Glutamine Regulates Skeletal Muscle Immunometabolism in Type 2 Diabetes

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Abstract
Dysregulation of skeletal muscle metabolism influences whole body insulin sensitivity and glucose homeostasis. We hypothesized that type 2 diabetes-associated alterations in the plasma metabolome directly contribute to skeletal muscle immunometabolism and the subsequent development of insulin resistance. To this end, we analyzed the plasma and skeletal muscle metabolite profile and identified glutamine as a key amino acid that correlated inversely with body mass index (BMI) and HOMA-IR index in men with normal glucose tolerance or type 2 diabetes. Using an in vitro model of human myotubes and an in vivo model of diet-induced obesity and insulin resistance in male mice, we provide evidence that glutamine levels directly influence the inflammatory response of skeletal muscle and regulates the expression of the adaptor protein GRB10, an inhibitor of insulin signaling. Moreover, we demonstrate that a systemic increase of glutamine levels in a mouse model of obesity improves insulin sensitivity and restores glucose homeostasis. We conclude that glutamine supplementation may represent a potential therapeutic strategy to prevent or delay the onset of insulin resistance in obesity by reducing inflammatory markers and promoting skeletal muscle insulin sensitivity.

Keywords: type 2 diabetes, obesity, metabolomics, skeletal muscle, inflammation, glutamine, Grb10.
Introduction

Type 2 diabetes is a chronic disease characterized by insulin resistance and disturbed glucose homeostasis that currently affects more than 400 million people world-wide. Obesity is a major risk factor for developing type 2 diabetes (1; 2). Skeletal muscle accounts for the majority of insulin-stimulated whole-body glucose disposal (3). Thus, dysregulation of skeletal muscle metabolism profoundly influences whole body insulin sensitivity and glucose homeostasis (3). While the molecular links between obesity and type 2 diabetes remain incompletely understood, chronic inflammation is strongly correlated with the development of insulin resistance (4). Nevertheless, the role of inflammation in modulating skeletal muscle metabolism and whole body insulin sensitivity remains to be fully appreciated.

Obesity increases systemic and local inflammatory processes with an infiltration of immune cells, such as macrophages and T cells, into the peripheral tissues controlling whole body glucose homeostasis, including skeletal muscle (5). This dynamic crosstalk between immune and metabolic processes is referred to as immunometabolism (6; 7). Alterations of metabolic pathways in immune or metabolic cells can drive inflammatory responses that lead to the production and release of cytokines, thereby exerting autocrine, paracrine and/or endocrine effects on glucose and energy homeostasis. In the context of overnutrition and obesity, an overload of nutrients and metabolites directly contributes to the low-grade inflammatory state associated with these conditions (8). For example, several lipid species such as saturated fatty acids, ceramides or lipid-derived eicosanoids activate immune cells, and induce inflammation in myocytes, thereby leading to insulin resistance (9; 10). Furthermore, in obese insulin resistant adipose tissue, alterations in amino acid levels, in particular reduced glutamine concentrations, are linked to increased glycolysis and inflammation in adipocytes resulting in dysregulated immunometabolism (11). Thus, altered levels of intercellular
metabolites, including lipids and amino acids, may influence peripheral insulin sensitivity in obesity and type 2 diabetes.

Recent advances in metabolomic techniques have revealed that elevated lactate and branched chain amino acids (BCAA), as well as an altered lipid composition, constitute a plasma metabolomic signature of type 2 diabetes (12). Changes in metabolite levels, such as BCAA, reflect both dietary intake and disturbed catabolism in various organs (13). However, few studies have characterized the metabolomic signature of both peripheral tissues and plasma in people with normal glucose tolerance or type 2 diabetes. Alterations in plasma metabolite levels in type 2 diabetes may affect the function of peripheral tissues controlling glucose and energy homeostasis through the modulation of the extracellular environment. Both an excess or an insufficient amount of metabolites can affect the cellular metabolic state, which may in turn impact transcriptional activity via intermediate metabolites that constitute substrates or co-substrates for enzymes modifying chromatin and/or the activity of transcriptional regulators (14). Through a comparative analysis of the plasma and skeletal muscle metabolomic signature of well-phenotyped subjects, specific metabolites involved in the pathophysiological mechanism underlying the development of type 2 diabetes can be identified.

We hypothesized that type 2 diabetes-associated alterations in the plasma metabolome are not only biomarkers of the disease, but directly contribute to an altered immunometabolic state in skeletal muscle and subsequent development of insulin resistance. In this study, we identified glutamine as a key amino acid that is inversely correlated with BMI and HOMA-IR index in men with normal glucose tolerance or type 2 diabetes. Using an in vitro model of human myotubes and an in vivo model of diet-induced obesity and insulin resistance in male mice, we provide evidence that extracellular glutamine levels can directly influence the inflammatory response of skeletal muscle and regulate the expression of the adaptor protein...
GRB10. Moreover, we demonstrate that a systemic increase of glutamine levels improves peripheral insulin sensitivity and restores glucose homeostasis.
Research design and methods

Participants

The study was approved by the regional ethics committee of Stockholm and conducted according to the Declaration of Helsinki. Informed written consent was obtained from each subject. Male volunteers with type 2 diabetes or normal glucose tolerance (NGT) were matched for age and BMI (Exclusion criteria and study design presented in Supplemental Figure 1). Clinical characteristics of the participants are presented in Table 1. The groups are subsets of a larger study designed to characterize the plasma and skeletal muscle metabolome in men with normal glucose tolerance or type 2 diabetes (15). Plasma and vastus lateralis skeletal muscle biopsies were collected after an overnight fast. Daily oral glucose-lowering medications were taken after the collection of skeletal muscle and plasma samples to reduce direct drug-related effects. However, longer effects of the medication on the metabolome may persist. Untargeted metabolomic analysis was performed by Metabolon, Inc (Durham, NC). Microarray analysis was performed on total RNA and hybridized to an Affymetrix array (ThermoFisher Scientific).

