Dietary supplementation with chitosan derived from mushrooms changes adipocytokine profile in diet-induced obese mice, a phenomenon linked to its lipid-lowering action

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A R T I C L E   I N F O

Article history:
Received 26 January 2009
Received in revised form 27 February 2009
Accepted 27 February 2009

Keywords:
Adipocytokine
Adiposity
Chitosan
Mushrooms
Obesity

A B S T R A C T

Recent data reported that chitosan reduces high-fat (HF) diet-induced obesity in mice without describing the metabolic consequences of such an effect. The aim of this study was to investigate the capacity of chitosan derived from edible mushrooms to modify adipocytokine levels and to assess the relevance of this effect on the development of fat mass, and on glucose and lipid metabolism in obese mice. Mice were fed a HF diet or a HF diet supplemented with 5% fungal chitosan for ten weeks. HF-induced hypertriglyceridaemia, fasting hyperinsulinaemia and fat accumulation in liver, muscle and white adipose tissue (WAT) were reduced after chitosan treatment. The higher lipid content in the caecum following treatment with chitosan suggested that this dietary fibre reduced lipid absorption. We postulated that the lower triglyceridaemia observed upon chitosan treatment could also be the result of the lower FIAF (fasting-induced adipose factor) expression observed in visceral adipose tissue. IL-6, resistin and leptin levels decreased in the serum after chitosan treatment. The higher lipid content in the caecum following treatment with chitosan suggested that fungal chitosan counteracts some inflammatory disorders and metabolic alterations occurring in diet-induced obese mice since it decreases feed efficiency, fat mass, adipocytokine secretion and ectopic fat deposition in the liver and the muscle.

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1. Introduction

Obesity, diabetes and cardiovascular diseases, clustered in a single word “the metabolic syndrome”, have emerged as a major health problem. Evidence has accumulated suggesting that, as a result of adipocyte hypertrophy during the development of obesity, white adipose tissue (WAT) function is compromised. Obesity changes the morphology and composition of WAT, leading to alteration in the production and secretion of specific proteins. Leptin, resistin, plasminogen activator inhibitor-1 (PAI-1), monocyte chemoattractant protein-1 (MCP1) and proinflammatory cytokines such as IL-6 and TNF-α are adipocytokines thought to provide an important link between obesity and related inflammatory and metabolic disorders [1–3]. The fasting-induced adipose factor (FIAF) — also known as angioptoein-like protein 4 — has recently been considered as an interesting adipocytokine since it acts as a powerful signal governing plasma lipid levels through its capacity to inhibit lipoprotein lipase (LPL) activity [4,5]. Moreover, it is well established that obesity causes not only hypertriglyceridaemia, but also peripheral insulin resistance which leads to hyperinsulinaemia [6].

Obesity also provokes structural and metabolic alterations in other organs, including skeletal muscle and liver. Nowadays, abnormal fat storage in the liver and in the muscle is considered as a major risk factor for the development of insulin resistance [7–12]. Identifying the link between excess body fat and impaired insulin sensitivity in the liver and muscle, has been difficult. It has recently been suggested that adipocytokines act as regulators of fatty acid metabolism, especially in the skeletal muscle [10].

A prime therapeutic approach to prevent and treat metabolic syndrome involves lifestyle changes. Increasing the intake of dietary fibres could play an interesting role in the management of metabolic syndrome. Their health promoting effects in this context may involve different mechanisms (e.g. decreases in blood lipids, modulation of inflammation, satiating effect) related to their food origin, their specific chemical structure and physical properties, and /or to their fermentation in the gut [13,14]. The discovery of novel sources of dietary fibres is thus interesting, but requires adequate studies in order to study the physiological targets modulated by specific dietary fibres.
In recent years, the consumption of mushrooms, either as whole mushrooms or extract supplements has increased. Mushrooms contain substances — mainly polysaccharides, polysaccharopeptides and polysaccharide proteins — with antitumour, antiviral and antibacterial properties [15,16]. However, the exact components/nutrients responsible for the potential health benefits of white mushrooms are still to be determined [16,17]. Chitin is well known as the main component of insect and crustacean cuticle, but interestingly, chitin combined with beta-glucan is the main component of the fungal cell exoskeleton. For the purpose of this study, we selected a deacetylated form of chitin — i.e. chitosan — extracted from white mushroom exoskeleton (Agaricus bisporus), with specific molecular weight and N-acetyl-D-glucosamine/D-glucosamine ratio. This so-called “fungal chitosan” has recently obtained a substantial equivalence to shellfish derived chitosan in Europe, and is consequently authorized for use in dietary supplements (http://ec.europa.eu/food/food/biotechnology/novelfood/notif_list_en.pdf).

