Oral Glutamine Supplementation Protects Female Mice from Nonalcoholic Steatohepatitis¹–³
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Abstract

Background: Genetic factors, a diet rich in fat and sugar, and an impaired intestinal barrier function are critical in the development of nonalcoholic steatohepatitis (NASH). The nonessential amino acid glutamine (Gln) has been suggested to have protective effects on intestinal barrier function but also against the development of liver diseases of various etiologies.

Objective: The effect of oral Gln supplementation on the development of Western-style diet (WSD)-induced NASH in mice was assessed.

Methods: Female 6- to 8-wk-old C57BL/6J mice were pair-fed a control (C) diet or a WSD alone or supplemented with 2.1 g L-Gln/kg body weight for 6 wk (C+Gln or WSD+Gln). Indexes of liver damage, lipid peroxidation, and glucose metabolism and endotoxin concentrations were measured.

Results: Although Gln supplementation had no effect on the loss of the tight junction protein occludin, the increased portal endotoxin and fasting glucose concentrations found in WSD-fed mice, markers of liver damage (e.g., nonalcoholic fatty liver disease activity score and number of neutrophils in the liver) were significantly lower in the WSD+Gln group than in the WSD group (−47% and −60% less, respectively; P < 0.05). Concentrations of inducible nitric oxide synthase (iNOS) protein and 3-nitrotyrosin protein adducts were significantly higher in livers of WSD-fed mice than in all other groups (−8.6- and −1.9-fold higher, respectively, compared with the C group; P < 0.05) but did not differ between WSD+Gln-, C-, and C+Gln-fed mice. Hepatic tumor necrosis factor α and plasminogen activator inhibitor 1 concentrations were significantly higher in WSD-fed mice (−1.6- and −1.8-fold higher, respectively; P < 0.05) but not in WSD+Gln-fed mice compared with C mice.

Conclusion: Our data suggest that the protective effects of oral Gln supplementation on the development of WSD-induced NASH in mice are associated with protection against the induction of iNOS and lipid peroxidation in the liver. J Nutr 2015;145:2280–6.

Keywords: glutamine, hepatic inflammation, iNOS, reactive oxygen species, nonalcoholic steatohepatitis

Introduction

During the past decades the prevalence of nonalcoholic fatty liver disease (NAFLD)⁷ has increased markedly worldwide, and the prevalence of its main risk factors (i.e., overweight, obesity, and insulin resistance) has reached almost epidemic proportions (1, 2). A recent survey that reviewed 260 epidemiologic studies published in Europe during the past 5 y showed that NAFLD, a disease comprising a continuum of liver damage ranging from simple steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis, is now the most frequent chronic liver disease in Europe (3). Previous results from our own and other groups suggest that, in addition to diet, alterations of intestinal barrier function associated with increased translocation of bacterial endotoxin, elevated formation of reactive oxygen species (ROS) in the liver, and induction of TNF-α may be critical in the development of NAFLD [for an overview, see (4–6)]. Results of animal models of NAFLD indicate that TNF-α can alter insulin-dependent signal cascades, subsequently leading to the induction of plasminogen activator inhibitor 1 (PAI-1) and alterations in hepatic lipid export (7). However, despite intense research efforts, molecular

Notes

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§ Supplemental Tables 1 and 2 and Supplemental Figures 1 and 2 are available from the “Online Supporting Material” link in the online posting of this article and from the same link in the online table of contents at http://jn.nutrition.org.
¶ Abbreviations used: ACTB, β-actin; ALT, alanine aminotransferase; Arg1, arginase 1; C, control; E%, percentage of energy; F4/80, macrophage membrane protein; iNOS, inducible nitric oxide synthase; Insr, insulin receptor; Irs, insulin receptor substrate; M2D88, myeloid differentiation primary response gene 88; NLFD, nonalcoholic fatty liver disease; NAS, nonalcoholic fatty liver disease activity score; NASH, nonalcoholic steatohepatitis; PAI-1, plasminogen activator inhibitor 1; ROS, reactive oxygen species; TLR, Toll-like receptor; WSD, Western-style diet; 3-NT, 3-nitrotyrosin protein adducts; 4-HNE, 4-hydroxynonenal protein adducts.
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mechanisms involved in the disease onset and even more so in its progression are not fully understood, and universally accepted therapies or prevention strategies are lacking.

