the postabsorptive net catabolism of 36 µmol/min (Pereira et al., 2008), or 44 g endogenous proteins [based on 590 µmol leucine/g protein (Wolfe, 1992)] over 12 hours of overnight fast (also assuming constant catabolic rate). Thus by assuring ample protein intake, well distributed during the day, as in this protocol, type 2 diabetic individuals should be able to achieve 24 h protein equilibrium, even taking account of probable overestimation of the totals by such calculations. This could explain the paucity of evidence of clinical signs and abnormal standard clinical laboratory tests showing impaired protein metabolism in type 2 diabetes. These results do not, however, negate the fact that such equilibrium is at the cost of long-term abnormality ("inefficiency") of kinetics of protein metabolism, nor that a negative daily balance, too small to be detected by our methodology, could lead to the accelerated muscle loss in type 2 diabetes, as recently shown (Kim et al., 2010. Park al., 2009) et



	Hyper-2/Hyper-3	Hyper-1	р
n	8	8	
Age (y)	57±2	55±2	0.537
Height (cm)	177.5±2.1	177.5±1.9	0.995
Weight (kg)	107.7±7.3	95.9±5.2	0.206
BMI (kg/m^2)	34.0±1.6	30.3 ± 1.3	0.106
FFM (kg)	71.7±3.9	67.3 ± 3.1	0.393
Body fat (%)	34.9±2.2	29.5±1.4	0.187
Waist circumference (cm)	118.6±4.8	108.2±3.7	0.108
A1C (%)	7.1±0.2	7.0±0.3	0.642
Diabetes duration (y)	9 ± 2	6 ± 2	0.233

Table 3.1. Subject characteristics

Data are means \pm SEM. BMI: body mass index. FFM: fat free mass measured by BIA.

Table 5.2. Trophammes am	no acia composition	
	grams per 100ml	mmol/l
Total	10	765.0
Essential amino acids	5.26	388.8
Isoleucine	0.82	62.5
Leucine	1.4	106.7
Lysine	0.82	56.1
Methionine	0.34	22.8
Phenylalanine	0.48	29.1
Threonine	0.42	35.3
Tryptophan	0.2	9.8
Valine	0.78	66.6
Non-essential amino acids	4.7	376.2
Alanine	0.54	60.6
Arginine	1.2	68.9
Proline	0.68	59.1
Serine	0.38	36.2
Glycine	0.36	48.0
L-Aspartic Acid	0.32	24.0
L-Glutamic Acid	0.5	34.0
Tyrosine	0.24	13.2
Histidine	0.48	30.9
Cysteine	0.016	1.3

Table 3.2. Trophamine® amino acid composition

		Hyperinsulinaemia		
Variable and Protocol Descline		Isoaminoacida	Hyperaminoacid	
	Dasenne	emia	aemia	
REE (kJ/min)				
Hyper-2/Hyper-3	6.1 ± 0.3^{a}	6.1 ± 0.3^{a}	6.4 ± 0.3^{b}	
Hyper-1	5.6±0.2	5.6±0.3		
RQ				
Hyper-2/Hyper-3	0.80 ± 0.02	0.81 ± 0.02	0.81 ± 0.02	
Hyper-1	$0.79{\pm}0.02^{a}$	$0.85 {\pm} 0.02^{b}$		
Glucose (mmol//l)†				
Hyper-2/Hyper-3	8.38±0.61	7.95±0.04*	7.93±0.02	
Hyper-1	8.75 ± 0.62^{a}	5.51 ± 0.03^{b}		
TAA (μmol/l)				
Hyper-2/Hyper-3	2499±95 ^a	2564±138 ^a	3381 ± 182^{b}	
Hyper-1	2669±47	2531±37		
IAA (µmol/l)				
Hyper-2/Hyper-3	966±51 ^a	1013 ± 49^{a}	1548 ± 66^{b}	
Hyper-1	1012 ± 22	1005 ± 20		
BCAA (µmol/l)				
Hyper-2/Hyper-3	454 ± 23^{a}	470 ± 19^{a}	770 ± 25^{b}	
Hyper-1	451±19	440 ± 18		
Leucine (µmol/l)†				
Hyper-2/Hyper-3	136±11 ^a	156 ± 8^{b}	312 ± 14^{c}	
Hyper-1	144±7	146±7		
NEFA (µmol/l)				
Hyper-2/Hyper-3	462 ± 74^{a}	114 ± 28^{b}	145 ± 45^{b}	
Hyper-1	547 ± 48^{a}	185 ± 26^{b}		
Insulin (pmol/l)				
Hyper-2/Hyper-3	187 ± 40^{a}	630 ± 68^{b}	659 ± 71^{b}	
Hyper-1	126 ± 19^{a}	542 ± 21^{b}		
C-peptide (pmol/l)				
Hyper-2/Hyper-3	1523 ± 303^{a}	1602 ± 279^{a}	1793 ± 300^{b}	
Hyper-1	ND	ND		
Glucagon (pmol/l)				
Hyper-2/Hyper-3	36 ± 6^{a}	32 ± 6^{b}	46 ± 9^{c}	
Hyper-1	30±5	27±4		

Table 3.3. REE, RQ, and circulating substrate and hormone concentrations at baseline and during clamps

Data are means±SEM. Within the same row, values with different letter superscripts are significantly different (paired t-test or repeated measures ANOVA and LSD posthoc test).*p<0.05 vs. Hyper-1 clamp (independent t-test). † significant group effect in response to clamp (repeated measures ANOVA). REE: resting energy expenditure. RQ: respiratory quotient. TAA: total amino acids. IAA: Indispensable amino acids. BCAA: branched-chain amino acids. NEFA: Non-esterified fatty acids. ND: not determined

Figure 3.2

a











3.6. Figure legends

Figure 3.1 Hyperinsulinaemic, euglycaemic (Hyper-1) and hyperglycaemic (Hyper-2) clamp protocols with postabsorptive aminoacidaemia. Hyper-2 only was followed by postprandial aminoacidaemia (Hyper-3) in men with type 2 diabetes mellitus.

Figure 3.2 Clamp whole body leucine kinetics in men with type 2 diabetes mellitus. Total R_a : total leucine flux, including leucine infusion; endogenous R_a : index of protein breakdown; nonoxidative R_d : index of protein synthesis; net balance: synthesis minus breakdown. Data expressed per FFM.

(a) Effect of hyperglycaemia during hyperinsulinaemia and

isoaminoacidaemia in subjects from this study, Hyper-2 (8 mmol/l) (black bars) vs. Hyper-1 (5.5 mmol/l) (white bars) (data of 8 subjects from[1]). Independent samples t-test * p=0.05.

(b) Effect of hyperaminoacidaemia during hyperinsulinaemia and hyperglycaemia Hyper-2 (black bars) vs. Hyper-3 (white bars); clamps performed sequentially in the same subjects. Paired t-test p<0.01 vs. Hyper-2 clamp. *p<0.001 vs. Hyper-2 clamp.

Figure 3.3 Effect of type 2 diabetes on leucine kinetics during Hyper-3 clamps; Type 2 diabetic men (white bars) vs. lean, nondiabetic men (black bars adapted from ref (Adegoke *et al.*, 2009)).

3.7. Acknowledgements

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Bridge 2

In the first manuscript, it was proposed that abundant protein intake might be needed to normalize protein metabolism in T2DM and compensate for the insulin resistance of protein. However, many studies in the literature challenge this conclusion. High protein consumption has been associated with increased risk of insulin resistance and type 2 diabetes in observational studies (Liese *et al.*, 2009, Promintzer and Krebs, 2006, Schulze *et al.*, 2003). This might be due to amino acid-induced blunting of glucose disposal that was demonstrated in healthy men (Adegoke *et al.*, 2009, Krebs *et al.*, 2007, Krebs *et al.*, 2002, Pisters *et al.*, 1991, Tremblay *et al.*, 2005b). However, no data yet exist on the effect of amino acids on glucose uptake in hyperinsulinemic clamps in T2DM. This is why the following clamp study was designed to assess the effect of only the variable of interest, i.e. hyperaminoacidemia vs. isoaminoacidemia on glucose uptake using ³H-glucose. Other variables, namely hyperglycemia and hyperinsulinemia were kept constant and comparable in the 2 steps of the clamp.

CHAPTER 4. MANUSCRIPT 2

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Hyperaminoacidaemia at postprandial levels does not modulate glucose metabolism in type 2 diabetes mellitus

Maya Bassil, Sergio Burgos, Errol B. Marliss, José A. Morais, Stéphanie Chevalier and Réjeanne Gougeon

McGill Nutrition and Food Science Centre, Montreal, QC, Canada, H3A 1A1.

Corresponding author: Réjeanne Gougeon

McGill Nutrition and Food Science Centre MUHC/Royal Victoria Hospital 687 Pine Avenue West, H6.61 Montreal, QC H3A 1A1 Canada Tel: (514) 843-1665 Fax: (514) 843-1706 E-mail: rejeanne.gougeon@muhc.mcgill.ca

Short running title: Effects of amino acids on glucose metabolism in type 2 diabetes

Keywords Glucose disposal, Glucose metabolism, Glucose turnover,

Hyperaminoacidaemia, Hyperinsulinaemic clamp, Insulin resistance, Type 2

diabetes

Abbreviations

BCAA	branched-chain amino acids
FFM	fat-free mass
IRS	insulin receptor substrate
LSD	least significant difference
MCR	metabolic clearance rate
mTORC1	mammalian target of rapamycin complex 1
NEFA	Non-esterified fatty acids
R _a	rate of appearance
R _d	rate of disappearance
rp	ribosomal protein
S6K	S6 kinase
Ser	Serine
TBS-T	Tris-buffered saline containing 0.1% Tween 20
Thr	Threonine

4.1. ABSTRACT

Aims/hypothesis Hyperaminoacidaemia attenuates glucose disposal during hyperinsulinaemic clamps in healthy lean individuals, an effect thought to be mediated by negative feedback on insulin signalling, downstream of the mammalian target of rapamycin (mTOR) signalling pathway. This has been interpreted as amino acids causing insulin resistance in healthy people, and contributing to it in type 2 diabetes. However, the effect of hyperaminoacidaemia on glucose disposal in type 2 diabetic individuals remains to be determined.

Methods Eight obese men with type 2 diabetes underwent a two-step hyperinsulinaemic-hyperglycaemic (8 mmol/l) clamp, first with amino acids at

postabsorptive concentrations, followed by postprandial concentrations. Wholebody glucose turnover was assessed using 3-D-[³H]glucose. Vastus lateralis biopsies were obtained at baseline and during each step of the clamp to determine the phosphorylation states of AKT, mTOR, ribosomal protein (rp) S6, and insulin receptor substrate (IRS)-1.

Results Rates of glucose infusion $(1.30\pm0.19 \text{ vs } 1.15\pm0.13 \text{ mmol/min})$, endogenous glucose production $(0.48\pm0.06 \text{ vs } 0.53\pm0.05 \text{ mmol/min})$ and disposal $(1.24\pm0.17 \text{ vs } 1.17\pm0.14 \text{ mmol/min})$ did not differ between postabsorptive and postprandial amino acid concentrations (*p*>0.05). Whereas phosphorylation of AKT^{Ser473}, AKT^{Thr308} mTOR^{Ser2448} and rpS6^{Ser235/236} increased (*p*<0.05) with elevated amino acids, that of IRS-1^{Ser636/639} and IRS-1^{Ser1101} did not change.

Conclusions/interpretation Postprandial circulating amino acid concentrations do not worsen the already attenuated glucose disposal in hyperglycaemic type 2 diabetic men, and cell-signalling events are consistent with this. Our results do not support recommendations to restrict dietary protein in type 2 diabetes.

4.2. Introduction

Optimal protein intake in type 2 diabetes is not clearly defined. Evidence cited to recommend decreasing intake includes observational studies reporting that high protein consumption is associated with impaired glucose metabolism (Skilton *et al.*, 2008), risk of increasing insulin resistance and also precipitating type 2 diabetes (Liese *et al.*, 2009, Promintzer and Krebs, 2006, Schulze *et al.*, 107

2003). Furthermore, amino acids blunt glucose uptake *in vitro* (Traxinger and Marshall, 1989, Tremblay *et al.*, 2005a, Tremblay and Marette, 2001) and in healthy humans at the whole-body (Adegoke *et al.*, 2009, Krebs *et al.*, 2007, Krebs *et al.*, 2002, Pisters *et al.*, 1991, Tremblay *et al.*, 2005b) and tissue levels (Pisters *et al.*, 1991, Schwenk and Haymond, 1987). In healthy men, during hyperinsulinaemic-euglycaemic clamps, whole-body glucose uptake was lower when amino acids were infused to maintain basal (Flakoll *et al.*, 1992, Pisters *et al.*, 1991) or twice-basal plasma amino acid concentrations (Flakoll *et al.*, 1992, Krebs *et al.*, 2007, Krebs *et al.*, 2002, Tremblay *et al.*, 2005b). We have reported lower glucose metabolic clearance rate (MCR) in healthy men during hyperinsulinaemic clamps when glucose and amino acids were raised to postprandial concentrations compared to hyperinsulinaemic-euglycaemic clamps with amino acids maintained at fasting concentrations (Adegoke *et al.*, 2009).

Findings from clamp studies in healthy subjects were extrapolated to insulin resistant states to postulate that increased protein intake in type 2 diabetes could contribute to peripheral insulin resistance of glucose metabolism (Krebs *et al.*, 2002). Fasting branched chain amino acids (BCAA) may be elevated in type 2 diabetes (Felig *et al.*, 1970, Pereira *et al.*, 2008) and were suggested to induce insulin resistance in a similar manner (Krebs *et al.*, 2002). By contrast, short-term intervention studies have reported improved glycaemic control in type 2 diabetes with higher protein diets (Gannon and Nuttall, 2004, 2006, Nuttall and Gannon, 2006, Nuttall *et al.*, 2008b). This was mainly attributed to protein-induced insulin secretion which, unlike for glucose, appears to be preserved (Gannon *et al.*, 2003,

Manders *et al.*, 2005) or even potentiated (Nuttall *et al.*, 1984) in persons with type 2 diabetes. It is still not clear whether hyperaminoacidaemia alters glucose disposal in that population, independently from its effect on insulin secretion.

Molecular mechanisms implicated in amino acid-induced insulin resistance of glucose disposal in skeletal muscle include activation of ribosomal protein (rp) S6 kinase 1 (S6K1) (Tremblay *et al.*, 2007a), a downstream effector of the mammalian target of rapamycin complex 1 (mTORC1) signalling pathway (Krebs *et al.*, 2007), which phosphorylates the insulin receptor substrate (IRS)-1 at specific serine residues (Tremblay *et al.*, 2007a, Tremblay *et al.*, 2005b). This, in turn, inhibits IRS-1 function by reducing IRS-1 associated phosphatidylinositol 3-kinase activity (Patti *et al.*, 1998, Tremblay and Marette, 2001) and deactivates downstream components of the insulin signalling cascade. This ultimately results in diminished translocation of GLUT-4 to the cell membrane and attenuated glucose uptake. Some of the multiple IRS-1 serine residues identified in humans, whose inhibitory phosphorylation is associated with mTOR/S6K1 activation, are Ser312, Ser636/639, and Ser1101.

The current protocol was designed to study hyperglycaemic men with type 2 diabetes during hyperinsulinaemic clamps and compare glucose turnover rates with amino acids clamped first at postabsorptive (isoaminoacidaemic, IsoAA) to those with amino acids raised to postprandial (hyperaminoacidaemic, hyperAA) concentrations. Muscle biopsies were obtained at baseline and at each step of the clamp for quantitation of relevant intracellular signalling molecules.

4.3. Methods

Eight white obese men with type 2 diabetes, aged <65 years and conventionally treated, were studied. They were screened with fasting blood and urine samples, electrocardiogram, chest X-ray and a complete physical examination. Participants signed the study consent form approved by the institutional Human Ethics Review Board. Exclusion criteria included smoking, unstable weight for the previous 6 months, insulin therapy, abnormal dietary habits assessed by a 24 h recall, and any significant hepatic, haematological, renal, pulmonary, thyroid or cardiovascular dysfunction.

Participants were admitted for 4 days to the McGill University Health Centre/Royal Victoria Hospital Clinical Investigation Unit. All participants were taking oral antihyperglycaemic drugs: metformin (eight participants); sulfonylureas (six participants); and repaglinide (one participant). Five men were treated with statins and six with antihypertensive agents. All medications except antihypertensives were held on the clamp day until the end of the experiment. Each person consumed a formula-based (80% Ensure and 20% Glucerna; Abbott, Saint Laurent, QC, Canada) isoenergetic, protein-controlled diet for 6 days (3 days at home) to assure nitrogen balance at equilibrium before the clamp experiment. This provided 1.7 g protein (kg fat-free mass [FFM])⁻¹ day⁻¹ (16% of energy), 59% of energy from carbohydrates and 25% from fat. The calculated energy requirement for weight maintenance was based on resting metabolic rate by indirect calorimetry (Deltatrac; SensorMedics, Yorba Linda, CA, USA), multiplied by ~1.6. An energy supplement [2/3 glucose polymer (Polycose;

Abbott) and 1/3 vegetable oil] was given to correct for energy lost as the preceding day's measured glycosuria. Waist circumference was measured according to World Health Organization 1995 criteria (World Health Organization, 1995). Body composition was determined by bioimpedance analysis (RJL-101A Systems, Detroit, MI, USA) and dual-energy x-ray absorptiometry (Lunar Prodigy Advance; GE Healthcare, Madison, WI, USA). Any pre-meal capillary glucose (Accuchek III; Boehringer Ingelheim, Mannheim, Germany) >15 mmol/l was treated by small doses of insulin, except during the 15 h before the clamp.

On the clamp day, at 08:00 h, with participants in the postabsorptive state, catheters were inserted in an antecubital vein for infusions, and in the opposite hand vein, which was kept in a heated box at 65-70°C to arterialise the venous blood for blood sampling. The protocol began 20 min later. Glucose turnover was determined with a primed (814 kBq) continuous (8.14 kBq/min) infusion of 3-D- $[^{3}H]$ glucose (PerkinElmer, Boston, MA, USA). Concurrently, a primed infusion of regular human insulin (0.95 mU [kg FFM]⁻¹ min⁻¹) (Humulin R; Eli Lilly Canada, Toronto, ON, Canada) was started. Glucose (20%) in water (Avebe, Foxhol, the Netherlands) and a 10% amino acid solution (TrophAmine 10% without electrolytes, B. Braun Medical, Irvine, CA, USA) were infused to maintain concentrations at 8 mmol/l for glucose and at each individual's fasting BCAA concentrations, based on arterialised venous concentration measurements made every 5–10 min. Three hours after the start of insulin, BCAA levels were increased to, and maintained at ~750 μ mol/l to match peak postprandial 111

concentrations reached during a meal test (2,981 kJ, 30 g protein) done in lean, healthy participants (data not shown). The intravenous insulin infusion was decreased by 17% to adjust for the stimulation of endogenous insulin secretion from increasing amino acid concentrations (Manders et al., 2005). The hyperAA clamp lasted for an additional 3 h, after which all infusions were stopped. Steady states of glucose and amino acid concentrations and infusion rates were achieved within 120 min and maintained until 180 min at each step of the clamps. Glucose turnover was calculated during the last 30 min ('plateau') of each step. Indirect calorimetry was performed for 20 min at baseline and during both plateaus. Blood samples for substrates, hormones and glucose turnover measurements were collected at baseline every hour until 50 min before the end of each step and at 10 min intervals thereafter. At baseline and 2 h after the start of each step, ~100 mg vastus lateralis muscle biopsies were obtained with a Bergström needle under anaesthesia (2% Xylocaine, Astra-Zeneca Canada, Mississauga, ON, Canada) using sterile techniques and immediately frozen in liquid nitrogen.

Assays Plasma glucose concentration was measured by glucose oxidase (GM7 Micro-Stat; Analox Instruments USA, Lunenberg, MA, USA) and BCAA by an enzymatic, fluorometric assay (FP-6200; Jasco Corporation, Tokyo, Japan) (Chevalier *et al.*, 2004) . Serum insulin, C-peptide and glucagon were determined by radioimmunoassay (Millipore, Billerica, MA, USA) (Sigal *et al.*, 1994). Glucose turnover was calculated with OOPSEG (Bradley *et al.*, 1993). Non-esterified fatty acids (NEFA) were measured by a colorimetric assay (NEFA C; Wako Chemicals USA Inc., Richmond, VA, USA) and reverse-phase HPLC was

used to determine individual plasma amino acid concentrations after pre-column derivatisation with o-phtalaldehyde (Chevalier *et al.*, 2005b).