Animal experiments

C57BL/6J male mice were purchased from Charles River (Germany). Experimental procedures were approved by the Stockholm North Animal Ethical Committee. At 6-8 weeks of age, mice were fed either a standard chow diet (4% kcal from fat, R34; Lantmännen) or a 60% fat diet (Research Diet, New Brunswick, NJ). Three separate experiments were performed with mice fed high fat diet (HFD) for either 17 weeks (Experiments 1 and 2) or 15 weeks (Experiment 3). Prior to termination mice received daily intraperitoneal injection of glutamine (1 g/kg body weight) or PBS (20 mL/kg body weight) for 14 days. To assess body composition (Experiment 1), total lean and fat mass was determined in conscious mice using the EchoMRI-100 system (Echo Medical Systems). At day 15, blood glucose was measured after 4 h of fasting (One
Touch Ultra 2 Glucose Meter; Lifescan) and animals were then euthanized under general anesthesia. Plasma insulin level were quantified using Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem). To assess insulin tolerance (Experiment 2), mice were fasted for 4 hours and 0.75 IU/kg body weight of insulin was injected intravenously, and the rate of glucose disappearance between 0 to 15 min was measured as described (16). To assess glucose tolerance (Experiment 3), the mice were fasted 4 h, glycemia was monitored during 150 min after intraperitoneally injection of 1.5g/kg body weight of glucose.

**Cell culture experiments**

Primary cells were isolated from vastus lateralis skeletal muscle biopsies derived from healthy volunteers as described (17). For the high/low glutamine experiment, DMEM glutamine free medium was used and supplemented to 0.5 mM or 10 mM final concentration (glutamine, Life Technologies) during the 8-days differentiation period, or after differentiation for 48h. BPTES, a glutaminase 1 inhibitor, was purchased from Sigma-Aldrich (SML0601) and added to the cell medium at a concentration of 10 µM for 24 h. For the silencing experiments, differentiated cells were transfected with 10 nmol/L of either silencer select Negative control No.2 (no. 4390847) or a siRNA against GRB10 (silencer select siRNA s6125, Ambion, Life Technologies). Lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (L2630) and added to the cell medium at a concentration of 100 ng/mL for 24 h.

**Insulin signaling and Western blot analysis**

Extensor digitorum longus (EDL) (Experiment 1) and soleus (Experiment 3) muscles were incubated with Krebs-Henseleit buffer under continuous gassing (95% O₂/5% CO₂) at 30°C in the absence (basal) or presence of a submaximal concentration (0.36 nmol/L) of insulin (Actrapid; Novo Nordisk) for 20 minutes. Western blot analysis was performed as described (18). The primary antibodies used are: anti-GRB10 (C-11) (Santa Cruz); anti-phospho AS160
Thr<sup>642</sup> (#8881, Cell Signaling Technology); anti-phospho AKT Thr<sup>308</sup> (#4056, Cell Signaling Technology); anti-phospho AKT Ser<sup>473</sup> (#9270, Cell Signaling Technology).

**Statistical analysis**

Analyses were performed using either R 3.5.2 or GraphPad Prism 8.1 software (GraphPad Software Inc.). The statistical test used are indicated in the figure legends.

**Data availability**

The datasets generated during and analysed during the current study are available from the corresponding author upon reasonable request.

**Results**

*Plasma glutamine levels are associated with BMI and HOMA-IR*

We performed a metabolomic analysis of plasma and *vastus lateralis* skeletal muscle biopsies from a cohort of 14 normal glucose tolerant men and 19 men with type two diabetes, separated in “lean” and “overweight” subgroups based on BMI. Anthropometric and clinical parameters are shown in Table 1. Plasma metabolites were correlated with BMI, HOMA-IR and the waist/hip ratio WHR (Figure 1A-B, Supplemental Figure 1B-C). Of these, monosaccharides, including glucose and mannose, were the metabolites that most strongly associated positively with the clinical parameters (Figure 1A-B). The amino acids glutamine and glutamate also correlated with BMI and HOMA-IR index (glutamine: r=-0.42, p=0.015 for both BMI and HOMA-IR; glutamate: r=0.54, p=0.0001 and r=0.62, p=0.001, respectively for BMI and HOMA-IR), with glutamine and glutamate showing an inverse correlation (Figure 1A-B). The BCAA isoleucine, which has previously been identified as biomarker of type 2 diabetes (19), increases in parallel with HOMA-IR and BMI. In skeletal muscle, the BCAAs isoleucine and
leucine displayed a positive association with BMI and HOMA-IR, while glutamine was negatively associated with HOMA-IR ($r=-0.38$, $p=0.03$, Figure 1C-D). When comparing the plasma metabolome between men with NGT or type 2 diabetes, we found a trend for decreased glutamine in type 2 diabetes, while the glutamate level was significantly increased (Supplemental Figure 1D-K). To further dissect the relationship between plasma glutamine/glutamate levels and glucose metabolism independently of adiposity, the participants were stratified based on BMI, revealing that glutamine and glutamate levels were selectively altered in overweight type 2 diabetic men (Figure 1E-F). This relationship was not recapitulated in the lean type 2 diabetic participants, suggesting that modulation of plasma glutamine/glutamate level is not a marker of glucose dysregulation or overweight per se, but rather a reflection of the differences in the combined adiposity and insulin resistance between the groups.

