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Effects of long term soluble vs. insoluble dietary fiber intake on high fat diet induced obesity in C57BL/6J mice

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Abstract

Although most of the proposed beneficial effects of fiber consumption have been attributed to viscous and gel-forming properties of soluble fiber, it is mainly insoluble cereal fiber and whole grains that are strongly associated with reduced diabetes risk in prospective cohort studies, indicating that other unknown mechanisms are likely to be involved.

We performed a long-term study investigating potential protective effects of adding soluble guar fiber (10% w/w) vs. insoluble cereal fiber (10% w/w) to an isoenergetic and macronutrient matched high-fat diet (control) in obesity prone C57BL/6J mice. After 45 weeks, mice fed soluble vs. insoluble fiber showed significantly increased body weight (41.8 ± 3.0 vs. 33.6 ± 1.5 g, $P = 0.03$), and markers of insulin resistance were increased. In mice fed soluble fiber, energy loss via the feces was significantly lower and colonic fermentation with production of short chain fatty acids (SCFA) was markedly increased. Gene expression analysis in white adipose tissue showed significantly increased levels of the fatty acid target G-protein coupled receptor-40 (Gpr40) in soluble fiber fed mice. Liver gene expression in the insoluble fiber group showed a pattern consistent with increased fatty acid oxidation. The present results show that soluble vs insoluble dietary fiber added to a high-fat, high-fat Western-style diet differently affected body weight and insulin sensitivity in obesity prone mice. Soluble fiber intake with increased SCFA production significantly contributed to digested energy, thereby potentially outweighing the well known short-term beneficial effects of soluble fiber consumption.

Introduction

Recent meta-analyses of prospective cohort studies report markedly reduced diabetes risk in subjects consuming diets high in insoluble cereal dietary fiber and whole grains [1, 2]. In contrast, and surprisingly, there is no strong support that soluble viscous fibers from fruits and vegetables play a key role in this context [1-3], although most of the proposed protective mechanisms of fiber consumption are either more likely to be relevant with soluble fiber, or they are shared by soluble and insoluble fiber [3]. For instance, viscous and gel-forming properties of soluble fibers are involved in hindering of macronutrient absorption, slowing of gastric emptying, reducing postprandial glucose responses, and reducing total and LDL cholesterol levels. Colonic fermentation of naturally available high fiber foods with the production of short chain fatty acids (SCFA) can also be mainly attributed to soluble fiber consumption, whereas effects on inflammatory markers [4] and moderate weight loss due to low energy density and increased satiety have been reported to be comparable with both soluble and insoluble fiber [3, 5]. Therefore, it is likely that further mechanisms are involved in conveying reduced diabetes risk after long term insoluble fiber intake. These might include improved whole-body insulin sensitivity, as recently shown in short-term randomized controlled intervention studies [6-8] and several cross-sectional studies in humans [9]. However, no causal relationships can be stated from cross-sectional studies, and long term dietary interventions in humans face the problem of controlling confounding factors and maintaining dietary adherence [10]. Difficulties investigating signalling pathways in the liver are another problem in human studies. Several relatively short-term studies in animal models reported soluble fiber such as guar gum or psyllium being superior in improving insulin sensitivity, in comparison to insoluble cellulose [11, 12]. In a study investigating male Wistar rats, short-term feeding with guar gum vs. cellulose or bran had favourable effects on body weight and carbohydrate tolerance. However, and importantly, in the long-term these effects

were absent, with a tendency to reduced body weight and significantly lower pancreatic insulin and glucagon concentrations in the cellulose fed rats after 67 weeks [13]. However, different effects may be observed in other species, and long-term effects of supplementing a Western-style high-fat diet with soluble vs. insoluble fiber in obesity prone mouse models are unknown. Potential fiber induced influences on transcription factors in liver and adipose tissue are of further interest and might provide important insights for the understanding of beneficial effects of fiber consumption. Therefore, in the present study we investigated in C57BL/6J mice whether a long term supplementation of a high-fat, Western-style diet with soluble viscous guar fiber vs. highly purified insoluble cereal fiber differently affects body weight, liver fat, insulin sensitivity, and gene expression of metabolic markers in liver and fat tissue.

Methods and Materials

Animals

The protocol for all animal experiments was approved by the local governmental animal ethic review board (State of Brandenburg, Germany). The animals were kept in accordance with the NIH guidelines for care and use of laboratory animals. After 45 weeks, mice were sedated using ether inhalation and sacrificed by decapitation.

Experiments were performed in adult (16 weeks old) male C57BL/6J mice obtained by Charles River, Germany. Animals were housed individually at a temperature of 22°C with a 12:12-h light-dark cycle in cages with soft wood bedding. Animals were divided into three groups (n = 7 per group) and received three different diets (Table 1) with unlimited access to chow and liquids over 45 weeks. Metabolizable macronutrients of diets were calculated according to the following energy contents: casein 15.7 kJ/g, carbohydrates 16 kJ/g, and fat 38 kJ/g. After the experimental period animals were sacrificed in fed state. Organs were

isolated after rapid preparation. Epididymal white adipose tissue and liver tissue were submerged in nitrogen and immediately stored at -80 °C until further RNA preparation.

