Supplementation of sodium butyrate protects mice from the development of non-alcoholic steatohepatitis (NASH)

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Abstract

Overnutrition, insulin resistance and an impaired intestinal barrier function are discussed as critical factors in the development of non-alcoholic fatty liver disease. Not only butyrate-producing probiotics as well as supplementation of sodium butyrate (SoB) have been suggested to bear protective effects on liver damage of various aetiologies. However, whether an oral consumption of SoB has a protective effect on Western-style diet (WSD)-induced non-alcoholic steatohepatitis (NASH) and if so molecular mechanism involved has not yet been determined. Eight-week-old C57BL/6J mice were pair-fed either a liquid control or WSD ± 0.6 g/kg body weight SoB. After 6 weeks, markers of liver damage, inflammation, toll-like receptor (TLR)-4 signalling, lipid peroxidation and glucose as well as lipid metabolism were determined in the liver tissue. Tight junction protein levels were determined in the duodenal tissue. SoB supplementation had no effects on the body weight gain or liver weight of WSD-fed mice, whereas liver steatosis and hepatic inflammation were significantly decreased (e.g. less inflammatory foci and neutrophils) when compared with mice fed only a WSD. Tight junction protein levels in duodenum, hepatic mRNA expression of TLR-4 and sterol regulatory element-binding protein 1c were altered similarly in both WSD groups when compared with controls, whereas protein levels of myeloid differentiation primary response gene 88, inducible nitric oxide synthase, 4-hydroxynonenal protein adducts and F4/80 macrophages were only significantly induced in livers of mice fed only the WSD. In summary, these data suggest that an oral supplementation of SoB protects mice from inflammation in the liver and thus from the development of WSD-induced NASH.

Key words: Non-alcoholic fatty liver disease: Sodium butyrate: Hepatic inflammation: Inducible nitric oxide synthase: Reactive oxygen species

During the last decades, the prevalence of non-alcoholic fatty liver disease (NAFLD), comprising a wide spectrum of liver diseases ranging from simple hepatic steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and even hepatocellular carcinoma(1), has dramatically increased. Indeed, Blachier et al.(2) has recently reported in a survey reviewing 260 epidemiological studies published in Europe during the last 5 years that NAFLD is by now the most frequent liver disease in Europe. However, in spite of intensive research efforts during the last decades, molecular mechanisms underlying the development of NAFLD remain mostly unclear, and universally accepted treatment strategies besides a lifestyle modification, such as weight-reduction diets and/or increases in physical activity are not yet available.

Butyrate, a naturally occurring SCFA in the body mainly produced by intestinal bacteria and found in various foods like cheese and butter, has in recent years been suggested to play an important role in maintaining intestinal homoeostasis(3). Indeed, it has been shown in in vitro studies that butyrate through AMP-kinase- and p38 mitogen-activated protein kinase-dependent signalling cascades may ameliorate ethanol-induced intestinal barrier dysfunction(4,5). Recent animal studies further suggest that sodium butyrate (SoB) may also possess protective effects on liver damage of various aetiologies(6–8).

For instance, it has been shown that rats treated before or at the onset of ischaemia reperfusion (I/R) with butyrate were markedly protected from I/R-induced liver injury not only through mechanisms involving inhibition of histone deacetylase and heat shock protein 70 induction in the liver as well as by maintaining the intestinal barrier structure(9). In line with these findings, Cresci et al.(9) reported that pretreatment of mice with tributyrin, an ester composed of butyric acid and glycerol, protects mice from acute but not chronic alcohol-induced liver damage through mechanisms involving a protection against the...
loss of tight junction proteins induced by acute alcohol exposure. Furthermore, Mattace et al.\(^{(6)}\) showed that butyrate and its synthetic derivative N-(1-carbamoyl-2-phenyl-ethyl) butiramide may protect rats from the development of insulin resistance and NAFLD. However, not only whether butyrate also possesses protective effects on the development of NAFLD in other models of NAFLD, as well as the molecular mechanisms involved has not yet been determined.

Using a mouse model in which mice were pair-fed a fat, fructose and cholesterol-enriched liquid diet to induce NAFLD, the main objective of the present study was to test the hypothesis that an oral supplementation of SoB protects mice from the development of NASH and if so, to determine underlying molecular mechanisms involved.

