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RESEARCH ARTICLE

Amino acids stimulate Akt phosphorylation, and reduce IL-8 production and NF-κB activity in HepG2 liver cells

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Hepatic insulin resistance and inflammatory cytokine production contribute to the manifestation of the metabolic syndrome. As amino acids have been implicated in modulating insulin signaling and inflammation, we have investigated the effects of glutamine, leucine and proline on markers of inflammation and insulin sensitivity in HepG2 liver cells. Cells were incubated with IL-1β (5 ng/mL) to stimulate IL-8 production. After 24 h, glutamine inhibited IL-8 production significantly ($p<0.05$) at 2, 5 and 10 mM (to 82, 73 and 72% of control), whereas leucine reduced IL-8 production significantly only at 10 mM (66%) and proline at 5 and 10 mM (71 and 52%). Glutamine, leucine and proline all reduced NF-κB activity after 3 h of IL-1β stimulation at 2, 5 and 10 mM ($p<0.001$). Insulin-induced (1 nM) Akt phosphorylation was reduced in cells treated with tumour necrosis factor-α (10 ng/mL) for 24 h, but was partly restored by simultaneous incubation with glutamine, leucine and proline (25 mM). Phosphorylation of glycogen synthase kinase-3β was unaffected by insulin stimulation and amino acid treatment. Our results indicate that glutamine, leucine and proline attenuate IL-8 production, probably through inhibition of NF-κB, and that they increase Akt phosphorylation in HepG2 cells.

Keywords:
Amino acids / Cytokine / Insulin sensitivity / Liver / NF-κB

1 Introduction

The metabolic syndrome covers numerous factors that enhance the risk for cardiovascular disease and type 2 diabetes mellitus. These risk factors, including dyslipidemia, insulin resistance, hypertension, a prothrombotic state and chronic inflammation, result from a complex interplay between multiple tissues in the body [1]. Of these tissues, the liver is one of the most important organs involved, because of its central role in lipid and glucose homeostasis. In fact, a strong correlation exists between obesity and nonalcoholic fatty liver disease, which is considered to be a hepatic manifestation of the metabolic syndrome [2]. An increased flux of plasma free fatty acids and elevated levels of inflammatory cytokines, both often the result of an enlarged fat mass, may cause hepatic insulin resistance and subsequent disturbances in hepatic fat and glucose handling. In addition, the inflammatory response of the liver itself is often enhanced in metabolic diseases and results in increased production of pro-inflammatory cytokines and acute phase proteins [3].

Diet is one of the environmental factors that play a part in the development of these metabolic disturbances. Amino acids are one group of nutrients that may be of interest in their prevention and treatment. Amino acids, as provided by proteins, are an important component of the Western diet and form 10–15 energy percent of the total food intake. They are highly abundant in dairy, meat and other high-protein foods. Several amino acids have been reported to modulate

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Abbreviations: FBS, fetal bovine serum; **GSK-3β,** glycogen synthase kinase-3β; **MEM,** minimal essential medium; **NEAA,** nonessential amino acids; **NF-κB,** nuclear factor-κB; **TNFα,** tumour necrosis factor-α

the inflammatory response. For example, glutamine decreased the release of the proinflammatory cytokines IL-8 and IL-6 in human intestinal cells *in vitro* [4–6]. Another amino acid, histidine, was also found to inhibit the production of IL-8 in intestinal epithelial cells [7]. In addition, some studies indicated that amino acids might modulate insulin action in isolated adipocytes [8, 9]. In rat heart tissue, amino acid supplementation was found to upregulate glucose transporter GLUT-4 [10]. Branched chain amino acids were reported to increase glucose uptake in skeletal muscle and hepatocytes in rodents [11, 12]. Moreover, branched chain amino acid-enriched supplements have been found to improve insulin resistance in patients with chronic liver disease [13]. These findings may implicate a role for amino acids in the modulation of insulin resistance as well. Furthermore, dairy products, which are a rich source of proteins, have been associated with a lower prevalence or incidence of the metabolic syndrome and its components [14–18]. It is possible that the amino acids derived from dairy proteins are (partly) responsible for any possible positive effects of dairy products on metabolic abnormalities.