Analysis of phosphorylation state of signalling proteins Muscle samples (25 mg wet weight) were homogenised on ice for 30 s in 10 volumes of homogenisation buffer (final concentration: 20 mmol/l HEPES, pH 7.4, 2 mmol/l EGTA, 50 mmol/l NaF, 100 mmol/l KCl, 0.2 mmol/l EDTA, 10 mmol/l Na₄PO₇, 50 mmol/l β -glycerophosphate) supplemented with 0.5 mmol/l Na₃VO₄, 1 µmol/l microcystin LR, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride and 10 µg/ml each of aprotinin, leupeptin and pepstatin using a hand-held homogeniser (Tissuemiser; Fisher Scientific, Mississauga, ON, Canada). The homogenate was cleared by centrifugation at $15,000 \times g$, at 4°C, for 15 min. A sample of homogenate was used to measure protein concentration by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as standard (Bradford, 1976). A sample of the remaining supernatant fraction was mixed with an equal volume of 2× Laemli sample buffer and then boiled for 5 min. Samples (25 µg of protein/lane) were resolved by SDS-PAGE and then transferred onto polyvinylidine fluoride membranes (GE Healthcare). The membranes were blocked in 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 1 h and then incubated with phospho-specific primary antibodies against AKT (Thr308), AKT (Ser473), IRS-1 (Ser1101) and (Ser636/639), mTOR (Ser2448) and rpS6 (Ser235/236) at 4°C overnight. Membranes were washed three times for 5 min in TBS-T and subsequently

incubated with secondary antibody in TBS-T containing 5% non-fat dried milk at room temperature for 1 h. Immunoblots were developed by enhanced chemiluminescence using ChemiDOC XRS Multi-Imager system (Bio-Rad Laboratories). After detection of the phospho-specific signal, the antibodies were stripped off the membrane by incubation in 62.5 mmol/l Tris-HCl, 100 mmol/l βmercaptoethanol, and 2% SDS at 50°C for 30 min. The membranes were then washed, blocked and reprobed with primary antibodies against total AKT, IRS-1, mTOR and rpS6. All antibodies were from Cell Signaling Technology (Danvers, MA, USA). Results are expressed as ratios of phosphorylated to total protein.

4.4. Statistical analysis

Results are presented as means \pm SEM. The effects of the three metabolic states (baseline vs isoAA vs hyperAA) were compared by repeated measures analysis of variance (ANOVA) and least significant difference (LSD) post-hoc test, or paired t-test (isoAA vs hyperAA) [SPSS 17.0 for Windows (SPSS, Chicago, IL)]. Based on a crossover design (Lachin, 1981) and SD (Krebs *et al.*, 2002), 8 individuals are sufficient to detect a 25% difference in glucose disposal between isoAA and hyperAA (two-tailed $\alpha = 0.05$, $\beta = 0.1$).

4.5. Results

Of the eight men, one was overweight and the rest obese (Table 1). Adiposity markers (BMI, percent body fat and waist circumference) were above reference values (World Health Organization, 1995) and according to the homeostatic model assessment (HOMA) index, all were insulin resistant (Esteghamati *et al.*, 2010). Plasma glucose and BCAA concentrations and infusion rates during the clamp are presented in Figure 4.1. Participants were hyperglycaemic at baseline (time 0) and plasma glucose was maintained at 8 mmol/l for the duration of the clamp. Fasting BCAA ($454\pm23 \mu mol/l$) were clamped during IsoAA then raised and maintained at postprandial concentrations (780±24 µmol/l) during HyperAA. NEFA and hormone concentrations are presented in Figure 2. During isoAA, insulin increased from 187±40 pmol/l to typical postmeal concentration ($630\pm68 \text{ pmol/l}$) that was maintained during hyperAA ($659\pm71 \text{ pmol/l}$), by design, despite a 12% increase in C-peptide (p=0.049). Plasma glucagon concentration decreased from baseline during IsoAA (p=0.008) and then increased by 45% during HyperAA. NEFA were suppressed to the same extent in both steps of the clamp.

Baseline and clamp plasma amino acid concentrations are shown in Table 2. Most amino acids as well as the total were maintained at their baseline concentrations during isoAA. There was a slight but significant increase in leucine and arginine concentrations by 22% and 14%, respectively, and an 18% decrease in tyrosine concentration. During hyperAA, concentrations of all amino acids except tyrosine and asparagine were higher such that total amino acids were increased by 34%.

Rates of endogenous glucose production (endogenous rate of appearance $[R_a]$), glucose infusion and the total, oxidative and non-oxidative glucose disposal (rate of disappearance $[R_d]$) during hyperAA did not differ from those of isoAA (Table 4.3).

In the muscle biopsies (Fig. 4.3a-e), during isoAA, there was a significant increase from baseline in the phosphorylation of AKT at Ser473 (p=0.002) and Thr308 (p=0.001), mTOR (p=0.002) and rpS6 (p=0.035). During hyperAA, AKT phosphorylation at Thr308 and Ser473 decreased but remained 87% (p=0.09) and 74% (p=0.029) higher than baseline, respectively. Hyperaminoacidaemia was associated with an additional 57% increment in mTOR phosphorylation compared with isoAA. rpS6 phosphorylation remained significantly higher than baseline during hyperAA (p=0.004). Importantly, IRS-1 phosphorylation at Ser636/639 and Ser1101 did not differ across the three time points (Fig. 4.3d,e).

4.6. Discussion

The present study shows that, unlike that previously shown in healthy lean individuals, increasing plasma amino acids does not lower the already blunted glucose disposal in men with type 2 diabetes in hyperinsulinaemichyperglycaemic conditions. Such conditions prevail postprandially in a large proportion of people with conventionally treated type 2 diabetes. The study was postprandial-level effect designed to test the of one variable. hyperaminoacidaemia on glucose turnover during hyperAA, while maintaining the other two conditions constant compared with isoAA. Therefore, branchedchain and total amino acids were raised to peak concentrations observed after a mixed meal in healthy lean participants. Insulin was clamped at the postprandial peripheral venous concentration we have found in type 2 diabetes (Mourad *et al.*, 2009), and was maintained unchanged during hyperAA, because we decreased the insulin infusion rate. This compensated for amino acid stimulation of endogenous insulin secretion, as inferred from the C-peptide rise. However, this infers higher portal vein insulin concentration. Although glucagon was slightly increased by amino acids during hyperAA, it did not affect endogenous glucose production compared with isoAA (Table 4.3). This could be because of the higher portal insulin overriding any effect glucagon might have on endogenous R_a . In addition, with insulin being 3.5-fold higher, the glucagon to insulin ratio was minimally altered (0.07±0.01 vs 0.05±0.01 in isoAA). Insofar as NEFA might play a role in postprandial insulin resistance in type 2 diabetes, in the present case this potential effect appears to be minimal, as NEFA were suppressed to identical concentrations in both phases of the clamp.

The whole-body glucose R_d did not change despite doubling of BCAA and leucine concentrations vs. baseline during HyperAA. This therefore, does not support the proposition that postprandial-level hyperaminoacidaemia would worsen insulin resistance of glucose metabolism in type 2 diabetes, an effect that had been extrapolated from findings in lean healthy participants (Krebs *et al.*, 2007, Krebs *et al.*, 2002, Tremblay *et al.*, 2005b) of a clamp protocol analogous to ours. These healthy individuals were studied during hyperinsulinaemic– euglycaemic pancreatic clamps, first without then with amino acids infused to reach and maintain hyperaminoacidaemia. The hyperaminoacidaemic phase of the clamp was associated with lowered glucose uptake. Our results also contrast with what we found previously in healthy lean men who had lower glucose MCR during hyperAA compared with a matched group studied during a 117

hyperinsulinaemic–euglycaemic–isoaminoacidaemic clamp (euglycaemic isoAA) (Adegoke et al., 2009). Glucose turnover rates from those studies are presented in Figure 4.4: Nine healthy men (BMI $21.3 \pm 0.4 \text{ kg/m}^2$; age $26 \pm 1 \text{ yrs}$; and FFM 59 \pm 1 kg, all p<0.05 vs. the type 2 diabetic men) underwent a euglycemic IsoAA protocol with insulin (449±24 pmol/l), glucose (5.50±0.01 mmol/l) and BCAA $(412\pm14 \mu mol/l)$ clamped at lower concentrations (p<0.05) than those of type 2 diabetic men during IsoAA. Another group of eight healthy men (BMI 21.5 ± 0.6 kg/m²; age 29 \pm 3 yrs; and FFM 59 \pm 2 kg; all *p*<0.05 vs. type 2 diabetic men) underwent a HyperAA protocol with glucose and amino acid concentrations similar to our study but with greater hyperinsulinaemia $(788\pm83 \text{ pmol/l})$ due to a larger C-peptide response. Postprandial hyperinsulinaemia did not completely suppress endogenous Ra in diabetes in either clamp phase, as it did in lean nondiabetic subjects (Figure 4.4), despite lesser hyperinsulinaemia in the euglycemic IsoAA participants. This confirms hepatic insulin resistance in type 2 diabetes. Whole-body R_d in nondiabetic HyperAA did not differ from that of Hyper-1 despite their higher glucose and insulin concentrations. Therefore glucose MCR was significantly lower in nondiabetic hyperAA, suggesting that hyperaminoacidaemia impeded glucose disposal. In contrast, glucose R_d and MCR in type 2 diabetic men did not decrease between IsoAA and HyperAA.

We postulate that hyperaminoacidaemia does not further impede glucose disposal in diabetes because glucose R_d and MCR are already markedly low compared with non-diabetic groups (Figure. 4.4) such that hyperaminoacidaemia is unlikely to have further notable suppression effect. Abnormalities in serine

phosphorylation of IRS-1 associated with impaired insulin signalling are already established in diabetes (Frojdo et al., 2009, Gual et al., 2005, Muoio and Newgard, 2008a). Thus any additional perturbation via amino acid signalling might not add to the degree and/or patterns of IRS-1 serine phosphorylation to further reduce glucose uptake. The data from muscle biopsies of the present study (Figure 4.3) support this. Both Ser 1101 (Tremblay et al., 2007a) and 636/639 (Tremblay et al., 2005a) are sites implicated in the amino acid-induced negative feedback phosphorylation of IRS-1 through mTOR activation and the subsequent blunting of glucose disposal in healthy lean men. Whereas the sum of insulin and hyperaminoacidaemia caused increased phosphorylation of mTOR and downstream to rpS6 in HyperAA, IRS-1 Ser phosphorylation on both sites did not change, suggesting that it might already be stoichiometrically phosphorylated. Though our protocol lacked a healthy control group, Bouzakri et al (Bouzakri et al., 2003) have found more than twofold higher basal IRS-1 Ser⁶³⁶ phosphorylation in a primary culture of skeletal muscle from diabetic subjects compared with cells from healthy controls. Furthermore, Tremblay and colleagues have reported that while baseline state of IRS-1 serine phosphorylation on Ser312 and Ser636/639 was barely detectable, co-infusion of insulin and amino acids increased IRS-1 Ser636/639 phosphorylation by six fold in lean healthy men (Tremblay *et al.*, 2005b). In our study, baseline phosphorylation of IRS-1 on both Ser 1101 and Ser636/639 was detectable but did not change significantly with hyperinsulinaemia and hyperaminoacidaemia. Taken together, these data support our hypothesis that, unlike healthy individuals, there is an elevated baseline IRS-1

serine phosphorylation associated with reduced peripheral glucose uptake in type 2 diabetes and that acute elevation of amino acids does not further impair wholebody glucose R_d in hyperglycaemic type 2 diabetic men.

Although hyperinsulinaemia stimulated AKT phosphorylation during isoAA at Thr308 and Ser473 (Fig. 4.3a,b), increasing amino acids simultaneously during hyperAA was associated with a lower AKT phosphorylation at both sites. This could be because of time-dependent desensitisation of insulin signalling as the muscle biopsy during hyperAA was obtained 5 h after the start of insulin infusion. This could also be attributed to a negative feedback loop by mTORC1 through a mechanism not involving IRS-1 as reviewed by Hay (Hay, 2005)

Several aspects of our study influence interpretation of the results and the comparison with studies performed in healthy lean men (Krebs *et al.*, 2007, Krebs *et al.*, 2002, Tremblay *et al.*, 2005b). First, the hyperAA steady-state does not mimic the temporal non-steady state changes in substrate and hormone concentrations that occur during meal absorption. Thus, it remains to be shown whether similar findings, or magnitude of effects, would be seen with oral feeding. Second, isoAA preceded hyperAA and thus the time of exposure to exogenous insulin and amino acids may have influenced the response to the second phase, including the phosphorylation state of molecules in the muscle intracellular signalling pathways. Third, serum insulin was a combination of both endogenous and exogenous sources and the proportions differed in the 2 phases of the clamp. This is in contrast to clamp studies (Krebs *et al.*, 2007, Krebs *et al.*, 2002, Tremblay *et al.*, 2005b) in which somatostatin was infused so circulating

insulin and glucagon were most probably exogenous. Fourth, we maintained fasting amino acid concentrations during isoAA by exogenous amino acid infusion, unlike others (Krebs et al., 2007, Krebs et al., 2002, Tremblay et al., 2005b) in which amino acids were not infused during the first hyperinsulinaemic clamp. Hence, plasma concentrations dropped below fasting levels. Furthermore, during hyperinsulinaemic-euglycaemic clamps, merely infusing amino acids to maintain plasma concentrations close to baseline (versus no infusion), decreased glucose uptake (Pisters et al., 1991). Therefore it is possible that by lowering the amino acid concentrations, there is some release of the negative feedback signal that would *improve* glucose uptake. Last, although BCAA levels in the studies by others (Krebs et al., 2007, Krebs et al., 2002, Tremblay et al., 2005b) were comparable to those achieved during our HyperAA, other individual as well as total amino acids [6000 µmol/l in (Krebs et al., 2007, Krebs et al., 2002, Tremblay *et al.*, 2005b) vs.3381 µmol/l in the present study] were markedly higher. This is due to the lower BCAA proportion of the total amino acids in solutions used in those studies. Individual amino acids, other than leucine, were found to suppress glucose transport in L6 myotubes (Tremblay and Marette, 2001). It is therefore difficult to exclude whether the supraphysiological increases in some amino acids in these studies (Krebs et al., 2007, Krebs et al., 2002, Tremblay *et al.*, 2005b) would contribute to the blunted glucose uptake. This effect, if present, would be less in our study. We selected the specific commercial amino acid solution TrophAmine® (B. Braun Medical), because it is particularly rich in leucine, the other BCAA, and essential amino acids, as well as arginine. This probably contributed to the significant increase in leucine and arginine in isoAA (Table 4.2). TrophAmine® (B. Braun Medical) induced protein anabolism in our previous clamp studies (Adegoke *et al.*, 2009, Chevalier *et al.*, 2005a, b), while maintaining physiologically relevant levels of other amino acids.

In conclusion, we have demonstrated that abnormal glucose turnover is not aggravated by postprandial hyperaminoacidaemia in hyperglycaemic type 2 diabetic men. These findings, together with the evidence of impaired protein metabolism in diabetes (Pereira *et al.*, 2008) that is overcome by generous protein provision (Bassil *et al.*, 2010), do not support recommendations to restrict protein intake.

n	8
Age (years)	57±2
Height (cm)	177.5±2.1
Weight (kg)	107.7±7.3
BMI (kg/m^2)	34.0±1.6
FFM (kg)	69.6±4.1
Body fat (%)	34.9±2.2
Waist circumference (cm)	118.6±4.8
HOMA-IR	11.51±2.53
Diabetes duration (years)	9 ± 2

Table 4.1. Participant Characteristics

Data are means \pm SEM. FFM: fat free mass measured by DXA. HOMA-IR: homeostatic model of assessment: insulin resistance

Table 4.2.	Plasma	concentration	IS (µmol/l)	of individ	ual and	total	amino	acids
at baseline	e and du	ring the clam	p steady	states				

Amino acids	Baseline	IsoAA	HyperAA
Essential amino acids	966±51	1013±49	$1547 \pm 66^{*\dagger}$
Histidine	83±5	95±5	$131 \pm 8^{*\dagger}$
Isoleucine	70±5	73±4	$162 \pm 7^{*\dagger}$
Leucine	137±11	156±8*	$312\pm14^{*\dagger}$
Lysine	181±15	199±18	$277 \pm 19^{*\dagger}$
Methionine	28±3	30±2	$57 \pm 3^{*\dagger}$
Phenylalanine	63±4	65±3	$94 \pm 8^{*\dagger}$
Threonine	99±8	94±8	$130\pm10^{*\dagger}$
Tryptophan	58±4	61±4	$84\pm5^{*\dagger}$
Valine	248±7	241±7	$301 \pm 12^{*\dagger}$
Non-essential amino acids	1507±64	1519±104	1833±130* [†]
Alanine	290±7	285±9	$296\pm10^{\dagger}$
Arginine	76±6	93±6*	$182 \pm 10^{*\dagger}$
Asparagine [‡]	46±3	41±5	$34\pm5^{*^{\dagger}}$
Aspartic acid	26±6	33±8	49±11* [†]
Citrulline	39±2	37±4	$50\pm5^{\dagger}$
Glutamine [‡]	482±23	461±29	483±28
Glutamic acid	90±9	107±9	135±12* [†]
Glycine	173±15	177±20	$200\pm 23^{\dagger}$
Ornithine [‡]	83±13	96±20	147±29* [†]
Serine	89±11	99±10	$126\pm12^{\dagger}$
Taurine‡	43±5	44±5	53±5* [†]
Tyrosine	96±5	79±5*	79±4*
Total amino acids	2473±99	2532±137	$3381 \pm 182^{*\dagger}$

Data are means±SEM Repeated measures ANOVA and LSD post-hoc test. p<0.05 vs. baseline. p<0.05 vs. IsoAA. ‡ Asparagine, glutamine, ornithine and taurine are not present in the infused amino acid formulation. Tyrosine is present as N-acetyl-L-tyrosine
	IsoAA (mmol/min)	HyperAA (mmol/min)
Endogenous R _a	0.53 ± 0.05	0.48±0.06
Infusion rate	1.15 ± 0.13	1.34±0.18
Total R _d	1.68 ± 0.11	1.82±0.15
Oxidative R _d	0.52±0.10	0.58±0.12
Nonoxidative R _d	1.17±0.14	1.24±0.17

Table 4.3. Clamp glucose kinetics

Data are means \pm SEM. Paired t-test; R_a, rate of appearance; R_d, rate of disposal.





a







IsoAA



Figure 4.3







4.7. Figure legends

Figure 4.1. Glucose (a) and amino acid (b) concentrations (black squares) and infusion rates (black diamonds) during isoAA vs. hyperAA phases of the clamp in men with type 2 diabetes. AA: amino acids. BCAA: branched chain amino acids. Paired t-test * p < 0.05 vs. IsoAA

Figure 4.2. Hormone and NEFA concentrations at baseline and during isoAA and hyperAA phases of the clamp in men with type 2 diabetes. (a) Insulin (black diamonds) and C-peptide (black squares) concentrations. p<0.05 vs isoAA. (b) Glucagon (black squares) and NEFA (black diamonds) concentrations. p<0.05 vs isoAA

Figure 4.3. Phosphorylation of AKT at Ser473 (**a**) and Thr308 (**b**), mTOR (**c**), rpS6 (**d**), IRS-1 at Ser636/639 (**e**) and Ser1101 (**f**) at baseline and during isoAA and hyperAA. Data are expressed as the ratio of phosphorylated to total protein. Representative western blots are shown. p<0.05 vs baseline, $^{\dagger}p<0.05$ vs isoAA

Figure 4.4. Rates of endogenous glucose production (R_a), glucose disposal (R_d) and glucose MCR presented per kg FFM in men with type 2 diabetes during isoAA (black bar) and hyperAA (white bar), and in lean non-diabetic men during euglycaemic isoAA (grey bar) and hyperAA (hashed bar) (adapted from (Adegoke *et al.*, 2009)). Independent t-test p<0.05 *vs. lean nondiabetic euglycemic IsoAA and HyperAA; †vs. lean nondiabetic HyperAA

4.8. Acknowledgements

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Bridge 3

In manuscripts 1 and 2, subjects with T2DM were hyperglycemic and thus the derangements in glucose and protein metabolism could be partly due to "glucotoxicity" (Del Prato, 2009). Improving glycemia was shown to ameliorate the insulin resistance of glucose in T2DM (Ciaraldi et al., 2002, Kirwan et al., 2009, Pratipanawatr et al., 2002). Furthermore it was previously demonstrated in our laboratories that administration of insulin (Gougeon et al., 1998) or oral antidiabetic agents (Gougeon et al., 2000) normalized the 24-hour integrated fedfasted whole-body protein kinetics in obese T2DM subjects to levels not different from healthy weight- and age-matched controls. However, no study appears to have examined the effect of glycemic control in T2DM on insulin resistance of protein metabolism. To test this, hyperinsulinemic clamps were used in two groups of T2DM men, with different intensities of glycemic control. Results of whole-body glucose turnover and protein kinetics as well as data on cellular mechanisms from muscle biopsies are presented in the following manuscript, manuscript 3.

