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Does high dietary protein intake contribute to the increased risk of developing prediabetes and type 2 diabetes?

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Title: Does high dietary protein intake contribute to the increased risk of developing prediabetes and type 2 diabetes?

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Abstract

Insulin resistance is a complex metabolic disorder implicated in the development of many chronic diseases. While it is generally accepted that body mass loss should be the primary approach for the management of insulin-resistance related disorders in overweight and obese individuals, there is no consensus among researchers regarding optimal protein intake during

dietary restriction. Recently, it has been suggested that increased plasma branched-chain amino acid (BCAAs) concentrations are associated with the development of insulin resistance and T2D. The exact mechanism by which excessive amino acid availability may contribute to insulin resistance has not been fully investigated. However, it has been hypothesised that mammalian target of rapamycin complex 1 (mTORC1) hyperactivation in the presence of amino acid overload contributes to reduced insulin-stimulated glucose uptake due to insulin receptor substrate (IRS) degradation and reduced Akt-S160 activity. In addition, the long-term effects of high-protein diets on insulin sensitivity during both weight stable and weight loss conditions require more research. This review focusses on the effects of high-protein diets on insulin sensitivity and discusses the potential mechanisms by which dietary amino acids can affect insulin signaling.

Novelty bullets: Excess amino acids may over-activate mTOR, resulting in desensitisation of IRS-1 and reduced insulin-mediated glucose uptake.

Keywords: Type 2 diabetes, insulin resistance, mTOR/S6K1, high-protein diet, branched-chain amino acids.

Introduction

Dietary protein has a central role within a healthy eating pattern, as evidenced by the fact that protein is the only macronutrient often represented on educational food guides (e.g., USDA's

MyPlate) and that a minimal amount in the diet is necessary for meeting the body's growth and regenerative requirements (National Academy of Sciences. The Institute of Medicine. Food and Nutrition Board 2005). Moreover, the protein content of foods is often a consideration of consumers when shopping at grocery markets (Li and Dando 2019). As such, it is crucial to not only consider the health benefits of eating a higher proportion of total daily energy intake from protein, but also consider the potential negative consequences on disease risk. From a clinical perspective, Type 2 diabetes (T2D) prevalence is predicted to reach 6.1% of the world's population by 2025 (Stumvoll et al. 2005) thus placing a major socio-economic burden on health care systems for decades to come. Indeed, T2D is a multifactorial disease characterised by impaired β -cell function, reduced insulin sensitivity, and increased hepatic glucose production (Kahn et al. 2014). There are a number of suggested causes of insulin resistance in skeletal muscle; including hyperinsulinaemia, hyperglycaemia & hyperlipidemia, all of which have been shown to decrease Akt protein activity and induce some form of hyperglycaemia (Chalkley et al. 1998; Frühbeck et al. 2001; Tremblay et al. 2001; Gonzalez-Castillo et al. 2015). The resulting hyperglycaemia increases the risk of secondary complications, including macrovascular and microvascular diseases (Stumvoll et al. 2005). One of the most important lifestyle factors in controlling and preventing T2D is dietary manipulation. Many different protein centric diets have been proposed to improve metabolic health, such as diets high in protein content (>30% of total energy intake) and low in carbohydrates. The popularity of controversial high-protein diets, such as Zone and Atkins diets, has revealed that high-protein diets may beneficially affect body composition (i.e., higher ratio of lean body mass to fat mass) and weight management by leading to a greater overall body weight loss (Anton et al. 2017). Moreover, high-protein diets have been advocated as an effective dietary intervention to improve insulin sensitivity. While the effect of carbohydrates and fats on glucose metabolism has been thoroughly investigated over the past three decades

(Hollenbeck and Coulston 1991; Kodama et al. 2009; Jung and Choi 2017), the role of dietary proteins on glucose homeostasis and insulin sensitivity is yet to be established. Overall results from several meta-analyses and systematic reviews have indicated that the effect of acute or chronic high-protein diet on insulin resistance remains inconclusive (Ajala et al. 2013; Dong et al. 2013; Schwingshackl and Hoffmann 2013; Yu et al. 2019). Moreover, in the long term, high-protein diets are thought to be linked to insulin resistance and increased risk of cardiovascular diseases and cancer (Song et al. 2004). Sluijs et al. (2010) concluded, based on self-reported dietary assessments, that replacing 5 energy % from carbohydrate or fat with 5 energy % from protein increases diabetes risk by ~30%, but it was left unclear if the potential harmful effects were due to the protein content or other nutrients within the protein meals, such as iron. On the other hand, the Nurses' Health Study suggested that diets high in protein and fat from plant sources were associated with modestly reduced risk of T2D, when compared to animal sources (Halton et al. 2008). Clearly observational data and intervention studies do not reach the same conclusion. It is not clear whether high-protein diets are harmful, neutral, or beneficial for people with T2D. Therefore, this review will explore the mechanisms by which dietary proteins and amino acids affect insulin resistance and glucose homeostasis, and discuss short-term and long-term effects from physiological and controlled studies.