**Methods**

**Animals and treatments**

Eight-week-old female C57BL/6J mice (Janvier SAS), which were indicated to be more susceptible to a fructose-induced NAFLD compared with the male mice by our own group\(^{(10)}\), were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The local Institutional Animal Care and Use Committee approved all procedures. During a 6-week feeding period, mice (n 6/group) were pair-fed either a standard liquid diet (control, 15.7 MJ/kg diet; 69% from carbohydrates, 12% from fat and 19% from protein) or a liquid Western-style diet (WSD; fortified with fructose, fat and cholesterol; 17.8 MJ/kg diet: 60% from carbohydrates, 25% from fat and 15% from protein with 50%, w/w fructose and 0.155%, w/w cholesterol) (Sniff) ±0.6 g/kg body weight/d SoB (Sigma-Aldrich) (for details also see online Supplementary Table S1). All mice had free access to plain tap water. Diet consumption was assessed and adjusted daily, and body weight was registered weekly. After 4 weeks of feeding, mice were fasted for 6 h to obtain fasting blood samples from retrobulbar venous plexus for blood parameter of liver damage and ELISA

Alanine aminotransferase (ALT) activity was determined by a colorimetric reaction (Architect, Fa.; Abbott) in a routine laboratory. Fasting blood glucose levels were measured using a blood glucose meter (Contour; Bayer Vital GmbH). Protein concentration of TNF-α in the liver tissue was determined using a commercially available ELISA kit (Mouse TNF-alpha ELISA Kit; Assaypro) as detailed before\(^{(14)}\).

Blood parameter of liver damage and ELISA

Paraffin-embedded liver tissue sections (4 µm) were stained for myeloid differentiation primary response gene 88 (MyD88), 4-hydroxynonenal (4-HE) protein adducts, inducible nitric oxide synthase (iNOS), F4/80 and cluster of differentiation 86 (CD86)-positive cells using polyclonal antibodies (MyD88: Santa Cruz Biotechnology; 4-HE: NEN; F4/80: Abcam; CD86: Santa Cruz Biotechnology) as previously detailed\(^{(15,16)}\). Paraffin-embedded duodenal tissue sections (4 µm) were stained for the tight junction proteins occludin and zonula occludens 1 (ZO-1) using polyclonal primary antibodies (Life Technologies GmbH) as described previously\(^{(11,17)}\). In brief, to detect the binding of target protein to the specific primary antibody, tissue sections were incubated with a peroxidase-linked secondary antibody and diamobenzidine (Peroxidase Envision Kit; Dako). Pictures of eight fields of each tissue section (×200 of each liver tissue; ×400 of each duodenum tissue) were captured using a digital camera integrated in a microscope (Leica DM4000 B LED), and the extent of staining in tissue sections was defined as per cent of field area within the default colour range determined by an analysis system incorporated in microscope. Mean values of eight sections were used per tissue section to determine means.

**Histological evaluation of liver sections**

Frozen sections of the liver fixed in OCT (10 µm) were stained with Oil red O (Sigma) as described previously\(^{(11)}\). Representative photomicrographs were captured at a 100× magnification using a system incorporated in microscope (Leica DM4000; Leica). Paraffin-embedded liver sections (4 µm) were stained with haematoxylin–eosin (Sigma) to evaluate histology. Representative photomicrographs were captured using a camera integrated in a microscope (Leica DM4000 B LED; Leica) at 200× magnification. NAFLD activity score was used to determine liver damage\(^{(12)}\). To determine number of neutrophilic granulocytes, liver sections (4 µm) were stained with Naphthol AS-D Chloroacetate Esterase kit (Sigma), and number of neutrophils was determined as previously described in detail\(^{(11)}\). Collagen deposition in the liver tissue was stained with Picrosirius red (Sigma) and counterstained with fast green (Sigma) to determine fibrosis. Quantitative evaluation of staining was carried out as previously described in detail\(^{(15)}\).