CHAPTER 5. MANUSCRIPT 3

In preparation for submission to Diabetes Care

Insulin resistance of protein metabolism in type 2 diabetes: effect of two levels of glycemic control.

Maya Bassil, Sergio Burgos, Errol B. Marliss, José A. Morais, Stéphanie Chevalier and Réjeanne Gougeon

McGill Nutrition and Food Science Centre, Montreal, QC, Canada, H3A 1A1.

Corresponding author: Réjeanne Gougeon

McGill Nutrition and Food Science Centre MUHC/Royal Victoria Hospital 687 Pine Avenue West, H6.61 Montreal, QC H3A 1A1 Canada Tel: (514) 843-1665 Fax: (514) 843-1706 E-mail: <u>rejeanne.gougeon@muhc.mcgill.ca</u>

5.1. Abstract

Aims/hypothesis Hyperglycemia in type 2 diabetes (T2DM) is associated with insulin resistance of protein as well as glucose metabolism. We hypothesized that intense glycemic control using oral antidiabetic agents improves the insulin sensitivity of both.

Methods 7 normoglycemic and 8 hyperglycemic (A1C 6.1 ± 0.2 vs. 7.1 ± 0.2 % p<0.01) men with T2DM underwent a 2-step hyperinsulinemic clamp, with amino acids at postabsorptive concentrations (IsoAA) and with glucose at 5.5 mmol/L in the normoglycemic and at 8 mmol/L in the hyperglycemic group. This was followed by a hyperinsulinemic, hyperglycemic (8 mmol/L) clamp with amino acids at postprandial concentrations (HyperAA). Whole-body glucose turnover was assessed using ³H-glucose, and protein kinetics using ¹³C-leucine. Vastus lateralis biopsies were obtained at baseline and during IsoAA and HyperAA to determine the phosphorylation states of proteins in the protein synthesis pathways.

Results Leucine flux was lower in the normoglycemic group (p<0.05) during IsoAA (3.14±0.16 vs. 4.53±0.16 µmol/LBM.min) and HyperAA (2.72±0.07 vs. 4.10±0.08 µmol/LBM.min) but leucine oxidation and net balance did not differ. Phosphorylation states of Akt and PRAS40 increased from baseline (p<0.05) in IsoAA and HyperAA with no difference between groups. rpS6 phosphorylation increased in response to HyperAA but tended to be lower (p=0.06) in the normoglycemic group, as was leucine nonoxidative R_d(p<0.05). *Conclusions/Interpretations* Intense glycemic control (vs. standard control) in T2DM does not improve the protein anabolic response to insulin. It remains to be determined whether physical activity and weight loss that improve insulin resistance of glucose can improve that of protein metabolism.

5.2. Introduction

Hyperglycemic men with type 2 diabetes (T2DM) had blunted wholebody protein anabolic response to insulin that was shown using leucine tracer methodology during a hyperinsulinemic clamp with amino acids infused to maintain baseline concentrations (isoaminoacidemia), compared with lean and weight-matched healthy controls (Bassil *et al.*, 2010, Pereira *et al.*, 2008). Insulin resistance of protein metabolism strongly correlated with that of glucose (Pereira *et al.*, 2008).

Hyperglycemia, per se, aggravates insulin resistance of glucose metabolism in T2DM. This "glucotoxicity" occurs in the liver with reduced suppression of endogenous glucose production by hyperinsulinemia (Campbell *et al.*, 1988, Firth *et al.*, 1987, Groop *et al.*, 1989a, Turk *et al.*, 1995) and at the periphery with blunted insulin-stimulated glucose uptake (Ciaraldi *et al.*, 2005, Pigon *et al.*, 1996, Staehr *et al.*, 2001, Vaag *et al.*, 1995). Acute lowering of glycemia, using insulin, in hyperglycemic subjects with T2DM normalized their hepatic insulin resistance (Kirwan *et al.*, 2009, Miyazaki *et al.*, 2001). Longer term good glycemic control using oral antidiabetic agents improved their glucose

uptake during hyperinsulinemic-euglycemic clamps (Ciaraldi *et al.*, 2002, Kirwan *et al.*, 2009, Pratipanawatr *et al.*, 2002).

Acute lowering of postabsorptive hyperglycemia in diabetic men to normal concentrations (5.5 mmol/L) during hyperinsulinemic isoaminoacidemic clamp (IsoAA) was associated with slower protein turnover rate. Subjects had lower rates of synthesis and breakdown compared to a group of diabetic men who were kept hyperglycemic (8 mmol/L) during the clamp (Bassil *et al.*, 2010). However, net protein anabolism, or synthesis minus breakdown, was comparable between the 2 diabetic groups and was lower than that of lean and obese controls, an indication of insulin resistance of protein metabolism (Bassil *et al.*, 2010). Whether maintaining euglycemia for a longer period would improve this resistance as it does for glucose is still not known.

At the cellular level, insulin stimulates muscle protein synthesis by activating the mammalian target of rapamycin complex 1 (mTORC1) via an Akt dependent signaling pathway. One downstream target of mTORC1 is the phosphorylation of 4E-BP1, which causes dissociation of the complex 4E-BP1-eIF4E. The freed eIF4E binds to eIF4G to form the eIF4F complex, which then binds to mRNA to proceed with translation in the ribosome subunit (Kimball *et al.*, 2002, Kimball *et al.*, 1997, Kimball *et al.*, 1994). Another downstream signaling target of mTOR is the phosphorylation of the ribosomal protein S6 protein kinase (S6K1), which gets activated and leads to hyperphosphorylation of a particular class of mRNA. Amino acids, especially the branched chain amino acid, leucine,

stimulate protein synthesis by activating mTORC1 through a yet unknown mechanism (Anthony *et al.*, 2001, Drummond and Rasmussen, 2008, Kim *et al.*, 2002, Kimball *et al.*, 1999). Few reports studied the effect of T2DM and intensity of glycemic control on the phosphorylation state of intracellular molecules involved in muscle protein synthesis, which warrants further investigation. One animal study showed a reduced insulin mediated protein synthesis in skeletal muscles of diabetic rats associated with lower dissociation of 4E-BP1.eIF4E and activation of S6K1 and S6 compared with controls (Anthony *et al.*, 2002). Orally administered leucine restored protein synthesis to normal levels without increasing 4E-BP1/S6K1 phosphorylation, suggesting an mTOR-independent protein synthesis pathway (Anthony *et al.*, 2002). Another human study found decreased total mTOR and eIF2 α phosphorylation in T2DM subjects vs. controls (Drummond and Rasmussen, 2008).

This study was designed to examine whether long term glycemic control in type 2 diabetes improves glucose and protein metabolism in IsoAA.

We have reported that raising and maintaining amino acids at postprandial concentrations concurrently with hyperglycemia during a hyperinsulinemic clamp (HyperAA) in hyperglycemic men with T2DM, increased net protein anabolism to levels not different from healthy lean controls (Bassil *et al.*, 2010). Thus, the effect of more intense glycemic control on glucose turnover and protein kinetics was also assessed during a second-step hyperinsulinemic-hyperglycemic clamp with amino acids infused to raise and maintain concentrations at postprandial levels, or hyperaminoacidemia (HyperAA). Muscle biopsies were obtained at

baseline and at each step of the clamp for quantification of relevant intracellular signaling molecules. Results have been presented in part in abstract form (CDA, 2010)

5.3. Methods

Fifteen T2DM Caucasian overweight or obese men (8 hyperglycemic and 7 normoglycemic), <65 years of age and conventionally controlled, were studied. Subjects were screened with fasting blood and urine samples, electrocardiogram, chest x-ray and a complete physical examination. Subjects signed the study consent form approved by the institutional Human Ethics Review Board. Exclusion criteria included smoking, unstable weight for the previous 6 months, insulin therapy, abnormal dietary habits assessed by a 24h-recall, and any significant hepatic, hematological, renal, pulmonary, thyroid, or cardiovascular dysfunction.

Subjects started the study diet for 3 days at home and then were admitted for 4 days to the McGill University Health Centre/Royal Victoria Hospital Clinical Investigation Unit. All but one were taking oral antihyperglycemic drugs: metformin, 14; sulfonylureas, 9; and repaglinide, one. Five subjects were treated with statins and six with antihypertensive agents. All medications except antihypertensives were held on the clamp day until the end of the experiment. Subjects consumed a formula-based (80% Ensure® and 20% Glucerna®, Abbott, Saint Laurent, QC, Canada) isoenergetic, protein-controlled diet for six days to assure nitrogen balance is at equilibrium before the clamp experiment. It provided 1.7 g protein kg/FFM·d (16% of energy), 59% of energy from carbohydrates and 25% from fat. The calculated energy requirement for weight maintenance was based on RMR by indirect calorimetry (Deltatrac, SensorMedics, Yorba Linda, CA, USA), multiplied by ~1.6. An energy supplement [2/3 glucose polymer (Polycose®, Abbott, Saint Laurent, QC, Canada) and 1/3 vegetable oil] was given to correct for energy lost as the measured preceding day's glycosuria in the hyperglycemic group. Waist, hip, chest, calf and thigh circumferences were measured according to World Health Organization 1995 criteria (World Health Organization, 1995). Body composition was determined by bioimpedance analysis (RJL-101A Systems, Detroit, MI, USA) and Dual Energy X-Ray Absorptiometry (DXA; Lunar Prodigy Advance, GE Healthcare, Madison, WI, USA). Any premeal capillary glucose (Accuchek III; Boehringer Ingelheim, Mannheim, Germany) > 15 mmol/L in the hyperglycemic group was treated by small doses of insulin, except during the 15h prior to the clamp.

On the clamp experiment day, at 8h00, with subjects in the postabsorptive state (fasting for 15 h), catheters were inserted in an antecubital vein for infusions and in the opposite hand vein, which was kept in a heated box at 65-70°C to arterialize the venous blood, for blood sampling. Glucose turnover was determined with a primed (22 μ Ci) continuous (0.22 μ Ci/min) infusion of 3-[³H]-D-glucose (PerkinElmer Inc., Life and Analytical Sciences, Boston, MA, USA). At the same time a bolus of 0.1 mg/kg of oral NaH¹³CO₂ (MassTrace Inc., Woburn, MA) and of 0.5 mg/kg of intravenous L-1-[¹³C]leucine (Isotech, Sigma-Aldrich, St Louis, MO) was given, followed by a constant infusion rate of 0.008

mg kg⁻¹ min⁻¹ for leucine kinetic determination. Concurrently, a primed infusion of regular human insulin (0.95 mU kg FFM⁻¹ min⁻¹) (Humulin R; Eli Lilly Canada Inc., Toronto, Canada) was started. Glucose (20%) in water (Avebe b.a., Foxhol, the Netherlands) and 10% amino acid solution (TrophAmine[®] 10% without electrolytes, B. Braun Medical Inc., Irvine, CA, USA) were infused. Plasma concentrations of glucose were maintained at 5.5 mmol/L for the normoglycemic and at 8 mmol/L for the hyperglycemic group and those of branched chain amino acids (BCAA) at each individual's fasting BCAA concentrations based on arterialized venous concentration measurements done every 5-10 min. Insulin infusion continued for 2.5h (normoglycemic) or 3h (hyperglycemic) after which BCAA levels were increased to, and maintained at ~750 µmol/L to match peak postprandial concentrations reached during a meal test (2981 kJ, 30 g protein) in lean, healthy subjects (data not shown). In HyperAA, plasma glucose in the normoglycemic group was raised to, and in the hyperglycemic group maintained at, 8 mmol/L. In order to maintain insulin concentrations at the same levels as IsoAA, intravenous insulin infusion was decreased by 50% in the normoglycemic and 17% in the hyperglycemic group to adjust for the stimulation of endogenous insulin secretion by increasing glucose and amino acid concentrations (Manders et al., 2005). The HyperAA clamp lasted for an additional 2.5-3h, after which all infusions were stopped. Blood samples for substrates, hormones, and glucose turnover measurements were collected at baseline, every hour until 30-50 min prior to the end of each step and at 10 min intervals thereafter. Steady states of glucose and amino acid

concentrations and infusion rates were calculated during the last 30 min ("plateau") of each step. Indirect calorimetry was performed for 20 min at baseline and during both plateaus. Leucine kinetics were measured during the last 30 min of the IsoAA clamp in both groups and for 30 min, 2 hours after the start of HyperAA clamp. In the normoglycemic group only, IsoAA clamp was preceded by a 2.5h infusion of tracers to quantify postabsorptive glucose turnover and leucine kinetics (data not shown). At baseline and 100-120 min after the start of each step, ~100 mg vastus lateralis muscle biopsies were obtained with a Bergström needle under local anesthesia (2% Xylocaine, Astra-Zeneca Canada, Mississauga, ON, Canada) using sterile techniques and immediately frozen in liquid nitrogen.

L-[¹³C]-leucine kinetics were calculated according to (Matthews *et al.*, 1980b), using plasma [¹³C]- α -ketoisocaproic acid (KIC) enrichment (reciprocal model) (Matthews *et al.*, 1982), providing leucine total rate of appearance (R_a , flux), endogenous R_a (protein breakdown), oxidation and non-oxidative endogenous rate of disappearance (R_d , protein synthesis). The ¹³C enrichment of expired CO₂ and VCO₂ from indirect calorimetry was used in calculating leucine oxidation. The recovery factor, the proportion of ¹³CO₂ generated during oxidation that is exhaled (Cynober, 1995, Matthews *et al.*, 1980b) was 0.799 during IsoAA steady states and 0.824 during HyperAA, based on previous control studies done in our laboratory. In the calculation of leucine oxidation rates, correction was made to ¹³CO₂ enrichment because low ¹³C glucose solutions dilute the natural enrichment, as previously described (Chevalier *et al.*, 2004). A

factor of 7.0% was used as determined by additional clamp studies done in obese subjects with or without type 2 diabetes during which ¹³C leucine was omitted.

Assays Enrichment of plasma ${}^{13}C-\alpha$ -KIC was determined by GC-MS (5988A; Hewlett-Packard, Palo Alto, CA) after derivatization with N-methyl-N-(tert-butyldimethylsilyl) trifluoroacetamide (Regis Technologies Inc., Morton Grove, IL) as detailed previously (Chevalier *et al.*, 2005a). Expired ${}^{13}CO_2$ enrichment was measured by isotope ratio mass spectrometry (Vacuum Generators, Winsforce, United Kingdom) and plasma glucose concentration by glucose oxidase (GM7 Micro-Stat; Analox instruments USA, Lunenberg, MA, USA). BCAA were measured during the clamp by an enzymatic, fluorometric assay (FP-6200, Jasco Corporation, Tokyo, Japan) (Chevalier et al., 2004) . Serum insulin, C-peptide and glucagon were determined by radioimmunoassay (Millipore, Billerica, MA, USA). Glucose turnover was calculated with OOPSEG (Bradley et al., 1993). Free fatty acids (FFA) were measured by a colorimetric assay (NEFA C; Wako Chemicals USA Inc., Richmond, VA, USA) and reversephase HPLC was used to determine individual plasma amino acid concentrations after pre-column derivatization with o-phtaldehyde (Chevalier et al., 2005b).

Analysis of phosphorylation state of signaling proteins Muscle samples (25 mg wet weight) were homogenized on ice for 30 sec in 10 volumes of homogenization buffer (final concentration in mmol/L: 20 HEPES, pH 7.4, 2 EGTA, 50 NaF, 100 KCl, 0.2 EDTA, 10 Na₄PO₇, 50 β -glycerophosphate) supplemented with 0.5 mmol/L Na₃VO₄, 1 µmol/L microcystin LR, 1 mmol/L dithiotreitol, 1 mmol/L phenylmethylsulfonyl fluoride and 10 µg/ml aprotinin,

leupeptin and pepstatin each using a hand-held homogenizer (Tissuemiser; Fisher Scientific, Mississauga, Canada). The homogenate was cleared by centrifugation at 15,000 \times g, at 4°C, for 15 min. An aliquot of homogenate was used to measure protein concentration by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as standard (Bradford, 1976). An aliquot of the remaining supernatant was mixed with equal volume of 2× Laemli sample buffer and then boiled for 5 min. Samples (25 µg of protein/lane) were resolved by SDS-PAGE and then transferred onto polyvinylidine fluoride membranes (GE Healthcare, Madison, WI, USA). The membranes were blocked in 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 1 h and then incubated with phospho-specific primary antibodies against Akt (Ser473), PRAS40 (Thr246), 4EBP1(Thr37-46), and rpS6 (235/236) at 4°C overnight. Membranes were washed three times for 5 min in TBS-T and subsequently incubated with secondary antibody in TBS-T containing 5% non-fat dried milk at room temperature for 1 h. Immunoblots were developed by enhanced chemiluminescence using ChemiDOC XRS Multi-Imager system (Bio-Rad Laboratories). After detection of the phospho-specific signal, the antibodies were stripped off the membrane by incubation in 62.5 mmol/L Tris-HCl, 100 mmol/L β -mercaptoethanol, and 2% SDS at 50°C for 30 min. The membranes were then washed, blocked and reprobed with primary antibodies against total Akt, PRAS40, 4EBP1 and rpS6. All antibodies were from Cell Signaling Technology (Danvers, MA, USA). Results are expressed as ratios of phosphorylated to total protein.

5.4. Statistical analysis

Results are presented as means \pm SEM. Repeated measures ANOVA (general linear model) was used to assess clamp effects on leucine kinetics, glucose turnover, hormones and substrates within-subject (baseline vs. IsoAA vs. HyperAA, with Bonferroni post-hoc test) and between-group responses to the clamp (hyperglycemic vs. normoglycemic). Data comparisons between hyperglycemic and normoglycemic groups were made using independent t-tests. Levene's test and Mauchly's test were used to check for equality of variances. HOMA was non-normally distributed, according to Shapiro-Wilk test, and was thus log-transformed. Lean body mass (LBM) tended to be higher in the hyperglycemic group (Table 2). Furthermore, it correlated positively with glucose disposal (r= 0.52, p=0.046) and leucine turnover rate (r=0.819, p<0.001). Thus, glucose turnover and leucine kinetics were expressed per LBM. Based on independent samples (Lachin, 1981) and SD from (Gougeon et al., 2000), 8 individuals are needed to detect a 95% difference in net leucine balance between the 2 groups (two-tailed $\alpha = 0.05$, $\beta = 0.2$). A *p*-value less than 0.05 was considered significant. Analyses were performed using SPSS 17.0 for Windows (SPSS, Chicago, IL).

5.5. Results

Age, height, weight and body composition did not differ significantly between the 2 groups (Table 5.1). By design, normoglycemic subjects had lower

A1C, InHOMA-IR and mean AC meal glycemia. Their diabetes duration was shorter.

Baseline metabolic data and responses to each step of the clamp are presented in Table 5.2. Plasma glucose was lower in the normoglycemic group at fasting and in IsoAA, by design. Glycemia in HyperAA was clamped at the same concentrations in both groups, though compared to IsoAA, it was raised from 5.5 mmol/L in the normoglycemic but maintained at 8 mmol/L in the hyperglycemic group. Total (TAA) and branched chain (BCAA) amino acids in IsoAA were kept similar to baseline in the 2 groups. During HyperAA, TAA increased by 33% and BCAA by 63-70% in both groups. Compared to baseline values, leucine did not differ in IsoAA in normoglycemic group while it increased slightly (15%) but significantly (p=0.003) in the hyperglycemic group. There were no differences in TAA, BCAA or leucine concentrations between the 2 groups at baseline and at each step of the clamp. Free fatty acids (FFA) were not different between groups at baseline and were suppressed in both groups in response to clamp. FFA did not differ between groups during both clamp steps. Resting energy expenditure (REE) and respiratory quotient (RQ) did not change in response to clamp in the hyperglycemic group, but REE increased significantly by 9% in the normoglycemic group in HyperAA vs. baseline and IsoAA.