High-protein diet and metabolic health

The phrase 'high-protein diet' can often be difficult to interpret as there is no standardized diet definition and simply refers to an eating pattern that emphasizes the consumption of protein dense foods (e.g., ≥ 1.0 g protein/kg/d or the upper range of the Acceptable Macronutrient Distribution Range at ≥ 20 -35% of total daily energy intake from protein; see Table 1). Moreover, the term 'protein' in itself is non-descriptive and does not take into account that not all protein foods are created equal in terms of their ability to supply dietary requirements of essential amino acids (e.g., protein quality) (Phillips et al. 2015) or the energy intake required

to meet the minimal essential amino acid requirement from various protein foods. Specifically, the ingestion of animal-based proteins are more efficient to achieve dietary essential amino acid requirement without the excessive ingestion of non-protein calories (Wolfe et al. 2018). In addition, animal products also vary in terms of their food matrices (nutrient-nutrient interactions), cooking preparation (grilled or deep fried, for example), home cooked vs. fast food, each of which can be manipulated to induce drastically different metabolic effects despite all falling under the same general umbrella of a high-protein diet. For example, on the other hand, plant-based protein sources, such as pulses or nuts, are also high in fibre and phytonutrients that may improve parameters of insulin sensitivity by themselves (Kim et al. 2017; Clark et al. 2018; Weickert and Pfeiffer 2018). The fat content of a plant or animal based protein diet could also influence insulin sensitivity, as it known that n-6 polyunsaturated fats- the predominant dietary fatty acid being linoleic acid, and saturated fats, such as palmitic acid, have divergent effects on insulin sensitivity (Summers et al. 2002; Bjermo et al. 2012). In summary, there are many factors that need to be considered when evaluating the effects of a high protein diet on tissue insulin sensitivity. In the next sections we will review the acute and long-term effect of high protein studied in the human literature.

Nonetheless, a number of studies have demonstrated key beneficial effects of eating a higher proportion of total daily energy intake from dietary protein, including fat mass loss coupled with lean mass retention and improved satiety, during energy restriction (Halton and Hu 2004; Anton et al. 2017). Body mass loss is attributed to a higher satiety, preservation of resting metabolic rate, and perhaps even contribution from a greater thermic effect of high-protein diet compared with low-protein diet (Halton and Hu 2004). Diet-induced thermogenesis is related to the energy required for the intestinal absorption, metabolism, and food storage during the postprandial period. Hence, a high-protein diet exerts a larger effect on energy expenditure (23-30%) compared with carbohydrates (5%-10%) and lipids (2%-3%) (Nair et

al. 1983). This is thought to be explained by the high adenosine triphosphate (ATP) cost for protein synthesis, amino acid oxidation, and urea production (Robinson et al. 1990). Additionally, high-protein diets are linked to increased secretion of incretin peptides, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) which play an important role in postprandial insulin response (Fehse et al. 2005). Rizi et al. (2018) reported that a high protein (51.4 % energy from protein) compared to a high carbohydrate meal induced a significantly lower postprandial response in ghrelin concentration and higher in GLP-1, which was maintained significantly higher 6 hours after the meal intake. This suggests that an increase in protein density of the meal will promote greater satiety throughout the postprandial period. These key incretins potentiate glucose disposal by increasing insulin secretion from β -cells. GLP-1 also inhibits gastrointestinal motility and secretion thus augmenting satiety (Edholm et al. 2010). Other studies have shown that ingesting a protein-rich meal also increases GLP-1 and insulin levels leading to reduced postprandial hyperglycaemia and increased satiety (Blom et al. 2006; Akhavan et al. 2014). Numerous studies have shown that high-protein diets may also help to preserve lean body mass during weight loss in overweight and/or obese and T2D individuals (Larsen et al. 2010; Pignone 2011; Tang et al. 2013) and correct accelerated protein catabolism (Gougeon et al. 2000; Bell et al. 2006). Hence, it is important to focus on ‘high quality’ weight loss, which is aimed at maintaining adequate skeletal muscle mass with a higher ratio of fat mass loss to ensure long-term weight management and physical performance.