In skeletal muscle, glutamine and glutamate content were not statistically altered in overweight and/or men with type 2 diabetes (Figure 1G-H). No differences in the gene expression of the enzymes responsible for glutamine synthesis (encoded by $GLUL$) or hydrolysis (encoded by $GLS$) (Supplemental Figure 2B-C) were noted. Nevertheless, skeletal muscle glutamine, but not glutamate, correlated with its respective plasma concentration (Figure 1I), suggesting a direct link between plasma and intramuscular glutamine concentration. Plasma and skeletal muscle glutamine levels had similar associations with indices of glucose metabolism including fasting insulin and glucose, C-peptide and HOMA-IR (Supplemental Figure 2A). Accordingly, participants with the highest plasma glutamine level (quartile 4) displayed lower BMI and HOMA-IR, while lower plasma glutamine level (Q3 to Q1) was progressively associated with increased BMI and HOMA-IR (Figure 1J-K). Based on these associations, we hypothesized that high plasma glutamine levels may be protective with respect to obesity-induced insulin resistance.
To assess whether increased glutamine levels could reverse or ameliorate the development of obesity-induced metabolic disturbances, three independent groups of C57BL/6J mice were fed a HFD for 15-17 weeks (Experiments 1-3). During the last 14 days of the diet, the mice were injected daily intraperitoneally with either PBS or 1g/kg of glutamine. Chow-fed mice injected with PBS were used as a control for the effects of the HFD (Figure 2A). Seventeen-weeks of HFD led to the development of obesity, with increased body weight, fat mass percentage and fasting glycemia (Figure 2B-D). Glutamine administration did not alter body weight or fat mass percentage in the HFD fed mice (Figure 2 B-C). Despite this, 14-days glutamine administration reversed the fasting hyperglycemia in HFD fed mice (p<0.05; Figure 2D). This reduction of hyperglycemia was accompanied by a reduction in the fasting insulin level and HOMA-IR as compared to PBS-HFD fed mice (Figure 2E-F). In a separate experiment (Experiment 3) of mice treated with HFD for 15 weeks, glucose tolerance was improved in glutamine-treated versus PBS-treated mice (Figure 2G-H). Furthermore, insulin sensitivity (Experiment 2) was improved, as indicated by the increased plasma glucose disappearance rate (KITT) after an intravenous insulin injection (Figure 2I-J). Given that skeletal muscle is a major insulin-sensitive organ, we assessed insulin signaling in isolated glycolytic EDL and oxidative soleus muscles. Submaximal insulin-stimulated (0.36 nM) phosphorylation of AKT at Ser\textsuperscript{473} was decreased in EDL muscle from HFD fed mice as compared to control chow fed mice, and glutamine administration tended to lessen this HFD-induced insulin signaling impairment (Figure 2K). Phosphorylation of AKT at Thr\textsuperscript{308} showed a similar pattern (Figure 2L). As a proxy for AKT activity, we determined phosphorylation of AS160, a direct AKT substrate that plays a role in GLUT4 translocation (20). Insulin-stimulated phosphorylation of AS160 was increased in EDL muscle of glutamine-treated HFD fed mice as compared to chow or PBS-HFD fed mice (respectively p=0.02; p=0.006) (Figure 2M), indicating that glutamine treatment
increased EDL muscle AKT activity. Conversely, glutamine did not affect insulin-stimulated AKT or AS160 phosphorylation in soleus muscle (Supplemental Figure 3A-D), suggesting a fiber-type difference in the response to glutamine treatment.

Glutamine suppresses gene transcripts annotated to inflammation-related pathways

To further decipher the molecular mechanisms by which glutamine administration affects the skeletal muscle profile, we performed a transcriptomic analysis on mouse quadriceps skeletal muscle. Microarray analysis showed that HFD treatment altered the skeletal muscle transcriptome in PBS- or glutamine-treated mice as compared to chow mice, with 1,300 and 1,290 genes altered, respectively between treatments (FDR<0.2, Figure 3A-B). Fifty-one genes were differentially regulated between PBS- and glutamine-treated HFD skeletal muscle, with most of the transcripts decreased in response to glutamine (Figure 3C-D). Moreover, genes altered by the glutamine treatment (FDR<0.2 HFD-glutamine versus HFD-PBS) displayed a negative correlation with their fold change in response to high-fat diet (HFD-PBS versus Chow-PBS), suggesting that glutamine treatment reversed the HFD-induced upregulation of gene expression (Figure 3F). Interestingly, a pathway enrichment analysis revealed that glutamine treatment decreased mRNA expression of genes annotated to pathways linked to the regulation of inflammation and extracellular matrix organization as compared to PBS-HFD and chow fed conditions (Figure 3E). Accordingly, gene expression analysis by qPCR showed that the expression of the inflammatory chemokine Ccl2 was decreased by glutamine treatment as compared to PBS-HFD fed mice, while Tnf (TNF-α) and Cxcl1 followed a similar pattern. Gene encoding macrophage surface markers such as Cd68, Adgre1 (F4/80) and Itgax (CD11c) were decreased in glutamine-treated HFD fed mice and were not significantly different from chow mice (Figure 3G). Collectively, our results provide evidence to suggest that the beneficial effects of glutamine on skeletal muscle metabolism may be conferred by downregulating inflammation and immune cell infiltration.
Skeletal muscle expression of the adaptor protein GRB10 is associated with plasma glutamine levels