RNA isolation and real-time RT-PCR

Total RNA from the liver tissue was isolated with Trizol (peqGOLD Trifast, Peqlab), and 1-0 µg total RNA was reverse transcribed (cDNA synthesis kit, Promega). SYBR Green® Supermix (Agilent Technologies) was used to prepare the PCR mix, and relative mRNA expression was determined using a MX QPCR System (Agilent Technologies) as previously detailed\(^{(14)}\). PCR primers for chemokine (C-C motif) ligand 2 (CCL-2), toll-like receptor 4 (TLR-4), insulin receptor (IR), 18S ribosomal RNA (18S), IGF-1, FAS, eukaryotic translation elongation factor 2 (eEF-2) as well as 18S were designed using Primer 3 software.
(Whitehead Institute for Biomedical Research). Sequences were listed in Table 1. The comparative cycle threshold (Ct) method was used to determine the amount of target, normalised to an endogenous reference (Eef-2) and relative to a calibrator (2^-\Delta\Delta C\text{t}). The purity of the PCR products was verified by melting curves and gel electrophoresis.

**Western blot**

A Dignum A buffer (1 mol/l HEPES, 1 mol/l MgCl2, 2 mol/l KCl, 1 mol/l dithiothreitol) containing a protease inhibitor mix (Roche) was used to homogenise liver tissue samples, and blots were prepared as previously detailed(18). Blots were probed with antibodies against inhibitor of NF-κB kinase (IKK) subunit β and phosphorylated IKK subunit α and β (Ser 176/180) (pIKKα/β) (both Cell Signaling Technology), and bands were visualised using a Super Signal Western Dura kit (Thermo Scientific). Equal loading of blots was ensured by Ponceau Red staining (Roth). Using a ChemiDoc MP System (Bio-Rad Laboratories), protein bands were detected and analysed.

**Statistical analysis**

All data were presented as means values with their standard errors. One-way ANOVA with Tukey’s post hoc test was used to determine the statistical differences between the treatment groups (Graph Pad Prism version 6.0; GraphPad Software). Outliers were identified using Grubbs’s test. P value <0.05 was considered to be significant.

**Results**

**Effect of an oral sodium butyrate supplementation on liver injury and markers of glucose metabolism**

As expected, mice fed the WSD developed massive macrovesicular steatosis associated with marked inflammatory alterations (see Fig. 1). In contrast, despite a similar energy intake, body weight gain and even a significantly higher liver: body weight ratio, mice fed a WSD supplemented with SoB (WSD + SoB) had a significantly lower number of fat infiltrated hepatocytes and displayed predominantly microvesicular fat accumulation in the liver (see Table 2, Fig. 1(a)–(d)). However, number of fat infiltrated hepatocytes in the WSD + SoB group was still significantly higher than that in both control groups (see Fig. 1(d)). Furthermore, number of not only inflammatory foci as well as neutrophils and F4/80 positive cells were only found to be significantly higher in livers of mice fed a WSD alone (P<0.05 in comparison with all other groups) (see Table 3 and Fig. 1(e)). In line with these findings, number of CD8α-positive cells in livers of mice receiving only WSD was also significantly higher than in those of mice fed the control diet or WSD + SoB. However, despite not showing any signs of inflammation, number of CD8α-positive cells was significantly higher in livers of mice fed the control diet supplemented with SoB (C + SoB) (see Table 3). Expression of CCL-2 mRNA was only found to be significantly higher in livers of WSD-fed mice when the WSD alone in comparison with control mice (P = 0.065) (see Table 3). In line with these findings, concentration of pIKKα/β was only significantly higher in mice fed the WSD alone (see Fig. 3(g) and (h)). A similar effect of the WSD feeding was not found in mice concomitantly fed SoB; however, data varied considerably in some groups. Expression of IL-1β mRNA was significantly higher in livers of mice fed only a WSD in comparison with controls; however, expression of IL-1β mRNA was also significantly higher in livers of both groups receiving SoB, irrespective of the diet fed (see Table 3). Sirius red staining of collagen deposition revealed no differences between the different treatment groups (see Table 3 and Fig. 2(a)). Expression of TGF-β1 mRNA in the liver of WSD-fed mice was significantly higher than that in both control groups. In livers of mice fed the WSD + SoB, TGF-β1 mRNA expression was only significantly higher than that in those of mice fed only the control diet (see Table 3). As mice only displayed the beginning of NASH and measurements varied considerably, ALT plasma levels did not differ between groups (see Table 2). Furthermore, fasting glucose levels did not differ between groups (see Fig. 1(f)). In contrast, expression of IRS was markedly higher in livers of mice fed the WSD + SoB; however, as expression varied considerably in the control groups, differences only reached the level of significance for the comparison of the two WSD groups (see Table 2). Furthermore, although IRS-1 expression was significantly lower in livers of mice of all groups in comparison with controls, expression of IRS-1 did not differ between groups treated with SoB, irrespective of the diet fed (see Table 2).