Branched-chain amino acids and insulin resistance

Certain dietary proteins are more effective at providing target amounts of essential amino acids into circulation than others (animal based > plant based), especially the branched chain amino

acids (BCAAs). BCAAs, which include leucine, isoleucine, and valine, are oxidised in peripheral tissues, mainly in skeletal muscle, and have diverse physiological and metabolic roles. In addition to their role as substrates for protein synthesis, BCAAs also act as anabolic signalling molecules, which are involved in signal transduction pathways essential in the regulation of protein synthesis and gene transcription (Dennis et al. 2011). Moreover, BCAAs are involved in lipolysis, lipogenesis, glucose metabolism and glucose transportation (Zhang et al. 2017).

BCAAs have emerged as a dietary component of interest in the development of insulin resistance due to their association with both insulin resistance and T2D (Nie et al. 2018). For example, Newgard et al. (2009) study, using metabolomic approach, reported that obese people have increased catabolism of BCAAs, when compared to healthy weight controls, that was associated with insulin resistance. Similar findings were also reported in a cross-sectional study of subjects with metabolic syndrome (Huffman et al. 2009) and in cohorts of Chinese and Asian-Indian men (Tai et al. 2010). Wang et al. (2011) reported that increased baseline plasma concentrations of BCAA and aromatic amino acids such as phenylalanine, tyrosine and tryptophan were associated with a higher chance of developing diabetes in the future. Likewise, systematic review and meta-analysis summarised current metabolomic studies and concluded that not only BCAA and aromatic amino acids are elevated in pre-diabetics and diabetics, but it is also suggested that elevated plasma BCAA concentrations are linked to higher risk of developing T2D (Guasch-Ferré et al. 2016), which could potentially be used as novel biomarkers. However, not all studies have shown that elevated plasma BCAA profiles are reliable predictors of T2D (Kouw et al. 2015) or insulin resistance (Beals et al. 2016, 2018). Moreover, habitual physical activity levels should be considered when evaluating the impact of blood BCAA concentrations and their link to disease risk.

While many clinical measures have been implicated as risk factors for T2D, including inflammatory cytokines, blood lipids and obesity, it is not known whether elevated plasma BCAAs are related to these factors. Wang-Sattler et al. (2012) demonstrated that elevated BCAAs were predictive for T2D even after adjustment for BMI, physical activity, smoking and HDL cholesterol, indicating that BCAAs may contribute to insulin resistance. Conversely, Mahendran et al. (2017) suggested that high plasma BCAAs concentrations have no causal effect on insulin resistance and that it is the insulin resistance that is a causal factor for increased circulating fasting BCAA concentrations. However, this study was completed using associations between genetic risk scores calculated from fasting insulin levels and genetic variants associated with BCAA levels, without a detailed mechanism that may explain the link between the two. Furthermore, it has been reported that BCAAs clearance (Marchesini et al. 1991) and branched-chained alpha-keto acid dehydrogenase complex (BCKDC) activity are decreased in people with T2D (Adams 2011). BCKDC catalyses an irreversible step in the catabolism of BCAAs to their respective ketoacids. BCKDC is inhibited by branched-chain α -ketoacid dehydrogenase kinase (BCKDK) and activated by the mitochondrial isoform of protein phosphatase 1K. The activity and the expression of these enzymes are affected in the obese and insulin resistance state thus contributing the BCAAs dysmetabolism (Bajotto et al. 2009).

Karusheva et al suggested that postprandial insulin sensitivity was improved during a 4 week intervention study, where the participants were randomly assigned to either have all BCAA or none in alternate weeks, as measured by mixed meal tolerance test but not during hyperinsulinemic-euglycaemic clamp, which suggests that whole body insulin sensitivity was not affected (Karusheva et al. 2019). In support of this, 20g of BCAA for 4 weeks did not change insulin concentrations of people with prediabetes (Woo et al. 2019). Therefore, large differences in BCAA concentration might be required to meaningfully alter insulin sensitivity.

It is not clear whether diet itself can induce the marked changes in BCAA even observed in these studies. Similarly, chronic consumption of whey does not increase circulating BCAA in an 8-week weight loss trial (Piccolo et al. 2015), and neither did self-reported reduced or increased dairy protein did not change circulating free AA, including BCAA concentrations after a month (Prodhan et al. 2018). Weight loss itself alters the circulating amino acid profile, which is an added confounding factor (Tochikubo et al. 2016). Therefore, it is possible that while protein foods can increase BCAA acutely, but this effect does not persist over time.

Despite the evidence of elevated BCAAs level in obese and diabetic individuals, it is still not well understood whether high plasma BCAA concentrations are causally implicated in T2D or whether it is a consequence of pathophysiology of the disease. In summary, it is possible that increased level of BCAAs in the insulin-resistant state is a consequence of altered appearance and disappearance rate of BCAAs that is coupled with decreased activity of catabolic enzymes. Nevertheless, it has been proposed that high-protein diet, rich in BCAAs, can contribute to insulin resistance via hyperactivation of mammalian target of rapamycin or mechanistic target of rapamycin (mTOR, also known as RAPT, FRAP, RAFT) (Um et al. 2004). This is discussed in the following sections.