The nonessential amino acid glutamine (Gln) has been shown to improve intestinal barrier function in various situations. Indeed, supplementation of Gln has been shown to ameliorate LPS-induced mucosal injury and inflammation (8–10). In addition, Gln has been suggested to regulate inflammatory processes and innate immune response, probably at least in part through modulating levels of glutathione and antioxidant defense systems (11, 12). Furthermore, results of animal studies that used models of diet-induced NAFLD and alcohol-induced liver diseases suggested that oral supplementation of Gln may have protective effects on the development of these types of liver diseases (13). In these studies, protective effects on the liver were associated with protection against alterations of glutathione concentrations, the activation of hepatic NF-κB, and the induction of TNF-α expression (14, 15). However, whether the protective effects of oral supplementation of Gln found in the latter studies stemmed from direct effects of the amino acid on the gut and subsequently the liver or from direct effects of Gln on the liver has not yet been fully clarified. Therefore, with the use of a mouse model in which mice were pair-fed a fat-, fructose-, and cholesterol-enriched so-called Western-style diet (WSD) to induce NAFLD, the main objective of the present study was to test the hypothesis that oral Gln supplementation protects mice from the development of NASH and, if so, to determine the underlying molecular mechanisms.

Methods

Animals and treatment. Female 6- to 8-wk-old C57BL/6J mice (Janvier S.A.S.), which were previously shown to be more susceptible to fructose-induced NAFLD than male mice (16), were housed in a specific-pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. All procedures were approved by the local institutional animal care and use committee. Mice (n = 8/group, total of 4 groups) had free access to plain tap water and were pair-fed either a liquid standard diet [control (C); 15.7 MJ/kg diet: 69% of energy (E%) from carbohydrates, 12 E% from fat, and 19 E% from protein] or a liquid WSD [17.8 MJ/kg diet: 60 E% from carbohydrates, 25 E% from fat, and 15 E% from protein with 50% wt:wt fructose and 0.16% wt:wt cholesterol] (both Ssniff) for 6 wk, respectively (Supplemental Table 1). Some of the mice were additionally fed 2.1 g/L Gln/kg body weight per day, which was added to the C diet and the WSD (C+Gln and WSD+Gln, respectively). Body weight was determined weekly. To ensure equal caloric intake between groups, the feed consumption of each group was determined daily and average caloric intake per group and day was calculated. The amount of diet and calories, respectively, fed to the different groups was then adjusted to the group with the lowest caloric intake for the next day to ensure pair-feeding of groups. The group with the lowest caloric intake was fed ad libitum. In week 4, mice were feed-deprived for 6 h to obtain fasting blood samples from the retrobulbar venous plexus for glucose measurements. At the end of the 6-wk feeding period, mice were anesthetized by intraperitoneal ketamine (100 mg/kg) and xylazine (16 mg/kg) injection, and blood was collected from the portal vein just before they were killed. Portions of the liver and duodenum were either snap-frozen immediately or fixed in neutral-buffered formalin.

Clinical chemistry and histologic evaluation of liver sections. Alanine aminotransferase (ALT) activity was determined by colorimetric reaction (Architect, Fa.; Abbott). Fasting blood glucose was measured by using a standard glucometer (Contour; Bayer Vital GmbH). Paraffin-embedded sections of liver (5 μm) were stained with hematoxylin and eosin (Sigma Aldrich Chemie GmbH) and analyzed as previously described (17). Neutrophil granulocytes were stained in paraffin-embedded liver sections (5 μm) by using a commercially available naphthol AS-D chloroacetate esterase staining kit (Sigma Aldrich Chemie GmbH) and counted as detailed previously (17).

Immunohistochemical staining of MYD88, iNOS, 4-HNE, 3-NT, and F4/80-positive cells in the liver and of the tight junction protein occludin in duodenum. Paraffin-embedded liver sections (4 μm) were stained and analyzed for myeloid differentiation primary response gene 88 (MYD88) protein, inducible NO synthase (iNOS) protein, 4-hydroxynonenal protein adducts (4-HNE), 3-nitrotyrosine protein adducts (3-NT), and the macrophage membrane protein F4/80, respectively, by using polyclonal antibodies (MYD88: Santa Cruz Biotechnology; iNOS: ThermoFisher Scientific; 4-HNE: AG Scientific; 3-NT: Cell Signaling Technology; F4/80: Abcam). Paraffin-embedded duodenal sections (4 μm) were stained by using a specific antibody against the tight junction protein occludin (Invitrogen). Staining intensities in all liver and duodenal sections were determined as previously detailed (17).

To determine means, data from 8 fields (liver: 200× magnification; duodenum: 400× magnification) of each tissue section (e.g., liver or duodenum) were used.