In this study, we therefore investigated the effects of glutamine, leucine and proline, the most abundant amino acids in dairy products [19], on markers reflecting inflammation and insulin signaling in a liver cell model.

2 Materials and methods

2.1 Cell culture

The liver cell line HepG2 was kindly provided by Dr. S. Braesch-Andersen (Mabtech, Nacka Strand, Sweden). Cells were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum (FBS), 1% sodium pyruvate, 1% L-glutamine, 1% nonessential amino acids (NEAA) and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The cells were grown under standard culturing conditions at 37°C in a 5% CO_2 humidified atmosphere.

2.2 Cytokine production assay

Cells were seeded in 24-well plates (2×10^5 cells/well). After 32 h, the medium was replaced by MEM without FBS, L-glutamine and NEAA, and the cells were maintained on this medium for 16 h before the start of the experiments. To stimulate cytokine (tumour necrosis factor-α (TNFα), IL-6 and IL-8) production, the cells were incubated for 24 h with IL-1β (5 ng/mL) (Sigma-Aldrich, St. Louis, MO, USA). In a pilot experiment, IL-1β was found to be a stronger inducer of IL-8 production than IL-6 and TNFα in these cells. Simultaneously, the amino acids glutamine, leucine and proline (Sigma-Aldrich) were added to the media to final concentrations of 2, 5 and 10 mM. For all experiments, amino acids were dissolved in MEM. Glucose, also dissolved in MEM, was added at the same concentrations to control for energy effects. After 24 h, supernatants were collected and stored at −80°C for further analysis. During experiments, appearance of the cells was checked microscopically. The protein content of the samples was determined using the BCA-method according to the manufacturer's manual (Pierce Biotechnology, Rockford, IL, USA).

The levels of cytokines secreted were measured using ELISA (Hycult Biotechnology, Uden, The Netherlands). Briefly, 96-well plates were coated with monoclonal murine anti-human TNFα/IL-6/IL-8 antibodies. Recombinant human cytokines were used for standard curves. Immobilized cytokines were detected using specific biotinylated rabbit anti-human TNFα/IL-6/IL-8 polyclonal antibodies, followed by addition of peroxidase-conjugated streptavidin and tetramethylbenzidine substrate. Absorption was measured at 450 nm.

2.3 NF-κB activity assay

Cells were seeded in 24-wells plates (2×10^5 cells/well) and grown for 24 h. Transient transfections were performed using FuGENE® HD Transfection Reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. The NF-κB-tk-luciferase vector was kindly provided by Dr. David Gius (National Cancer Institute, Bethesda, MA, USA) [20] and was used as a reporter for the activity of nuclear factor-κB (NF-κB). Cells were incubated with the FuGENE®-DNA mix for 6 h (0.1 μg pcDNA+0.1 μg NF-κB vector *per* well).

Cells were placed on media without FBS, L-glutamine and NEAA for 16 h before start of the experiments. Cells were pre-incubated with glutamine, proline or leucine (2, 5 and 10 mM) for 24 h. Glucose was added at the same concentrations to control for energy effects. To induce NF-κB activation, IL-1β (5 ng/mL) was added to the media. After 3 h incubation, cells were placed on ice, supernatants were removed and cells were lysed with Reporter Lysis Buffer (Promega, Madison, WI, USA). Lysates were stored at −80°C. NF-κB transcriptional activity was measured using a luminometer (Gomex, 96 microplate luminometer, Promega) and luciferase assay substrate (Promega) according to the user manual.