The role of mTOR in insulin signalling

mTOR is a highly conserved Ser/Thr protein kinase that is a central regulator of various cellular processes, such as cellular metabolism, cell growth and proliferation, protein synthesis, transcription and autophagy (Dobashi et al. 2011). mTOR is inhibited by rapamycin, which is known to induce anti-proliferative and immunosuppressive activities in cells (Visner et al. 2003; Janes and Fruman 2009). mTOR integrates the input of upstream pathways, such as insulin, growth factors IGF-1, IGF-2 and amino acids. mTOR assembles into two multi-component complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).

mTORC2 is less sensitive to rapamycin and its stimulus is not well understood as only growth factors have been shown to induce mTORC2 activity. On the other hand, there is a greater understanding about the function of mTORC1 as it is nutrient sensitive. mTORC1 integrates several intracellular and extracellular signals and plays a key role in the activation of protein translation (Dobashi et al. 2011).

The role of mTORC1 in activating and regulating insulin signalling has been comprehensively investigated. Insulin signalling in skeletal muscle is initiated following binding of the insulin to insulin receptor (IR) that then binds to IR substrate (IRS-1/2). IR is a heterotetrameric bifunctional complex that belongs to the receptor tyrosine kinase superfamily and consists of two extracellular α and two transmembrane β subunits with tyrosine kinase activity (Knudsen et al. 2011). Insulin binding to IR stimulates structural changes to the α subunit that leads to autophosphorylation of a tyrosine kinase. Tyrosine phosphorylation leads to IRS-1 and -2 proteins interacting with the p85-regulatory subunit of phosphoinositide-dependent protein kinase-1 (PI3K). Activation of this enzyme results in the activation of the p110 catalytic subunit of PI3K and production of the second lipid messenger phosphatidylinositol-3,4,5-triphosphate (PIP₃). PIP₃ then binds to the pleckstrin homology domain of Akt [Also known as protein kinase B (PKB)] (Mackenzie and Elliott 2014). Akt is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes, such as cell growth, proliferation, apoptosis, cell migration and glucose metabolism. There are three homologous isoforms of Akt that have been identified – Akt1/PKB α , Akt2/PKB β and Akt3/PKB γ (Manning and Toker 2017). Among these isoforms, Akt2 plays a central role in insulin-stimulated glucose uptake in muscle tissues. Binding of PIP₃ to Akt results in conformational change in Akt that is then followed by its phosphorylation at Thr³⁰⁸ by PDK1, inducing about 10% of the kinase activity (Manning and Toker 2017). Subsequently this leads to phosphorylation of specific serine residue (Ser⁴⁷³) of the carboxyl-terminal hydrophobic motif of Akt by mTORC1 (Wick

et al. 2000) (Fig. 1). This promotes full Akt activation and its translocation to the cytoplasm, mitochondria and nucleus where it phosphorylates its many substrates. Akt substrate 160 (AS160) is thought to be the most important signalling molecule for glucose uptake. Upon activation, AS160 is phosphorylated at Thr⁶⁴² by Akt leading to reduction in Rab GTPase-activating proteins (Rab-GAP) activity and promoting glucose transporter-4 (GLUT4) translocation (Sakamoto and Holman 2008; Naufahu et al. 2018). GLUT4 is a glucose transporter containing 12-transmembrane domains, that is sequestered into specialised intracellular compartments known as GLUT4 storage vesicles (GSVs) in unstimulated cells. In response to insulin stimulation, GLUT4s are translocated from GSVs to the plasma membrane via targeted exocytosis to facilitate glucose uptake in muscle and fat tissue.

Amino acids and insulin resistance: proposed mechanisms

Dietary protein derived amino acids, primarily leucine, are potent activators of mTORC1 (Kimball et al. 2016; Son et al. 2019). Indeed, most of the evidence on the interaction between elevated postprandial dietary amino acid availability in circulation (or intracellularly) and the regulation of mTORC1 activation is based on free amino acid ingestion and/or isolated protein sources (Van Vliet et al. 2019). These methods usually lead to a rapid and transient pattern of aminoacidemia; neither of which are indicative of postprandial blood amino acid profiles after the ingestion of mixed meals or protein dense food sources (e.g., chicken, eggs, beef). In our hands, meal combinations and/or the ingestion of protein dense foods do not elicit such strong activation of mTORC1 or associated downstream targets (i.e., p70S6K or 4E-BP1) (Beals et al. 2016; Sawan et al. 2018; Van Vliet et al. 2018; Van Vliet et al. 2019). In any case, based on *in vitro* culture models, intracellular amino acid availability regulates mTORC1 via the RAS-related GTP-binding protein (Rag) family of small GTPases: RagA, RagB (RagA/RagB) and Rag C, Rag D (RagC/RagD) (Zoncu et al. 2011; Han et al. 2012). In the presence of amino acids, regulator complex and vacuolar H⁺ adenosine triphosphatase (v-ATPase) undergo