**Table 1. Primer sequences used for real-time RT-PCR detection**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward (5′–3′)</th>
<th>Reverse (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL-2</td>
<td>GCC AGA CGG GAG GAA GGC CA</td>
<td>TGG ATG CTC CAG CGG GCA AC</td>
</tr>
<tr>
<td>TLR-4</td>
<td>AGC CAT TGC TGC CAA CAT CA</td>
<td>GCT GCC TCA GCA GGG ACT TC</td>
</tr>
<tr>
<td>IR</td>
<td>CAT CCC GAA AGC GAA GAT CC</td>
<td>GAG TCC TGA TTG CAT GCC TGC</td>
</tr>
<tr>
<td>IRS-1</td>
<td>GTC ACC ACC CCT AGA CAA AA</td>
<td>GCT CTA GTG CTT CCG TGC TT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGG CTG TGG AGA AGC TGG GG</td>
<td>GTC CGA CAG CAG GAG GCT TT</td>
</tr>
<tr>
<td>FAS</td>
<td>TCT GGG CCA ACC TCA TTG GT</td>
<td>GAA GCT GGG GGT CCA TTG TT</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>ACC GGC TAC TTG GGC ACT GC</td>
<td>AGA GCA AGA GGG TGC CAT CG</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>GTG TGC GAC CCT GCC CTC AT</td>
<td>TTG CAG GCG AGC ATC AT</td>
</tr>
<tr>
<td>Eef-2</td>
<td>GTG ACA GCT GCC TTG CTT GT</td>
<td>GAT GGC CTG CAA GGT CTT GT</td>
</tr>
<tr>
<td>18S</td>
<td>GTA ACC CGT TGA ACC CCA TT</td>
<td>CCA TCC AAT CAG TAG CAG</td>
</tr>
</tbody>
</table>

CCL-2, chemokine (C-C motif) ligand 2; TLR-4, toll-like receptor 4; IR, insulin receptor; IRS-1, insulin receptor substrate 1; FAS, fatty acid synthase; SREBP-1c, sterol regulatory element-binding protein 1c; TGF-β1, transforming growth factor β1; Eef-2, eukaryotic translation elongation factor 2.
Effect of oral sodium butyrate supplementation on tight junction protein levels in duodenum and on markers of the toll-like receptor 4 signalling cascade in the liver

As butyrate has been suggested to at least in part mediate its beneficial effects on the development of liver diseases through altering intestinal barrier function (19), we determined protein levels of the tight junction protein occludin and ZO-1 in tissue obtained from duodenum and mRNA expression of the endotoxin receptor TLR-4 as well as its adaptor protein MyD88 in the liver. Protein levels of occludin and ZO-1 were significantly lower in the duodenum of both groups fed a WSD irrespective of additional treatments when compared with both control groups (see Fig. 2(b) and (c), Fig. 3(a) and (b)). Expression of
TLR-4 mRNA was significantly higher in livers of mice fed the WSD alone and in both groups fed diets supplemented with SoB when compared with controls (see Fig. 3(c)). In contrast, protein levels of MyD88 were only significantly induced in livers of mice fed a WSD, whereas MyD88 protein levels of all other groups were at the level of controls (see Fig. 2(d) and Fig. 3(d)).

Effect of oral sodium butyrate supplementation on inducible nitric oxide synthase protein levels and markers of lipid peroxidation

As it has been shown that TLR-4 mediates its effects also through an activation of iNOS subsequently leading to an increase in the formation of reactive oxygen species, we determined iNOS and 4-HNE protein adduct levels in livers of mice. In line with the findings for MyD88, protein levels of iNOS were only found to be significantly induced in mice only fed a WSD, whereas in livers of those concomitantly treated with SoB while being fed the WSD, protein levels of iNOS were almost at the level of controls (see Fig. 2(e) and Fig. 3(e)). Similar results were also found for levels of 4-HNE protein adducts, also only being significantly higher in livers of mice fed a WSD when compared with controls (see Fig. 3(f)).