Diets

The macronutrient composition of the experimental diets is shown in Table 1. The diet enriched with soluble fiber contained 10% (w/w) guar gum, which is an established model for the investigation of soluble fiber diets (64% soluble fiber, 13% insoluble fiber) (Kumar J Nutr Biochem 2002, Owusu-Asiedu J Anim Sci 2006). The diet enriched with insoluble fiber contained 10% of the insoluble fraction of oat fiber, as previously used [8, 14, 15] providing a mixture of insoluble fibers as found in cereal fiber and whole grain products (3 % soluble fiber, 93 % insoluble fiber (cellulose 70%, hemicelluloses 25%, lignin 3-5%). Food intake rate was recorded every week.

Digestibility of diets

Feces of all animals were collected at week 5 and food intake was recorded for further analysis of energy balance. After drying, energy content of diet samples and feces was determined by bomb calorimetry (IKA C5003, IKA Werke, Germany) and digested energy (defined as diet energy intake (kJ/g) minus energy loss via the feces (kJ/g)) was calculated for all dietary groups. Digestibility of the diet (%) was defined as $[(\text{digested energy (kJ/g)}/\text{diet energy intake (kJ/g)}) \cdot 100]$. Cumulative digested energy was calculated over the experimental period of 45 weeks, by multiplying diet energy intake with digestibility, as measured over one week.

Body composition

Body composition (fat mass, lean mass, and free fluids) was measured every four to six weeks using nuclear magnetic resonance spectroscopy (Mini Spect MQ 10 NMR Analyser Bruker, Karlsruhe, Germany). Body weight was recorded every week.

Hydrogen (H₂) breath test as a marker of colonic fermentation

This test was performed twice in all mice in the fed state between 08:00 and 09:00. In order to collect hydrogen exhalation samples animals were placed individually into 140 ml syringes. After 1 min of equilibration 2 samples of 30 ml air breath were drawn of for further analysis. Air breath samples were collected and H₂ concentrations were measured using a breath hydrogen analyser (Quinton Model-12i-Microlyzer; Quintron Instruments, Milwaukee, USA).

Analysis of hepatic triacylglycerol

Frozen liver tissue was ground in liquid nitrogen to a homogenous powder. 100 mg of tissue was homogenized in 5 ml of 10 mM sodium phosphate buffer containing 1 mM EDTA and 1% polyoxyethylene 10 tridecylethan, using an Ultra-Turrax (IKA Werke, Germany). Samples were centrifuged (10 min, 20.000 g) and the supernatant was incubated at 70 °C for 5 min. Triacylglycerols (triglyceride reagent, SIGMA) and protein (DC protein assay, Bio-Rad) levels were analysed in triplicates.

Insulin tolerance test

Insulin sensitivity was estimated after 45 weeks of dietary intervention in fed mice by intraperitoneal (i.p.) injection of insulin (0.75 IU/kg body mass, Actrapid®, Novonordisk) as described previously [16]. There was a six days period between ITT and sacrifice of the animals. Glucose concentrations were measured in tail blood at 0, 15, 30, and 60 min after insulin injection.

Glucose tolerance test

Glucose tolerance tests were performed by intraperitoneal glucose injection, as described (Isken Horm Metab Res 2006). Plasma was collected before and 10, 30, and 60 min after glucose challenge and immediately frozen at -80°C for measurement of glucose and insulin.

Plasma analyses

Animals were investigated in the over-night fasted state. Mouse plasma insulin levels were measured by ELISA for rat insulin using a mouse insulin standard (both from Crystal Chem Inc., Chicago, Illinois, USA), as described [17]. Blood was obtained from the retro-orbital sinus during anaesthesia using Isoflurane® (Baxter, Unterschleissheim, Germany). Plasma glucose, plasma triacylglycerols, and plasma total cholesterol were measured using commercial kits (glucose HK 125; triacylglycerols and total cholesterol: ABX Pentra, Montpellier France), by using an autoanalyzer (Cobas Mira S, Hoffmann La Roche, Switzerland).

RNA extraction and real time RT-PCR

Total RNA was extracted from liver and epididymal white fat tissue of animals in non fasted state by RNeasy lipid tissue kit® (QIAGEN GmbH, Germany). DNA digestion was performed using RNase free DNase® (QIAGEN GmbH, Germany). Total RNA (1 µg) was reverse-transcribed to first-strand cDNA using high capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to manufacturer's protocols. Each cDNA sample was applied at a concentration of 0.626 ng of total RNA to the qRT-PCR assay wells, using optical 384-well plates, and labelled with Power SYbr® Green master mix (Applied Biosystems, Germany). All samples were measured in triplicates, and non template controls were used to confirm specificity. The quantity of target and the housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) were calculated according to a

standard curve. In liver tissue, expression of forkhead transcription factor Foxa2, Ppar γ coactivator β (Pgc-1 β), L-carnitine palmitoyl transferase1 (Cpt-1), acyl-CoA oxidase (Aox), diacylglycerol acetyltransferase-2 (Dgat-2), uncoupling protein2 (Ucp-2), and peroxysome proliferator-activated receptor- α (Ppara) were measured. Expression of G protein-coupled receptors 40, 41, and 43 (Gpr40 (free fatty acid receptor (Ffar-1)), Gpr41 (Ffar-3), and Gpr43 (Ffar-2)) was analysed in white adipose tissue.