conformational change resulting in stimulation of guanine nucleotide exchange factor (GEF) (Willoughby 2015). GEF promotes GTP-nucleotide charging that activates Rag proteins resulting in the recruitment of mTORC1 to the lysosomal surface where mTORC1 is activated by Rheb (Kim et al. 2013). Activation of mTORC1 by amino acids is essential for many cellular functions, including anabolic process, cell growth and proliferation (Fig. 1). However, it has been hypothesised that mTORC1 hyperactivation, stimulated under non-physiological amino acid overload may contribute to insulin resistance (Hay and Sonenberg 2004; Um et al. 2004; Tzatsos and Kandror 2006). *In vitro* studies have shown that chronic activation of the mTOR/S6K1 pathway by amino acids can promote insulin resistance in muscle cells via increased IRS-1 Ser³⁰⁷ phosphorylation and degradation thus leading to the impairment of PI3K stimulation (Haruta et al. 2000; Tremblay and Marette 2001) (Fig. 1). PI3K is a key effector in insulin's metabolic action via the downstream activation of Akt, which is essential for GLUT4 translocation and glucose uptake. In addition, Um and colleagues reported that hyperactivation of mTORC1 has been shown to drive S6K1-mediated feedback inhibition of insulin signaling, thus reducing glucose uptake in skeletal muscles of obese rodents (Um et al. 2004). Other studies conducted in myotubes and isolated rat skeletal muscles also suggest that leucine may interfere with insulin signaling, given that leucine stimulates mTOR/S6K1 and IRS-1 Ser³⁰⁷ phosphorylation (Tzatsos and Kandror 2006; Iwanaka et al. 2010). Collectively, this research suggests that over-activation of mTORC1 due to amino acid availability may contribute to insulin resistance in muscle. In contrast some research supports an opposing hypothesis that leucine improves insulin sensitivity and glucose control. Macotela et al. (2011) demonstrated that mice fed a high-fat diet showed that, serine phosphorylation of IRS-1 was reduced, despite an increase in phosphorylation of S6K1, with chronic leucine supplementation for 8 weeks. In addition, leucine supplementation correlated with improved insulin sensitivity, a finding that is consistent with studies of obese rodents (Guo et al. 2010; Eller et al. 2013; Li et al. 2013).

The differences in study design, including the metabolic phenotypes of obese rodents and protein supplementation dose and type, may explain some of the inconsistencies. While amino acids are potent activators of mTORC1, some suggest that amino acid associated activation of mTOR may not be sufficient to induce insulin resistance (Houde et al. 2010; Li et al. 2013). It is possible that BCAAs may elicit different effects on glucose homeostasis, depending on the prevalence of anabolic and catabolic states of the organism (positive or negative energy balance) and thus making it difficult to determine the exact effect of BCAAs on insulin signalling (Bifari and Nisoli 2017). Further detailed work examining amino acids and insulin resistance is required to determine key mechanistic pathways explaining the role of high-protein diet on glucose homeostasis.

Human studies examining the role of mTOR/S6K1 pathway in T2D are very limited. Bandyopadhyay et al. (2005) reported that people with T2D have greater basal phosphorylation of IRS-1 at Ser307 that corresponded with lower insulin-stimulated IRS-1 tyrosine phosphorylation. Additionally, this study found that diabetics and obese individuals had lower PI3K and Akt activity compared with healthy participants. Similar findings were also reported in studies with prediabetics (Storgaard et al. 2001) and people with T2D (Kim et al. 1999; Krook et al. 2000; Karlsson et al. 2005). However, studies examining the effect of amino acids on mTOR/Akt and insulin resistance in human participants report inconsistent results. The effects of amino acids were shown to be associated with inhibitory phosphorylation of IRS-1 and inactivation of PI3K in healthy human participants (Tzatsos and Kandrór 2006). Tremblay et al. (2005) studied healthy men and found that intravenous (IV) infusion of amino acids decreased insulin-stimulated glucose disposal, which the authors attributed to elevated IRS-1^{Ser312} phosphorylation and blunted PI3K, while failing to increase insulin-induced phosphorylation of Akt. Conversely, Bassil et al. (2011) investigated the effects of a hyperinsulinaemic-hyperglycaemic clamp, starting with postabsorptive amino acid