Effect of oral sodium butyrate supplementation on markers of hepatic lipid metabolism

Expression of SREBP-1c mRNA in the liver did not differ between WSD groups and was significantly higher than that in livers of control groups (see Fig. 4(a)). In contrast, expression of FAS mRNA was significantly higher in livers of WSD-fed mice when compared with all other groups. However, although being significantly lower in livers of mice fed WSD alone, FAS mRNA expression in livers of WSD + SoB-fed mice was still significantly higher than that in both control groups (see Fig. 4(b)).

Table 2. Effect of an oral supplementation of sodium butyrate (SoB) on body weight, liver-body weight ratio and clinical parameter of liver damage as well as markers of insulin resistance in mice fed a Western-style diet (WSD) or control diet (C) (Mean values with their standard errors)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>WSD</th>
<th>C + SoB</th>
<th>WSD + SoB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
</tr>
<tr>
<td>Daily energy uptake (kJ/g of body weight)</td>
<td>2.1 (0.17)</td>
<td>2.1 (0.04)</td>
<td>2.01 (0.04)</td>
<td>2.01 (0.04)</td>
</tr>
<tr>
<td>Daily energy uptake (kcal/g of body weight)</td>
<td>0.50 (0.04)</td>
<td>0.50 (0.01)</td>
<td>0.48 (0.01)</td>
<td>0.48 (0.01)</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>21.4 (0.6)</td>
<td>22.1 (0.3)</td>
<td>21.3 (0.9)</td>
<td>22.2 (0.2)</td>
</tr>
<tr>
<td>Absolute weight gain (g)</td>
<td>2.4 (0.5)</td>
<td>2.6 (0.4)</td>
<td>3.1 (0.6)</td>
<td>2.5 (0.6)</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.1 (0.0)</td>
<td>1.4± (0.0)</td>
<td>1.1 (0.1)</td>
<td>1.4± (0.0)</td>
</tr>
<tr>
<td>Liver:body weight ratio (%)</td>
<td>4.9 (0.1)</td>
<td>6.4± (0.2)</td>
<td>5.1 (0.1)</td>
<td>6.2± (0.1)</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>15.2 (1.0)</td>
<td>18.9 (2.4)</td>
<td>23.1 (2.9)</td>
<td>21.6 (3.1)</td>
</tr>
<tr>
<td>IR (fold induction)</td>
<td>4.0 (0.1)</td>
<td>1.5 (0.2)</td>
<td>1.7 (0.0)</td>
<td>2.4± (0.2)</td>
</tr>
<tr>
<td>IRS-1 (fold induction)</td>
<td>4.4± (0.4)</td>
<td>2.4 (0.3)</td>
<td>2.3 (0.5)</td>
<td>2.6 (0.2)</td>
</tr>
</tbody>
</table>

ALT, alanine aminotransferase; IR, insulin receptor; IRS-1, IR substrate 1.
* P < 0.05 compared with mice fed with control diet.
† P < 0.05 compared with mice concomitantly fed WSD diet.
‡ P < 0.05 compared with mice concomitantly fed control diet and SoB (0.6 g SoB/kg body weight/d).
§ P < 0.05 compared with mice concomitantly fed WSD diet and SoB (0.6 g SoB/kg body weight/d).
|| IR and IRS-1 mRNA expressions were normalised to Ef-2 mRNA expression.

Table 3. Effect of an oral supplementation of sodium butyrate (SoB) on markers of inflammation and fibrosis in livers of mice fed a Western-style diet (WSD) or control diet (C) (Mean values with their standard errors)

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>WSD</th>
<th>C + SoB</th>
<th>WSD + SoB</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
<td>Mean (SEM)</td>
</tr>
<tr>
<td>Neutrophils (number/field)</td>
<td>1.8 (0.3)</td>
<td>4.4± (0.6)</td>
<td>1.5 (0.2)</td>
<td>1.1 (0.2)</td>
</tr>
<tr>
<td>F4/80 (number/field)</td>
<td>6.4 (0.7)</td>
<td>14.7± (1.9)</td>
<td>7.4 (1.7)</td>
<td>8.5 (1.3)</td>
</tr>
<tr>
<td>CD80 (number/field)</td>
<td>14.4 (2.8)</td>
<td>27.6± (2.8)</td>
<td>25.0± (3.2)</td>
<td>10.1 (1.3)</td>
</tr>
<tr>
<td>CCL-2 (fold induction)</td>
<td>3.2 (0.6)</td>
<td>14.6± (4.6)</td>
<td>2.3 (0.6)</td>
<td>3.4 (0.5)</td>
</tr>
<tr>
<td>TNF-α (ng/mg protein)</td>
<td>0.30 (0.04)</td>
<td>0.48 (0.06)</td>
<td>0.32 (0.03)</td>
<td>0.29 (0.06)</td>
</tr>
<tr>
<td>IL-1β (fold induction)</td>
<td>2.5† (0.6)</td>
<td>6.2 (0.5)</td>
<td>4.8 (0.5)</td>
<td>6.4 (0.7)</td>
</tr>
<tr>
<td>TGF-β (fold induction)</td>
<td>1.5 (0.2)</td>
<td>3.5± (0.2)</td>
<td>2.4 (0.3)</td>
<td>3.2± (0.4)</td>
</tr>
<tr>
<td>Sirius red (% per microscopic field)</td>
<td>0.47 (0.07)</td>
<td>0.65 (0.08)</td>
<td>0.51 (0.08)</td>
<td>0.59 (0.13)</td>
</tr>
</tbody>
</table>