The oligonucleotide specific primers were:

Hprt:

up5'-CAGTCCCAGCGTCGTGATTA-3', lo5'-AGCAAGTCTTTCAGTCCTGTC-3',

Foxa2:

up5'-GCGGCCAGCGAGTTAAAGTAT-3', lo5'-TCATTCCAGCGCCCACATA-3',

Pgc-1 β :

up5'-ATGAAGGCGACACACCATCCT-3', lo5'-TGCCATCCACCTTGACACAAG-3',

Cpt-1:

up5'-CCTGCATTCCTTCCCATTG-3', lo5'-CCCATGTCCTTGTAATGTGCG-3',

Aox:

up5'-TACTTGAATGACCTGCCGAGC-3', lo5'-GCAGCAATTTCTACCAATCTGG-3',

Dgat-2:

up5'-CCAAGAAAGGTGGCAGGAGAT-3', lo5'-GCAGGTTGTGTGTCTTCACCA-3',

Ucp-2:

up5'-CCAACAGCCACTGTGAAGTTCC-3', lo5'-TGACTCTCCCCTTGGATCTGCA-3',

Ppara:

up5'-CAGTGCCCTGAACATCGAGTGT-3', lo5'-TTCGCCGAAAGAAGCCCTT-3',

Gpr40:

up5'-TGGCTAGTTTCATAAACCCGG-3', lo5'-TCCCAAGTAGCCATGGACCAGT-3'

Gpr41:

up5'-CTTGTATCGACCCCCTGGTTTT-3', low5'-GCTGAGTCCAAGGCACACAAGT-3'

Gpr43:

up5'-TG TTCAGTTC CCTCAATGCCA-3', lo5'-CAGGATTGCGGATCAGTAGCA-3'

Statistical analysis

Quantitative data are presented as means \pm SEM. Data were analysed using one-way ANOVA with Bonferroni post hoc test, or two-tailed Student's t test for unpaired samples (SPSS 14, Chicago, USA). Not normally distributed data were calculated using non parametric Mann Whitney U-test. Six of seven mice in the fiber groups, but only three of seven mice in the control group reached the end of the intervention in week 45, indicating that minimum fiber contents in mouse diet are essential. Therefore, because of low number of animals in the control group further analysis was restricted to comparisons between fiber groups. $P < 0.05$ was considered significant.

Results

Different effects of soluble guar fiber vs. insoluble cereal fiber on body weight gain and insulin resistance in mice fed a Western-style diet

Fig. 1A shows changes in body weight during the experiment. The observed drops in body weight at weeks 9/10 and 15/16 were induced by overnight fasting procedure for the performance of GTTs, with AUC glucose showing no difference between soluble and insoluble fiber fed animals ($P = 0.68$ and $P = 0.4$, respectively). After 45 weeks, mice fed the soluble fiber diet showed significantly increased body weight, in comparison to mice fed the insoluble fiber diet (41.8 ± 3.0 g 33.6 ± 1.5 g, $P = 0.03$). Increased body weight was accompanied by a tendency to increased body fat (Fig. 1B, $P = 0.14$). The exact body composition (free fluid, lean mass and fat mass) in week 43 is shown in Fig 1C. In agreement

with the observed change in body weight, measurements of insulin sensitivity after i.p. insulin administration at week 45 were significantly lower after soluble vs. insoluble fiber consumption (Fig. 2; $P = 0.044$). AUC tended to be lower in insoluble fiber fed animals (298.8 ± 26.2 vs. 344.5 ± 15.8 mmol x min, $P = 0.19$). No significant differences were detected in fasting concentrations of plasma cholesterol, plasma triacylglycerols, plasma glucose, and plasma insulin (data not shown).

Increased energy digestion with soluble vs. insoluble fiber

For analysis of digestibility, fecal excretion and food uptake were recorded at week 5 for further analysis of digested energy. Energy content of the respective diets measured in a calorimeter was 19.5 kJ/g in the fiber free control group, 18.8 kJ/g in the group fed with soluble fiber and 17.6 kJ/g in the group fed with insoluble fiber. Food intake was multiplied with these values. Fig. 3A shows no significant difference in diet energy intake during a one week period. However, fecal excretion was significantly increased with the insoluble fiber diet, both compared to soluble fiber ($P < 0.0001$) and fiber free control ($P < 0.0001$) (Fig. 3B). The difference in feces weight between soluble fiber and fiber free control was also significant ($P = 0.0002$). Energy content in feces was significantly higher both in soluble fiber fed mice ($P < 0.0001$) and in insoluble fiber fed mice ($P < 0.0001$), as compared to the fiber free control group, but not significantly different between the fiber groups ($P = 0.16$).