concentration then followed by infusion of amino acids matching postprandial concentration in T2D individuals. This work showed that infusion of amino acids increased phosphorylation of Akt^{Ser473}, Akt^{Thr308} and mTOR^{Ser2448}, yet no changes were observed in the phosphorylation of IRS-1^{Ser636/639} and IRS-1^{Ser1101}. Additionally, no changes were observed in glucose infusion rates, glucose disposal or in endogenous glucose production with IV amino acid infusion, suggesting that amino acid availability does not alter whole-body glucose control. Yet, with the oral ingestion of leucine and whey proteins, Smith et al. (2016) showed that the positive effects of a weight loss diet with normal protein intake were blunted by an increase in protein intake, in obese, postmenopausal women. This was reflected in the decrease in glucose disposal and phosphorylation of Akt^{Ser473} when compared to the normal protein diet group. Such a finding is not consistent with the finding that there are no differences in postprandial muscle protein synthesis rates, an mTORC1 mediated event, after the ingestion of a meal-like amount of dietary protein between T2D patients vs. normoglycemic controls (Kouw et al. 2015). Taken together, these data suggest that excessive amino acids availability may contribute to insulin resistance via mechanisms yet to be determined, in humans with similar metabolic characteristics.

The effect of short and long-term high-protein diets on insulin resistance

The synergistically stimulating effect of protein and carbohydrate co-ingestion on insulin concentration in healthy adults was first reported in the 1960s (Rabinowitz et al. 1966; Pallotta and Kennedy 1968). Van Loon et al. (2003) demonstrated that amino acid ingestion with carbohydrates increased plasma insulin response 2-3-fold in patients with T2D. While an increase in the insulin concentration is itself sufficient to cause insulin resistance, this effect requires prolonged hyperinsulinemia (Del Prato et al. 1994).

However, studies examining long-term effect of high protein diets during energy restriction have reported a variety of beneficial effects, including medication reduction, better adherence and improvements in haemoglobin A1c (HbA1c) (Table 1). Most of the studies that have examined the effect of increasing dietary protein have done so in the context of energy restriction. While weight loss itself can improve insulin sensitivity, comparisons with the control group (provided that weight loss was matched) can provide insight into the metabolic effect of high protein on insulin sensitivity. In obese participants, energy restriction and high-protein diet have been shown to be more effective in reducing insulin resistance when compared to low protein diet (Farnsworth et al. 2003; Claessens et al. 2009; De Luis et al. 2015; Mateo-Gallego et al. 2017). On the other hand, some studies suggested that there are no differences between high-carbohydrate and high-protein diets (34% from total energy derived from protein and respectively 1.34 g protein/kg of body weight/day), and beneficial outcomes for insulin sensitivity are attributed to body mass reduction (Noakes et al. 2005; Campos-Nonato et al. 2017). In contrast, Smith et al. (2016) reported that a high-protein diet (1.2 g protein/kg of body weight/day) may blunt beneficial effects of weight loss and prevent improvements in muscle insulin signalling through adverse effects on the metabolic pathways involved in oxidative stress, without any changes in p-mTOR^{Ser2448} or p-AMPK^{Thr172} in muscle. Similar disagreements about high-protein diet effect on insulin sensitivity are evident in studies with diabetic participants. While Sargrad et al. (2005) reported no effect of high-protein diets on fasting insulin and glucose levels, Gannon et al. (2003) demonstrated that high-protein diet decreased HbA1c after 5 weeks, with no associated weight change during the study. The discrepancy may be due to the fact that protein may primarily affect postprandial insulin and glucose concentrations. It is also worth noting that all the foods within this study were provided by the researchers for the duration of the intervention, which is likely to have increased compliance to dietary protocol. Research that incorporated energy restriction during high-

protein diet intervention reported a variety of beneficial outcomes, such as decreased fasting glucose and insulin requirements (Luger et al. 2013) as well as a reduction in HbA1c (Larsen et al. 2011). While high-protein diets are feasible and safe for individuals with T2D, they do not always provide superior long-term metabolic benefit over a standard protein intake during energy restriction. The inconsistent findings may be attributed to the confounding effects of differences in protein sources in diet (e.g. consumption of dairy and meat products versus plant proteins), differences in weight loss, participant characteristics, overall energy balance state and other lifestyle factors often cited in several systematic reviews and meta-analysis (Schwingshackl and Hoffmann 2013; Rietman et al. 2014). There is also the issue that many long-term trials, which are included within meta-analysis, lack the internal validity required to assess physiological efficacy, unless they provide the intervention foods/diet and/or can objectively assess compliance. Studies that have used oral or IV amino acids can provide mechanistic insight that may overcome some of these limitations, and therefore inform the food-based literature.