Insulin concentrations were maintained 2.5-3 fold during the clamps with no difference between IsoAA and HyperAA or between groups, confirming the appropriateness of the decreases in insulin infusion rates during HyperAA. There was a significant group effect in C-peptide response to the clamp. Although comparable across time points in the hyperglycemic group, C-peptide concentrations were 31% lower than baseline in IsoAA, and increased by 1.4 fold in response to HyperAA (vs. IsoAA, p=0.004) in the normoglycemic group. Fasting glucagon was lower in the normoglycemic group and there was a group effect in response to the clamp. Compared to baseline, concentrations did not change during IsoAA, but increased in HyperAA in the normoglycemic group. However, in the hyperglycemic group, glucagon concentrations decreased by 11% (p<0.05) during IsoAA and rose more (43% vs. 28%) during HyperAA. Glucagon to insulin ratio decreased in response to IsoAA in both groups and was significantly higher in the hyperglycemic group (p=0.049). During HyperAA it increased significantly vs. IsoAA in the hyperglycemic group following the trend of glucagon (p=0.002)

Results of glucose turnover and substrate oxidation during the clamp are presented in Table 5.3. Glucose endogenous rate of appearance (Ra, glucose production) tended to be lower in the normoglycemic group in IsoAA (p=0.11) and HyperAA (p=0.05). In the normoglycemic group, glucose infusion rate, total rate of disappearance (R_d) and non-oxidative R_d in HyperAA were 54%, 34% and 41% higher, respectively, than those in IsoAA. None of the latter variables was different between clamp steps in the hyperglycemic group. Glucose metabolic clearance rate (MCR), defined as total R_d divided by glycemia (in mg/dL), tended to be higher (p=0.07) in IsoAA in the normoglycemic compared with hyperglycemic group. There were no group or clamp effects on the rates of glucose and fat oxidation. Table 5.4 presents leucine kinetics during the clamp. Leucine flux (total R_a) was lower during both steps of the clamp in normoglycemic compared with hyperglycemic subjects. This is consistent with lower rates of synthesis (nonoxidative R_d) and breakdown (endogenous R_a) in the normoglycemic group in IsoAA and HyperAA, with statistical significance reached only for HyperAA synthesis rate (*p*=0.006). Leucine infusion rate in the normoglycemic group in IsoAA was 14% lower (*p*=0.031) than that in the hyperglycemic group. Net balance (synthesis-breakdown) was not different between groups in both steps of the clamp. Leucine flux, rates of infusion, oxidation and synthesis as well as net balance increased, while rates of breakdown decreased in response to HyperAA in both groups.

Data from muscle biopsies showed an increase from baseline in the phosphorylation state of Akt (Figure 1.a) and its substrate PRAS40 (Figure 1.b) in response to IsoAA in both groups, with no change in the latter in HyperAA. Akt phosphorylation decreased in HyperAA compared to IsoAA in the 2 groups and remained significantly different from baseline in the normoglycemic but not hyperglycemic group. There was no group effect on the phosphorylation state of Akt and PRAS40 at fasting and in response to clamp. mTOR efferent, 4EBP1 phosphorylation at Thr37-46 (Figure 2.a) did not change in response to clamp and was not different between groups at the 3 time points. Phosphorylation of rpS6 (Figure 2.b), another protein downstream of mTOR, increased 4.5 fold from baseline in IsoAA in the normoglycemic group (p<0.001) and 1.4 fold in the hyperglycemic group (p=0.073). Compared with IsoAA, rpS6 phosphorylation in

HyperAA did not change in the normoglycemic group but was 78% higher in the hyperglycemic group such that it tended to be different between groups (p=0.06).

5.6. Discussion

The study showed that net protein balance is not different between hyperglycemic and normoglycemic men with T2DM during hyperinsulinemic clamps with amino acids maintained at individuals' baseline concentrations, and glycemia at mean fasting concentrations (IsoAA). Importantly, hyperglycemic men with T2DM had lower net anabolism compared to weight- and sex-matched healthy controls in IsoAA (0 vs. 10% change from baseline) (Pereira *et al.*, 2008). Taken together, these demonstrate that insulin resistance of protein metabolism is not different between the 2 levels of glycemic control studied.

This is in contrast to our reports on 24h whole-body protein kinetics in subjects with poorly controlled T2DM (A1C, 12.2%). When their glycemia was improved with oral antidiabetic agents (Gougeon *et al.*, 1997a, Gougeon *et al.*, 2000) or normalized with insulin (Gougeon *et al.*, 1998, Gougeon *et al.*, 1997a) for seven days prior to metabolic studies, their net protein balance was increased to levels not different from obese controls. Apart from using different methods (¹⁵N glycine vs. ¹³C leucine) to assess different endpoints (whole body protein kinetics over 24h vs. 3h hyperinsulinemic clamp), a potential factor that could explain these discrepant findings is the difference in the glycemic control between the 2 studies. In the hyperglycemic group of the present study, antidiabetic medications were decreased 6 days prior to the clamp and stopped

only for one day such that their hyperglycemia was short term. Consistently, although significantly different between groups, A1C, a marker of long term glycemic control was at the recommended guidelines of 7% (Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2008) in the hyperglycemic group. Therefore it remains to be determined whether longer term poorer control could further aggravate insulin resistance of protein metabolism in T2DM.

The moderate glycemic control in our hyperglycemic group could explain the absence of significant difference in glucose turnover in IsoAA between groups. There was only a nonsignificant trend for greater suppression of endogenous glucose production in the normoglycemic group, which could be due to lower glucagon to insulin ratio and higher MCR compared with the hyperglycemic group (table 3). Glucose MCR was used instead of R_d to account for the differences in glycemia between groups and in non-insulin dependent glucose uptake (Radziuk and Lickley, 1985).

At the muscle level, results from the biopsies are consistent with predictions from the whole-body data during IsoAA: Akt-dependent mTOR activation was associated with higher rpS6 phosphorylation (Figure 5.3.b), a molecule downstream of mTOR that stimulates the protein translational machinery. Although our protocol lacked a control group, hyperinsulinemia at comparable levels to this clamp was associated with 3.5-4.0 fold increases in Akt phosphorylation in healthy lean subjects (Adegoke *et al.*, 2009, Karlsson *et al.*, 2005), a response significantly higher than the 1.4-1.7 fold observed in both

groups during IsoAA in the present study. Indeed Akt, PRAS40 (substrate of Akt) and rpS6 phosphorylations did not differ between groups. This is in agreement with insulin resistance data i.e. whole-body glucose R_d and net protein balance that were comparable in both groups.

Tighter control was, however, associated with a slower protein turnover rate (lower flux, synthesis and breakdown) in IsoAA. This is in line with our findings in hyperglycemic men with T2DM whose glycemia was acutely normalized to 5.5 mmol/L at the beginning of a similar IsoAA clamp protocol. Their leucine flux was lower compared with a matched diabetic group whose hyperglycemia was maintained during the clamp (Bassil *et al.*, 2010). The lower leucine infusion rate in IsoAA of the present study in the normoglycemic group is consistent with this. It emphasizes the better "efficiency" in utilizing amino acids, such that less amino acid administration is needed to maintain fasting concentrations and achieve the same net balance.

There was a marked anabolic response to hyperaminoacidemia that was comparable between groups. Net balances increased from negative values in IsoAA to a positive net anabolism in HyperAA (table 4). We have shown recently that protein kinetics of hyperglycemic men with T2DM in HyperAA were comparable to those of younger lean healthy men studied during a similar clamp (Bassil *et al.*, 2010). These data thus support that hyperaminoacidemia can overcome the insulin resistance of protein anabolism in obese T2DM men, that has been well documented in IsoAA clamps (Pereira *et al.*, 2008) In the normoglycemic group, protein flux and synthesis rates remained lower than the hyperglycemic group with HyperAA. This was in response to hyperinsulinemia since there was no group effect in the response to increased amino acids. Consistent with this, rpS6 phosphorylation was significantly increased in HyperAA in the hyperglycemic group and tended to be higher than that of the normoglycemic group (figure 5.3.b). However, 4EBP1 phosphorylation at Thr37-46 was not affected by hyperinsulinemia with or without hyperaminoacidemia in both groups (Figure 5.3.a). This phosphorylation site does not appear to be nutrient-sensitive as our group has previously observed in healthy young and elderly subjects who were studied during a similar HyperAA protocol (unpublished data).

Glucose total and non-oxidative R_d increased in response to HyperAA compared with IsoAA in the normoglycemic but not in the hyperglycemic group. This is mainly due to the plasma glucose that was raised from 5.5 to 8 mmol/L in the normoglycemic group while it was maintained at 8 mmol/L in the hyperglycemic group. Higher glucose concentrations in the former group create more mass action and thus higher glucose R_d that is partly not insulin-mediated as previously suggested (Del Prato *et al.*, 1997). The increase in glucose and amino acids in hyperAA in the normoglycemic group versus only raising amino acids in the hyperglycemic group may have also contributed to higher endogenous insulin compared with isoAA, reflected by the higher increments in C-peptide (Table 5.2). This also explains the higher REE in HyperAA vs. IsoAA in the normoglycemic group only, due to more substrate administration. That the insulin

infusion was lowered by 17% in the hyperglycemic as opposed to 50% in the normoglycemic group when starting the hyperAA step of the clamp successfully resulted in comparable mean insulin concentrations in both groups. This assured that most tissues would be perfused at the same level of hyperinsulinemia.

Apart from glycemic control, other aspects of the protocol that differed between groups could have also influenced the interpretation of the results. First, the duration of each clamp step was 2.5 hours for the normoglycemic versus 3 hours for the hyperglycemic group. Furthermore, muscle biopsies in the normoglycemic group were obtained 100 min and in the hyperglycemic group 120 min after the start of each clamp step. Therefore, the time of exposure to hyperinsulinemia with or without hyperaminoacidemia prior to plateau and muscle biopsy was shorter in the normoglycemic group. This could explain why the phosphorylation state of Akt, upstream of the insulin-stimulated protein synthesis pathway, remained elevated in HyperAA vs. IsoAA (Figure 5.1) in the normoglycemic group. Conversely, phosphorylation of rpS6, further down in the pathway was lower (Figure 5.2) compared with hyperglycemic group. Second, the study tested a small difference in prior metabolic control (as inferred by A1C) and thus poorer control in the hyperglycemic group might have revealed bigger differences. Since in our pool of potential recruits, standard diabetes control achieved good A1C, we did not consider justified to purposely allow it to worsen for many weeks prior to study.

In conclusion, intense glycemic control achieved by using oral antidiabetic agents does not improve insulin resistance of protein metabolism.

The effect on protein metabolism of other interventions including dietary (Boden *et al.*, 2005) and exercise (Borghouts and Keizer, 2000), which improve insulin resistance of glucose, still needs to be investigated.

	Hyperglycemic	Normoglycemic	p
n	8	7	
Age (years)	57±2	51±2	0.089
Height (cm)	177.5±2.1	173.2±3.1	0.259
Weight (kg)	107.7±7.3	93.5±2.5	0.103
BMI (kg/m ²)	34.0±1.6	31.2±0.9	0.182
LBM (kg)	66.1±4.0	58.6±2.1	0.138
Body fat (%)	34.9±2.2	33.8±1.8	0.711
Waist circumference (cm)	118.6±4.8	111.2±2.5	0.218
Mean AC meal glucose (mmol/L)	9.2±0.4	7.1±0.2	<0.001
A1C (%)	7.1±0.2	6.1±0.2	0.006
LnHOMA-IR	2.33±0.16	1.87±0.09	0.051
Diabetes duration (vears)	9±2	3±1	0.011

 Table 5.1. Subject characteristics

Data are means \pm SEM. BMI: body mass index. LBM: lean body mass measured by DXA. LnHOMA-IR: log of Homeostatic Model Assessment-Insulin Resistance. AC meal glucose: premeal capillary glucose during the 6 days preceding the clamp, mean of 26-30 readings per subject. *P* value, based on independent samples t-test

Variable and Protocol	Baseline	IsoAA	HyperAA
Glucose (mmol//L)§			
Hyperglycemic	8.38±0.61*	7.95±0.04*	7.93±0.02
Normoglycemic	6.19 ± 0.49^{a}	5.53±0.03 ^a	7.98 ± 0.03^{b}
TAA (µmol/L)			
Hyperglycemic	2499±95 ^a	2532±137 ^a	3381 ± 182^{b}
Normoglycemic	2771 ± 112^{a}	2757 ± 72^{a}	3687 ± 131^{b}
BCAA (µmol/L)			
Hyperglycemic	454 ± 23^{a}	470 ± 18^{a}	770 ± 25^{b}
Normoglycemic	479 ± 20^{a}	494 ± 22^{a}	843 ± 46^{b}
Leucine (µmol/L)			
Hyperglycemic	136±11 ^a	156±8 ^b	312 ± 14^{c}
Normoglycemic	156 ± 6^{a}	163±9 ^b	352 ± 17^{c}
FFA (µmol/L)			
Hyperglycemic	448 ± 56^{a}	127±23 ^b	158±35 ^b
Normoglycemic	347 ± 17^{a}	84 ± 8^{b}	77 ± 14^{b}
Insulin (pmol/L)			
Hyperglycemic	187 ± 40^{a}	630 ± 68^{b}	659±71 ^b
Normoglycemic	148 ± 15^{a}	635 ± 44^{b}	685 ± 105^{b}
C-peptide (pmol/L)†§			
Hyperglycemic	1523±303	1602±279	1793±300
Normoglycemic	1355 ± 76^{a}	929±130 ^b	2234 ± 244^{c}
Glucagon (pmol/L)†			
Hyperglycemic	$36\pm 6^{a}*$	32 ± 6^{b}	46 ± 9^{a}
Normoglycemic	22 ± 2^{a}	21 ± 2^{ab}	27 ± 3^{b}
Glucagon/insulin ratio			
Hyperglycemic	0.208 ± 0.023^{a}	$0.049 \pm 0.005^{b} *$	0.068 ± 0.007^{c}
Normoglycemic	0.157 ± 0.021^{a}	$0.034{\pm}0.004^{b}$	0.046 ± 0.010^{b}
REE (kcal/LBM.min)			
Hyperglycemic	32.0±0.1	32.3±1.3	33.6±1.1
Normoglycemic	30.4 ± 1.1^{a}	30.8 ± 1.2^{a}	33.5 ± 1.4^{b}
RQ			
Hyperglycemic	$0.80{\pm}0.02$	0.81±0.02	0.81±0.02
Normoglycemic	$0.79{\pm}0.02$	0.82 ± 0.02	0.83±0.02

Table 5.2. REE, RQ, and circulating substrate and hormone concentrations at baseline and during clamps

Data are means \pm SEM. Within the same row, values with different letter superscripts are significantly different (p < 0.05, repeated measures ANOVA and Bonferroni posthoc test).*p < 0.05 vs. normoglycemic (independent t-test). Significant group effect; † in response to IsoAA clamp vs. baseline, § in response to HyperAA vs. IsoAA (repeated measures ANOVA). REE: resting energy expenditure. RQ: respiratory quotient. TAA: total amino acids. IAA: Indispensable amino acids. BCAA: branched-chain amino acids. FFA: free fatty acids.

	Hyperglycemic		Normoglycemic		
	IsoAA	HyperAA [§]	IsoAA	HyperAA [§]	ANOVA P value Interaction
Endogenous R _a , mg/LBM.min	1.47±0.14	1.33±0.20	1.15±0.12	0.85±0.08†	0.398
Infusion rate, mg/LBM.min	3.18±0.36	3.70±0.51	2.71±0.28	4.19±0.37*	0.074
Total R _d , mg/LBM.min	4.65±0.34	5.02±0.43	3.88±0.21	5.17±0.31*	0.072
MCR, ml/LBM.min	3.25±0.25	3.52±0.30	3.91±0.22	3.60±0.22	0.786
Oxidative R _d , mg/LBM.min	1.43±0.30	1.59±0.34	1.60±0.29	1.95±0.39	0.717
Nonoxidative R _d , mg/LBM.min	3.22±0.38	3.43±0.50	2.28±0.27	3.22±0.42*	0.072
Fat oxidation, mg/LBM.min	1.19±0.15	1.21±0.16	1.08±0.18	1.11±0.24	0.835

Table 5.3. Hyperinsulinemic clamp glucose turnover and substrate oxidation

Values are means±SEM; R_a , rate of appearance; R_d , rate of disappearance; MCR, metabolic clearance rate (total R_d /glycemia). [§]Glycemia during HyperAA was clamped at 8.0mmol/L in both groups. *p<0.05 vs. IsoAA (within the same group), by paired *t*-test. †p=0.05 vs. Hyperglycemic, by independent *t*-test. Repeated measures ANOVA to compare responses from IsoAA to HyperAA between the 2 protocols

	Нуре	rglycemic	Normo	glycemic	
(µmol/LBM.min)	IsoAA	HyperAA [§]	IsoAA	HyperAA [§]	ANOVA <i>P</i> value Interaction
Total R _a (flux)	3.14±0.16	4.53±0.16*	2.72±0.07†	4.10±0.08*†	0.961
Oxidation	0.71±0.03	1.49±0.09*	0.65±0.44	1.56±0.04*	0.170
Endogenous R _a (breakdown)	2.51±0.17	2.07±0.16*	2.20±0.07	1.83±0.06*	0.011
Infusion rate	0.63±0.03	2.47±0.15*	0.54±0.12†	2.25±0.08*	0.520
Nonoxidative R _d (synthesis)	2.43±0.15	3.04±0.13*	2.07±0.07	2.53±0.08*†	0.345
Net balance (synthesis – breakdown)	-0.07±0.04	0.97±0.09*	-0.13±0.04	0.71±0.08*	0.159

 Table 5.4. Hyperinsulinemic clamp leucine kinetics

Values are means±SEM. [§]Glycemia during HyperAA was clamped at 8.0mmol/L in both groups. *p<0.05 vs. IsoAA, by paired *t*-test. †p<0.05 vs. Hyperglycemic, by independent *t*-test. Repeated measures ANOVA to compare responses from IsoAA to HyperAA between the 2 protocols

Figure 5.1













5.7. Figure legends

Figure 5.1. Phosphorylation of Akt (a) and PRAS40 (b) at baseline and during IsoAA and HyperAA in the 2 groups. Data are expressed as the ratio of Phosphorylated to total protein. Representative western blots are shown. [§]Glycemia during HyperAA was clamped at 8.0mmol/L in both groups. *p<0.05 vs. baseline, † p<0.05 vs. IsoAA (repeated measures ANOVA with Bonferroni post-hoc test).

Figure 5.2. Phosphorylation of 4EBP1 (a) and rpS6 (b) at baseline and during IsoAA and HyperAA in the 2 groups. [§]Glycemia during HyperAA was clamped at 8.0mmol/L in both groups. Data are expressed as the ratio of Phosphorylated to total protein. Representative western blots are shown. *p<0.05 vs. baseline (repeated measures ANOVA with Bonferroni post-hoc test).
CHAPTER 6. OVERALL SUMMARY AND CONCLUSIONS

Of the four hypotheses proposed in this thesis, the first manuscript (chapter 3) addressed two: 1) that hyperglycemia aggravates insulin resistance of protein metabolism in T2DM, compared with euglycemia during a hyperinsulinemic clamp with amino acids maintained at fasting concentrations (Hyper-2 vs. Hyper-1); 2) that protein anabolism will be most impaired in a clamp simulating the fed state (Hyper-3) when it should be at its maximal rate.

