The possible role of leucine in modulating glucose homeostasis under distinct catabolic conditions

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Abstract
Branched-chain amino acids (BCAA) (especially leucine) have been shown to activate protein synthesis pathways, decrease proteolysis and increase insulin sensitivity. Furthermore, it appears that leucine can be used as a nutritional therapy to avoid sarcopenia and skeletal muscle atrophy due to immobilization or glucocorticoid treatment. However, it is of note that all of these conditions are related to insulin resistance to varying degrees and affect different tissues, particularly skeletal muscle. Additionally, evidence from recent studies demonstrate that a combination of protein containing high levels of leucine with nutrients containing saturated fatty acids or an excess of leucine are capable of inducing insulin resistance. From this discussion, a few major questions arise. First, what is the role of a combination of macronutrients in inducing insulin resistance? Second, in insulin resistance, does leucine supplementation follow the same path observed under healthy conditions? Finally, what are the dose-dependent outcome and the latency of leucine effect under such conditions? The present article discusses these questions based on data from the literature and experiments performed by our group.

Introduction
Leucine supplementation has been considered to be a physiopharmacological entity because several of its effects are independent of other amino acids (AA) [1]. During healthy states, leucine supplementation has been shown to activate protein synthesis pathways, decrease proteolysis and increase insulin sensitivity [1–4]. Moreover, leucine appears to be the signal acting together with resistance exercise leading to increased signaling mechanisms of muscle remodeling [5].

On the other hand, it appears that leucine can be used as a nutritional therapy to avoid sarcopenia [6] and skeletal muscle atrophy due to immobilization [7] or glucocorticoid treatment [8]. It is of note that all of these conditions are related to varying degrees of insulin resistance and affect different tissues, particularly skeletal muscle. From this discussion, a few major questions arise. First, what is the role of a combination of macronutrients in avoiding or to inducing such catabolic, insulin resistant states? Second, in an insulin resistant state, does leucine supplementation follow the same paths observed under normal conditions? Finally, what are the dose-dependent outcome and the latency of leucine effect under such conditions? The aim of the present article is to discuss these questions based on the data from the literature and experiments performed by our group. Of note, given the lack of well controlled studies and the limitation of some experimental designs in humans (i.e., use of glucocorticoids in healthy humans), we focused primarily on animal studies.

Protein intake and insulin resistance
Insulin resistance is a common disorder of type 2 diabetes mellitus (DM2), affecting millions of people worldwide, reaching epidemic proportions [9]. DM2 patients display increased whole body insulin resistance, mainly in skeletal muscle, liver, and white adipose tissue, which leads to hyperglycemia due to reduced insulin-stimulated glucose disposal and increased endogenous glucose production. Therefore, understanding the molecular mechanism of peripheral insulin resistance in adipose tissue, liver and skeletal muscle is one of the major challenges in science today.

Since skeletal muscle is responsible for almost 75% of the insulin-stimulated glucose uptake, it is a suitable target for an increase
in insulin sensitivity, which in turn delays the progression from insulin resistance to DM2 [10]. Although it is clear that pancreatic beta cell dysfunction or failure is essential to development DM2, reduction of peripheral insulin resistance is able to prevent or delay beta cell failure and subsequent DM2 development [11]. Indeed, studies that aimed to reduce insulin resistance using insulin-sensitizing agents suggest that those therapies are able to delay or prevent the development of DM2 and avoid beta cell failure [11–14].

Among other interventions, diet composition appears to play a role in DM2, since there is a lower incidence of this disease in people who consume a high quantity of fish-based protein [15]. In fact, some studies have shown that both human and rodents fed with cod protein displayed an improvement in insulin sensitivity. Lavigne et al. [16] showed that rats fed a high fat diet (HFD) in combination with cod protein displayed higher whole body insulin sensitivity when compared with rats fed a HFD combined with casein protein. Additionally, in this study, an improvement in insulin sensitivity was accompanied by increased glucose uptake in skeletal muscle. These results were observed without any significant changes in body weight and plasmatic TNF-α suggesting that cod protein has a direct effect on insulin sensitivity. A potential beneficial effect of cod protein in humans was also observed in other studies. Ouellet et al. [17] showed that humans who ate cod protein for 4 weeks displayed increased insulin sensitivity when compared with humans who ate other types of protein, such as lean beef, eggs, pork, milk, and milk products. Moreover, as observed in rats, these results occurred without changes in body weight. A suitable explanation for the effects of a cod protein diet on insulin sensitivity is that BCAA are found at lower concentrations in cod protein than other kinds of protein, such as casein and soy protein. Other evidence suggesting that AA directly affects insulin sensitivity include that some AA, such as leucine, are increased in the plasma from obese individuals [18].