We next compared genes altered by glutamine administration in HFD fed mice (p<0.05) with the genes altered in skeletal muscle of humans with high or low plasma glutamine levels (p<0.05) and identified a small subset of genes responding similarly in both species (Figure 4A, supp. table 1). Amongst this subset, we focused on GRB10, a gene encoding an adaptor protein that interacts with the insulin receptor, for its role in the development of insulin resistance in adipocytes and myotubes (21). Human GRB10 expression was lower in skeletal muscle of individuals with higher plasma glutamine levels and inversely correlated with plasma glutamine levels (Figure 4B-C). The same pattern was noted in mouse models, where Grb10 gene expression was increased in quadriceps of PBS-HFD fed mice and was decreased after glutamine administration (Figure 4D). Grb10 protein content followed a similar profile (Figure 4E). Grb10 expression also significantly correlated with HOMA-IR in mouse models, with a similar relationship observed in humans (Figure 4F). Gene silencing of GRB10 in cultured human skeletal muscle cells increased insulin-stimulated glucose uptake, as well as insulin-stimulated AKT phosphorylation (21), suggesting that the reduction in GRB10 protein may partly account for the enhanced insulin signaling in GLN-treated HFD fed mice. Since the gene ontology analysis revealed that the glutamine effect was primarily related to pathways controlling inflammation, we treated skeletal muscle cells with LPS for 24 h to determine whether there is an association between inflammation and GRB10 expression. As expected, LPS induced a strong increase in IL6 and CCL2 expression, however GRB10 mRNA was unchanged (Figure 4G-H-I). Furthermore, GRB10 silencing (Supplemental Figure 3E) did not affect the LPS-induced upregulation of IL6 and CCL2 expression (Figure 4J-K), suggesting these transcriptional events are regulated in an independent manner in response to glutamine.

Glutamine metabolism by glutaminase regulates IL6 and GRB10 transcription in myotubes

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To decipher the molecular mechanism by which glutamine regulates the skeletal muscle transcriptional profile and exerts a beneficial effect on metabolism, we differentiated primary human skeletal muscle cells during 8 days in presence of 0.5 mM (low) or 10 mM (high) glutamine (Figure 5A). Gene expression and secretion of the inflammatory cytokines *IL6* and *CCL2* was decreased in myotubes exposed to high glutamine (Figure 5B, C, Supplemental Figure 3F-G), indicating that glutamine directly affects the expression of inflammatory cytokines in skeletal muscle. In myotubes, glutamine can be used as a substrate for several cellular pathways, including the hexosamine pathway, the TCA cycle, and the glutathione system. Modulation of these metabolic pathways can ultimately regulate transcription factors activity (22). In order to identify potential pathways by which glutamine modulates gene expression, we incubated myotubes with an inhibitor of glutaminase 1 activity, BPTES. BPTES inhibits the conversion of glutamine to glutamate, which is the precursor for glutaminolysis and glutathione synthesis (Figure 5D). A 24 h BPTES exposure increased the expression of *IL6*, but not *CCL2* (Figure 5E-F). *GRB10* expression was decreased in response to high glutamine exposure (p=0.03) and upregulated after BPTES treatment (Figure 5G). Presumably, increased glutamine integration into glutathione could affect the cellular oxidative stress status and therefore the regulation of inflammatory cytokines. Therefore, we measured the expression of the antioxidant enzyme glutathione peroxidase 1 (*GPX1*) and of the transcription factor nuclear factor erythroid 2–related factor 2 (*NRF2*), which is activated in response to oxidative stress and regulates the expression of cytoprotective genes. *NRF2* mRNA level showed a trend to decrease in response to high glutamine (p=0.07) and was increased after BPTES treatment, while GPX1 show an opposite pattern (Figure 5H, Supplemental Figure 3H). Similarly, in differentiated myotubes exposed to a high glutamine medium for 48 h, we found decreased *CCL2* expression, a trend for reduced *GRB10* expression, and unchanged *IL6* expression (Supplemental Figure 3I-L). Collectively, our results suggest that modulation of extracellular...
glutamine level regulates \textit{GRB10} expression and inflammation in myotubes. Moreover, the effect of glutamine on transcriptional activity is partly mediated through glutamine metabolism by glutaminase and its role as a substrate in intermediary metabolism.
**Discussion**

Here we provide evidence that plasma glutamine levels are associated with insulin resistance and BMI in men. Moreover, glutamine administration directly improves high-fat diet-induced impairments in glucose homeostasis in a mouse model. Our data suggests that the beneficial effect of glutamine treatment is mediated through modulation of skeletal muscle immunometabolic profile, leading to decreased inflammation and improved insulin sensitivity (Figure 5I). Comparative analysis of the skeletal muscle transcriptomic profile in humans and mouse models identified that GRB10, an adaptor protein involved in insulin signaling, is associated with glutamine levels and insulin sensitivity. These glutamine-dependent changes in inflammatory cytokines and GRB10 expression require glutamine metabolism by glutaminase, the first enzyme in glutaminolysis.

Analysis of paired samples of plasma and *vastus lateralis* skeletal muscle from men with either normal glucose tolerance or type 2 diabetes allowed us to compare circulating and skeletal muscle metabolomic signatures of the same individual. With this comparative analysis we have earlier identified changes in plasma amino acids (15), and here reveal that low glutamine levels and high glutamate levels are associated with BMI and HOMA-IR. This is consistent with evidence that a high glutamine/glutamate ratio is associated with reduced risk of incident type 2 diabetes in a large-scale population cohort (23), and insulin sensitivity in obese patients (11). Of note, plasma glutamine levels were unaltered in the lean men with type 2 diabetes, possibly indicating that glutamine levels are implicated in the development of peripheral insulin resistance, whereas impaired glucose homeostasis in lean people with type 2 diabetes is often associated with defective insulin secretion (24; 25). We found that plasma glutamine, but not glutamate, correlated with skeletal muscle levels, in line with previous reports showing that skeletal muscle is the major contributor for circulating glutamine level
Thus, glutamine availability may be of importance in the pathogenesis of obesity-related type 2 diabetes.