2.4 Akt and glycogen synthase kinase-3β phosphorylation assay

Cells were seeded in 6-well plates (5×10^5 cells/well). After 32 h, the medium was replaced by MEM without FBS, L-glutamine and NEAA, and the cells were maintained on this medium for 16 h before the start of the experiments. To evaluate the effect of amino acids on phosphorylation of Akt,

a key protein involved in the signaling pathway following insulin stimulation, we first incubated the cells with TNFα (10 ng/mL) for 24 h. Prolonged exposure to TNFα down-regulates IRS-1 and prevents the occurrence of the insulin signaling cascade, including phosphorylation of Akt, in HepG2 cells, creating a situation reflecting insulin resistance [21]. Cells were incubated simultaneously with or without 25 mM of glutamine, leucine and proline. Glucose was added at the same concentration to control for energy effects. The cells were then exposed to 1 nM of insulin for 5 min to induce Akt phosphorylation. Cells were placed on ice, supernatants were removed and cells were washed twice with ice-cold PBS. Lysates of the cells were obtained by adding 200 μL cold lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 1% Nonidet, 1 mM DTT, 1 mM NaVanadate, 1 mM PMSF, 10 μg/mL leupeptin, 1% aproptenin). After incubation for 30 min on ice, cells were scraped and lysates were cleared by centrifugation and mixed with SDS-PAGE sample buffer (125 mM Tris (pH 6.8), 4% SDS, 20% glycerol, 200 mM DTT, 0.02% bromophenol blue), heated for 5 min at 95°C and stored at −20°C.

Western blotting was used to visualize the amount of Akt, phosphorylated Akt (P-Akt), glycogen synthase kinase-3β (GSK-3β), phosphorylated GSK-3β (P-GSK-3β) and β-actin (loading control for equal protein loading) in the lysates. GSK-3β, an enzyme that inhibits glycogen synthesis, is inhibited by insulin and is a downstream target of P-Akt. Routinely, 30 μg of protein sample was electrophoresed through 10% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes at 100 mA overnight at 4°C and visualized by Ponceau S reagent. The blots were blocked with 5% nonfat dry milk/0.05% TBS-Tween-20 for 1 h at room temperature. The blots were probed with rabbit anti-Akt, anti-P-Akt, anti-GSK-3β, anti-P-GSK-3β and anti-β-actin antibodies (Cell Signaling Technology, Boston, USA) and visualization was performed using chemiluminiscence reagent (Pierce Biotechnology).

2.5 Statistics

All statistical analyses were performed using SPSS 16.0 for Macintosh OS X (SPSS, Chicago, IL, USA). Data were evaluated using univariate analysis of variance, adjusted for multiple comparisons using the Bonferroni's *post-hoc* test.

3 Results

3.1 Effect of amino acids on IL-1β-induced cytokine production

After stimulation with IL-1β, the concentrations of TNFα and IL-6 were below the detection limit (60 and 40 pg/mL, respectively) and effects of the amino acids on the production of these cytokines could therefore not be evaluated (data not shown). Stimulation with IL-1β increased IL-8 production almost 30-fold after 24 h of stimulation ($p < 0.001$). After the experiment, cells' appearance had not changed, as checked microscopically. The protein content was not different between the various conditions. IL-8 production is given as percentage of control. As shown in Fig. 1, incubation with 2, 5 and 10 mM glutamine reduced IL-8 production significantly to 82, 73 and 72% of control levels ($p = 0.009$, 0.001 and 0.001), respectively. IL-8 production was unaffected by incubation with 2 and 5 mM of leucine (110%, $p = 0.125$ and 92%, $p = 0.232$ respectively), although it was significantly reduced by the addition of 10 mM of leucine (66%, $p = 0.001$). Proline did not affect IL-8 production at the 2 mM condition (99%, $p = 0.909$), but inhibited production of IL-8 to 71 and 52% at 5 and 10 mM ($p = 0.001$ and < 0.001), respectively. The addition of 2, 5 and 10 mM glucose did not alter IL-8 production (106, 103 and 104%, respectively, $p = 1.00$).