The studies cited above showed that the type of protein is able to directly affect insulin sensitivity in both human and rodents, and suggests, in turn, that AA may be modulating agents of insulin signaling and glucose metabolism. Among these, it appears that the BCAA concentration plays a pivotal role in modulating such response since it corresponds to 35–40% of daily intake of essential AA.

Verhoeven et al. [19] supplemented elderly men with 7.5 g/d of leucine (3 daily doses of 2.5 g) during 3 months and observed no improvement in skeletal muscle mass or strength nor in indexes of whole-body insulin sensitivity (assessed by oral glucose insulin sensitivity index and by the homeostasis model of insulin resistance). Since sarcopenia is a well-known condition characterized by insulin resistance [20], it is plausible that leucine supplementation did not present any therapeutic effects in glucose homeostasis and did not aggravate aging-induced insulin resistance. Thus, it can be concluded that leucine supplementation is safe in modulating glucose homeostasis in humans even in a condition characterized by insulin resistance. This same research group tested the effects of leucine supplementation (the same protocol) on elderly type 2 diabetic men but during 6 months [21]. The authors observed no change in lean tissue body mass, body fat percentage, muscle strength and muscle fiber type characteristics. In addition, oral glucose insulin sensitivity did not differ between the leucine-supplemented and the placebo group. Although no therapeutic effects were observed in the later study, it is also possible that, in humans, leucine does not aggravate insulin resistance in conditions characterized by impaired insulin signaling.

**Direct effects of AA on insulin signaling in skeletal muscle**

Skeletal muscle is the major tissue responsible for glucose uptake stimulated by insulin [10]. This effect occurs when insulin binds to its receptor (IR) on the skeletal muscle cell membrane surface, thus triggering IR tyrosine kinase activity. Activated IR is able to phosphorylate insulin receptor substrates (IRS-1 and IRS-2) at tyrosine residues, allowing for these substrates to associate and activate phosphoinositide 3-kinase (PI3 K), leading to phosphorylation of PI3P (phosphoinositide 2 phosphate) and subsequent increase in phosphoinositide 3 phosphate (PIP3) cellular content. The downstream protein that is activated by increased PIP3 content is Akt, whose activation leads to increased GLUT4 translocation to plasma membrane through 160 kDa Akt-substrate (AS160), and increased glucose uptake [22]. Another pathway activated by Akt, as opposed to the GLUT4 pathway, is the mTOR/p70S6K pathway, which is important in skeletal muscle protein synthesis. On the other hand, the mTOR/p70S6K pathway is a negative regulator of insulin signaling, leading to phosphorylation of serine residues in IRS-1 decreasing its association with PI3K [23].

The mTOR/p70S6K pathway is thought to be an AA concentration sensor because several studies with cultured cells observed that AA were able to activate elements involved in protein synthesis translation initiation [24,25]. In fact, it was observed that AA acutely stimulates p70S6K phosphorylation, and this effect was completely inhibited by rapamycin, an mTOR inhibitor [26]. Additionally, it was shown that AA-deprived cells display reduced p70S6K phosphorylation, suggesting an important role for AA in the mTOR/p70S6K pathway activation [27]. These effects were also observed in cultured skeletal muscle and in vivo infusion, in both human and rodents [26–28]. One of the major AA responsible for the mTOR/p70S6K activation is the BCAA leucine [27].

Due to its effect on mTOR/p70S6K activation, leucine can impair glucose metabolism in skeletal muscle by reducing the tyrosine phosphorylation of IR and IRS-1, and reduce the activity of PI3K [29] (Fig. 1). Recently it has been shown that the role of p70S6K in insulin resistance occurs in part by delaying activation of the IR/IRS-1/PI3K in the postprandial state [30]. The role of p70S6K in insulin resistance has been shown in other studies. Um et al. [31] showed that p70S6K-deficient mice (also known as S6K1) were protected against both age- and high fat diet-induced insulin resistance. Additionally, it was demonstrated that p70S6K1 is able to directly phosphorylate IRS-1 on serine residues, reducing insulin-stimulated IRS-1 tyrosine phosphorylation [32]. Corroborating these results, Zheng et al. [33] showed that improved insulin sensitivity by calorie restriction in obese Zucker rats is associated with decreased p70S6K phosphorylation. This has also been observed in humans, since women with gestational diabetes mellitus have chronically increased S6K1 activation [34]. Recently, our group demonstrated that rats treated with the synthetic glucocorticoid dexamethasone and supplemented with leucine during 7 days presented increased insulin resistance in plantaris muscle. It was observed that dexamethasone-treated animals developed hyperglycemia and insulin resistance and that leucine supplementation aggravated such effects through impaired GLUT-4 translocation to the sarclemma in the post-prandial state. We also observed that this impaired translocation occurred through decreased phosphorylation of Akt. However, we did not find significant differences in p70S6K phosphorylation, suggesting a possible p70S6K-independent mechanism [35].