Digestibility of the diets was: 94% with soluble fiber, 89% with insoluble fiber, and 97% with fiber free control. Digested energy between groups was not significantly different in the short term (Fig. 3D), as measured over one week (soluble fiber 365.0 ± 8.0 vs. insoluble fiber 340.8 ± 10.8 kJ/week, $P = 0.1$). However, long term cumulative energy digestion over 45 weeks was significantly different between soluble and insoluble fiber consumption (AUC 350577 ± 3919 kJ/mouse vs. 316367 ± 6569 kJ/mouse, $P = 0.0025$) (Fig. 3E).

Increased colonic fermentation with soluble fiber

Colonic fermentation of each diet was estimated by using hydrogen breath tests at week 4 (Fig. 4A). As could be expected, hydrogen exhalation was significantly higher in mice fed the soluble fiber diet, both in comparison with mice fed insoluble fiber ($P = 0.002$) or fiber free control ($P = 0.003$).

Fiber-induced changes in adipose tissue mRNA expression of G-protein coupled receptors

The G-protein coupled receptor (Gpr) family Gpr40, Gpr41, and Gpr43 shares approximately 30% identity among members [18] and has recently been shown to be specifically activated by SCFA [18, 19]. In agreement with significantly increased colonic fermentation in mice fed soluble vs. insoluble fiber, expression in white adipose tissue of Gpr40 was significantly increased after 45 weeks of dietary intervention ($P = 0.042$), (Fig. 4B). Gpr41 was not detectable in adipose tissue, as also observed by others [20]. No significant differences were observed in Gpr43 (Fig. 4B, $P = 0.47$).

Expression of hepatic transcription factors of fat metabolism

After 43 week hepatic triacylglycerol contents tended to be higher in mice fed with soluble fiber (Fig. 5A), $P = 0.11$. We further investigated fiber induced changes of factors involved in the regulation of liver fat metabolism. Foxa2 is an important transcription factor in liver and fat tissue, regulating e.g. beta oxidation, triacylglycerol synthesis, and fat cell differentiation [21]. After intervention with insoluble fiber expression of Foxa2 was significantly increased, as compared with the soluble fiber fed animals (Fig. 5B, $P = 0.031$). In agreement with this, there was also significantly increased expression of Pgc-1 β (Fig. 5B, $P = 0.006$), which is another transcription factor known to be recruited by Foxa2. Expression of key enzymes of beta-oxidation and triacylglycerol synthesis was further analysed. Dgat-2 was significantly increased in liver tissue of mice fed with insoluble fiber ($P = 0.045$), whereas Cpt-1 ($P =$

0.07), Aox ($P = 0.37$), and Uxp-2 ($P = 0.12$) were comparable between groups (Fig. 5B). The expression of Ppara α , which plays a key role in fatty acid catabolism was significantly higher in insoluble fiber fed animals ($P = 0.042$).

Discussion

A high fiber intake is emphasised in the recommendations of most nutritional and diabetes associations. Although most of the proposed beneficial effects of fiber consumption have been attributed to viscous properties of soluble, fermentable sorts of fiber, results from prospective cohort studies consistently show that insoluble cereal fiber and whole grain consumption, but not soluble fiber intake is strongly linked to reduced risk of diabetes [1-3, 22]. We present novel findings showing that long-term supplementation of a Western-style diet with soluble, highly fermentable guar fiber vs insoluble, moderately fermentable cereal fiber resulted in an obese phenotype in C57BL/6J mice. Weight gain and insulin resistance were significantly different between groups, although dietary energy intake was comparable. Mice in the present study had free access to chow. Therefore, significantly lower energy loss via the feces in the soluble fiber group together with comparable dietary energy intake in all mice suggests that more metabolizable energy was extracted from the soluble, highly fermentable fiber diet, without affecting satiety and feeding behaviour. This is supported by earlier studies showing that fiber induced increases of SCFA do contribute to energy intake both in rodents and humans [23]. Interestingly, colonization of germ-free gnotobiotic mice with a prominent saccharolytic member of the normal human gut microbiota together with the dominant human methane producing germ results in markedly improved colonic fermentation and is associated with an obese phenotype in the host [3, 24]. As could be expected, H₂-measurements indicated that colonic fermentation with the production of SCFA was largely increased in mice consuming soluble fiber, but not in animals ingesting insoluble cereal fiber. Fiber induced increases of SCFA production are commonly assumed to be beneficial, e.g. by reducing hepatic glucose output and beneficially affecting blood lipid profiles [25, 26]. Increased insulin sensitivity after relatively short term consumption of highly fermentable resistant starch has also recently been shown [7]. However, both highly fermentable insoluble