CD80, cluster of differentiation 80; CCL-2, chemokine (C-C motif) ligand 2; TGF-β1, transforming growth factor β1.
* P < 0.05 compared with mice fed with control diet.
† P < 0.05 compared with mice fed with WSD diet.
‡ P < 0.05 compared with mice concomitantly fed WSD diet and SoB (0.6 g SoB/kg body weight/d).
§ P < 0.05 compared with mice concomitantly fed control diet and SoB (0.6 g SoB/kg body weight/d).
|| CCL-2, IL-1β and TGF-β1 mRNA expressions were normalised to 18S mRNA expression.
Discussion

Up to now, life-style- or pharmaceutical-based interventions aiming to prevent the development but even more so the progression of NAFLD are still limited. Animal-based models resembling alterations found in patients with simple steatosis as well as NASH have been found to be useful tools to investigate possible molecular mechanisms involved in the development and progression of the disease and to evaluate new potential therapeutic and preventive strategies\(^1\). In the present study, a mouse model using a liquid fructose-enriched WSD was used to induce not only the early metabolic but also molecular changes associated with the development of NASH (e.g. steatosis, inflammation and insulin resistance). Indeed, chronic feeding of this type of diet produced pathological changes in the liver and also at the level of the intestine that resembles many of the early alterations found in humans with beginning NASH\(^1\). Despite these similarities with the human situation, it needs to be emphasised that chronic intake of a pair-fed liquid diet containing 50\% fructose, 25\% fat and 0\% cholesterol by no means mimics all alternations found in humans with NAFLD; however, this kind of dietary model offers the possibility to study molecular mechanisms underlying the development of NASH. Furthermore, as dietary intake of animals can be adjusted between groups, this kind of model can also serve to determine efficacy of nutritional supplements or drugs in a more accurate way as differences in energy intake associated with different treatments can be accounted for. Here,
this dietary model was used to determine whether an oral supplementation of SoB can protect mice from the development of NASH. Despite a similar intake of energy and weight gain in all feeding groups and no differences in ALT plasma levels, mice fed a WSD supplemented with SoB were markedly protected from the development of NASH. Indeed, these mice displayed less macrovesicular fat accumulation and almost no inflammatory foci in their livers. However, animals fed the WSD regardless of additional treatments did not yet display any marked histological signs of fibrosis in their livers, and TGF-β1 mRNA expression was also only slightly increased. Indeed, it was shown in a recently published study that mice fed a WSD in...
combination with drinking water enriched with high-fructose maize syrup for 16 weeks only showed mild signs of fibrosis, suggesting that feeding time in the present study might not have been long enough yet to induce fibrotic changes in the liver. In line with the histological findings with regard to fat accumulation and inflammation, a number of neutrophils and F4/80-positive cells as well as expression of CCL-2 mRNA and to a lesser extend protein levels of TNF-α and phosphorylation of IKKβ were also only markedly higher in livers of mice fed the WSD alone. These findings are in line with those of others, suggesting that the progression of hepatic injury to later stages – for example, NASH and fibrosis – largely depends on a CCL-2/CXCr2 (chemokine (C-C motif) receptor 2) -dependent recruitment of ‘inflammatory’ Ly-6Chi expressing monocytes into the liver(24). Results of the in vitro studies of Ohira et al. indicate that butyrate may reduce CCL-2 and TNF-α expression. In line with our findings, results of others also suggest that the hepatoprotective effects of butyrate might at least in part stem from an inhibition of the NF-κB signalling cascade(7). In contrast, the induction of the expression of IL-1β found in WSD-fed mice was not attenuated in mice fed a WSD supplemented with SoB; however, supplementation of SoB per se seemed to have altered the expression of this pro-inflammatory cytokine in the liver. Indeed, expression of IL-1β in livers of controls fed SoB was almost at the level of that found in both WSD-fed groups. In line with these findings, the number of CD8α-positive cells shown to be recruited to the liver by IL-1β-dependent mechanisms was also not only higher in the liver of WSD-fed mice but also in controls fed SoB. However, no