Along with the evidence cited above, AA are thought to modulate insulin signaling through the mTOR/p70S6K pathway followed by glucose metabolism. Activated p70S6K was able to inhibit glucose uptake stimulated by insulin in L6 skeletal muscle cells and leucine was one of the AA that induced insulin resistance in those cells [26]. Indeed, Patti et al. [25] showed that AA inhibited early steps of insulin signaling (IRS-1/2 and PI3K) in liver cells and muscle cells associated with increased p70S6K phosphorylation. These results were largely due to BCAA, (primarily leucine) and were blunted by rapamycin, while wortmannin (an inhibitor of PI3K)
did not have any effect, suggesting that leucine is able to activate mTOR/p70S6K pathway independently of the IRS/PI3K/Akt pathway.

Interestingly, the potential effects of leucine on insulin signaling were not only observed in cultured cells but were also observed in rats skeletal muscle, where they reduced the time of insulin-stimulated IRS-1-associated PI3K activity [36]. Additionally, AA infusion in humans demonstrated increased p70S6K phosphorylation and reduced whole body glucose uptake. Greiwe et al. [37] showed that leucine infusion in humans for 2 h increased the p70S6K phosphorylation in skeletal muscle. Tremblay et al. [29] also showed that AA infusion in humans for 6 h increased p70S6K1 activity, decreased whole body glucose uptake and increased endogenous glucose production. These results were associated with increased IRS-1 phosphorylation on serine residues and reduced PI3K activity.

Although it has been well established that AA, in particular leucine, are able to activate the mTOR/p70S6K pathway, stimulate protein synthesis and possibly induce insulin resistance, it is not well understood how AA may induce the activation of this signaling pathway. One possible mechanism by which leucine may be able to induce mTOR/p70S6K activation could be through AMPK inhibition (Fig. 1). Recently, it was reported that rat EDL muscle incubated with leucine displayed an increase in protein synthesis accompanied by increased mTOR/p70S6K activation. Additionally, in this work, it was observed that mTOR/p70S6K activation by leucine incubation was associated with a decrease in AMPK activity and that the co-incubation of EDL with leucine and AICAR, a pharmacological activator of AMPK, prevented all effects of leucine. Although the work of Saha et al. [38] presents strong evidence for the role of AMPK on leucine-stimulated mTOR/p70S6K activation, it is unknown whether the decrease in AMPK activity induced by leucine is needed to observe the effects on protein synthesis and mTOR/p70S6 K activation. Other possible mechanism it is through increase in Ca2+ concentration in muscle cell, which stimulates the direct binding of Ca2+/Calmodulin to an evolutionary conserved motif of human vacuolar protein sorting 34 (hVps34) rather than through the canonical class I PI3k pathway used by growth factors and hormones [39].