ELISAs for TNF-α and PAI-1 and endotoxin measurement. By using a commercially available mouse TNF-α or PAI-1 ELISA kit according to the manufacturers’ instructions (TNF-α: Assaypro; PAI-1: Molecular Innovations), protein concentrations of TNF-α and PAI-1 were determined in liver homogenate of mice. Plasma endotoxin concentrations were measured as previously described (18).

RNA isolation and real-time RT-PCR. For insulin receptor (Insr), insulin receptor substrate (Irs) 1 and 2 and arginase 1 (Arg1) expression measurement, RNA isolation and real-time RT-PCR were performed as previously described by Kanuri et al. (19). Primer sequences are shown in Supplemental Table 2.

Western blot. Protein isolation and Western blot were performed as previously detailed by Spruss et al. (6). Blots were probed with antibodies against Toll-like receptor (TLR) 4 (Abcam) and β-actin (ACTB) (Cell Signaling Technology), and bands were detected by using the Super Signal Western Dura kit (ThermoFisher Scientific). All blots were stained with Poncēau red (Roth) to ascertain equal loading, and protein bands were densitometrically analyzed by using Image Lab Software (Bio-Rad Laboratories). TLR-4 protein concentrations were normalized to ACTB concentrations.

Statistical analyses. Results are reported as means ± SEMs. Grubb’s test was used to identify outliers before statistical analysis (GraphPad Prism Software). Two-factor ANOVA with Tukey’s post hoc test was used to determine significance between treatment groups (GraphPad Prism Software). Homogeneity of variances was tested with Bartlett’s test, and raw data were log-transformed in cases of unequal variances. The significance level chosen a priori was P ≤ 0.05.

Results

NASH development. As expected, mice fed the WSD developed massive macrovesicular steatosis associated with marked inflammatory alterations [NAFLD activity score (NAS): ~14.8-fold higher in WSD mice compared with C mice; P < 0.01] (Figure 1). Caloric intake, body weight gain, and the number of fat-infiltrated hepatocytes (~45% of hepatocytes when determined by NAS; data for steatosis are not shown separately) were similar between WSD groups. Liver weight and liver to body weight ratio were higher in WSD+Gln-fed mice than in WSD-fed mice (P < 0.01) (Table 1). In contrast, NAS was significantly lower in WSD+Gln-fed mice compared with WSD-fed mice. However, the NAS in livers of WSD+Gln-fed mice was still significantly higher than that for C- and C+Gln-fed mice (P < 0.05). Furthermore, numbers of neutrophils and F4/80-positive cells were significantly higher in livers of WSD-fed mice than in all other groups (P < 0.05). TNF-α and PAI-1 protein concentrations were also significantly higher in livers of mice fed the WSD compared with both C and C+Gln.
groups ($P < 0.05$). TNF-α and PAI-1 protein concentrations in livers of WSD+Gln-fed mice did not differ from mice fed the WSD, C, and C+Gln diets ($P \geq 0.05$) (Figure 1, Table 2). The expression of Arg1 mRNA in liver was not different between C-, C+Gln-, and WSD-fed mice; however, in livers of mice fed WSD+Gln, Arg1 expression was significantly higher than in livers of all other groups ($P < 0.01$). ALT plasma concentrations did not differ between groups (Table 1).

Glucose metabolism and insulin signaling. Although C+Gln-fed mice had significantly lower fasting glucose concentrations than all other groups ($P < 0.01$ for all groups), fasting plasma glucose concentrations did not differ between both WSD groups and mice fed the C diet alone (Table 1). In contrast, fasting plasma insulin concentrations did not differ between groups. Expression of Insr, Irs1, and Irs2 in liver did not differ between mice fed the WSD or C diets. In contrast, the expression of Insr and Irs1 mRNA was significantly higher in livers of mice fed the WSD+Gln diet than in C+Gln-fed mice ($P < 0.05$ for both variables), whereas the expression of Irs2 did not differ between these 2 groups.