signs of inflammation were seen in livers of these control mice. In vitro studies of Ohira et al., it was shown that an incubation of macrophages with butyrate especially in the presence of endotoxin is associated with an increased expression of IL-1β. Furthermore, Mattace et al. also reported a non-significant approximately 2-fold increase in IL-1β expression and cyclooxygenase-2 (COX-2) protein levels in livers of control rats fed a diet enriched with butyrate (20 mg/kg body weight). However, similar to our own findings, in spite of the induction of IL-1β mRNA and COX-2 protein levels, Mattace et al. also reported that an oral supplementation of SoB protected rats from the development of a high-fat diet-induced NAFLD. In accordance with our findings, the protective effects in this study were predominantly associated with a protection against the development of hepatic inflammation rather than lipid accumulation. Further studies are needed to unravel molecular mechanisms involved in the anti-inflammatory effects of butyrate in spite of the induction of some pro-inflammatory cytokines and increased number of CD8α-positive cells found in the liver when SoB is supplemented. The lack of differences in ALT levels between groups found in the present study could be attributed to the fact that mice only displayed early signs of NASH and no overall impaired glucose intolerance. Indeed, it has recently been suggested that adipose tissue insulin resistance, high liver TAG content and low plasma adiponectin are major factors in the elevation of plasma aminotransferase levels in patients with NAFLD, although hepatic insulin resistance seems to be of lesser importance in this respect(29). In the present study, blood glucose levels were similar between groups, whereas, in line with earlier findings of our group, expression of IR in liver was slightly lower in livers of mice fed a WSD, indicating that mice suffered from hepatic insulin resistance(30). In contrast, in livers of mice concomitantly fed a
WSD and SoB, IR expression was markedly higher than that in those only fed a WSD, suggesting that the supplementation of SoB protected mice from the development of hepatic insulin resistance. Interestingly, expression of IRS-1 was per se lower in livers of mice fed SoB regardless of the diet fed, which suggests that SoB may alter insulin signalling in the liver independent of other dietary factors. These findings need to be further investigated in future studies. However, contrary to the findings in the groups not receiving SoB, expression of IRS-1 in the liver did not differ between controls and WSD-fed mice. These findings are in line with those of other groups showing that supplementation of SoB may modulate insulin signalling and thereby may protect against the development of insulin resistance\(^{30,31}\). Indeed, it has recently been shown that treating chicken with 0·25 g/kg body weight SoB resulted in a marked decrease in protein levels of the IR β subunit in the liver and overall systemic insulin sensitivity\(^{31}\). Furthermore, in line with the findings of our study for the liver, it has also been shown in mice fed a high-fat diet, that supplementation of 5%, w/w SoB protected animals markedly from the development of insulin resistance\(^{30}\). Taken together, our data suggest that SoB supplementation in spite of the induction of some pro-inflammatory markers in the liver may protect mice from the development of NASH and hepatic insulin resistance.

The protective effect of a sodium butyrate supplementation is associated with a protection of mice from the induction of the toll-like receptor 4 signalling cascade, lipid peroxidation and the induction of fatty acid synthase in the liver.

It has been repeatedly suggested by not only the results of human studies of our own lab but also those of other groups that an increased translocation of bacterial endotoxin and activation of TLR-dependent signalling cascades in the liver may be involved in the development of NAFLD\(^{32–34}\). Results of recent studies suggest that SoB may exert its protective effect on the liver and maybe also on metabolic alterations through its effects on intestinal barrier function\(^{35,36}\). Furthermore, we and others showed before that the development of NAFLD is associated with a loss of tight junction proteins in the upper parts of the small intestine, increased bacterial endotoxin levels in the portal vein, the elevated expression of TLR in the liver and with the activation of dependent signalling cascades\(^{14,35}\).