Glutamine supplementation is beneficial for diseases associated with hypercatabolic state such as critical illness or sepsis (27). Glutamine administration is also beneficial during high intensity exercise, where oxidative stress and transient inflammation occurs, through increased levels of the antioxidant glutathione and an inhibition of the proinflammatory NF-κβ pathway (28). In metabolic diseases, oral glutamine supplementation induces weight loss and improves glucose metabolism in rodent models of HFD-induced obesity (23; 29; 30), and obese humans (31; 32). However, glutamine is primarily metabolized by the intestine when administrated orally (27), and part of its beneficial effect may be mediated through changes in the microbiome (33) and/or incretin release. In contrast, parenteral glutamine administration increases circulatory glutamine and glutamine availability for peripheral organs such as skeletal muscle (27; 34). In the present study, two-week glutamine treatment of HFD-fed mice improved glucose tolerance and insulin sensitivity, concomitant with enhanced EDL muscle insulin signaling and increased AKT activity. The insulin-sensitizing effect of glutamine appears to be lower in oxidative soleus muscle, which may be due to an intrinsic fiber-type difference in insulin signaling or metabolism (35). Other organs, such as adipose tissue could also contribute to the glutamine-associated improvements in whole-body glucose homeostasis. Conversely, glutamine administration did not affect body weight or body fat composition, suggesting that the improvements in insulin sensitivity are independent of adipose tissue loss. Using a shorter high fat diet exposure, we have shown that a similar 2-week glutamine treatment protocol resulted in an attenuation of fat mass gain as compared to the HFD fed control group, with reductions in adipocyte size, decreased immune cell infiltration and improved glucose metabolism (11). In the current study, the 2-week glutamine treatment was initiated after a longer period (15 weeks) of HFD, in severely obese mice. Thus, we cannot exclude the
possibility that a longer glutamine treatment, or an earlier intervention would have a more beneficial effect on adiposity and prevent obesity (11; 29).

Low grade inflammation during the early stages of obesity may be triggered by nutrient overload and metabolic dysfunction and thereby cause peripheral insulin resistance (5). Here we report that glutamine treatment improved the skeletal muscle expression profile of genes related to inflammatory responses as compared to HFD fed mice, with decreased expression of the cytokine \( \text{Tnf} \) and the chemokine \( \text{Ccl2} \). Accordingly, the expression of the monocyte marker \( \text{Cd68} \) and M1 macrophage marker \( \text{Cd11c} \) were lower in glutamine-treated HFD fed mice, suggesting a diminution in macrophage infiltration and/or activation in response to glutamine (36). In parallel, we show that glutamine treatment can directly regulate the expression and secretion of the cytokines IL6 and CCL2 in human myotubes. Interestingly, inhibition of glutaminase activity by BPTES profoundly increased \( \text{IL6} \) expression in myotubes, suggesting that glutamine catabolism is important for its anti-inflammatory effect. Glutamine conversion by glutaminase generates glutamate, which can undergo metabolism in the TCA cycle (glutaminolysis) or be used as a precursor for the synthesis of glutathione, an antioxidant. Thus, the modulation of \( \text{NRF2} \) expression in response to glutamine suggests that lower oxidative stress level may account for the decrease in cytokine expression in high glutamine condition. Conversely, CCL2 expression, despite significant downregulation after a 48h exposure of cultured cells to high glutamine, is not increased after inhibition of glutaminase activity. This suggests a differential temporal and mechanistic glutamine-dependent regulation of IL6 and CCL2 expression in myocytes, consistent with earlier report in isolated adipocytes where IL6, but not CCL2 was regulated by glutamine-induced O-GlcNAcylation (11). Collectively, our data indicate that increased glutamine availability may modulate cytokine secretion by myotubes and thereby reduce immune cell recruitment or pro-inflammatory activity. Of clinical
relevance, dietary glutamine supplementation may potentially delay the onset of insulin resistance by modulating skeletal muscle metabolism towards an anti-inflammatory phenotype.

Glutamine levels can influence the inflammatory response of skeletal muscle and regulate the expression of multiple transcripts. In the current study, a limited number of transcripts were similarly altered in skeletal muscle following stratification by glutamine exposure in humans and mice. This could reflect both the cohort size and interspecies differences in muscle transcriptome (37; 38). Therefore, the identified genes are likely “glutamine-responsive genes” that are conserved across species. Interestingly, we noted that \textit{GRB10}, an adaptor protein that interacts with tyrosine kinase receptors, was downregulated in skeletal muscle of both humans and mice in conjunction with elevated glutamine levels. Furthermore, \textit{Grb10} mRNA correlates with HOMA-IR in mice, with a similar profile observed in humans. A GWAS has identified the \textit{GRB10} locus associated with type 2 diabetes in an Amish population (39). Of functional relevance, disruption of \textit{GRB10} in peripheral tissues improves insulin sensitivity in skeletal muscle and adipose tissue (40-42). Moreover, downregulation of \textit{GRB10} mRNA in human skeletal muscle cells increases insulin-induced PI3K/AKT signaling and glucose uptake (21), which is consistent with the increased AKT activity detected in the EDL muscle of glutamine-treated HFD fed mice. \textit{GRB10} can also be upregulated by mTORC1 activation (43). In our study, LPS treatment of primary human myotubes, which activates the mTORC pathway (44), did not increase \textit{GRB10}, suggesting that a different mechanism is involved. In skeletal muscle cells, high glutamine treatment downregulated \textit{GRB10} expression, and BPTES inhibition of glutaminase 1 increased \textit{GRB10} levels, indicating that \textit{GRB10} expression is regulated through glutamine metabolism by glutaminase. Thus, transcription factors sensitive to metabolites such as CTCF (regulated by alpha ketoglutarate, an intracellular metabolite of glutamine) are potential candidates for glutamine dependent \textit{GRB10} regulation (45; 46). While we cannot exclude the possibility that
changes in glutaminase activity increase GRB10 levels in skeletal muscle from men with type 2 diabetes, glutamine supplementation of myotube cultures and HFD-fed mice were associated with reductions in GRB10 expression in skeletal muscle. Therefore, glutamine modulation of GRB10 could represent a potential target to improve skeletal muscle insulin sensitivity in type 2 diabetes. Nevertheless, further validation by proteomic-based approaches may elucidate the link between glutamine and GRB10 regulation. Additional studies in both men and women may also clarify whether there is a sex-dependent effect of glutamine treatment on insulin sensitivity and inflammation.