3.2 Effect of amino acids on IL-1β-induced NF-κB activity

Stimulation of the cells with IL-1β increased NF-κB promoter-induced luciferase production, as a marker of NF-κB activity, approximately sevenfold ($p < 0.001$). Effects of the amino acid incubations on luciferase expression from the NF-κB promoter reporter construct are shown in Fig. 2. The addition of glutamine reduced IL-1β-induced luciferase expression to 75, 76 and 66% of control at 2, 5 and 10 mM, respectively ($p < 0.001$). Luciferase expression was decreased to 89, 87 and 78% by 2, 5 and 10 mM of leucine and to 83, 71 and 57% by 2, 5 and 10 mM of proline (all $p < 0.001$). Incubation with glucose had no effect on NF-κB promoter-driven luciferase expression (96% ($p = 0.395$), 98% ($p = 0.706$) and 92% ($p = 0.133$) at 2, 5 and 10 mM, respectively).

3.3 Effect of amino acids on Akt phosphorylation and GSK-3β phosphorylation

Glutamine preincubation for 24 h increased basal Akt phosphorylation at 25 mM (Fig. 3A). In cells treated with

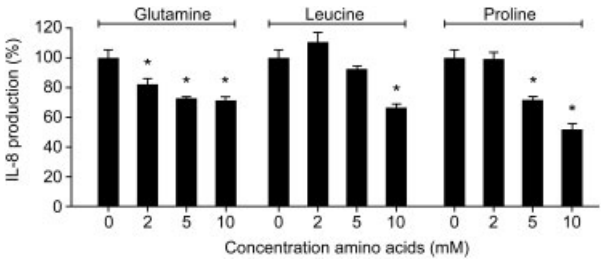

Figure 1. Effect of amino acids on IL-8 production in HepG2 cells. Cells were treated with IL-1β (5 ng/mL) and the amino acids glutamine, leucine and proline for 24 h. Values are presented as mean ± SD ($n = 6$). *Statistically different from control ($p < 0.05$).

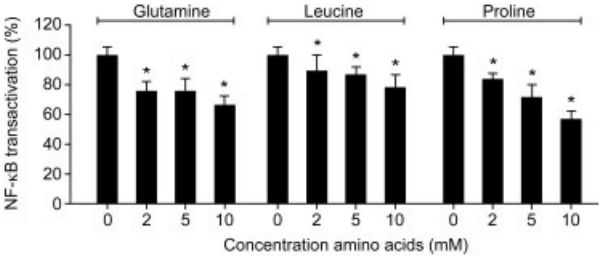

Figure 2. Effect of amino acids on NFκB activity, as measured using a NF-κB promoter driven luciferase construct, in HepG2 cells after stimulation with IL-1β (5 ng/mL) for 3 h. Values are expressed as percentage of control and are presented as mean ± SD ($n = 12$). *Statistically different from control ($p < 0.05$).

both TNFα and glutamine, Akt phosphorylation was higher than in cells treated with TNFα alone, but lower than in cells treated with only glutamine. Preincubation with leucine and proline did not change basal Akt phosphorylation (Figs. 3B and C).

As expected, insulin treatment (1 nM) for 5 min increased Akt phosphorylation. Preincubation with TNFα for 24 h reduced insulin-stimulated phosphorylation of Akt. All three amino acids were able to (partly) restore the TNFα-decreased Akt phosphorylation to the level of Akt phosphorylation in the insulin-stimulated condition without TNFα. The addition of glucose had no effect on Akt phosphorylation in the absence or presence of insulin and TNFα.

Levels of phosphorylated GSK-3β were not changed after insulin stimulation, nor were they affected by amino acid treatment.

4 Discussion

In this study, we showed that the amino acids glutamine, leucine and proline may reduce IL-1β-stimulated inflammation, probably through inhibition of NF-κB, and that they might be able to modulate insulin signaling in the HepG2 liver cell model.