resistant starch [7, 27] and only moderately fermentable insoluble cereal fiber [8, 14] increased insulin sensitivity in humans, indicating that a dose-dependent relation between fermentability of dietary fibers and insulin sensitivity is unlikely [3]. Further, available studies indicate that SCFA could contribute to increased de novo lipogenesis [24], probably by stimulating adipogenesis through Gpr43 [18], although in the present study Gpr43 expression was not significantly increased in fermentable fiber fed mice. However, expression of Gpr40 representing another recently identified target of fatty acids including SCFA [19] that is mainly expressed in pancreas and brain, but also in monocytes and in adipose tissue, was significantly increased in the soluble fiber group. Loss of Gpr40 protects mice from obesity-induced hyperinsulinemia, hepatic steatosis, increased hepatic glucose output, hyperglycemia, and glucose intolerance [28]. Vice versa, increased Gpr40 in soluble fiber fed mice could provide a potential mechanism contributing to the observed obese phenotype in these animals. A further novel finding of the present study was liver gene expression in the insoluble fiber group showing a pattern consistent with increased fatty acid oxidation. Mice fed insoluble fiber showed significantly increased expression levels of Foxa2 and Pgc-1b in comparison to soluble fiber fed animals. These transcription factors are known to play an important role in the regulation of hepatic lipid homeostasis. Co-expression of Foxa2 and Pgc-1b has been reported to concomitantly increase gene expression of key enzymes of mitochondrial beta-oxidation [29]. Significantly increased expression levels in liver of Ppara and Dgat-2 in insoluble fiber fed mice in the present study were in good agreement with these findings, thus potentially contributing to increased beta-oxidation and reduced fat mass in mice fed insoluble cereal fibers. Differences in liver gene expression were likely caused by fiber induced differences in body weight and insulin resistance, although a direct influence of fiber types on liver fat metabolism can not be finally excluded.

It has been suggested that soluble fibers such as guar gum increase postprandial fullness and reduce food intake, e.g. by increasing the viscosity of the bowel content [30]. However, in a

meta-analysis of randomized controlled trials in humans the conclusion was drawn that guar gum was not efficacious for reducing body weight [30]. High SCFA production by the gut microbiota might increase available energy, thereby potentially outweighing the well known short-term beneficial effects of soluble fiber consumption. Further studies are needed to elucidate whether long term consumption of soluble, highly fermentable fiber unfavourably affects energy balance also in humans.

In conclusion, long-term supplementation with soluble guar gum lead to an obese phenotype in obesity prone mice fed a Western-style diet. In contrast, supplementing the same diet with insoluble cereal fiber resulted in significantly lower weight gain and improved insulin sensitivity, and was further associated with a pattern in liver gene expression consistent with increased fatty acid oxidation. Increased energy digestion with soluble, highly fermentable fiber consumption and increased expression of SCFA target genes might unfavourably affect energy homeostasis in the long term.

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Legends

Table 1:

Macronutrient composition of experimental diets

Figure 1:

Change in body composition over 45 weeks of dietary intervention in three groups (starting with $n = 7$ per group). (A) Changes in body weight. (B) Body fat contents were measured by NMR and are exemplarily shown at weeks 0, 15, 27, 35 in animals fed soluble vs. insoluble fiber. (C) Body composition measured by NMR after 45 weeks.

Figure 2:

Increased insulin resistance under diet with soluble vs. insoluble fiber. Glucose levels after i.p. insulin administration during insulin tolerance test in fed state after 45 weeks of intervention in mice fed soluble vs. insoluble fiber ($n = 6$ per group), * indicates P -value < 0.05 .

Figure 3:

Energy digestion during one exemplary week, and cumulative energy digestion during whole experimental period. (A) Diet energy intake at week 5 in groups of animals fed soluble fiber, insoluble fiber, and fiber free control ($n = 7$ per group). (B) Feces excretion during week 5 of intervention. (C) Feces energy content in the three treatment groups. (D) Energy digestion at week five in the dietary intervention groups. (E) Extrapolated cumulative digested energy calculated from diet energy intake over 45 weeks of treatment. Head down arrows indicate

significant differences between soluble vs. insoluble fiber fed mice. * indicates P-value < 0.05, ** indicates P-value < 0.01, *** indicates P-value < 0.001.

Figure 4:

Marker of colonic fermentation. (A) Hydrogen breath test in week 4 in mice fed soluble fiber, insoluble fiber, and fiber free control (n = 7 per group). (B) Real-time RT-PCR analysis of SCFA targets Gpr40 and Gpr43 in epididymal white adipose tissue after 45 weeks of intervention in mice consuming diets supplemented with soluble vs. insoluble fiber. Results were normalized to internal control HPRT and the intervention group fed soluble fiber was set to 100% (n = 5-6 each group). * indicates P-value < 0.05, ** indicates P-value < 0.01.

Figure 5:

Hepatic triacylglycerol contents and real-time RT-PCR analysis of transcription factors and enzymes regulating liver fat metabolism in fed state.

(A) Hepatic triacylglycerol contents after 45 weeks (n = 6 per group). (B) RT_PCR results were normalized to internal control HPRT and the intervention group fed soluble fiber diet was set to 100%. Foxa-2, Pgc-1 β , Cpt-1, Aox, Dgat-2, Ucp-2, and Ppara α were measured in liver tissue of mice fed soluble vs. insoluble fiber (n = 5-6 per group). * indicates P-value < 0.05, ** indicates P-value < 0.01

Table 1: Macronutrient composition of experimental diets

	without fiber	soluble fiber	insoluble fiber
<i>Diet (g/kg)</i>			
Casein ^a	222	200	200
Amylopectin ^b	433	390	390
Sucrose ^c	56	50	50
Palm kernel fat ^d	189	170	170
Thistle oil ^e	11	10	10
Linseed oil ^f	11	10	10
Guar fiber ^g		100	
Insoluble fraction of oat fiber ^h			100
Mineral mixture ⁱ	56	50	50
Vitamin mixture ^j	22	20	20
<i>Calculated macronutrient metabolizable energy (%)</i>			
Protein	18	18	18
Carbohydrates	41	41	41
Fat	41	41	41