The Hyper-2 results revealed a faster turnover rate associated with hyperglycemia in T2DM. It is the first report that examined the effect of hyperglycemia on insulin sensitivity of protein metabolism in T2DM. The results partially support the first hypothesis, as hyperglycemic men with T2DM appear to achieve the same anabolic response to hyperinsulinemia as euglycemic diabetic controls (same net balance) but with less "efficiency" (higher flux). An accelerated glucose turnover (higher R_a and R_d) was also observed with higher glycemia (R_a : 1.39±0.13 vs. 0.70±0.19 mg/kgFFM.min; p= 0.011 and R_d : 4.41 ± 0.31 vs. 3.57 ± 0.35 mg/kgFFM.min, p=0.096). The elevated rates of glucose R_d are most likely the result of mass action of glucose at higher glucose concentration (Del Prato et al., 1997). It can be proposed that increased endogenous glucose production (R_a) to sustain hyperglycemia induces higher protein turnover rate to supply substrates for de-novo glucose synthesis. A negative correlation between leucine flux and serum concentration of the gluconeogenic amino acid glutamine (r = -0.754; p = 0.031), found in this study,

could be consistent with this proposition. Increased rates of gluconeogenesis in T2DM were established previously (Woerle *et al.*, 2006) and appear to be commonly observed in more hyperglycemic subjects, and not affected by serum levels of insulin or glucagon (Boden *et al.*, 2001b, Nuttall *et al.*, 2008a). A positive correlation between fasting protein flux and gluconeogenesis was also demonstrated in obesity (Chevalier *et al.*, 2006b).

Respiratory quotient (Table 3.3) was not increased by hyperinsulinemia (Hyper-2) nor by added hyperaminoacidemia (Hyper-3), in the context of maintained hyperglycemia, which can be linked to defective mitochondrial function and blunted postprandial glucose oxidation reported previously in T2DM (Woerle *et al.*, 2006).

As regards clinical significance, these results reinforce the importance of improving and maintaining good glycemic control in T2DM in order to not only prevent diabetic complications but also correct abnormalities of glucose and protein metabolism.

The main finding of the first manuscript, though, is derived from Hyper-3 data which refute the second hypothesis. Rates of protein synthesis were markedly stimulated and those of breakdown suppressed by postprandial-level hyperaminoacidemia. This resulted in a positive net balance that was comparable to that of healthy lean men previously studied in the same laboratory with a comparable Hyper-3 protocol. Similar results were reported by others who also studied the effect of hyperaminoacidemia on protein anabolism in T2DM using either a clamp protocol (Luzi *et al.*, 1993) or a meal test (Manders *et al.*, 2008).

However it is the first report in which the concurrent stimulation of physiological postprandial hyperinsulinemia and hyperaminoacidemia at clinically relevant hyperglycemia was tested. Of note is that protein consumption prior to the clamp day (1.2 g/kg/d) and that administered during the "meal-like" Hyper-3 (25 g) reflected usual generous intake by T2DM subjects. The latter, in turn, might be needed to overcome insulin resistance of protein metabolism. Lower protein intakes are however recommended for diabetic patients with compromised renal function (Robertson *et al.*, 2007). Thus, it remains to be determined the extent to which intakes at the minimal recommendations of 0.8g/kg/d would alter protein metabolism in T2DM.

The results shown in the first manuscript are data from whole-body protein kinetics. It was assumed that most protein anabolism during the clamp occurs in the skeletal muscles especially that these are the primary tissues responsive to insulin. This is why molecular mechanisms at the muscular level explaining the whole-body results were proposed in the discussion. However insulin also modulates the metabolism of many plasma proteins; it stimulates the synthesis of albumin and antithrombin III and suppresses that of fibrinogen in healthy individuals (De Feo *et al.*, 1993). While albumin synthesis was found to be normal in T2DM in the postabsorptive and hyperinsulinemic states (Tessari *et al.*, 2006) that of fibrinogen was increased (Barazzoni *et al.*, 2003, Tessari *et al.*, 2006), consistent with an insulin resistance or failure in suppressing its synthesis. Therefore measuring the synthesis of these plasma proteins as well as others is

still needed during a clamp similar to the present study to explain whole-body metabolism, in addition to muscular contribution.

The third hypothesis of this thesis was that elevated amino acids with concurrent hyperinsulinemia impede peripheral glucose uptake in T2DM seen with hyperinsulinemia and sustained fasting amino acids. This possibility was raised from multiple previous reports (Tremblay et al., 2007a, Tremblay et al., 2005a, Tremblay et al., 2007b), demonstrating blunted insulin-mediated glucose uptake in response to an increase in amino acids in healthy lean individuals. It was important to test this hypothesis because, as reported in manuscript 1 (chapter 3), high protein intake might be needed to normalize whole-body protein metabolism in T2DM. The protocol of manuscript 1, described above, was used for this purpose and the results are presented in manuscript 2 (chapter 4). Here again, the data disproved the hypothesis as glucose R_d in HyperAA did not differ from that in IsoAA. This is the first study to examine the isolated effect of elevated amino acids on peripheral glucose uptake in T2DM. Noteworthy is that there was no further diminution in glucose uptake from the already considerably reduced rates, typical of obesity and diabetes. Amino acids can impact glucose metabolism indirectly by stimulating insulin secretion (Nuttall and Gannon, 1991) that, in turn, could enhance glucose uptake. However, this effect was accounted for in my protocol when exogenous insulin was lowered in HyperAA to successfully obtain similar peripheral insulin concentrations as IsoAA (Fig. 4.2).

One other strength of the protocol is the success in simulating postprandial hyperaminoacidemia by using the commercial amino acid solution

TrophAmine®, with concentrations during HyperAA highly comparable to those achieved during a typical meal. This was also obtained in a previous HyperAA study undertaken in the same lab by healthy lean subjects (Adegoke *et al.*, 2009) using the same solution. Asparagine and glutamine are not included in TrophAmine® and therefore plasma concentrations did not increase in HyperAA (Table 4.2). Citrulline and ornithine are also absent but their concentrations increased, probably because they are metabolized from arginine via the urea cycle. Although present as N-acetyl-L-tyrosine, plasma tyrosine concentrations did not change, possibly because it is not effectively deacetylated in adults (Magnusson *et al.*, 1989).

Of note is that, similar to healthy individuals, glucose R_d in T2DM subjects during a conventional hyperinsulinemic euglycemic clamp (4.37±0.45 mg/kg.min) (DeFronzo *et al.*, 1985) is higher than when amino acids are infused to maintain fasting concentrations during a similar clamp (2.62±0.22 mg/kg.min) (Pereira *et al.*, 2008). In the former clamp, however, plasma amino acids dropped below baseline values due to suppression of proteolysis by insulin (Fukagawa *et al.*, 1986). This indicates that sustained postabsorptive amino acids compared with hypoaminoacidemia attenuate glucose uptake. In contrast, as demonstrated in manuscript 2, postprandial hyperaminoacidemia does not add to this attenuation. Therefore these results, together with those of manuscript 1, are in discordance with dietary recommendations limiting protein intake in T2DM.

Muscle biopsies obtained at fasting and during both steps of the clamp supported the whole-body data. The western blots of mTOR and IRS-1 were not

of the best quality for commonly experienced technical reasons, but were comparable to those published previously (Tremblay et al., 2005b). S6K1 negative-feedback phosphorylation of IRS-1 on serine residues was suggested as the principal mechanism through which amino acids suppress glucose uptake in healthy individuals (Tremblay et al., 2007a, Tremblay et al., 2005a, Tremblay et al., 2007b). Western blotting for S6K1 was performed but the bands were not quantifiable. However, an increase in phosphorylation of mTOR and rpS6, the efferent of S6K1, during Hyper-3 (Fig. 4.3) is a good marker that the latter was indeed activated. Despite this, IRS phosphorylation on Ser 636/639 and Ser1101 did not change, consistent with an already elevated phosphorylation and attenuated whole-body glucose uptake. As acknowledged in manuscript 2, the protocol lacked a control group of healthy subjects. If similar to what was demonstrated in the literature (Tremblay et al., 2007a, Tremblay et al., 2005a, Tremblay et al., 2007b), had a healthy control group been studied with the same protocol as that in manuscript 2, it would be expected to have lower baseline IRS serine phosphorylation that increases with hyperinsulinemia and hyperaminoacidemia. This would be associated with lower whole-body glucose uptake compared with hyperinsulinemia and isoaminoacidemia.

The third manuscript (chapter 5) tested the hypothesis that tight glucose control improves insulin resistance of protein metabolism in T2DM as it does for glucose. The results demonstrated otherwise, as essentially normoglycemic men with T2DM (A1C = $6.1\pm0.2\%$, FPG = 6.2 ± 0.5 mmol/L) had the same net protein anabolism compared with age, sex and BMI-matched hyperglycemic diabetic

subjects (A1C = $7.1\pm0.2\%$, FPG = 8.4 ± 0.6 mmol/L) in response to hyperinsulinemia with and without hyperaminoacidemia.

It was acknowledged that the prior glycemic control of the "hyperglycemic" group was at the goal of treatment guidelines, and thus might have masked impairment associated with A1C levels above this target that are not uncommon clinically. We had difficulty recruiting such subjects, perhaps not surprisingly. Nevertheless, it can be stated with confidence that normoglycemia does not restore insulin-stimulated protein anabolism to levels of healthy controls. balance of normoglycemic T2DM men (-0.012 ± 0.04) Net protein umol/kgFFM.min) during hyperinsulinemic euglycemic isoaminoacidemic clamps was significantly lower (p=0.01) than that of matched obese men (0.06±0.05 µmol/kgFFM.min) studied previously in the same laboratory in the same conditions (Pereira et al., 2008). In addition to being novel, these data are in line with the recommendations to broaden the focus in the management of T2DM beyond using pharmacotherapy only to achieve good glycemic control, and include interventions that target body composition as well. This is supported by the significant correlations between net protein balance (expressed per kg FFM.min) in IsoAA studies of T2DM subjects pooled together (n=23) with markers of adiposity (% fat, r = -0.50, p = 0.02; waist circumference, r = -0.45, p =0.03) rather than those of diabetes control (A1C, r = 0.30, p = 0.16; LnHOMA-IR, r = -0.24, p = 0.28).

Normoglycemia versus hyperglycemia in T2DM was, however, associated with a 22% lower protein turnover rate in response to hyperinsulinemia (IsoAA,

Table 5.4). This was also demonstrated in manuscript 1 with acute lowering of postabsorptive hyperglycemia to 5.5 mmol/L by insulin during IsoAA clamps and in the 24h integrated fed-fasted state. Furthermore, clamping glucose at ~10 mmol/L during an IsoAA clamp in healthy lean subjects resulted in a 17% higher protein flux compared with a similar clamp at euglycemia (Flakoll *et al.*, 1993).

Chronic elevation of plasma glucose impairs both insulin action and insulin secretion (Del Prato, 2009, Lisato et al., 1992). This is thought to be mainly mediated through hyperglycemia-induced oxidative stress (Newsholme et al., 2007). Furthermore glucose suppresses, in a dose-dependent manner, the phosphatidylinositol 3-Kinase (PI3K)/Akt signaling pathway in murine 3T3-L1 cells through mTOR-mediated IRS-1 serine phosphorylation (Tzatsos and Kandror, 2006). IRS-1 phosphorylation at Ser636/639 was quantified at baseline and in IsoAA and HyperAA in normoglycemic and hyperglycemic T2DM men. While IRS-1^{Ser636/639} phosphorylation was not different among time points in hyperglycemic group (manuscript 2, Figure 4.3d), it was 2 fold higher than baseline (data not shown) in IsoAA and remained unchanged in HyperAA in the normoglycemic group. This could indicate, in the former group, an already elevated baseline IRS-1 serine phosphorylation associated with hyperglycemia. However, since western blots of normoglycemic and hyperglycemic groups were performed on 2 different gels, between-group comparisons cannot be made.

Glucose turnover and protein kinetics of normoglycemic men with T2DM were also assessed in the fasting state but results were not presented in the manuscript. Protein flux (2.51 ± 0.07), rates of synthesis (1.99 ± 0.06), breakdown

(2.51±0.07), oxidation (0.53±0.02) and net balance (-0.53±0.02 μ mol/kgFFM.min) were comparable to those of matched hyperglycemic T2DM men (Pereira *et al.*, 2008). Furthermore, those kinetics were found not to be impaired compared with obese controls (Pereira *et al.*, 2008). This is consistent with an absence of a T2DM effect on fasting protein kinetics, reported in T2DM women (Pereira *et al.*, 2008, Staten *et al.*, 1986, Welle and Nair, 1990) and in a heterogeneous sample of T2DM men and women (Halvatsiotis *et al.*, 2002a, Luzi *et al.*, 1993, Umpleby *et al.*, 1990).

Postabsorptive glucose R_a of normoglycemic T2DM subjects (2.52±0.21) was not different (p>0.05) from that of obese controls (2.37±0.11 mg/kgFFM.min) (Pereira *et al.*, 2008), while that of the hyperglycemic group was 22% higher (p<0.05). This indicates an amelioration of hepatic insulin resistance of glucose with better glycemia, which was demonstrated previously (Boden *et al.*, 2001b, Jeng *et al.*, 1994) and could be linked to lower fasting glucagon in the normoglycemic group (22±2 vs. 36±6 pmol/L, p=0.04).

At the cellular level, muscle biopsy data were in concordance with the whole-body protein kinetics. However results included only the phosphorylation states of signaling proteins within the protein synthesis pathway. Identifying and quantifying relevant molecules in the protein breakdown pathway, whose activation can be modulated within the 2 to 3 hour period of the clamp, need further investigation. Muscle protein breakdown takes place mostly via the ubiquitin (Ub)-proteasome pathway (Goldberg, 2003, Lorite *et al.*, 2001, Mitch and Goldberg, 1996) and autophagy (Sandri, 2010). Both are suppressed by

insulin by Akt-mediated blocking of nuclear translocation of FOXO. This transcription factor is thus prevented from increasing the expression of ligases that catalyze the ubiquitination of specific proteins triggering their degradation by the 26S proteasome (Latres *et al.*, 2005, Wing and Banville, 1994). FOXO activation also induces muscle protein autophagy by regulating autophagy genes such as LC3 and Gabarap (Mammucari *et al.*, 2007). During autophagy, the cytoplasmic form of LC3 (LC3 I) is processed and recruited to the autophagosomes, where LC3 II is generated by site specific proteolysis and lipidation near to the C-terminus. Tracking the conversion of LC3-I to LC3-II is, at this time, the only reliable marker of autophagosomes (Sandri, 2010). This conversion was quantified in the present study using western blotting from muscle biopsies obtained in the protocol of manuscript 3. However, only one LC3-II band from one out of 42 muscle biopsies was detectable.

Additional strengths of the studies include using validated clamp and tracer techniques to quantify glucose turnover and protein kinetics (see appendix 1) and adjusting for many confounders that affect protein metabolism such as sex, age, weight, body composition, prior dietary and protein intake as well as physical activity.

As for the study limitations, other than those stated in the manuscripts, statistical power could have been improved by larger sample sizes. For manuscripts 1 and 2, sample sizes used were sufficient to be able to detect potential differences with adequate statistical power (90%) and accept or reject the null hypothesis. However for manuscript 3 studying more subjects would

increase the power from 77% especially that the *p* value for many comparisons was of borderline significance. Recruitment was difficult with the length and invasive nature of the study as well as the strict inclusion criteria especially for the normoglycemic T2DM group (manuscript 3) and the requirement to have A1C <7%. Of the 110 eligible screened subjects, 40 phone and in-person interviews and 30 potential candidate screenings, only 15 subjects participated and completed the study. Furthermore, data from 9 T2DM, 9 obese and 18 lean men who were studied previously in the same laboratory using similar clamp protocols were reanalyzed for comparison purposes. One possible recruitment target for future studies would be to obtain ethics approval to screen patients in family-medicine clinics where one would expect higher probability of finding candidates with tight control T2DM.

One confounding factor that was not accounted for in the first manuscript is the discrepant use of oral antidiabetic agents between groups. Although both T2DM groups had comparable types and dosages of medications prior to the study, Hyper-1 group stopped diabetes medications before admission while in the Hyper-2 group, medication dosages were reduced during the study and stopped only on the night prior to the clamp. It is conceivable that drugs might have contributed more to the kinetics of Hyper-2 clamp than to those of Hyper-1 clamp.

Another limitation is the use of liquid meal replacement Ensure® as the major constituent of the diet to control for energy and protein intake prior to the clamp. Although its macronutrient and micronutrient content is adequate, Ensure® is rich

in simple sugars (18g/250 kcal) and thus induced a slight increase in glycemia which was unfavourable in the normoglycemic group. In addition, the protein sources in Ensure® are milk and soy, which represent only two of the different types of dietary protein sources regularly consumed by adults. The use of solid foods, more representative of usual diets was not possible due to the lack of a metabolic kitchen in the research centre. Furthermore, it was important to be consistent for comparison with previous studies undertaken in the same laboratory by lean, obese and T2DM subjects who consumed Ensure®-based diets prior to their clamp studies.

Using archived data from previous studies is another limitation due to intermeasurer errors and the use of different equipments. For instance, different spectrofluorometer, GCMS and insulin assays were used in the two T2DM groups of manuscript 1. A different indirect calorimeter was used for each group in manuscript 3. However, for all the latter, validation studies had been conducted to verify that either methods or equipments produce comparable results.

In summary, the main findings of this thesis are that in obese men with T2DM, 1) insulin resistance of protein metabolism in T2DM is not normalized by short term intense glycemic control, 2) sustained postprandial hyperaminoacidemia that simulates a generous protein intake overcomes the insulin resistance of protein metabolism, and 3) hyperaminoacidemia with concurrent hyperinsulinemia does not aggravate peripheral insulin resistance of glucose.

It has been pointed out how this complex set of metabolic studies is pertinent to the understanding of the derangements of protein metabolism in T2DM. These observations point the way to the kinds of randomized clinical trials that would have to be designed to resolve the lack of strong evidence behind protein intake recommendations for the number of persons with T2DM whose number is growing to epidemic dimensions. Our findings further underscore that recommendations will have to be sex-specific, with iterative adjustments for current levels of A1C and the presence of other disorders in each person. Finally, although the hypothesis that poor metabolic control would be associated with correspondingly poorer ability to achieve protein anabolism in the fed state was not fully supported, this perhaps provide explanation for why we do not commonly encounter clinically apparent, uncontrolled lean tissue loss. Abundant protein intake, common in overweight T2DM individuals, seems able to overcome the thoroughly-documented insulin resistance of protein anabolism. Notwithstanding, there are likely many long-term, more subtle effects, and such insulin resistance may even ultimately be found to contribute to the development of the common co-morbidities.

Accordingly, further investigation is warranted to test whether 1) the protein anabolic response to a meal is similar to that obtained in a clamp simulating the fed state, 2) lower protein intakes in T2DM like those recommended in chronic renal disease would affect protein metabolism, 3) different diet and physical activity regimens used to manage weight or improve body composition in T2DM would mitigate insulin resistance of protein

metabolism as they do for glucose, 3) explore and identify cellular mechanisms especially within the muscle protein breakdown pathway that could explain whole-body protein kinetics data in T2DM.

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APPENDICES

Appendix 1. Methodology

Appendix 2. Newspaper advertisements

Appendix 3. Recruitment posters

Appendix 4. Phone interview questionnaire

Appendix 5. Study timeline and outline of the tests and measurements

Appendix 6. Consent forms

Appendix 7. 24-hour recall questionnaire

Appendix 8. Baecke questionnaire

Appendix 9. MOSPA questionnaire

Appendix 10. Hyperinsulinemic clamp

Appendix 1. Methodology

Subjects and ethical considerations

Fifteen overweight/obese Caucasian men with T2DM, in otherwise good health, and aged <65 years were recruited from newspaper advertisements (appendix 2), posters (appendix 3) and through scanning the patients charts on the Metabolic Day Centre of the Royal Victoria Hospital (permission obtained from MUHC Office of the Director of Professional Services and corresponding endocrinologists). Potential interested subjects underwent a phone or in-person interview during which responses to a general questionnaire (appendix 4) about medical history and lifestyle habits were collected and a brief introduction of the study was provided. The subsequent step (appendix 5) included admission to the McGill University Health Centre-Royal Victoria Hospital's Clinical Investigation Unit (CIU) where subjects were informed thoroughly of the nature, purpose and possible risks of the study and signed a consent form approved by the Hospital's Ethics Committee (appendix 6). On this same day, subjects were screened with a complete physical examination by one of the MDs (co-investigators in the protocol) and a 24-hour recall was collected (appendix 7). Weight and height were obtained for the calculation of BMI. Bioelectrical impedance (RJL-101A Systems, Detroit, MI) and indirect calorimetry (Deltatrac, SensorMedics, Yorba Linda, VA) were performed in order to estimate FFM and RMR respectively. CBC, biochemistry, A1C, lipids, TSH, serology for hepatitis and HIV, chest Xray, electrocardiogram and urine analysis were done. Exclusion criteria included very low carbohydrate diets, abnormal dietary habits assessed by a 24h-recall (appendix 7), substance abuse, uncontrolled hypertension, unstable weight for the last 6 months, cancer other than skin within 5 years, other significant medical diagnoses, serum creatinine > 120 μ mol/L, hemoglobin < 120 g/L, positive HIV or hepatitis serology, liver function test results > 2 X upper limits of normal, proteinuria and the following medications: β -blockers, bronchodilators, antianginals, anticoagulants, antiarrhythmics, oral steroids. After admission, subjects completed two validated questionnaires of habitual physical activity: Baecke (Baecke *et al.*, 1982) (appendix 8) and MOSPA (1997) (appendix 9).