In conclusion, we show that lower plasma glutamine is associated with high BMI and HOMA-IR index in men. Moreover, we provide evidence for a direct role of glutamine in the prevention of inflammation and insulin resistance in skeletal muscle. Dietary glutamine supplementation could represent a potential therapeutic strategy to prevent or delay skeletal muscle insulin resistance by maintaining the homeostatic control of immunometabolism and through the regulation of GRB10 expression.

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Declaration of Interests

J.H. is employee of Daiichi Sankyo Co., Ltd.

Contributions

LD and AK conceived the idea, planned the experiments, collected, and analyzed data and wrote the manuscript. NJP performed bioinformatic analysis. AMA collected cell data. MK, EC, DRR, LP and ED collected mouse data and analyzed tissue samples. H.K.K. assisted with recruitment of subjects and collection of human metabolite data. NJP, AMA, EC, HKK MB, JT and JH contributed to the scientific discussion. EN obtained human skeletal muscle biopsies and blood samples. JRZ, MR and A.K. supervised the study, reviewed, and edited the manuscript and acquired funding. All authors edited and reviewed the manuscript. AK is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
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| Clinical parameters | Lean NGT | Lean T2D | Overweight NGT | Overweight T2D | OWE effect | T2D effect |
|---------------------|----------|----------|----------------|----------------|-------------|------------|
| n size              | 7        | 6        | 7              | 13             | -           | -          |
| Age                 | 57.3 ± 9.5 | 65.3 ± 2.7 | 61.4 ± 10.2 | 63.6 ± 6.1 | p=0.721     | p=0.097    |
| BMI [kg/m2]         | 24.1 ± 0.58 | 24.6 ± 0.36 | 27.2 ± 1.65 | 27.6 ± 1.2 | p<0.001 *** | p=0.321    |
| W/H ratio           | 0.91 ± 0.04 | 0.98 ± 0.06 | 0.94 ± 0.05 | 0.99 ± 0.06 | p=0.296     | p=0.007*   |
| fP-glucose [mmol/L] | 5.27 ± 0.4 | 8.42 ± 2.28 | 5.36 ± 0.32 | 8.77 ± 1.76 | p=0.679     | p<0.001 ** |
| S-insulin [pmol/L]  | 40.1 ± 15.4 | 44.2 ± 17  | 64.8 ± 17.8 | 85.9 ± 37.5 | p=0.002 **  | p=0.193    |
| HbA1c [mmol/mol]    | 34 ± 5.32  | 51 ± 6.13  | 38.1 ± 2.54  | 51.9 ± 6.42  | 0.241       | p<0.001 ** |
| HOMA-IR             | 1.61 ± 0.5 | 3.03 ± 1.48 | 2.27 ± 0.73 | 5.51 ± 2.92 | p=0.033 *   | p=0.002*   |
| S-C-peptide [nmol/L]| 0.59 ± 0.12 | 0.69 ± 0.15 | 0.72 ± 0.131 | 0.99 ± 0.40 | p=0.035 *   | p=0.057    |
| P-TG [mmol/L]       | 0.89 ± 0.33 | 1.19 ± 0.44 | 1.21 ± 0.71  | 1.47 ± 0.78  | p=0.212     | p=0.240    |
| P-Chol [mmol/L]     | 4.94 ± 0.53 | 4.35 ± 0.85 | 5.56 ± 0.67  | 4.65 ± 0.76  | p=0.099     | p<0.005*   |
| P-HDL [mmol/L]      | 1.24 ± 0.13 | 1.42 ± 0.21 | 1.36 ± 0.26  | 1.21 ± 0.312 | p=0.523     | p=0.869    |
| P-LDL [mmol/L]      | 3.3 ± 0.54  | 2.43 ± 0.76  | 3.67 ± 0.44  | 2.77 ± 0.83  | p=0.174     | p=0.001*   |

Table 1 Clinical characteristics of the study participants.

The cohort is comprised of 7 lean NGT subjects, 6 lean subjects with type 2 diabetes, 7 overweight NGT subjects and 13 overweight subjects with type 2 diabetes (overweight: BMI ≥ 25). Effect of BMI (OVE) and type 2 diabetes (T2D) were measured by 2-way ANOVA. Results are mean ± SEM. No interactions were significant for the parameters displayed. *p<0.05; **p<0.01; ***p<0.001.
Figure Legends

**Figure 1: Plasma glutamine levels are associated with BMI and HOMA-IR.** Volcano plots of metabolites in plasma and skeletal muscle correlating with BMI and HOMA-IR. Spearman correlation and Kruskal-Wallis comparison across groups. Blue color indicates significance at p<0.05 (A-D). Plasma glutamine and glutamate levels. Kruskal-Wallis test with Dunn’s multiple comparison test (E-F). Skeletal muscle glutamine and glutamate levels. Kruskal-Wallis test (G-H). Spearman correlation of glutamine and glutamate in plasma and skeletal muscle (I). BMI of individuals ranked based on plasma glutamine quartiles. Ordinary one-way ANOVA with uncorrected Fisher’s LSD comparison (J). HOMA-IR of individuals ranked based on plasma glutamine quartiles. Kruskal-Wallis test with uncorrected Dunn’s test (K). n=9; 8; 8; 7 per quartiles.