Mechanistic studies with oral or intravenous amino acids

Manders et al. (2005) reported that co-ingestion of amino acid mixture with carbohydrates during hyperglycemic clamp resulted in 3-4- fold greater insulin response that improved postprandial glucose disposal and lowered plasma glucose concentration in patients with T2D. However, studies with healthy individuals reported a decrease in whole-body glucose disposal and greater insulin resistance in response to IV amino acid infusion during euglycaemic-hyperinsulinemic clamp (Pisters et al. 1991; Flakoll et al. 1992; Krebs et al. 2002; Tremblay et al. 2005). It is important to note that during hyperinsulinemic-euglycemic clamp, insulin-mediated decrease in amino acid concentration results in greater glucose disposal compared to when amino acids are maintained at their basal level, and they do not represent normal physiological concentration. As a result, glucose disposal during amino acid IV infusion is

compared to a physiological condition where plasma amino acid concentrations are below the baseline levels. This could potentially explain a greater glucose disposal in ‘control’ compared to amino acid infusion. Everman et al. (2015) designed a study in which BCAAs concentrations were maintained in the control group in a way that was not different from baseline/postabsorptive levels during insulin infusion. In this way, they were able to investigate the role of increased plasma BCAA concentration on glucose disposal. The study concluded that short-term increase in plasma BCAAs does not modify insulin sensitivity of glucose metabolism.

Conclusion

Glucose homeostasis involves a complex interplay and cross-talk between pancreatic β -cells and insulin sensitive peripheral tissues. Amino acids are nutrient signals that can induce a variety of direct and indirect effects at the cellular and organismal level. Despite the potentially favorable effects of high protein intake, controversy exists regarding the findings from short (< 6 month) and long-term interventional studies and the differences in participants’ characteristics. Moreover, there is a debate among nutrition experts on what the optimal amount of dietary protein is for those with T2D (Hamdy and Horton 2011). In contrast, it has been proposed that ‘excessive’ amino acids may hyperactivate the mTOR/S6K1 axis, which may present an inhibitory effect on insulin-mediated PI3K/IRS-1 activation, reducing insulin-stimulated glucose uptake and increasing insulin resistance. Indeed, understanding the molecular basis underpinning a macro-nutrient related disease risk is relevant to have a complete understanding of a recommendation providing dietary guidance; however, it is also important to consider the experimental model is not always indicative of a healthy eating pattern and thus dietary context is key for accurate interpretation. It is possible that a high-protein diet may contribute to hyperinsulinemia, yet, as a dietary strategy, it could be helpful for reducing body weight and subsequently increasing insulin sensitivity. Nevertheless, there is a need for more research exploring the effects of high-protein diets on both plasma BCAAs

concentrations and intracellular signaling in insulin-sensitive tissue, which could potentially indicate whether high BCAAs concentrations contribute to insulin resistance or are a consequence of the disease.

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Table 1. Overview of high-protein diets on insulin resistance in human studies

Participants	Duration	Dietary intervention	Main outcomes	References
Obese and overweight adults	12 weeks	≈30% restriction of total energy HP: 30 % of energy as protein	↔Fasting glucose ↓Fasting insulin ↓iAUC glucose	Farnsworth et al. 2003

Obese females	12 weeks	≈ 1300kcal per day HP: ≈34% of energy as protein	↓Fasting insulin ↓Fasting glucose	Noakes et al. 2005
Obese and overweight adults	12 weeks	500kcal per day for 5 weeks followed by normal ad libidum for 7 weeks HP: 25% of energy as protein	↔HOMA-IR ↔HbA1c	Claessens et al 2009
Obese adults	9 months	1050 cal/day, HP:34% of energy as protein	↓HOMA-IR ↓Fasting insulin ↓Fasting glucose	De Luis et al. 2015
Obese woman	6 months	Calorie restriction to induce 10% weight loss HP: 1.2 g protein/kg/day	↔Glucose R_a and R_d	Smith et al. 2016
Obese and overweight adults	6 months	Caloric restriction of 500 kcal less than the RMR HP: 1.34 g/kg body weight protein intake	↔HOMA-IR ↔HbA1c	Campo-Nonato et al.2017
Obese women	6 months	Caloric deficit of 600 kcal/day HP: 35% of energy as protein	↓HOMA-IR ↔HbA1c	Mateo-Gallego et al. 2017
Adults with T2D	5 weeks	No specific energy restriction HP: 30% of energy as protein	↓iAUC glucose ↔iAUC insulin	Gannon et al. 2003
Adults with T2D	8 weeks	No specific energy restriction HP: 30% of energy as protein	↔Fasting insulin ↔Fasting glucose	Sargrad et al. 2005
Adults with T2D	12 weeks	No specific energy restriction HP: 30% of energy as protein	↓Fasting glucose ↓Insulin dose	Luger et al. 2013