Clinical Investigation Unit Admission

The subjects screened with elevated A1C that reflected long term poor control were studied in the hyperglycemic protocol and were taken off some antidiabetic medications in hospital. The subjects whose A1C reflected good control were admitted and euglycemia was maintained. During the study, the subjects were confined to ambulation on hospital grounds and ward

Diet: Subjects were given a formula-based isoenergetic, protein controlled diet for 6 days to assure N balance does not differ from 0 before the clamp experiment. The prescribed diet started at home 3 days prior to admission and continued for 4 days in hospital. The diet was divided into 4 or 5 equal meals ending at 2000 h. N balance was measured once subjects were hospitalized. The diet consisted of complete formulas (Ensure[®]) and (Glucerna[®]) with applesauce, 150mL of milk (Québon, Natrel, Longueuil, QC) and 30 g All Bran cereal (All Bran, Kellogg Cabada Inc, Mississauga, ON), to provide 1.7 g protein/kg FFM/d

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(16 % of energy) and 59% of energy from carbohydrates and 25% from fat. The calculated energy requirement for weight maintenance is based on measured RMR, multiplied by 1.6 and compared to the 24h recall. An energy supplement [(2/3 glucose polymer (Polycose[®]) and 1/3 vegetable oil] was given to correct for energy loss as the measured preceding day's glycosuria in the hyperglycemic subjects, and protein intake was kept constant.

Monitoring: During hospitalization, overnight-fasted serum electrolytes, calcium, phosphorus, uric acid, liver and kidney function tests, and complete blood counts were performed on the fifth day. Subjects were weighed and their body temperature measured daily. Their body circumferences (waist, hip, chest, calf and thigh) and FFM by bioimpedance analysis were measured once as well as their body composition by Dual Energy X-Ray Absorptiometry (DEXA) using Lunar DPX machine at McGill Nutrition and Performance Laboratory. Capillary blood glucose was measured before each meal using the Chemstrip bG and Accuchek III meter system (Boehringer Mannheim). In the hyperglycemic protocol, capillary glucose that read > 15 mmol/L was treated by small doses of insulin. All 24-h urines were collected in containers and stored at 4°C. Aliquots were analyzed daily for glucose, urea, creatinine and electrolytes.

Hyperinsulinemic clamp

On the clamp day (appendix 10), at 07h05, an antecubital catheter was inserted in an arm for infusions and in retrograde contralateral hand vein of the opposite arm (Zello *et al.*, 1990) for blood sampling. The hand was placed in a heated box at 65-70°C to arterialize the venous blood. The first muscle biopsy and

baseline blood and breath samples were taken. Thereafter, RMR was measured by indirect calorimetry (Deltatrac® Metabolic Monitor, Sensormedics Corporation, Anaheim, CA for n=8 and TrueOne[®] 2400 Canopy System, Parvo Medics, Sandy, UT for n=7). Data were collected while the subjects breathed under a plastic canopy for 20 minutes. The average of the last 15-20 minutes was used for calculation of 24-hour resting energy expenditure according to the de Weir equation (Weir, 1949). At t0min, a primed infusion of $[{}^{3}H_{3}]$ glucose (PerkinElmer Inc., Life and Analytical Sciences, Boston, MA) with a 22 µCi bolus was given at the start and the bolus was adjusted according to the glycemia (Hoerr et al. 1989). It was followed by a 0.22 µCi/min infusion for the duration of the study, for glucose kinetics. Then, an intravenous bolus of 0.1 mg/kg of NaH¹³CO₂ (MassTrace Inc., Woburn, MA) and of 0.5 mg/kg of L-[¹³C]leucine (Isotech, Sigma-Aldrich, St Louis, MO), followed by a constant infusion rate of 0.008 mg/kg/min of ¹³C-leucine, was given for the first step of the clamp (2.5-3 hours). A primed infusion of biosynthetic regular human insulin (0.95-1.2 mU/kg FFM•min) (Humulin R; Eli Lilly Canada Inc, Toronto, Canada), 20% low ³Henriched glucose in water (Avebe b.a., Foxhol, the Netherlands) and amino acid solution (TrophAmine[®] 10% without electrolytes, McGaw Inc., Irvine, CA, USA) started at variable rates based on measurements of arterial plasma glucose by glucose oxidase and BCAA concentrations by fluorometry (Jasco FP-6200, jasco Corporation, Tokyo, Japan) done every 5-10 min to adjust infusion rates to maintain concentrations constant. At 100-120min, a second muscle biopsy was taken, indirect calorimetry repeated and blood and breath samples taken. VCO₂ was used for ¹³CO₂ production and RQ for substrate oxidation calculation. At 150-180 min, BCAA levels were increased to 700-800 µmol/L in both protocols, the ¹³C leucine rate increased by 50% to prevent a dilution effect in specific activity, glycemia increased to 8 mmol/L in those previously at 5.5 mM and intravenous insulin decreased by 17% in the hyperglycemic group and 50% in those previously at 5.5 mM to compensate for increased stimulation of endogenous insulin secretion due to increases in glucose and/or amino acid infusions. At 250-300 min a third biopsy was taken, followed by indirect calorimetry and more frequent sampling of blood and breath. Plateaus in glucose and amino acid infusion rates were achieved and maintained for 30 min in both states. The infusions were stopped at 300-360 min. Blood samples from the arterial line were collected for glucose, BCAA, insulin, glucagon, C-peptide, amino acid, FFA, tritiated glucose specific activity and $[^{13}C]$ leucine enrichment, at baseline and every hour until 30 min prior to the second step when it was done at 10 min intervals. Thereafter, sampling was hourly until the last 30 min, when it was resumed at 10 min intervals. In conjunction with blood samples, expired air samples were collected into a breath collection balloon. The expired air was then transferred to 10-mL Vacutainer tubes and stored at room temperature until analyzed for ¹³CO₂ enrichment by IRMS.

Blood samples were taken until 30 min after the end of the clamp to monitor blood glucose. A meal was served and a supplementation of potassium given. Serum potassium and capillary glucose were measured after the study to ensure that the values are within the normal range. Urine collection was done throughout for urea and glucose measurements.

Glucose Oxidase

During the clamp, plasma glucose concentrations were maintained constant by feedback adjustment after measuring them in blood samples withdrawn every 5-10 min, on a GM7 Micro-Stat glucose analyzer. β -D-glucose, in the presence of oxygen, is converted to gluconic acid, a chemical reaction catalyzed by glucose oxidase. The amount of oxygen consumed corresponds to the amount of glucose in the sample.

Fluorometric Assay for Branched Chain Amino Acids

To maintain plasma amino acids (AA) at constant postabsorptive (isoaminoacidemia) or postprandial levels (hyperaminoacidemia), plasma BCAA were measured as indicators of total AA since they are the ones mostly sensitive to insulin. Plasma BCAA were quantified every 5-10 min using a fluorometry technique, whereby each BCAA undergoes oxidative deamination to the corresponding ketoacid, catalyzed by the enzyme *Bacillus cereus* leucine dehydrogenase (E.C. 1.4.1.9, specific activity of 47.0 U/mg; Calbiochem-Novabiochem, La Jolla, CA) and in the presence of the cofactor NAD⁺ (β -NAD, Roche, Roche Diagnostics, Laval, QC). As a product, NADH was generated, whose fluorescence was measured on a spectrofluometer (Jasco FP-6200, Jasco Corporation, Tokyo, Japan) equipped with a temperature controller (Jasco ETC-272T, Jasco Corporation, Tokyo, Japan) during the first 3-4 minutes of the reaction, which occurred at 37°C and at wavelengths of 355 nm for excitation and

485 nm for emission. *Bacillus cereus* leucine dehydrogenase in a buffer of 25 mmol/L sodium phosphate (Fisher Chemicals, Fischer Scientific, St Laurent, QC) was stored at -70°C until the day of the clamp, when it was diluted in 25 mmol/L sodium phosphate buffer with 1 mg/mL of bovine serum albumin (Sigma, Sigma Chemical, St Louis, MO). β -NAD was dissolved in a buffer of 0.1 mol/L sodium carbonate (Fisher Chemicals, Fischer Scientific, St Laurent, QC). Each sample for analysis consisted of 25µL of plasma, 4 mmol/L of NAD, 0.5U of leucine dehydrogenase, and buffer (0.1mol/L potassium phosphate, 2 mmol/L EDTA, and 0.02% mercaptoethanol; Sigma, Sigma Chemical, St Louis, MO). A blank sample was run before each reading to establish background fluorescence and BCAA concentrations were determined from a standard curve that was plotted in the morning of the experiments using BCAA standard mixture (Sigma, Sigma Chemical, St Louis, MO)

Analyses and Calculations

Nitrogen balance

Urine Nitrogen (UrN) is the sum of nitrogen from urea, ammonia and creatinine, obtained from measurements of daily urine collections throughout admission by the RVH laboratory Nitrogen balance was determined by subtracting the sum of UrN, fecal losses (70 mg nitrogen per g nitrogen intake) (Gougeon *et al.*, 2000), and miscellaneous losses (5 mg nitrogen per kg BW) (Gougeon *et al.*, 2000) from nitrogen intake, determined from the study diet.

L-[1-¹³C] Leucine Kinetics

L-[1-¹³C] leucine kinetics methodology is based on a two pool model, an AA pool and a bigger protein pool, at the level of the whole body (Abrams and 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 exit 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 μ mol/min, and at steady state, the rates of appearance and disappearance of the tracer are equal according to the following equation (Abrams and Wong, 2003):

$$\mathbf{Q} = \mathbf{S} + \mathbf{O} = \mathbf{B} + \mathbf{I}$$

When L-[1-¹³C] leucine is used to represent the other AAs, S, O, B and I would refer to the rates at which leucine is incorporated into protein, oxidized, released from protein breakdown, or infused, respectively. This simplified stochastic model is a simplification (Cynober, 1995, Wolfe and Chinkes, 2005) and has various assumptions. For example, the isotope L-[1-¹³C] leucine is indistinguishable from the much more common L-[1-¹²C] leucine and hence, the tracer moves into the same compartments and undergoes the same biochemical reactions as the trace (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). In the intracellular compartments, BCAA transaminase catalyzes the reversible conversion of leucine to α -Ketoisocaproic acid (α -KIC).
Plasma ¹³C- α -KIC is used in the calculations of leucine kinetics since it was found to be a better indicator of intracellular leucine enrichment than plasma ¹³C-leucine (Matthews *et al.*, 1982, Thompson *et al.*, 1988). This is the reciprocal model, as opposed to the minimal model which uses plasma leucine enrichment. The ¹³C enrichment of expired CO₂ was ultimately used in calculating leucine O. The following steady state equations for leucine Q and O from Matthews et al. (1980a) have been adapted for the reciprocal model:

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

Enrichment is expressed as Atom Percent Excess (APE). E_i is the enrichment of L-[1-¹³C]leucine administered, as specified by the manufacturer and **i** is the rate of L-[1-¹³C]leucine infusion in µmol/kgBW·hr. E_p is the enrichment of ¹³C- α -KIC in plasma, according to the equation APE = 100(r_sr_b)/[(r_s-r_b)+1], where r is the ratio ¹³ α -KIC/¹² α -KIC, the subscript s refers either clamp steady state, and the subscript b refers to baseline (Wolfe and Chinkes, 2005). **V**_{13CO2} is the rate of ¹³CO₂ production in µmol/kgBW·hr, while **V**_{CO2} is the rate of CO₂ production in mL/min and **E**_{CO2} is the ¹³C enrichment of CO₂ exhaled. ¹³CO₂ enrichment is initially obtained as delta (δ) and is converted to APE (Wolfe and Chinkes, 2005); the ratio ¹³CO₂/¹²CO₂ 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 baseline (Wolfe and Chinkes, 2005).

The above equations also include conversion factors: 100 changes APE into a fraction, 44.6 in μ mol/mL converts the units of air from mL to μ mol 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 ¹³CO₂ 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.*, 1980a). Based on previous bicarbonate studies in our laboratory, **r** equals 0.799 during the hyperinsulinemic isoaminoacidemic clamp steady state and 0.824 in the clamp simulating the fed state (hyperinsulinemic hyperglycemic hypearminoacidemic clamp).

The values of Q and O for each steady state were calculated from the averages of ${}^{13}CO_2$ and ${}^{13}C-\alpha$ -KIC enrichments for each plateau. Since Q and O are calculable and I is known, S and B are determined by subtraction. B=Q-I and S=Q-O. The sum of B and I are known as rate of appearance (R_a) and B as endogenous 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 was also used to measure V_{CO2} . Breath samples were collected in Kendall tubes (Tyco Healthcare Group LP, Mansfield, MA). E_{CO2} was determined using Micromass 903D (Vacuum Generators, Winsforce, United Kingdom), an isotope ratio mass spectrometer.

Assessment of ¹³C- α -KIC enrichment in plasma involves a series of steps (Chevalier *et al.*, 2005b, Mamer and Montgomery, 1988). Protein was precipitated from the plasma sample and the supernatant was collected. At a basic pH, NaBH₄ 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).

Adjustments to ${}^{13}\text{CO}_2$ enrichment were necessary during the clamp because infusions of glucose and AAs dilute the 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 is 7.0% for T2DM subjects.

Glucose turnover using D-[3-³H] glucose

Glucose turnover studies are based on a single pool model and at steady state, the rate at which glucose appears in plasma, rate of appearance (R_a), equals the rate at which it leaves plasma to enter tissues, rate of disappearance (R_d) (Wolfe and Chinkes, 2005). The main assumptions include that D-[3-³H] glucose does not behave differently from the more abundant glucose isotope in the body and that recycling of D-[3-³H]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, adding tracer to the 20% glucose infusion minimized alterations in specific activity (SA) throughout the experimental protocol, which in turn reduced the probability of obtaining negative endogenous EGP values; this is known as the hot glucose infusion method, or "hot ginf" (Finegood *et al.*, 1987, 1988). The current experimental protocol used the hot gluff 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)

$$\begin{split} R_d(t) &= I/SA_p(t) - \left[(pV \cdot G(t) \cdot dSA_p(t)/dt)/SA_p(t)\right] + \left[SA_g \cdot Ginf(t)/SA_p(t)\right] - pV \cdot (dG(t)/dt) \end{split}$$

In the previous formula, R_a represents 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 μ Ci, divided by the total amount of glucose in a sample (Wolfe and Chinkes, 2005). SA_p(t) refers to SA in the plasma sample at time t in μ Ci/mg, SA_g refers to SA of the exogenous glucose solution in μ Ci/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 distribution in the body in dL/kg and it is 25% of body weight (Finegood *et al.*, 1987, Radziuk *et al.*, 1978), (Wolfe and 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 and 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 SAg was generated. It was assumed that R_a equaled 2 mg/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 clamp plateaus equaled 0 (definition of an isotopic steady state). Ultimately, the goal was to maintain plasma glucose SA.

A smoothing program, OOPSEG, is 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

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probable magnitude of measurement error (Bradley *et al.*, 1993). 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 selecting the last 30 minutes of 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).

Measurements of plasma D-[3-³H]glucose radioactivity was made using a radioactive assay, according to Finegood et al. (1987). Briefly, plasma proteins are precipitated, the supernatant isolated and dried, water and scintillation cocktail (ICN Biomedical, Irvine, CA) are added, followed by analysis in a Beckman scintillation counter (Beckman Coulter LS6500 Multi-Purpose Scintillation Counter, Fullerton, CA).

Other assays Insulin, Glucagon, Free Fatty Acids, D-[3-³H]glucose, and Amino Acids (HPLC)

A radioimmunoassay (RIA) utilizing [¹²⁵I] 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 and C-peptide 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 analysed by a gamma counter; using standard curves, the level of radioactivity in samples was interpolated to establish hormone concentrations. Serum FFA concentrations was 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.*, 2005a). Ion-exchange HPLC was used to determine plasma AA concentrations (Chevalier *et al.*, 2005a). 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 and Cummings, 1991). All of the above substrate and hormone measurements, except HPLC AA measurements, were done in duplicates.

Cellular Mechanisms of protein synthesis:

Muscle samples (25 mg wet weight) were homogenised on ice for 30 sec in 10 volumes of homogenisation buffer (final concentration in mmol/l: 20 HEPES, pH 7.4, 2 EGTA, 50 NaF, 100 KCl, 0.2 EDTA, 10 Na₄PO₇, 50 βglycerophosphate) supplemented with 0.5 mmol/l Na₃VO₄, 1 µmol/l microcystin LR, 1 mmol/l dithiothreitol, 1 mmol/l phenylmethylsulfonyl fluoride and 10 µg/ml aprotinin, leupeptin and pepstatin each using a hand-held homogeniser (Tissuemiser; Fisher Scientific, Mississauga, Canada). The homogenate was cleared by centrifugation at 15,000 × g, at 4°C, for 15 min. An aliquot of homogenate was used to measure protein concentration by the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA) using BSA as standard (Bradford, 1976). An aliquot of the remaining supernatant was mixed with equal volume of $2\times$ Laemli sample buffer and then boiled for 5 min. Samples (25 µg of protein/lane) were resolved by SDS-PAGE and then transferred onto polyvinylidine fluoride membranes (GE Healthcare). The membranes were

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blocked in 5% non-fat dried milk in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at room temperature for 1 h and then incubated with phospho-specific primary antibodies against Akt (Ser473), PRAS40 (Thr246), 4EBP1(Thr37-46), IRS-1 (Ser1101) and (Ser636/639), mTOR (Ser2448) and rpS6 (235/236) at 4°C overnight. Membranes were washed three times for 5 min in TBS-T and subsequently incubated with secondary antibody in TBS-T containing 5% non-fat dried milk at room temperature for 1 h. Immunoblots were developed by enhanced chemiluminescence using ChemiDOC XRS Multi-Imager system (Bio-Rad Laboratories). After detection of the phospho-specific signal, the antibodies were stripped off the membrane by incubation in 62.5 mmol/l Tris-HCl, 100 mmol/l β-mercaptoethanol, and 2% SDS at 50°C for 30 min. The membranes were then washed, blocked and reprobed with primary antibodies against total Akt, PRAS40, 4EBP1, IRS-1, mTOR and rpS6. All antibodies were from Cell Signaling Technology (Danvers, MA, USA). Results are expressed as ratios of phosphorylated to total protein.

Appendix 2. Newspaper advertisements





Appendix 3. Recruitment posters



The McGill Nutrition & Food Science Centre is seeking men 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)



Principal Investigator: Réjeanne Gougeon, PhD If you:

I 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 4 days

yyyalkia kampak

For more information call: Connie Nardolillo or Maya Bassil at 514– 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!

nnie Nardolilo 514-843-1665 -	aya Bassil	onne Nardollo - 514 843-1665 aya Bassil	onnie Nardolilo 514- 843-1665 aya Bassil	nnie Nardolilo 514-843-1665 aya Bassil	onnie Nardolilo 514- 843-1665 aya Bassil	nnie Nardolilo 514-843-1665 aya Bassil	onnie Nardolilo 514- 843-1665 aya Bassil	onnie Nardolilo 514-843-1665 aya Bassil	nnie Nardolilo 514-843-1665 aya Bassil	onnie Nardoliko 514-843-16655 aya Bassil	onnie Nardoliko 514-843-1665 aya Bassil
Com	Maye	Maya	May ^e	Maye	May ^e	Maya Maya	Mays Mays	May ^e	<u>Conn</u> Maya	<u>Conn</u> Maye	Maya

Une étude sur le diabète de type 2

Le Centre de nutrition et des sciences de l'alimentation de l'université McGill recherche des hommes 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)



Investigateur Principale: Réjeanne Gougeon, PhD

Si vous:

- Souffrez du diabète de type 2
- + Avez moins de 65 ans
- N'avez aucun autre problème de santé majeur

Ŵ

 Étes prêt à passer 4 jours à l'Unité de recherche dinique de l'hôpital Royal Victoria,

yyy, dibio inang, sk

Pour plus d'information appeler Connie <u>Nardolillo</u> ou Maya <u>Bassil</u> au 514–843–1665 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 yous sera versée pour votre participation!