**Figure 2: Glutamine administration improves glucose homeostasis in high-fat diet fed mice.** Experimental plan (A). Final body weight (D15) expressed in grams (B). Total body fat percentage measured by MRI (C). Glycemia, plasma insulin level and calculated HOMA-IR after 4h fasting (n=6, 8, 8) (D-E-F). Statistical difference was determined using a one-way ANOVA or Kruskal-Wallis. Glucose tolerance test and 150’ AUC (n=8, 12, 12) (G-H). Insulin tolerance test and glucose disappearance rate KITT (n=8, 9, 9) (I-J). For both tests, statistical differences were determined using a 2-way ANOVA and unpaired t-test between the HFD groups. ☐: group effect; # time effect. Dashed lines represent the Chow-PBS group response. Akt phosphorylation (P-AKT) and AS160 phosphorylation (P-AS160) in lysates of EDL skeletal muscle incubated in the absence or presence of a submaximal dose of insulin (0.36 nmol/L) for 20 min (K-L-M). Data are mean ± SEM in figure G-I. *p<0.05, **p<0.01, ***p<0.001. Statistical effect was measured using 2-way ANOVA for figures K-M. $: insulin effect; ☐: group effect; &: interaction effect.
Figure 3: Glutamine suppresses gene transcripts annotated to inflammation-related pathways. Changes in gene expression between HFD-PBS and CD-PBS (A), HFD-GLN and CD-PBS (B), and HFD-GLN and HFD-PBS (C) conditions are represented in Volcano Plots. Colored dots represent genes significantly altered between conditions (FDR<0.2). Significantly regulated genes (FDR<0.2) overlapped in a Venn-Diagram (D). Gene ontology analysis performed based on genes with FDR<0.2 showing downregulated and upregulated pathways between conditions (E). Spearman correlation between the logarithm of fold change of the genes altered between HFD-GLN and HFD-PBS conditions (“Glutamine”, y axis), and fold change between HFD-PBS and CD-PBS conditions (“HFD”, x axis) (F). Gene expression analysis of cytokines and immune cells markers in quadriceps muscle as measured by qPCR (G). Data are mean ± SEM. *p<0.05. Statistical effect has been measured using one-way ANOVA or Kruskal-Wallis for figure G. n=8 mice per group.

Figure 4: Skeletal muscle expression of the adaptor protein GRB10 is associated with plasma glutamine levels. Correlation between the subset of genes altered between both skeletal muscle of subjects with high glutamine (Q4) versus low glutamine (Q1), and skeletal muscle of glutamine-treated versus PBS HFD fed mice (p<0.05) (A). Correlation between plasma glutamine level and human skeletal muscle expression of GRB10 (B). n= 27. GRB10 expression level measured by gene array in human skeletal muscle, per quartiles of plasma glutamine concentration (C). mRNA and protein level of GRB10 in quadriceps of mice, Kruskal-Wallis test with Dunn’s multiple comparison test (D-E). n=8. Spearman correlation between GRB10 gene expression and HOMA-IR in mice and humans (F). CCL2, IL6 and GRB10 mRNA level in skeletal muscle cells after 24h treatment with 100 ng/mL LPS (G-H-I). CCL2 and IL6 mRNA level in response to LPS after transfection with scramble siRNA (scr) or siRNA targeting GRB10 (siGRB10) (J-K). The dashed line represents the expression in scramble control condition. Paired Student’s t-test. n=5. *p<0.05, **p<0.01, ***p<0.001.
Figure 5: Glutamine metabolism by glutaminase regulates IL6 and GRB10 transcription in myotubes. Skeletal muscle cells were differentiated during 8 days in myotubes in low (0.5 mM) or high (10 mM) concentration of glutamine (A), and *IL6* (B) and *CCL2* (C) mRNA level were measured. N=6. BPTES is an inhibitor of glutaminase (GLS1) that converts glutamine to glutamate (D). BPTES was added for 24h and *IL6* (E), *CCL2* (F), *GRB10* (G) and *NRF2* (H) mRNA level were measured. n=7. Statistical effect has been measured using paired Students *t*-test or Wilcoxon test. *p<0.05, **p<0.01, ***p<0.001: difference between BPTES treatment and untreated. □ p<0.05, □□p<0.01, □□□p<0.001: difference between high glutamine and low glutamine condition (showed by the dashed line). Graphical abstract (I).
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190x169mm (200 x 200 DPI)
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Glutamine Regulates Skeletal Muscle Immunometabolism in Type 2 Diabetes

Lucile Dollet¹, Michael Kuefner¹, Elena Caria¹, David Rizo-Roca², Logan Pendergrast², Ahmed M. Abdelmoez², Håkan KR Karlsson², Emilie Dalbram³, Jonas Treebak³, Jun Harada⁴, Erik Näslund⁵, Mikael Rydén⁶, Juleen R. Zierath¹,², Nicolas J. Pillon¹, Anna Krook¹.