To investigate the effects of these amino acids on inflammation, we studied the production of IL-8. IL-8 is a major pro-inflammatory cytokine, whose main function is the attraction of leukocytes to the site of infection. IL-8 and other cytokines, such as IL-1, IL-6 and TNFα, have been found to be elevated in obese subjects [22–24]. In nonalcoholic fatty liver disease patients, circulating levels of IL-8 were increased and correlated with TNFα and IL-6 concentrations [25]. Moreover, IL-8 has been associated with the development of atherosclerosis and cardiovascular disease [26]. Adipose tissue is partly responsible for the increased production of these cytokines, but another part of this cytokine production originates from an increased inflammatory response of the liver. We showed that simultaneous incubation of HepG2 cells with amino acids and IL-1β attenuates the production of IL-8 induced by IL-1β. These

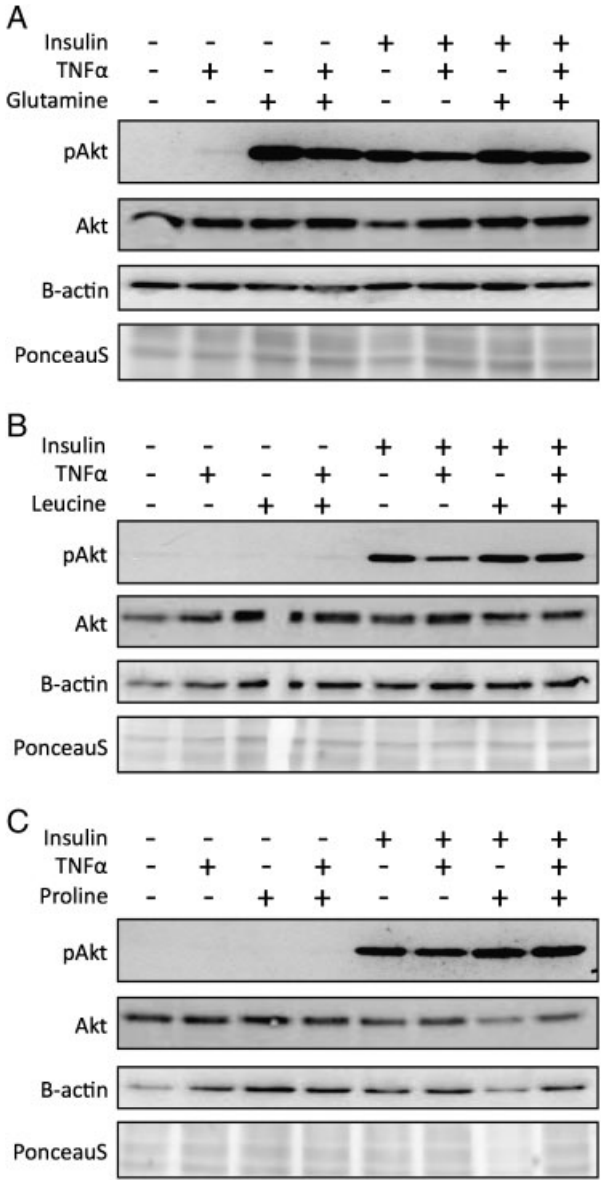

Figure 3. Representative image of the effects of amino acids on Akt phosphorylation in HepG2 cells. Cells were pretreated with glutamine (A), leucine (B) or proline (C) in the absence or presence of TNFα (10 ng/mL) for 24 h. Cells were then stimulated with insulin (1 nM) for 5 min.

observations are in agreement with experiments in human intestinal cells, in which several amino acids have also been described to decrease IL-8 production under basal conditions and after stimulation with cytokine mixtures or hydrogen peroxide [4, 7]. Here, we show this inflammation attenuating effect for the first time, to our knowledge, in a liver cell model.