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Appendix 4. Phone interview questionnaire

Questionnaire for potential subjects

Date:	Protocol:
 Name:	Phone#:
Age: Weight: BMI:	Height:
1. Currently in another study?	□ Yes - End date?
	□No
2. Previous participation?	□Yes □No
3. Student?	□Yes □ No
4. University/Faculty	
5. Stable weight for 6 months?	□Yes □No □Don't know
6. Allergies (Food/ Medications)?	□ Yes □ No
7. Smoker? □Yes	

8. Vegetarian (red meat, eg	ggs, cheo ed meat	ese, milk, tofu, vegetables, fish, poultry)? ?
9. Drink alcohol (wine, bee	er)?	□Yes – Less than 3 drinks/day?
	_	□No
9. Medicare card? □Yes □No		
10. RVH card?	□Yes □No	
11. Any diseases within the	e family	? 🗆 Yes
		□ No
12. Type 2 DM? □Yes	- For I	how many years?
- Medication (dosage, freq	uency)?	

 $\Box No$

13. Glycemic control:

	Date & Value				
Hemo A1C					
BS Monitoring					

14. \bigcirc Post-menopausal? \Box Yes - Last period?

□ No - Regular periods?

5. Surgery/Hospitalizati	on/ Accidents?
	□No
16 Pland transfusions?	Vog Why? Whon?

 \Box No

17. 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:		
(including HIV, Hep)		

	$2 \cup Yes - Which ones?$
□ No	
. Activities/Exercise:	
Where did you hear about the study?	
Poster - Where?	
Newspaper - Which one?	
Other	
omments:	

Participation in study:□UYesUNo - Why was subject excluded?

Questionnaire done by:

Treating Physician:

	Telephone interview Inclusion/exclusion	Screening CIU			S	tudy per	iod		
			Home/CIU				CIU		
	↓ 	↓ 						∧	
Physical	Exam	✓							
Blood a	nd urine tests	\checkmark							\checkmark
Chest X	-ray /ECG	\checkmark							
Indirect	calorimetry (RMR)	\checkmark							\checkmark
BIA		\checkmark						\checkmark	
Height		\checkmark							
Weight,	BMI	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark
Diet/24	hr-urine collection		\checkmark	\checkmark	\checkmark	\checkmark	\checkmark	\checkmark	
Blood P	ressure	\checkmark				\checkmark	\checkmark	\checkmark	\checkmark
Blood G	lucose Monitoring		\checkmark						
Temper	ature						\checkmark	\checkmark	\checkmark
DEXA/	body circumferences							\checkmark	
Hyperin	sulinemic clamp								\checkmark

Appendix 5. Study timeline and outline of the tests and measurements

Appendix 6. Consent forms

CONSENT FORM

McGILL UNIVERSITY HEALTH CENTRE

Royal Victoria Hospital: Clinical Investigation Unit

<u>INSULIN SENSITIVITY</u> <u>OF PROTEIN METABOLISM IN TYPE 2 DIABETES</u> <u>MELLITUS</u>

<u>Your participation in this study</u>: You have been invited by Dr. Réjeanne Gougeon to take part in a study on the effect of diabetes on how well insulin maintains your body proteins. This study takes eight (8) days. The first two (2) days of the study may be spent at home or in the hospital.

1) <u>Screening and Admission</u>: To be sure the study will be safe for you and that you are a good candidate, the following will be done first. You will have an interview, a physical examination, standard blood and urine tests, chest X-ray and electrocardiogram (ECG). A suitable time will then be set for you to have your body composition and your resting metabolic rate measured. The screening tests will take about 4 hours to complete. If the tests confirm that you should enter the study, you will then be admitted to the Clinical Investigation Unit (CIU) of the Royal Victoria Hospital.

2) <u>Special Diet</u>: You will be asked to stop your diabetes medications for at least three (3) days prior to admission, unless your usual blood glucoses are high. From day 1 of the study, you will consume the following diet: a breakfast with cereal and milk, then the rest of your food will be a liquid formula diet that contains all necessary nutrients in amounts exactly suited to your needs. It is important that you drink plenty of water (about 1-2 litres per day or 8 glasses per day) while you are on the study: You will have these meals every four (4) hours starting at 8 A.M. and ending at 8 P.M.

3) <u>Activity</u>: During the whole period of the study, you will stay on the ward except for short walks about the hospital grounds.

4) <u>Data Collection</u>: You will be asked to collect <u>all</u> your urine each day in suitable containers provided for you. It is important for the study that these urine collections be carefully and completely done. You will be weighed each morning in sleep wear after passing urine. Every day, you will take your temperature and

record your water intake. You will test your blood glucose four (4) to six (6) times daily. It will never be allowed to rise to very high levels. If required to keep your glucose controlled, insulin injections may be given. You will be asked to call the nurse if you feel uncomfortable or your blood glucose level is over 15 mmol/L.

5) Body Composition and Resting Metabolic Rate: your amounts of body fat, muscle and other tissues will be measured by several methods. 1) Bioelectrical impedance analysis. (BIA) is a safe and painless method that involves passing a weak electrical current via wires attached to your right hand and foot. 2) Four skinfold measurements are performed with a pair of calipers gently squeezing the skin of the front and back of the upper arm, back and abdomen. 3) Circumference measurements are performed with a measuring tape around the arm, chest, waist, hip and thigh. 4) Dual X-ray Absorptiometry (DEXA) uses an X-ray analysis that distinguishes the different tissues (fat, bone or muscle). It involves lying on a table for a few minutes only. 5) Magnetic resonance imaging (MRI) involves lying down on a narrow bed for about 20 minutes while the bed is passed through a scanner. Several views of your body will be taken. For the abdominal views, you will be asked to hold your breath for 15 to 25 seconds. You may experience discomfort by lying still in the same position for the duration of the test, the noise inside the scanner, or being asked to hold your breath. You will not be asked to have this test if you do not tolerate confined spaces. There is no radiation exposure in MRI. 6) Your resting metabolic rate is measured while you lie comfortably and quietly in bed. You will have a large transparent hood that is open to the air of the room, over your upper chest and head, for about 20 minutes. This measures your breathing as you rest. This is called "indirect calorimetry".

6) <u>Gluconeogenesis Test</u>: This test requires that you take deuterated water [(water that contains a stable isotope (not radioactive)] to measure the body's production of sugar. This test will start on day 5. For this, you will eat the same diet until 5:15 PM. There will be no food after this, until 11 AM the next day. You may have ordinary water, however. At 9:45, 10:15, 10:45, 11:15 PM, you will drink a 40 mL x 4 (5 ounces) glass of water with deuterium. For the rest of the night, you may drink deuterated water diluted at 0.5%. A blood sample (25 mL) will be collected at 11 AM the next day for this test. Deuterated water has caused slight, tolerable and short-lived dizziness in some people, but is otherwise the same as drinking ordinary water.

7) <u>Behaviour Modification Counseling</u>: During your stay in hospital, you may choose to have individual sessions with Dr. Gougeon for training in behaviour modification. This will be for your benefit, to learn how to make changes in your lifestyle.

8) <u>Study of the effects of insulin</u>: On day 8, the study of the effects of insulin on body protein will be performed at the Crabtree Laboratory of the Nutrition Centre which is located in the hospital. It will last approximately 8 hours. On the

morning of the test, while you are fasting and resting comfortably in bed, the nurse will insert two intravenous catheters in the veins of your arm and hand. Your hand catheter will allow for repeated painless blood samples to be taken. This catheter will be in a vein on the back on your hand or wrist. The hand will be placed in a warming box at 65° C to make the veins dilate so the blood in the vein will be similar to that of an artery. This is not a painful or uncomfortable procedure because the hand becomes used to the warm temperature. The other catheter will be in a vein of the opposite arm. It will be attached to an intravenous bag with water and salt. This solution will carry in the test substances. Tiny, trace amounts of radioactive (tritiated) glucose and of the safe stable isotope, ¹³Cleucine, will be given for the whole test. The amount of radioactive material received will be about 150 to 250 uCi (5.5-9.25 MBq). 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 urine) within 2 1/2 weeks of administration. Stable isotopes occur naturally and are so safe that they are even used for studies in infants, children and pregnant women. You will first receive an amount of 15 mL of tritiated glucose and 5.9 mL of labeled ¹³C-leucine followed by a continuous infusion of both at from 0.11 to 0.25 mL/min. You will be asked to drink a small amount of water with sodium ¹³C-bicarbonate (the same stable isotope). Insulin will be infused for the next 7 hours.

The mixture of amino acids (protein) solution and the glucose solution will be infused to ensure that your blood amino acid and glucose levels remain at levels that occur after a meal. Indeed, this whole test is like a meal taken through the vein. Blood samples of 1 mL (one-fifth of a teaspoon) will be taken every 5 minutes after the insulin infusion has been started; 12.5 mL (about one tablespoon) blood samples will also be taken every 30 minutes. Between 3 to 3 $\frac{1}{2}$ and 6 $\frac{1}{2}$ and 7 hrs, blood samples will increase to every 10 minutes. The total blood volume taken will be 280 ml, 2/3 of a blood donation.

Your resting metabolic rate will be measured 3 times during the insulin study. In addition, several times during the test you will be asked to give breath samples by blowing two breaths into a tube connected to a plastic bag.

9) <u>Muscle biopsies</u>: The procedure used is a standard one for studies and diagnosis. It is even done in athletes, who exercise immediately after the procedure. Three (3) samples will be taken at different times, (8:30, 10:30 and 2 PM) from the large muscle on the side of your thigh. The skin and tissue under it will be anesthetized (like a dentist's "freezing") before a skin incision of 0.7 cm (about the width of a pencil) is made. A needle (hollow cylinder of 5 mm diameter) will be inserted into your muscle to remove a piece of about one tenth of a gram (the size of a small pill). Once the biopsy is obtained the skin will be held together with sterile strips of adhesive tape and a protective dressing will be applied on top of this. Firm pressure will be applied to the area for 10 minutes to

prevent bruising. Because of the local anesthetic, you should feel no pain during the study.

10) Risks: The risks of muscle biopsy are considered more than minimal. You may feel some pain in the thigh following the study and for a few days, from the muscle biopsies. This should be controlled by acetaminophen (Tylenol[®]). There is a chance that the small scars on your skin will not disappear completely. The radiation from the DEXA and the labeled glucose is considered negligible. There is a risk of both high and low blood sugars but your blood glucose will be monitored to keep it stable to reduce the risk to minimum. The risks involved in consuming the diet and in blood sampling are considered to be minimal. You may experience some changes in your bowel movements with the diet, but special attention will be paid to minimize them. There may be slight pain or discomfort while having blood tests with a slight risk of bruising. The amount of blood drawn during the whole study will not exceed that in an ordinary blood donation. There is a small risk of dehydration if you do not drink enough fluid to match the amount of urine passed during the diet period. This will be carefully monitored by the research team. Your study will be supervised by experienced nurses and doctors, who will also remain available to you until your return to your usual state. At this time, there will be a follow-up of your diabetes management by the research team.

11) <u>Benefits</u>: You will benefit directly if you opt to have diet and activity counseling. Although the study is not expected to provide you any direct benefit, it is hoped that the information obtained will lead to a better understanding of the abnormal protein metabolism in diabetes. This should show how protein requirements differ in diabetes, and to help to define optimal intakes for weight maintenance and reduction. Any questions you may have about your diabetes, the diet and the study results will be answered by contacting Dr. Réjeanne Gougeon at 843-1665.

Both Dr. Errol B. Marliss at 843-1665 or pager 406-1746 or Dr. José A. Morais at 843-1665 or pager 406-0163 will be available to answer questions regarding your diabetes control and the muscle biopsy.

Should you have any question regarding your rights as a research subject, you may contact the Patient Representative of the Royal Victoria Hospital at (514) 934-1934, local 35655.

12) <u>Confidentiality</u>: The data obtained will be treated confidentially and it will not be possible to identify you personally by name or otherwise in any publication of the results, or in presentations at scientific meetings. As part of normal research practice it is important that information related to the study can be checked for accuracy. It may be necessary for Health Canada (Health Protection Branch),

other regulatory agencies, and the McGill University Health Centre Quality Assurance for research to review the information obtained from study documents and your medical records. In such circumstances, confidentiality will be maintained at all times.

You will receive \$35 for the morning screening visit and \$900 for the full study, only if it includes the study of the effects of insulin on day 8. These payments are to compensate for your time and loss of potential income during the eight day study.

Subjects Declaration of Consent

I,above description	, have	read	the		
member(s)	of	the	research	te	eam,

I fully understand the procedures, advantages, and disadvantages of the • study which has been explained to me.

I freely and voluntarily consent to participate in this project. •

• I understand that I may seek information about each test either before or after it is given, and that I am free to withdraw from the study at any time if I desire.

I understand that my personal information will be kept confidential. •

Early termination of this project, for any reason will not compromise my • medical care.

Dated at Montreal: (month) _		(day)	(year) 200	
	mm	dd		уу

PARTICIPANT: _________Signature

INVESTIGATOR:

FORMULAIRE DE CONSENTEMENT

Centre universitaire de santé McGill Hôpital Royal Victoria: Unité d'investigation clinique

<u>SENSIBILITÉ À L'INSULINE DU MÉTABOLISME DES PROTÉINES</u> <u>DANS LE DIABÈTE SUCRÉ DE TYPE 2</u>

<u>Votre participation dans cette étude:</u> Vous avez été invité(e) par le Dr. Réjeanne Gougeon à participer à une étude des effets du diabète sur l'action de l'insuline dans le maintien des protéines du corps. Cette étude dure huit (8) jours. Les deux (2) premiers jours de l'étude peuvent être passés à la maison ou à l'hôpital.

1) <u>Dépistage et admission</u>: Afin de s'assurer que l'étude est sécuritaire pour vous et pour déterminer votre éligibilité, vous devrez vous soumettre aux tests suivants: une entrevue avec un des membres de l'équipe pour discuter de l'étude et obtenir votre consentement, l'évaluation de votre état de santé par l'infirmière de recherche, des tests sanguins et urinaires de routine, une radiographie pulmonaire et un électrocardiogramme (ECG). Un temps optimal sera déterminé pour mesurer votre composition corporelle par impédance bioélectrique et votre dépense énergétique au repos, par calorimétrie indirecte. Les tests de dépistage dureront à peu près 4 heures. Si les tests confirment votre admissibilité à l'étude, vous serez alors admis à l'Unité d'investigation clinique de l'Hôpital Royal Victoria.

2) <u>Diète Spéciale:</u> Nous vous demanderons de cesser de prendre vos médicaments pour le diabète au moins trois (3) jours avant votre admission, à moins que votre taux de glucose sanguin habituel ne soit élevé. Au jour 1 de l'étude, vous suivrez le régime alimentaire suivant: le petit déjeuner quotidien sera composé de céréales et de lait, mais le reste de vos repas se présentera sous la forme d'une diète liquide contenant l'énergie, les protéines et tous les nutriments essentiels dans des quantités correspondant à vos besoins. Il est important de boire beaucoup d'eau (1-2 litres ou 8 verres par jour) pendant que vous faites partie de cette étude. La prise des repas se fera à chaque quatre (4) heures, et ce à partir de 8h00 jusqu'à 20h00.

3) <u>Activité physique</u>: Vous resterez dans l'hôpital pour la durée de l'étude, à l'exception de courtes promenades aux alentours de l'hôpital.

4) <u>Cueillette de données:</u> Nous vous demanderons de recueillir <u>toutes</u> vos urines chaque jour dans des contenants prévus à cette fin qui vous seront fournis. Il est important pour cette étude que vous recueilliez l'urine correctement et entièrement. Vous prendrez votre poids en pyjama chaque matin, après avoir recueilli votre urine. Vous prendrez votre température tous les jours et prendrez note de la quantité d'eau que vous ingérez. Vous testerez votre glucose sanguin de quatre (4) à six (6) fois par jour. Il ne sera jamais permis à ce taux d'atteindre de hauts niveaux. Des injections d'insuline seront administrées si le besoin se présente. Nous vous demanderons d'appeler l'infirmière si vous vous sentez mal ou si vos taux de glucose sanguin dépassent 15 mmol/L.

5) <u>Composition corporelle et dépense énergétique au repos</u>: votre quantité de gras corporel, de muscle et d'autres tissus sera mesuré pas diverses méthodes : 1) l'impédance bioélectrique (BIA) qui est une méthode sécuritaire et indolore et qui consiste à faire passer un faible courant électrique par l'intermédiaire d'électrodes apposées sur votre main et votre pied droit ; 2) la mesure des plis cutanés qui se fait avec un compas appliqué sur la peau autour des biceps, triceps (muscles du bras), du dos et de l'abdomen ; 3) la mesure des circonférences du bras, de la poitrine, de la taille, des hanches et de la cuisse; 4) l'absorptiométrie biphotonique de rayons X (DEXA) qui utilise l'analyse radiographique pour distinguer les divers tissus corporels (gras, os et muscles). Il vous faudra vous coucher sur une table pendant quelques minutes seulement; 5) l'imagerie par résonance magnétique (IRM) où il vous faudra vous coucher sur un lit étroit pendant une période d'à peu près 20 minutes, durant laquelle le lit passera à travers l'appareil d'IRM. Plusieurs images de votre corps seront prises. On vous demandera de retenir votre souffle de 15 à 25 secondes, pour prendre les images de l'abdomen. Il est possible que de rester allongé(e) dans la même position pour la durée du test, que le bruit à l'intérieur de l'appareil, ou que de retenir votre souffle vous rendent inconfortable. Nous ne vous demanderons pas de faire ce test si vous ne tolérez pas les espaces confinés. Il n'y a pas de radiation dans le IRM; 6) votre dépense énergétique au repos sera mesurée pendant que vous êtes couché(e) confortablement et calmement dans un lit. Nous placerons un grand casque transparent, qui est ouvert à l'air de la chambre, par-dessus votre tête et la partie supérieure de votre poitrine pendant environ 20 minutes. Cette méthode mesure votre respiration au repos. Elle se nomme "calorimétrie indirecte".

6) <u>Test de néoglucogénèse</u>: Ce test requiert que vous buviez de l'eau deutérée [de l'eau qui contient un isotope stable (non radioactif)] pour mesurer la production de glucose par le corps. Ce test commencera le onzième (11) jour. Pour ce test, vous mangerez la même diète mais jusqu'à 16h45. Après cela, vous n'aurez plus le droit de manger jusqu' au jour suivant. À 21h45, 22h15, 22h45 et 23h15, vous boirez 40 mL (x 4) ou 5 onces au total d'eau deutérée. Vous pourrez boire de l'eau deutérée diluée à 0,5% pour le reste de la nuit. Un échantillon de sang (25 ml) sera prélevé le lendemain à 9h45 pour ce test. L'eau deutérée a causé des étourdissements mineurs mais tolérables et de courte durée chez certaines personnes, mais à part ça, cette eau est identique à l'eau ordinaire.

7) <u>Consultation en modification du comportement</u>: Lors de votre séjour à l'hôpital, on vous offrira des sessions individuelles avec le Dr. Gougeon, sur la

modification du comportement qui peuvent vous aider à apporter des changements dans votre mode de vie.

8) L'étude des effets de l'insuline: le douzième (12) jour, l'étude des effets de l'insuline sur les protéines du corps se déroulera au laboratoire Crabtree du Centre de nutrition, qui est situé à l'hôpital. Il durera à peu près 8 heures. Le matin même du test, alors que vous êtes en état de jeûne et que vous vous reposez confortablement dans un lit, l'infirmière insèrera deux (2) cathéters intraveineux dans une veine du bras et une de la main. Le cathéter de la main permettra de prélever régulièrement des échantillons de sang, de façon indolore. Ce cathéter sera situé dans une veine du dos de la main et votre main sera placée dans une boîte chauffant à 65°C, pour dilater les veines et ainsi rendre le sang de la veine semblable à celui de l'artère. Cette procédure n'est pas jugée douloureuse ni inconfortable puisque la main s'habitue à la température tiède. L'autre cathéter sera placé dans une veine du bras opposé. Il sera attaché à un sac intraveineux contenant de l'eau et du sel. Cette solution servira à introduire les substances testées. Des quantités minimes de glucose radioactif (tritié) et de l'isotope stable, ¹³C-leucine, seront administrées pendant la durée entière du test. La quantité de substance radioactive administrée sera d'environ 150 à 250 µCi (5.5-9.25 MBq). Cette quantité équivaut à une exposition à la radioactivité reçue lors d'une radiographie pulmonaire. Cette dose est minime et disparaît complètement du corps (principalement par l'urine) environ 2 1/2 semaines suite à son administration. Les isotopes stables existent naturellement dans l'environnement et sont tellement sécuritaires qu'ils sont mêmes utilisés lors d'études chez les nouveaux-nés, les enfants et les femmes enceintes Vous recevrez 15 mL de glucose tritié et 5.9 mL de traceur ¹³C-leucine suivi d'une perfusion continue de ces deux substances à des taux de 0.11 à 0.25 mL/min. Nous vous demanderons de boire de l'eau contenant du ¹³C-bicarbonate de sodium (le même isotope stable). L'insuline sera administrée durant les 7 heures qui suivent.