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| Mouse gene | Ensembl reference | Human gene | Ensembl ref | mouse_logFC | mouse_P.Value | human_logFC | human_P.Value |
|------------|------------------|------------|-------------|-------------|---------------|-------------|---------------|
| Defa22     | ENSMUSG000000774443 | DEF5       | ENSG00000164816 | 0.092528    | 0.027769      | 0.140774    | 0.01513       |
| Defa28     | ENSMUSG000000774434 | DEF5       | ENSG00000164816 | -0.15117    | 0.016915      | 0.140774    | 0.01513       |
| Defa40     | ENSMUSG000000774441 | DEF5       | ENSG00000164816 | 0.084919    | 0.044609      | 0.140774    | 0.01513       |
| Fndc1      | ENSMUSG00000071984 | FNDC1      | ENSG00000164694 | -0.17304    | 0.011134      | -0.16488    | 0.022891      |
| Grb10      | ENSMUSG00000020176 | GRB10      | ENSG00000106070 | -0.14901    | 0.018904      | -0.15099    | 0.022287      |
| Hba-a1     | ENSMUSG00000069919 | HBA1       | ENSG00000206172 | -1.03272    | 0.000178      | -1.13577    | 0.000698      |
| Hba-a1     | ENSMUSG00000069919 | HBA2       | ENSG00000188536 | -1.03272    | 0.000178      | -1.27445    | 0.000917      |
| Mrgrpb2    | ENSMUSG00000050425 | MRGPRX4    | ENSG00000179817 | 0.112245    | 0.036967      | 0.217329    | 0.048118      |
| Ptx4       | ENSMUSG000000444172 | PTX4       | ENSG00000251692 | -0.22896    | 0.000259      | -0.20994    | 0.018986      |
| Rsad2      | ENSMUSG00000020641 | RSAD2      | ENSG00000134321 | -0.26204    | 0.00066       | -0.12742    | 0.02321       |
| Snca       | ENSMUSG00000025889 | SNCA       | ENSG00000145335 | -0.18844    | 0.005769      | -0.17318    | 0.009612      |
| Tas2r120   | ENSMUSG00000059382 | TAS2R31    | ENSG00000256436 | 0.087108    | 0.016376      | -0.19283    | 0.00415       |
| Tas2r120   | ENSMUSG00000059382 | TAS2R19    | ENSG00000212124 | 0.087108    | 0.016376      | 0.32795     | 0.005263      |
| Zfp992     | ENSMUSG00000070605 | ZNF34      | ENSG00000196378 | -0.08283    | 0.031277      | 0.10602     | 0.048793      |

**Supplemental Table 1:** List of the genes commonly altered by glutamine administration in HFD fed mice (p<0.05) and altered in skeletal muscle of humans with high or low plasma glutamine levels (p<0.05). Log fold-change and adjusted p-value are indicated for both mice and human dataset.
Supplemental figure 1

A

Assessed for eligibility (n=50)

Clinical parameters outside healthy range (n=1)

Do not meet inclusion criteria (impaired glucose tolerance, blood glucose >7.8mM, <11.1mM after OGTT) (n=9)

BMI >30 (n=7)

Normal Glucose Tolerant: n=14
Type 2 Diabetic: n=19

B

Plasma

\[ \text{WHR correlation, Spearman } r \]

C

Muscle

\[ \text{WHR correlation, Spearman } r \]

D

Plasma

\[ \text{WHR correlation, Spearman } r \]

E

Plasma

\[ \text{HOMA-IR correlation, Spearman } r \]

F

Muscle

\[ \text{BMI correlation, Spearman } r \]

G

Muscle

\[ \text{HOMA-IR correlation, Spearman } r \]

H

Plasma

\[ \text{Glutamine, a.u.} \]

I

Plasma

\[ \text{Glutamine, a.u.} \]

J

Muscle

\[ \text{Glutamine, a.u.} \]

K

Muscle

\[ \text{Glutamine, a.u.} \]
Supplemental Figure legends:

Supplemental Figure 1: Details of the cohort recruitment and criteria for inclusion/exclusion (A). Volcano plots of metabolites in plasma (B) and skeletal muscle (C) correlating with waist/hip ratio (WHR). Spearman correlation and Wilcoxon comparison across groups. Blue color indicates significance at p<0.05. Volcano plots of metabolites in plasma and skeletal muscle correlating with BMI and HOMA-IR (D-G). Spearman correlation and Wilcoxon comparison between NGT (lean+overweight) and T2D (lean+overweight) groups. Blue color indicates significance at p<0.05 (D-G). Plasma glutamine and glutamate levels (H-I) and skeletal muscle glutamine and glutamate levels (J-K). Mann-Whitney test between NGT and T2D groups.
Supplemental figure 2
Supplemental Figure 2: Correlations between clinical parameters and plasma (blue) and muscle (red) glutamine level. *p<0.05 for plasma, □p<0.05 for muscle (A). GLUL (B) and GLS (C) gene expression level in human skeletal muscle. n= 27.
Supplemental figure 3
**Supplemental Figure 3:**

Fold-change in Akt phosphorylation (P-AKT) and AS160 phosphorylation (P-AS160) in lysates of soleus skeletal muscle incubated in the absence or presence of a submaximal dose of insulin (0.36 nmol/L) for 20 min (A-D). Data are mean ± SEM. Kruskal-Wallis test with Dunn’s multiple comparison test. GRB10 mRNA expression level in myotubes 48h after transfection using either a scramble siRNA (scr) or a siRNA targeting GRB10 (siGRB10) (E). n=5. IL6 and CCL2 content in the medium collected after 24h from skeletal muscle cells differentiated during 8 days in low (0.5 mM) or high (10 mM) (F-G). n=5. BPTES was added for 24h and GPX1 (H) mRNA level were measured. n=7. Statistical effect was measured by paired Student’s t-test. *p<0.05; **p<0.01; ***p<0.001. Skeletal muscle cells were differentiated during 7 days in myotubes in classical 2mM glutamine medium, and then exposed to low (0.5 mM) or high (10 mM) concentration of glutamine during 48h (I), and CCL2 (J), GRB10 (K) and IL6 (L) mRNA level were measured. N=7. Statistical effect was measured by paired Student’s t-test. *p<0.05; **p<0.01; ***p<0.001.