The production of many pro-inflammatory cytokines, including IL-8, is mainly under control of the transcription factor nuclear factor κB (NF-κB) [27]. Following a pro-inflammatory stimulus, a signaling cascade is initiated

resulting in the translocation of NF-κB into the nucleus, where it regulates the transcription of its target genes. In our experiments we found that glutamine, leucine and proline are all able to significantly reduce the activity of NF-κB induced by IL-1β, as measured using a luciferase expression construct driven by the NF-κB promoter. Our results therefore suggest that the inhibition of IL-8 production by these amino acids is mediated through the inhibition of NF-κB transcriptional activity. Hubert-Buron *et al.* [6], who found a reduction in IL-8 production in human intestinal epithelial cells after glutamine pretreatment, reported a concomitant decrease in ubiquitination of IκBα, the cellular inhibitor of NF-κB. This suggests that amino acids may reduce cytokine production by limiting IκB ubiquitination. However, the exact mechanism behind these effects remains to be unravelled. In summary, our results suggest that it might be of interest to further explore the physiological effects of amino acids and proteins on inflammatory markers.

Furthermore, we studied the effects of amino acids on insulin signaling. Hereto, the cells were incubated with TNFα to induce a state of insulin resistance, as TNFα has been shown to downregulate IRS-1 and subsequently inhibit the insulin signaling cascade in HepG2 cells [28]. In our experiments, TNFα indeed reduced insulin-stimulated Akt phosphorylation. Addition of glutamine partly abolished this TNFα-induced inhibition. Leucine and proline showed similar effects. These results may imply that these amino acids could (partly) diminish the insulin resistant state induced by TNFα. This could be an important observation for the prevention of metabolic diseases, as these often involve elevated plasma levels of this cytokine. Interestingly, we also observed an increase in Akt phosphorylation after incubation with glutamine alone, without insulin, but not after incubation with leucine or proline only. Amino acids have been shown to downregulate IRS-1, but to upregulate GLUT-4 in rat heart tissue [10]. The authors hypothesized that although the expression of IRS-1, the direct downstream substrate of insulin, is reduced, amino acids may stimulate glucose transport and overcome insulin resistance by inducing the expression of GLUT-4 *via* other routes. However, no data on phosphorylation of IRS-1 or translocation of GLUT-4 were reported and physiological effects are therefore speculative. Our observations also imply that these nutrients may play a role in modulating the insulin signaling pathway and they therefore might be a useful nutrient to consider in the treatment of insulin resistance. Indeed, a high-protein diet may be helpful in controlling blood glucose in type 2 diabetic patients [29]. However, Akt is part of a very complex signaling pathway involving many molecules, and its function may vary from one organ to another. In fact, glucose uptake in the liver, facilitated by GLUT-2, is an insulin- and Akt-independent event [30]. Glycogen synthesis is a more downstream event in the liver that is dependent on insulin and Akt. Therefore, we also determined the effects of amino acids on levels of phosphorylated GSK-3β, the key enzyme in glycogen synthesis. However, we were not able to detect any differences in P-GSK-3β levels after insulin or amino acid treatment. Hence, although Akt phosphorylation is increased by amino acid treatment, the downstream effects of this phenomenon are not clear. It has also been proposed that a relationship exists between inflammation and insulin resistance. NF-κB is now thought to be involved in the development of obesity- and diet-induced insulin resistance, providing the link between inflammation and insulin resistance [31, 32]. Our observation that NF-κB activity is reduced by amino acid treatment could therefore also have implications for insulin sensitivity. In fact, the increased phosphorylation of Akt could also be a result of the NF-κB suppression exerted by the amino acids. The exact mechanism, as well as the *in vivo* effects, however, remains to be further investigated.

Taken together, our results indicate that the amino acids glutamine, leucine and proline reduce the production of IL-8 in HepG2 cells, probably by inhibiting NF-κB activity. We also found that these amino acids were able to restore the phosphorylation of Akt after TNFα treatment, but the physiological consequences of these effects remain to be elucidated. Therefore, further research is necessary to explore the *in vivo* effects of these amino acids and their possible role in the treatment of metabolic diseases.

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The authors have declared no conflict of interest.

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