Dose effects of leucine: Duality in healthy and insulin-resistant states

Based upon the above information it can be concluded that leucine supplementation is capable of exerting both insulin resistance and protein synthesis. What is the condition leading to each situation? Bohé et al. [40] have described that in humans, a single dose of 3.5 g of protein (not taking into account the AA uptake performed by splanchnic tissues) results in a “muscle full” situation (a maximal effect on muscle protein synthesis) [41] and for this reason it is unnecessary to supply more than the amount required to cause a “muscle full” situation. However, it has been shown that in old rats the slope of the relationship between muscle protein synthesis and the availability of leucine is shifted to the right, therefore demonstrating a decrease in sensitivity, capacity or both [41,42]. Moreover, an excess of nutrients is capable of inducing insulin resistant states [23]. What is the threshold of leucine supplementation needed to transform a normal state of physiological protein synthesis signal into an insulin-resistant one? To address this question, we observed that in healthy rats, supplementation with high doses of leucine (1.35 g/kg/bw twice a day) through gavage (Fig. 2A), did not induce an increase in the fasting glycemia (Fig. 2B) when compared with rats receiving lower doses of leucine (0.068 g/kg/bw) (Zanchi et al. unpublished results). Importantly, the high dosage was capable of maximally increasing muscle protein synthesis and insulin plasmatic levels (in a well defined pulsatile form), whereas the low dosage was not capable of increasing neither, muscle protein synthesis nor insulin plasmatic levels [2,43]. Collectively, these results suggest that healthy adult rats are capable of metabolizing very high amounts of leucine, and that the threshold of leucine supplementation needed to transform a
physiological protein synthesis signal into an insulin-resistant one is very high. Additionally, when adult rats were treated using both low and high leucine dosages during dexamethasone treatment (5 mg/kg/bw during 7 days) there were no differences when compared to the placebo group (Zanchi et al. unpublished results – Fig. 3). This result could explain why BCAA supplementation in addition to dexamethasone treatment was so effective in the treatment of skeletal muscle atrophy in the study conducted by Yamamoto et al. [8].

Latency effects of leucine supplementation during dexamethasone-induced resistance state conditions

Another interesting question related to leucine supplementation is the following: is there latency for the effect of leucine supplementation during pathological conditions? In other words, since the dosage effect was not different when comparing rats presenting insulin resistance mediated by dexamethasone treatment as shown above (Fig. 3), would frequent nutritional stimuli be different from that provided by a pulsatile pattern to aggravate insulin resistance caused by dexamethasone treatment?

To test this hypothesis, we supplemented two groups of dexamethasone-treated rats: the first group consumed the low dose of leucine (0.068 g/kg/bw twice a day exactly as described before) in a pulsatile form (via gavage), and the second group consumed the same dose in a non-pulsatile form (via drinking water). Our results were notable: rats supplemented through short periods of time (offered in drinking water), in a non pulsatile form presented a markedly higher fasting glycaemia compared with rats supplemented with the same daily dosage twice a day, in a pulsatile form (Fig. 4). Such results suggest that tissues need time to terminate the leucine signal. Moreover, these results show that the continuous presence of this AA in the whole body, in an insulin resistant state (dexamethasone-induced) would be capable of transforming insulin resistance into diabetes. Although yet unknown, this may be the result of a blockade in the IR/IRS-1/p70S6 K signaling pathway plus the glucocorticoid regulation of muscle BCAA metabolism, as described by Block & Buse [44]. Such a result is partly supported by Rieu et al. [45] who found that in old rats, an excess...
of glucocorticoid induced a prolonged leucine resistance on muscle protein synthesis.

Data from El-Kadi et al. [46] and Gazzanese et al. [47] indicates that different feeding strategies promotes distinct physiological responses. In piglets, intermittent orogastric tube feeding results in greater elevation of BCAAs concentration in plasma than in continuous feeding [47]. After a single bolus, insulin and amino acids levels rise concomitantly with protein synthesis rate, but returns to basal levels in 2–4 h [48]. Intermittent feeding promotes a greater amino acids uptake and protein synthesis, as well as a more pronounced phosphorylation in Akt, S6K1 and 4E-BP1, while protein degradation appears to be insensitive to feeding frequency [46].

This short latency pattern and transient response appears to be important to stimulation of Akt/mTOR pathway and protein accretion in skeletal muscle, which can be beneficial in insulin resistance and catabolic states.

Conclusions and perspectives

Leucine is an AA with particular effects during healthy conditions. However, from recent studies, it can be concluded that a combination of protein containing high levels of leucine with nutrients containing saturated fatty acids is capable to induce insulin-resistant states. On the other hand, when administered during a pre-existing insulin resistant state (i.e., dexamethasone induced) leucine supplementation demonstrates a completely different effect in the organism, and the latency of the effect becomes an important modulator of insulin resistance, being able to induce diabetes. Such a result has several implications: 1) will the results of prolonged leucine plus dexamethasone treatment in muscle cells (in a non-pulsatile form) apply to the whole body observing? 2) will patients receiving intravenous nutrition but suffering from insulin-resistant states (induced by glucocorticoid treatment) benefit from AA supplementation? The answers to such questions are still unknown and until further research clarifies the issue, the pros and cons will have to be weighed for each individual case.

Conflict of interest statement

None declared.

References