Abbreviations: Basal glucose rate of appearance (R_a) and glucose rate of disappearance (R_d); HbA1c, glycated haemoglobin; HOMA2-IR, homeostasis model assessment of insulin resistance index 2 to assess insulin resistance; HP, high-protein diet; iAUC, the incremental area under the curve; RMR, resting metabolic rate.

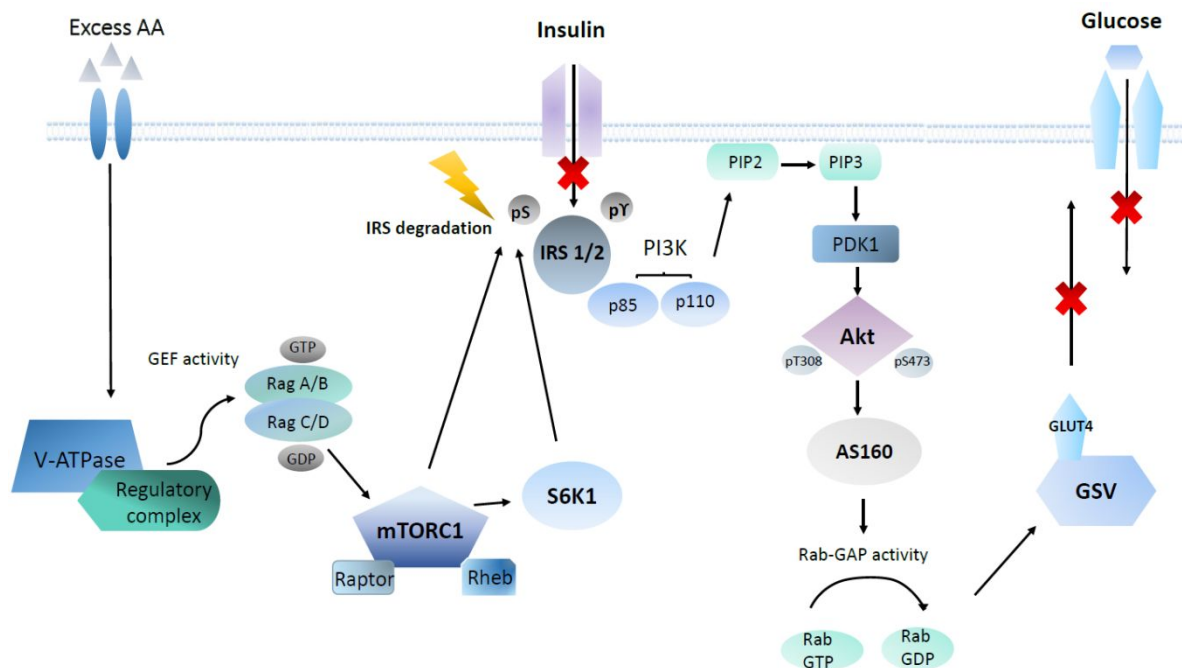


Figure 1: Potential mTOR/S6K1 signalling pathway in response to high intake of amino acids. These mechanisms are largely based on experimental models (e.g., *in vitro* and animal models) not commonly experienced in a healthy eating patterns such as meal combinations and protein dense foods.

Abbreviations: AA, amino acids; Akt, serine/threonine protein kinase; AS160, 160 kDa Akt substrate; GDP, Guanosine diphosphate; GEF, guanine nucleotide exchange factors; GLUT4, glucose transporter 4; GSV, GLUT4 storage vesicle; GTP, Guanosine-5'-triphosphate; IRS, insulin receptor substrate; PDK1, phosphoinositide-dependent protein kinase-1; PI3K, class IA phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol (4,5)-bisphosphate; PIP3, phosphatidylinositol 3,4,5-trisphosphate; Rab-GAP, Rab-GTPase-activating protein; Rab-GDP, guanosine-5'-diphosphate-loaded Rab; Rab-GTP, guanosine-5'-triphosphate-loaded Rab; Rag C/D, Rag heterodimer C/D; RagA/B, Rag heterodimer A/B; Raptor, Regulatory-associated protein of mTOR; S6K1, ribosomal protein S6 kinase beta-1; V-ATPase, vacuolar-type H⁺-ATPase.