Markers of lipid peroxidation and iNOS in liver. Concentrations of both 4-HNE and 3-NT were significantly higher in livers of mice fed a WSD alone than those in C-fed mice (4-HNE, ~150%; and 3-NT, ~88% higher in WSD mice compared with C mice; $P < 0.01$ for both). This effect of the WSD was significantly attenuated in livers of mice fed the WSD+Gln diet (4-HNE, ~47%; and 3-NT, ~44% less in WSD+Gln mice compared with WSD mice; $P < 0.01$) (Table 2, Supplemental Figure 2); however, 4-HNE concentrations were still significantly higher than in mice fed C or C+Gln diets (~32% and ~110% higher, respectively). Concentrations of 3-NIT in the liver did not differ between C-, C+Gln-, and WSD+Gln-fed mice. In line with these findings, protein concentrations of iNOS were found to be significantly induced in livers of mice fed the WSD in comparison to all other groups ($P < 0.01$ for all groups), whereas in livers of mice fed the WSD+Gln diet, iNOS protein concentrations were almost the same as those in controls.

Discussion

Until now, universally accepted therapeutic and preventive interventions, such as lifestyle or pharmaceutical-based interventions, that aim to prevent the development and progression of NAFLD have been lacking. Animal-based models displaying alterations found in patients with NAFLD were found to be useful tools to investigate possible molecular mechanisms involved in the different stages of the disease and to evaluate new potential treatment and prevention strategies. Here, through feeding a WSD, early metabolic as well as molecular changes associated with the development of NASH (e.g., steatosis, inflammation, and insulin resistance) were induced in mice. Chronic intakes of this WSD promote pathological changes in the liver as well as at the level of intestine, which are also found in many humans with early NASH [for an overview of alterations, see also (20–22)]. Of course, despite many similarities with the human situation, it needs to be emphasized that the chronic intake in mice of a pair-fed liquid diet containing 50% wt:wt fructose and 25 E% fat with 0.16% wt:wt cholesterol does not resemble all alterations found in humans with NAFLD (e.g., genetic predisposition, lack of physical inactivity). Still, this dietary model offers the possibility to determine molecular mechanisms involved in the development of NASH under a controlled dietary environment because caloric intake is adjusted between groups on a daily basis. This approach offers the possibility to overcome effects resulting from differences in caloric intake that are often found between single animals or groups of animals.

Using this dietary model we aimed to determine if supplementation of Gln protects mice from the development of NASH. Although total caloric intake, body weight gain, and ALT plasma concentrations were similar between all feeding groups and even slightly higher liver to body weight ratios in WSD+Gln fed mice not differ between WSD- and WSD+Gln-fed mice (Figure 2). In line with these findings, endotoxin concentrations were also significantly higher in both groups fed the WSD regardless of additional treatments ($P < 0.01$ for both groups). Protein concentrations of TLR-4 in liver were similar among groups. Protein concentrations of the TLR-4 adaptor protein MYD88 were significantly higher in livers of mice fed a WSD in comparison to C-fed mice ($P < 0.01$). MYD88 protein concentrations in livers of mice fed C+Gln and WSD+Gln diets were similar and did not differ from those fed C or WSD diets only (Table 1 and Supplemental Figure 1).
Glutamine protects mice from NASH

**TABLE 1** Body and liver weights, plasma ALT, markers of glucose metabolism in blood and liver, and hepatic TLR-4 and MYD88 protein concentrations in female mice fed a C diet or WSD with or without supplemental Gln for 6 wk

<table>
<thead>
<tr>
<th>Diet groups</th>
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<tr>
<td>C</td>
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<td>Caloric intake, kcal/mouse - d</td>
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<td>Weight gain, g/6 wk</td>
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<td>Liver weight, g</td>
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<td>Plasma ALT, µkat/L</td>
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<td>Blood glucose, mg/dL</td>
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<td>Plasma insulin, µg/L</td>
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<td>Hepatic Insr mRNA, % of control</td>
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<td>Hepatic Insr1 mRNA, % of control</td>
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<td>Hepatic Insr2 mRNA, % of control</td>
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<tr>
<td>Hepatic TLR-4: ACTB ratio</td>
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<td>Hepatic MYD88 protein, % per microscopic field</td>
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1 Values are means ± SEMs, n = 8, with the exception of TLR-4, Insr, Insr1, and Insr2 (n = 5–8). mRNA expressions of Insr, Insr1, and Insr2 were normalized to 18S expression, and TLR-4 protein concentrations were normalized to ACTB concentrations. Means without a common letter differ, P < 0.05. NS, P > 0.05. ACTB, β-actin; ALT, alanine aminotransferase; C, control; DE, diet effect; GE, glutamine effect; DE×GE, interaction between diet and glutamine; Insr, insulin receptor; Insr1, insulin receptor substrate; MYD88, myeloid differentiation primary response gene 88; TLR-4, Toll-like receptor 4; WSD, Western-style diet.