It was further shown that a protection against the loss of tight junction proteins in the upper parts of the small intestine is associated with lower endotoxin levels and a protection of the liver from the development of NAFLD\(^{31,37}\). However, in the present study, protein levels of the tight junction protein occludin and ZO-1 were both lower in mice fed a WSD regardless of additional treatments, suggesting that protective effects of the supplementation of SoB found in the present study did not primarily stem from a protection against the loss of intestinal integrity. Differences between the results of the present study and those of other groups might have resulted from differences in the aetiology of liver disease\(^{6,9}\), for example, acute alcohol-induced liver damage and I/R, respectively, v. long-term feeding of a WSD and animal species used (rats v. mice). Furthermore, Cresci et al.\(^{39}\) fed tributyrin in their experiments, and Endo et al.\(^{39}\) treated animals with a butyrate-producing probiotics, whereas in the present study mice were fed SoB. Results of the present study suggest that the protective effects that were found stem from an attenuation of the induction of TLR-4-dependent signalling cascade and herein especially the induction of iNOS and an increased lipid peroxidation (e.g. formation of 4-HNE protein adducts) found in livers of mice fed a WSD. Indeed, it was shown before that iNOS is critical in regulating not only NF-κB depending signalling cascades in the development of NAFLD but also the expression of the TLR-4 adapt protein MyD88\(^{20}\). In line with our findings, Qiao et al.\(^{37}\) showed that the protective effects of SoB treatment against hepatic I/R damage were associated with a protection against the activation of NF-κB depending signalling pathways in Kupffer cells. Mattace et al.\(^{60}\) also found that insulin resistance and NAFLD were associated with a marked attenuation of the induction of NF-κB depending signalling cascades in the liver; however, in this study the protective effect of the butyrate supplementation was associated with a protection against the induction of TLR-4.

Differences between the findings of Mattace et al.\(^{60}\) and our study might have resulted from differences in experimental setups – for example, high-fat diet fed to rats v. WSD fed to mice. However, Mattace et al.\(^{60}\) also found a marked protection of animals against the induction of iNOS in the liver. Results of Endo et al.\(^{39}\) further indicated that a dietary supplementation of butyrate-producing probiotics probably resulting in increased formation of butyrate in the intestine attenuates the induction of SREBP-1c mRNA in the liver. In our study, despite not affecting mRNA expression of SREBP-1c in livers of WSD-fed mice, treatment with SoB significantly attenuated the induction of FAS mRNA expression in the liver. Differences between the present study and that of Endo et al.\(^{39}\) might have resulted from differences in study design (e.g. WSD-induced NASH v. CDAA (choline-deficient L-amino-acid-defined diet)-induced NASH and oral feeding of sod v. feeding of butyrate-producing probiotics). Furthermore, Endo et al.\(^{39}\) determined SREBP-1c protein levels in nuclear extracts, whereas in the present study we only determined mRNA expression. Indeed, it has been shown before that SREBP-1c is regulated not solely at the level of expression but also through cleavage of its membrane-bound precursor\(^{40}\). Therefore, it might be that the lack of response of SREBP-1c mRNA expression in livers of WSD + SoB-fed mice despite the down-regulation of FAS mRNA expression in livers of these mice might have resulted from a regulation of this nuclear factor at the level of protein activation rather than a regulation at the level of transcription. Taken together, not only the results of our study as well as those of other groups\(^{6,9}\) suggest that butyrate may protect mice and rats from the development of liver damage; however, molecular mechanisms involved not only in the protection against the induction of iNOS and lipid peroxidation as well as in the down-regulation of FAS remain to be determined.

**Conclusion**

Taken together, our data suggest that an oral supplementation of SoB at least partially protects mice from the development of dietary-induced NASH. Our data further suggest that these protective effects not primarily resulted from a protection
against the enhanced intestinal permeability and associated translocation of bacterial endotoxins found in mice with NASH but rather resulted from an inhibition of the induction of iNOS and subsequently a decreased formation of reactive oxygen species and induction of dependent signalling cascades in the liver. However, additional studies are necessary to (1) further explore underlying mechanism and to (2) determine whether similar beneficial effects of an oral supplementation of SoB are found also in humans.

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Supplementary material

For supplementary material/s referred to in this article, please visit http://dx.doi.org/10.1017/S0007114515003621

References