^a Dauermilchwerk Peiting GmbH, Landshut Germany

^b National Starch and Chemical GmbH, Hamburg, Germany

^c EUCO GmbH, Hamburg, Germany

^d Kölln KGaA, Elmshorn, Germany

^e EUCO GmbH, Hamburg, Germany

^f Kunella-Feinkost GmbH, Cottbus, Germany

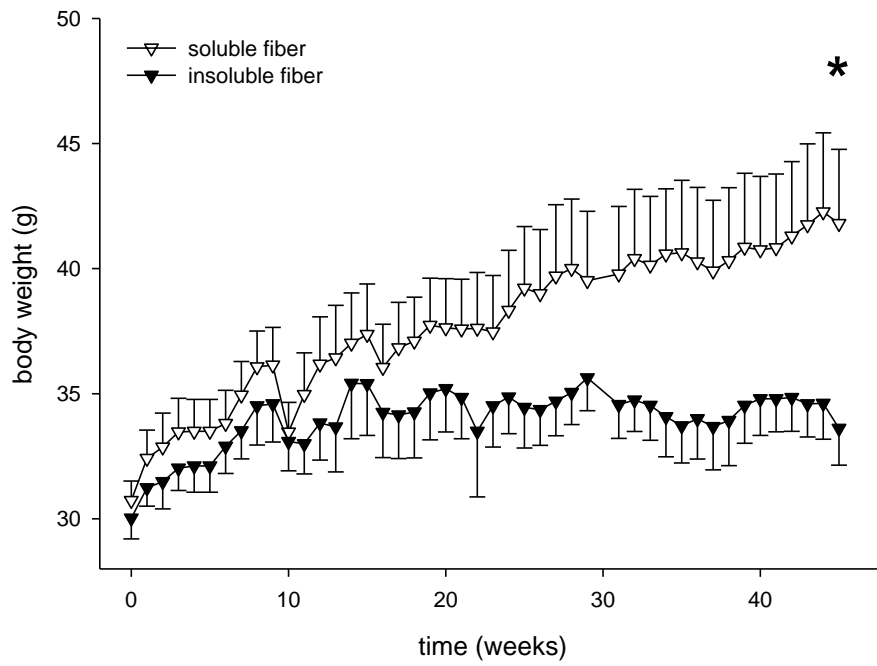
^g Roeper GmbH, Hamburg, Germany

^h Rettenmeier, Ellwangen, Germany

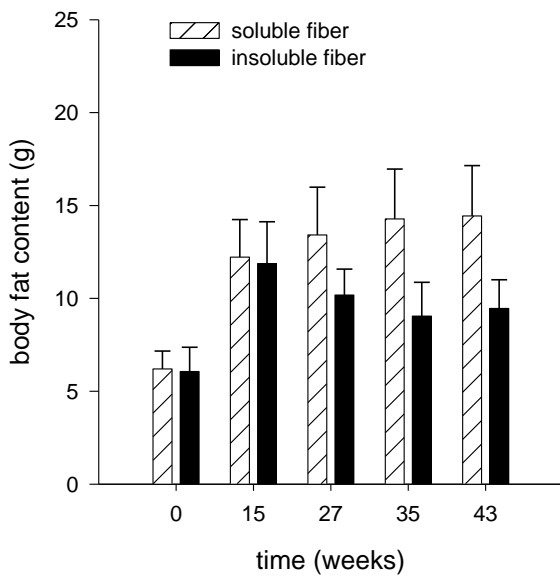
^{i+j} Altromin GmbH, Lage, Germany

Figure 1

A



B



C