**TABLE 2** Markers of inflammation and oxidative stress in livers of female mice fed a C diet or a WSD with or without supplemental Gln for 6 wk

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<th>Diet groups</th>
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<td>C</td>
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<tr>
<td>Hepatic F4/80-positive cells, % per microscopic field</td>
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<tr>
<td>Hepatic Arg1 mRNA, % of control</td>
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<td>Hepatic TNF-α, pg/mg protein</td>
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<td>Hepatic PAI-1, pg/mg protein</td>
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<td>Hepatic 4-HNE, % per microscopic field</td>
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<td>Hepatic iNOS protein, % per microscopic field</td>
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<td>Hepatic 3-NT, % per microscopic field</td>
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1 Values are means ± SEMs, n = 7–8, with the exception of Arg1 (n = 5–8); mRNA expression of Arg1 was normalized to 18S expression. Means without a common letter differ, P < 0.05. NS, P > 0.05. Arg1, arginase 1; C, control; DE, diet effect; GE, glutamine effect; DE×GE, interaction between diet and glutamine; iNOS, inducible nitric oxide synthase; PAI-1, plasminogen activator inhibitor 1; WSD, Western-style diet; 3-NT, 3-nitrotyrosine protein adducts; 4-HNE, 4-hydroxynonenal protein adducts.
WSD-fed mice supplemented with Gln, the expression of Insr, Irs1, and Irs2 was associated with an induction of NAFLD in mice. However, in the present study, oral supplementation of Gln had no effect on the loss of tight junction proteins in the upper parts of the small intestine and the elevated endotoxin concentrations in portal plasma found in mice fed a WSD. These data suggest that the protective effects found in the present study did not primarily result from an effect of the amino acid on intestinal barrier function. Differences between our results and those of others might have resulted from differences in animal models (e.g., models of inflammatory bowel disease, gastrointestinal infections, and mucositis vs. models of WSD-induced NAFLD) as well as the amount of Gln supplemented (here, 2.1 g/kg vs. 4.4% wt:wt or 450 mg/kg) and differences between species (e.g., mice vs. rats) (13, 33–35).

The results of our study suggest that the protective effects of oral supplementation of Gln on the liver resulted from a marked attenuation of the induction of MYD88, lipid peroxidation (e.g., formation of 4-HNE and 3-NT), and subsequently induction of TNF-α and PAI-1 found in the livers of mice fed a WSD. These findings are also supported by previous studies by our group showing that protection against the induction of iNOS and lipid peroxidation is associated with a marked protection in mice against the development of NAFLD (6). Indeed, we showed that iNOS, probably through NF-κB–dependent signaling cascades, is also involved in the regulation of MYD88 expression in the liver (6). Furthermore, the results of Kim et al. (36), Lu et al. (37), and Hammami et al. (38) suggest that Gln may directly alter iNOS activity in vitro. In accordance with these findings, Esposito et al. (39) also found that in ischemia/reperfusion models the beneficial effects of Gln supplementation on the liver were associated with protection against the induction of iNOS in the liver. Also in line with these findings, it was shown that the beneficial effects of Gln on the development of high-fat and high-cholesterol diet–induced NAFLD in rats was associated with protection from the enhanced formation of ROS (14). Lipid peroxidation was further shown to be a major trigger of redox-sensitive NF-κB and subsequently the release of proinflammatory cytokines such as TNF-α (40, 41).

Taken together, our data suggest that the protective effects of oral supplementation of Gln on the development of WSD-induced NAFLD in the present study may result from protection against the induction of iNOS; however, molecular mechanisms involved in the suppression of iNOS remain to be determined.

In summary, results of the present study suggest that oral supplementation of Gln at least partially protects mice from the onset of diet-induced NASH. However, whether the protective effects of Gln supplementation in settings of NAFLD persist over time remains to be determined. Our results further suggest that the beneficial effects of Gln are not primarily a result of protection against the enhanced intestinal permeability and subsequent increased translocation of bacterial endotoxins but rather result from inhibition of the induction of iNOS in the liver, subsequently leading to decreased formation of ROS and dependent signaling cascades. Further studies are warranted to 1) explore the
underlying mechanisms of iNOS suppression, 2) assess long-term effects of Gln supplementation in the setting of NAFLD in animals, and 3) determine if these protective effects are also found in humans.

Acknowledgments
J-PDB and IB designed the research; CS, CJJ, and CD conducted the research and analyzed the data; CS and IB wrote the manuscript; and IB had primary responsibility for final content. All authors read and approved the final manuscript.

References