Un mélange d'acides aminés (protéines) en solution et une solution de glucose seront perfusés afin de maintenir vos taux sanguins d'acides aminés et de glucose à des niveaux tels que l'on observe après un repas. En effet, le test entier est comme un repas pris par voie intraveineuse. Des échantillons sanguins de 1mL (un cinquième d'une cuiller à thé) seront prélevés à chaque 5 minutes après le début de la perfusion d'insuline. 12.5 mL (à peu près une cuiller à table) d'échantillons de sang seront aussi prélevés à chaque 30 minutes. La fréquence des échantillons de sang augmentera à une fois chaque 10 minutes entre 3 et $3\frac{1}{2}$ et $6\frac{1}{2}$ et 7 heures après le début de l'étude. Le volume de sang total qui sera prélevé sera de 280 mL, soit 2/3 d'un don de sang.

Votre dépense énergétique au repos sera mesurée à trois (3) reprises pendant l'étude. De plus, on vous demandera régulièrement pendant l'étude de nous donner des échantillons d'air et ce en expirant deux fois dans un tube qui sera connecté à un sac en plastique.

9) <u>Biopsie musculaire</u>: Cette procédure est souvent retrouvée (standard) dans des études et pour des diagnostics. Elle est même faite chez des athlètes qui continuent à s'entraîner immédiatement après. Trois (3) échantillons seront prélevés à divers moments (8h30, 10h30 et 14h00) à même le grand muscle sur le côté de votre cuisse. La peau et les tissus sous-jacents seront anesthésiés (comme une anesthésie chez le dentiste) avant qu'une incision de 0.7 cm (environ l'épaisseur d'un crayon) soit faite. Une aiguille à biopsie (un cylindre creux d'un diamètre de 5 mm) sera insérée dans votre muscle pour prélever rapidement un échantillon d'environ un dixième de gramme (la grandeur d'une petite pilule). Une fois la biopsie obtenue, la peau sera recouverte d'un bandage adhésif et d'un pansement protecteur. Une pression ferme sera appliquée à cette région pendant 10 minutes, afin d'éviter une ecchymose. Grâce à l'anesthésie locale, vous ne devriez pas ressentir de douleur lors de l'étude.

10) Risques: Les risques associés aux biopsies musculaires sont considérés plus que minimes. Toutefois, vous pourriez éprouver des douleurs dans la cuisse après la fin de l'étude, et ce pour quelques jours. Celles-ci devraient être contrôlables par l'acétaminophen (Tylénol[®]). Il est possible que la petite cicatrice sur la peau ne disparaisse pas complètement. La radiation à laquelle vous serez exposé lors du DEXA et par le glucose tritié est considérée négligeable. Il y a un risque que le glucose dans votre sang atteigne des taux trop élevés ou trop bas, mais le glucose sanguin sera régulé afin de le garder stable et de rendre ce risque minime. Les risques associés à la consommation de la diète et aux prélèvements de sang sont jugés minimes. Vous pourriez percevoir quelques changements dans vos habitudes de selles avec la prise de cette diète, mais une attention particulière sera apportée afin de minimiser cet effet. Une légère douleur ou de l'inconfort pourrait être ressenti lors des prélèvements de sang qui sont associés à un risque mineur d'ecchymose. La quantité totale de sang prélevé au cours de la semaine d'étude ne dépasse pas celle d'un don de sang. Il y a un faible risque de déshydratation si vous ne buvez pas une quantité de liquides comparable à vos pertes urinaires durant la période de l'étude. Ceci sera contrôlé de facon précise par l'équipe de recherche. Votre étude sera supervisée par des infirmières expérimentées et des médecins qui seront à votre disposition jusqu'à ce que vous reveniez à votre état normal. A ce moment là, vous aurez accès à un suivi du contrôle de votre diabète par l'équipe de recherche.

11) <u>Avantages</u>: Vous bénéficierez directement si vous décidez de recevoir une consultation sur la diète et l'activité. Même si cette étude n'est pas censée vous procurer d'avantages directs, il est à souhaiter que l'information que vous obtiendrez mènera à une meilleure compréhension du métabolisme anormal des protéines que l'observe dans le diabète. Cette étude devrait être en mesure de démontrer à quel point les besoins en protéines diffèrent dans le diabète et de définir l'apport optimal lors du maintien ou de la perte de poids. Le Dr Réjeanne

Gougeon répondra à toute question concernant votre diabète, votre diète, ou les résultats de cette étude en appelant au 843-1665.

Vous pouvez joindre le Dr Errol B. Marliss au 843-1665 ou télé-avertisseur 406-1746 ou le Dr José Morais au 843-1665 ou télé-avertisseur 406-0163 qui seront disponibles pour répondre à vos questions sur le contrôle de votre diabète et les biopsie musculaires.

Si vous avez des questions sur vos droits à titre de sujet de recherche, vous pouvez joindre l'ombudsman du Centre de santé universitaire McGill au (514) 934-1934, poste 35655.

12) <u>Confidentialité</u>: Les résultats obtenus seront traités de façon confidentielle et il ne sera pas possible de vous identifier par votre nom ou par toute autre information dans la publication des résultats, ou lors de nos présentations scientifiques. Il est de coutume, en recherche, que toute information tirée d'une étude soit révisée pour en évaluer l'exactitude. Il se pourrait que des instances gouvernementales ou académiques telles que Santé Canada ou le Centre d'assurance de la qualité en recherche du Centre universitaire de santé McGill révisent l'information obtenue à partir de vos données médicales. Si tel était le cas, la confidentialité serait assurée en tout temps.

Vous recevrez \$35 pour la visite de dépistage et \$900 pour votre participation dans l'étude entière, à condition que celle-ci comprenne l'étude des effets de l'insuline qui a lieu le douzième (12) jour. Ces paiements qui sont offerts à titre compensatoire servent à défrayer les pertes encourues pour le temps et le revenu potentiel au cours des douze jours d'étude.

Déclaration de consentement du sujet

Je,_____

_____, déclare avoir lu l'étude

décrite plus haut,

(Lettres moulées)

en compagnie d'un (des) membre(s) de l'équipe de recherche,

• Je comprends les méthodes, les avantages et inconvénients de l'étude qui m'ont été clairement expliqués.

• Je consens à participer à ce projet de façon libre et volontaire.

• Je comprends qu'il m'est possible d'obtenir de l'information au sujet de chaque test, soit avant ou auprès qu'il soit administré, et que je suis libre de me retirer de l'étude en tout temps.

• Je comprends que toute information personnelle à mon sujet sera traitée avec confidentialité.

• De me retirer de ce projet avant qu'il soit complété, pour toute raison que ce soit, ne compromettra en rien mon suivi médical.

Date à Montréal : (mois) _____(jour) ____(année) 200

PARTICIPANT :

Signature

CHERCHEUR PRINCIPAL :

Signature

24-HOUR FOOD F	RECALL			
Name:		Date:		Day of recall:
Duralifact				
Breaktast			time:	
Snack				
Lunch			time:	
Snack				
Dinner			time:	
Onesh				
БЛАСК				

Appendix 7. 24-hour recall questionnaire

PROTEIN-RICH FOODS

Food	Per Day	Per Week	Portion Size
red meat			
poultry			
fish & sea foods			
deli meat, sausages			
eggs			
dried beans			
tofu			
peanut butter			
nuts, seeds			
milk (% fat)			
cheese			
yogurt			
other			

ENERGY-DENSE FOODS

Food	Per Day	Per Week	Occasionally
fast-food restaurants			
sweet desserts			
chips & salty snacks			
candy bars, chocolate			
soft drinks			
alcohol			
other			

Notes:

Appendix 8. Baecke questionnaire

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Baecke Questionnaire of Habitual Physical Activity

1 What is your main occupation?	
1. What is your main occupation.	1-3-5
2. At work I sit	
never/seldom/sometimes/often/always_	1-2-3-4-5
3. At work I stand	
never/seldom/sometimes/often/always	1-2-3-4-5
4 At work I walk	
never/seldom/sometimes/often/always	1-2-3-4-5
5 At work I lift heavy loads	
never/seldom/sometimes/often/very ofte	en 1-2-3-4-5
6 After working I am tired	
very often/often/sometimes/seldom/nev	er 5-4-3-2-1
7 At work I sweat	
very often/often/sometimes/seldom/nev	er 5-4-3-2-1
8 In comparison with others my own age I th	ink my work is physically
much heavier/heavier/as heavy/lighter/m	uch lighter 5-4-3-2-1
9 Do vou play sport?	
ves/no	
If yes:	
-which sport do you play most frequent	lv? Intensity 0.76-1.26-1.76
-how many hours a week?	<1/1-2/2-3/3-4/>4 Time 0.5-1.5-2.5-3.5-4.5
-how many months a year?	<1/1-3/4-6/7-9/>9 Proportion 0.04-0.17-0.42-0.67-0.92
If you play a second sport:	
-which sport do you play most frequent	ly? Intensity 0.76-1.26-1.76
-how many hours a week?	<1/1-2/2-3/3-4/>4 Time 0.5-1.5-2.5-3.5-4.5
-how many months a year?	_<1/1-3/4-6/7-9/>9 Proportion 0.04-0.17-0.42-0.67-0.92
10. In comparison with others my own age I	think my physical activity during leigure time is
much merc/merc/the same/less/much le	5 4 2 2 1
11 During leigure time I gweet	5-4-5-2-1
very offen/often/sometimes/seldom/ner	5.4.2.2.1
12 During leisure time I play sport	5-4-5-2-1
never/seldom/sometimes/often/ very of	ten 1-2-3-4-5
13 During leigure time I watch television	
never/caldom/sometimes/often/ yery of	tan 1-2-3-4-5
14 During leigure time Lwalk	1-2-3-4-3
never/seldom/sometimes/often/ voru of	ten 1-2-3-4-5
15 During leisure time I cycle	1-2-5-4-5
never/seldom/sometimes/often/ yerry of	ten 1-2-3-4-5
16 How many minutes do you walk and/or or	wele per day to and from work school and shopping?
<5/5_15/15_30/30_45/>45	1_2_3_4_5
-515-15115-50150-4512-45	1-2-5-4-5

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	MOCRA Question	naire	
	MOSPA QUESCIO		
	CORE DATA	Numbers	<u>Columns</u>
. MOI	NICA Collaborating Center	_ _	1-2
. MO	NICA Reporting Unit	12121	3 - 4
. Se	rial number		5-10
. Nu	mber of the MONICA Population Survey	1_1	11
. Da	te of examination (day, month, year)	[_[_] [_[_] (day) (mos)	_ _ 12-17 (yr)
5. Da	te of birth (day, month, year)	<u> </u> <u> </u> (day) (mos)	_ _ 18-23 (yr)
7. Na	ame (for local use only)	-	

Appendix 9. MOSPA questionnaire

в.	Plea	se i	ndicate the category that best describes	
	your (Sel	ect	only ONE. If two or more apply, select the best one.):	
		1 2 3 4 5 6 7	student - go to question 9 homemaker - go to question 9 retired - go to question 9 disabled - go to question 9 unemployed - skip to question 17 employed - skip to question 10 other - go to question 9	
9.	Are	you 1 2	also employed? [] 25 yes - go to question 10 no - skip to question 17	

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MOSPA Questionnaire

.

10.	What is your occupation? (Choose one from the list)		26-27
	01Professional and technical workers07Non-farm laborers Non-farm laborers02Managers, officials and proprietors09Service workers ex 	workers ccept d anagers foremen	
11.	How many hours do you work during a typical week?	_ _ (hrs)	28-29
12.	How many days do you work during a typical week?	<u> </u> (days)	30
13.	On a typical day at work, how much time do you spend sitting or standing? (hrs) (Do not include time spent going to and from work. Do not include time spent walking, lifting or carrying moderately heavy objects or very heavy objects.)	_ _ (mins)	31-34
14.	On a typical day at work, how much time do you spend walking? (hrs) (Do not include time spent going to and from work. Do not include time spent lifting or carrying moderately heavy objects or very heavy objects.)	_ _ (mins)	35-38
15.	On a typical day at work, approximately how $ - - $ much time do you spend actually lifting or (hrs) carrying moderately heavy objects (about 5-10 kg) or doing activities of similar effort?	_ _ (mins)	39-42
16.	On a typical day at work, approximately how $ - - $ much time do you spend actually lifting or (hrs) carrying very heavy objects (more than 10 kg) or doing activities of a similar effort?	_ _ (mins)	43-46

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MOSPA Questionnaire

TRANSPORTATION TO AND FROM WORK, SCHOOL, AND SHOPPING

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17. Going to and from work, school or shopping, _ _ _ 47. how much time do you spend walking each day? (hrs) (mins) (Do not include hiking or walking for sport, exercise, or pleasure.)	-50
18. Going to and from work, school, or shopping, _ 51. how much time do you spend bicycling each day? (hrs) (mins) (Do not include bicycling for sport, exercise, or pleasure.)	- 54
WALKING DURING LEISURE-TIME FOR PLEASURE OR EXERCISE	
19. During an average week, how many hours do you spend walking? (hrs) (Do not include time spent at work, or going to and from work, school, or shopping.)	- 56
20. When you are walking, what usually happens to the 1 57 rate or depth of your breathing?	b.
1 no change 2 small increase 3 moderate increase 4 large increase	
HOUSEWORK	
21. On the average, how much time do you spend _ _ _ 58- every day doing moderately vigorous or very (hrs) (mins) vigorous chores at home such as sweeping, vacuuming, washing clothes, scrubbing floors, etc.?	·61
22. When you do these chores, what usually happens to the rate or depth of your breathing?	
1 no change 2 small increase 3 moderate increase 4 large increase	
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MOSPA Ques	tionnaire
28. When you did this sport or exercise happened to the rate or depth of yo	e, what usually _ 73 bur breathing?
1 no change 2 small increase 3 moderate increase 4 large increase	1-1 74
29. During the past 12 months, did you sport or do any other exercises at	play any other _ 74 least 12 times?
1 yes - go to question 30 2 no - skip to question 38	
30. What sport or exercise was it? (Choose ONE from the following lis	<u> </u> 75-76
<pre>01 aerobic exercises or</pre>	<pre>14 martial arts (judo,</pre>
31. During the past year, in how many did you do this sport or exercise	months $\left \begin{array}{c} - \\ - \\ - \\ \end{array} \right = \left \begin{array}{c} 77 - 78 \\ (mos) \end{array} \right $
32. In the months when you did this show many times per week did you u less than one time per we one time per week two times per week three times per week 	sually do it? ek 4 four times per week 5 five times per week 6 six times per week 7 seven or more times per week

			MOGPA Questionna	ire		
			MOSFR QUESCIONNA.			
33.	When time	you did	did this sport or exercise, how a you usually spend for each session	much on?	_ _ (hrs)	_ _ 80-83 (mins)
34.	When happ	n you bened	did this sport or exercise, what to the rate or depth of your breat	usual athing	lly g?	_ 84
		1 : 2 : 3 : 4	no change - skip to quest small increase - skip to quest moderate increase - skip to quest large increase - skip to quest	ion 38 ion 38 ion 38	8 8 8 8	
35.	PRIC spor skii or 12 t	OR to cts o ing, cycli times	the past 12 months, did you play r do any exercises such as runnin soccer, table tennis, gardening, ng for exercise or pleasure at le in one year?	any g, aerob: ast	ics,	_ 85
		1	yes - go to question 36			
		2	no - skip to question 38			
36.	What regi (Cho	2 t was ular oose	no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.)	lid on	a	<u> </u> 86-87
36.	What regu (Cho	2 ular oose 01	no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing	lid on 14	a martia kara	_ _ 86-87 ll arts (judo, ute, Tai chi)
36.	What regu (Cho	2 ular oose 01 02	<pre>no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing baseball or softball</pre>	lid on 14 15	a martia kara orient	_ _ 86-87 ll arts (judo, ate, Tai chi) ceering
36.	What regu (Cho	2 ular oose 01 02 03	<pre>no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing baseball or softball basketball, European handball,</pre>	lid on 14 15 16	a martia kara orient rowing	_ _ 86-87 ll arts (judo, ate, Tai chi) ceering
36.	What regu (Cho	2 ular oose 01 02 03	<pre>no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing baseball or softball basketball, European handball, or Australian netball</pre>	lid on 14 15 16 17	a martia kara orient rowing rugby,	_ _ 86-87 ll arts (judo, ate, Tai chi) ceering football
36.	What regu (Cho	2 t was ular oose 01 02 03 04	<pre>no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing baseball or softball basketball, European handball, or Australian netball bowling</pre>	14 14 15 16 17	a martia kara orient rowing rugby, (Ame	_ _ 86-87 al arts (judo, ate, Tai chi) ceering football erican, Australia
36.	What regu (Cho	2 t was ular oose 01 02 03 04 05	<pre>no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing baseball or softball basketball, European handball, or Australian netball bowling calisthenics or</pre>	lid on 14 15 16 17	a martia kara orient rowing rugby, (Ame or	_ _ 86-87 al arts (judo, ate, Tai chi) ceering football erican, Australia Canadian)
36.	What regu (Cho	2 t was ular oose 01 02 03 04 05	<pre>no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing baseball or softball basketball, European handball, or Australian netball bowling calisthenics or gymnastics aricket</pre>	lid on 14 15 16 17 18	a martia kara orient rowing rugby, (Ame or ice-sk bock	_ _ 86-87 al arts (judo, ate, Tai chi) ceering football erican, Australia Canadian) cating, ice- cey or roller ska
36.	What regu (Cho	2 t was ular 00se 01 02 03 04 05 06 07	<pre>no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing baseball or softball basketball, European handball, or Australian netball bowling calisthenics or gymnastics cricket cveling or biking</pre>	lid on 14 15 16 17 18 19	a martia kara orient rowing rugby, (Ame or ice-sk hock skiing	<pre>[_[_] 86-87 alter, Tai chi) ceering f football erican, Australia Canadian) cating, ice- tey or roller ska forcess country</pre>
36.	What regu (Cho	2 t was ular 00se 01 02 03 04 05 06 07 08	<pre>no - skip to question 38 the most recent sport that you d basis prior to this past year? ONE from the following list.) aerobic exercises or dancing baseball or softball basketball, European handball, or Australian netball bowling calisthenics or gymnastics cricket cycling or biking dancing</pre>	lid on 14 15 16 17 18 19 20	a martia kara orient rowing rugby, (Ame or ice-sk hock skiing skiing	<pre> _ _ 86-87 altarts (judo, atte, Tai chi) ceering f football erican, Australia Canadian) cating, ice- tey or roller ska g-cross country a-downhill</pre>
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MOSPA Questionnaire

SUMMARY QUESTION ABOUT VIGOROUS PHYSICAL ACTIVITY

- 38. Which of the following four activity classes best describes your present activity outside of your job? Please consider transportation to and from work, sporting activity and other physical effort during your leisure time, like gardening or dancing.
 - 1 No weekly physical activity.
 - 2 Only light physical activity in most weeks.
 - 3 Vigorous physical activity at least 20 minutes once or twice a week. (Vigorous activity causes shortness of breath, a rapid heart rate, and sweating.)
 - 4 Vigorous physical activity for at least 20 minutes three or more times per week.

INSTRUCTIONS

Self-explanatory instructions are included on the questionnaire. The participating MONICA centers may have translated their own questionnaire from the English version, which makes cultural biases a consideration.

Note: A copy of the scoring algorithm used for data analysis can be obtained from the CDC MOSPA Data Management Center (see primary source of information above).

STUDIES USING THE QUESTIONNAIRE

Some studies that have used the MOSPA questionnaire are included in the reference list (1,2).

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Appendix 10. Hyperinsulinemic clamp