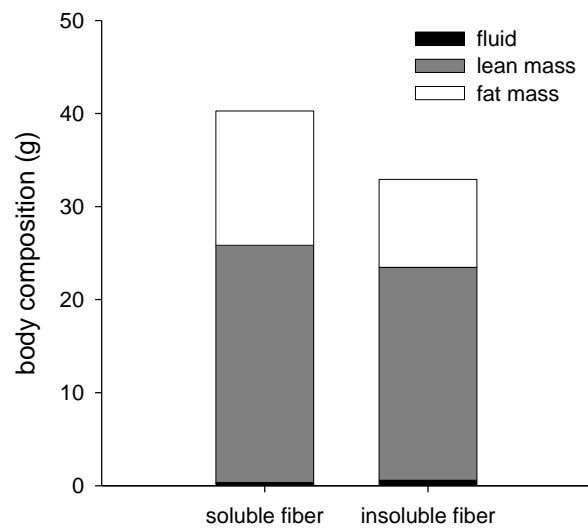


Figure 2

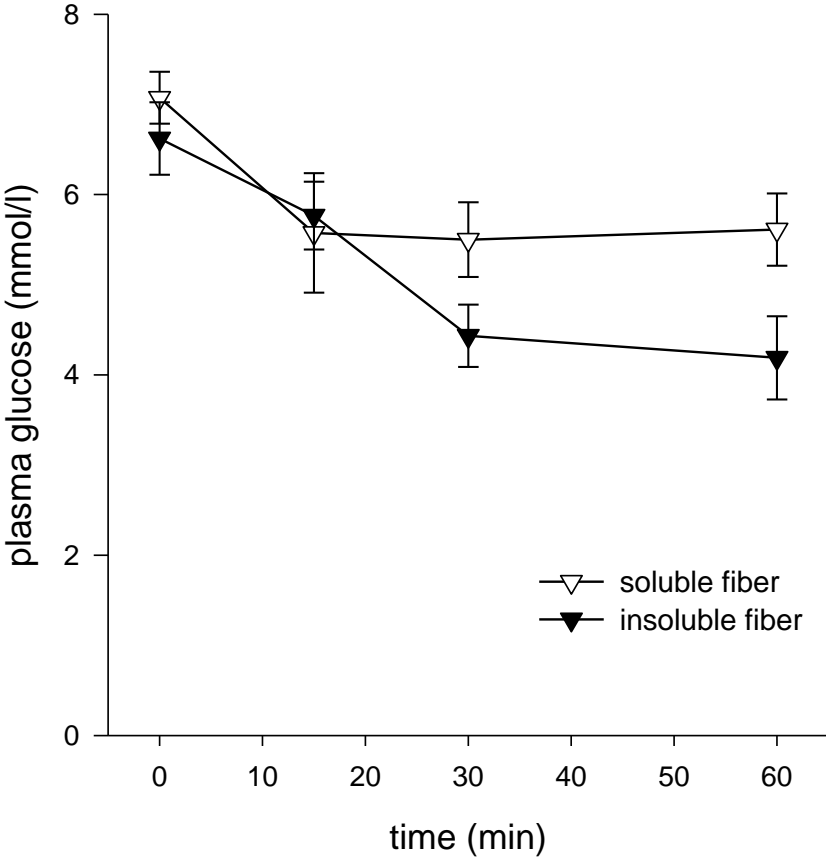


Figure 3

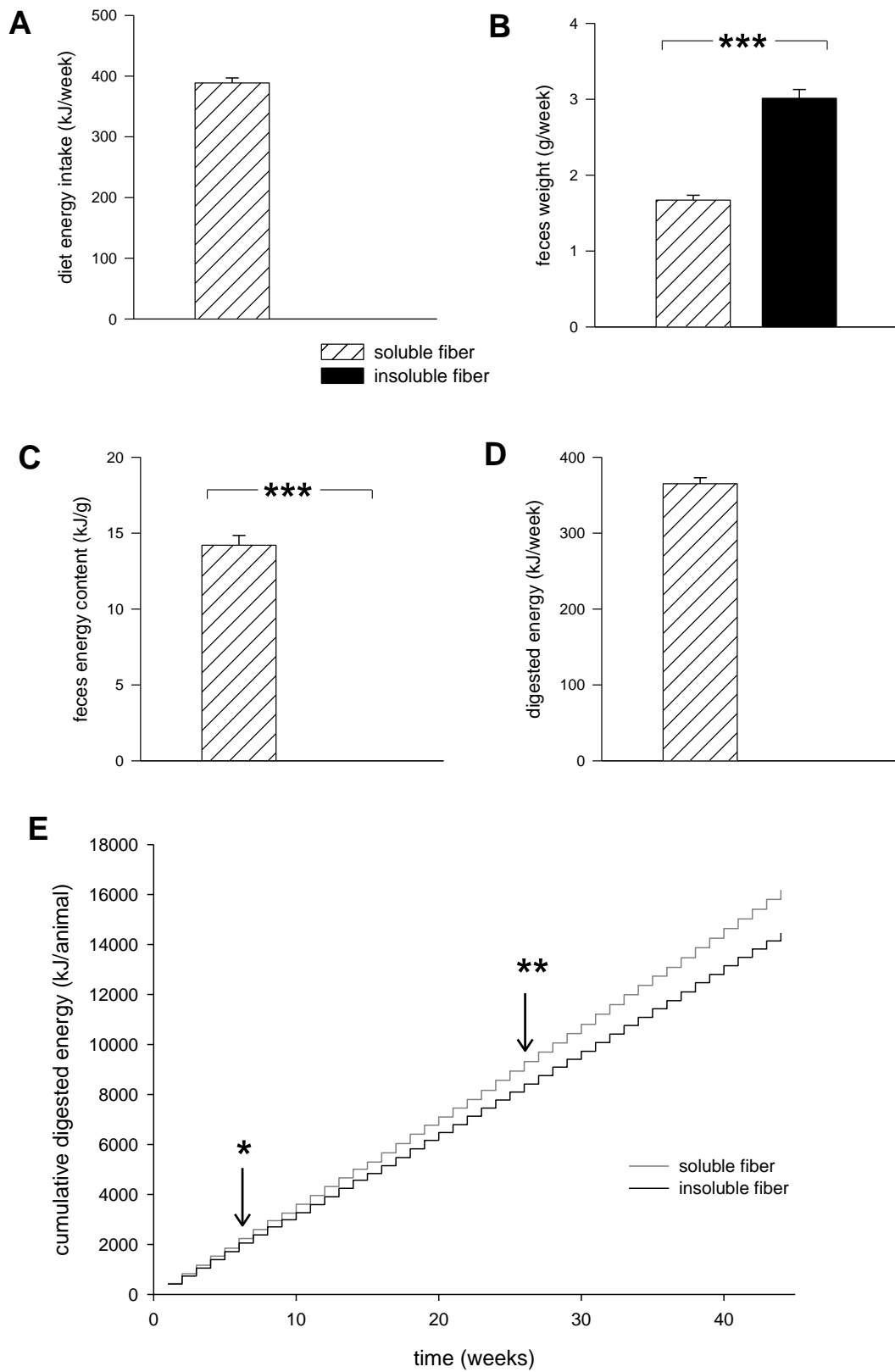


Figure 4

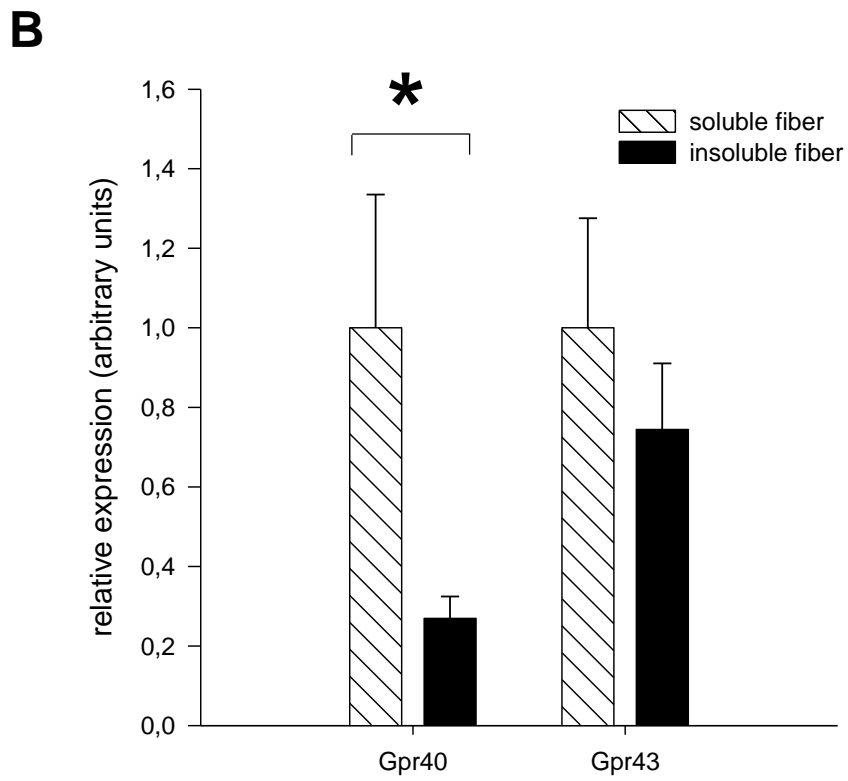
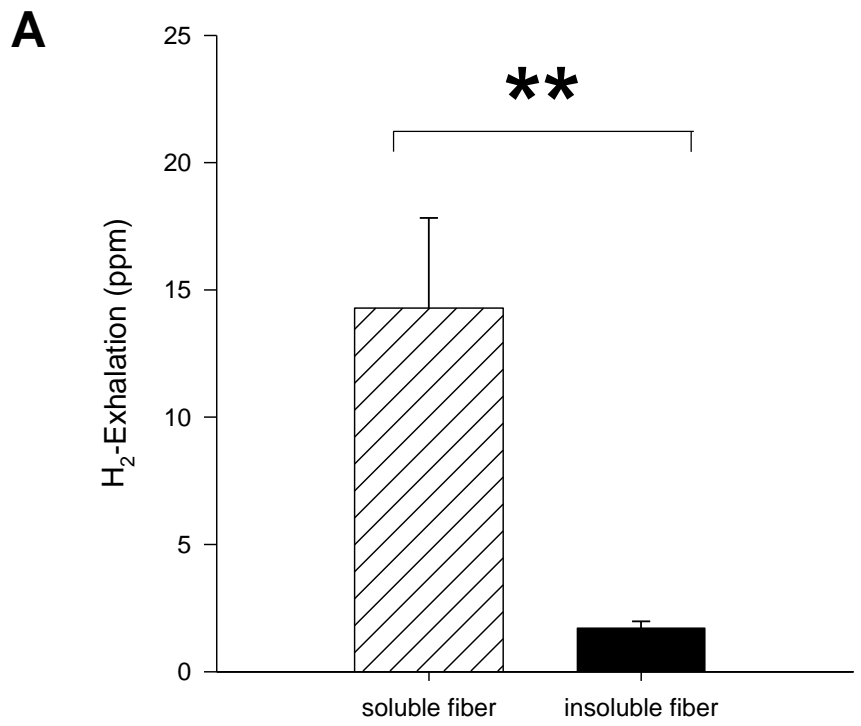


Figure 5