Fungal chitosan shares characteristics with dietary fibre, namely its resistance to mammalian digestive enzymes. Although some controversy still exists about the clinical relevance of chitosan for treatment against obesity in humans [18,19], several studies in animals and humans have reported that chitosan reduces both body weight and cholesterol [20–24]. The mechanism remains to be studied.

A HF carbohydrate-free diet is a classic nutritional model leading to disorders associated with obesity. The central and early pathological consequences of HF feeding include the development of insulin resistance [25,26]. The mechanisms impairing this function have not been fully identified but they could be related to inflammation [27], lipotoxicity [12,28], and chronic hyperinsulinaemia [29]; phenomena primarily affecting the liver [30] and the muscles [31].

The aim of the present study was to investigate the capacity of fungal chitosan (i) to counteract the alteration of several adipocytokines linked to the development of fat mass and (ii) to modulate glucose and lipid metabolism in HF diet-induced obese mice. As a consequence of the close relationship between these effects, this study was aimed to test the effect of fungal chitosan intake on metabolic disorders associated with obesity.

2. Animals and methods

2.1. Animals and diet

Sixteen male C57Bl6/J mice (9 week old at the beginning of the experiment, Charles River Laboratories, France) were housed in a controlled environment (12-hour daylight cycle, light out at 6pm) with free access to food and water in groups of 4 per cage. After one week for acclimatisation, the mice were divided into 2 groups (n = 8/group): HF group were fed with a HF diet containing 49.5% fat — corn oil and lard — (g/100g of total dry diet), 37% protein, 10% cellulose, 3.5% mineral/vitamin mixture. The energy content of the HF diet was 72% of fat, 28% protein, and <1% carbohydrates (UAR, Epinay-sur-Orge, France) and HF+chitosan group received a mix of 95% HF diet and 5% chitosan derived from the exoskeleton fungi (KiOUnutrine-C™ from KitoZyme sa, Belgium). In fact, we have tested the dose that had been used by other authors, and that was able to modify lipid metabolism in rats [21,32,33]. After 5 weeks of treatment, 60 µl of blood was collected from the tip of the tail vein in order to assess in parallel plasma glucose, triglycerides, NEFA and cholesterol concentration. The insulin resistance index was calculated by multiplying the area under the curve for glucose, and the area under the curve for insulin, calculated from — 30 min until 15 min after glucose challenge.

2.2. Oral glucose tolerance test (OGTT)

After 8 weeks of treatment, an oral glucose tolerance test was performed on 6 h-fasted mice. Glucose was administered orally (3 g/kg of glucose, 66% glucose solution) and blood glucose determined using a glucose meter (Roche Diagnostics) on 3.5 µl of blood collected from the tip of the tail vein both before (− 30 min and 0 min) and after glucose load (15, 30, 60, 90, and 120 min). Twenty µl of blood was sampled 30 min before and 15 min after the glucose load to assess plasma insulin concentration. The insulin resistance index was calculated by multiplying the area under the curve for glucose, and the area under the curve for insulin, calculated from — 30 min until 15 min after glucose challenge.

2.3. Blood parameters

Plasma insulin concentration was determined using the ELISA kit (Merckodia, Uppsala, Sweden) on 5 µl of plasma collected from tail blood during OGTT. Plasma glucose, triglycerides, cholesterol, NEFA and β-hydroxybutyrate concentrations were measured using kits combining enzymatic reaction and spectrophotometric detection of reaction end-products (Elitech diagnostics, Sées, France and Stanbio Laboratory, USA). Concentrations of IL-6, PAI-1, MCP-1, resistin, insulin and leptin were determined in 10 µl of plasma using a multiplex immunoassay kit (Mouse Serum Adipokine Lincoplex kit, Linco research, USA) and measured using Luminex technology (Bioplex, Bio-Rad, Belgium). Of note, ELISA kit was used for insulin determination during OGTT performed after 8 weeks of treatment whereas we used the Lincoplex kit to measure insulin in the plasma obtained from the vena cava after 10 weeks of treatment.

2.4. Lipid analysis in caecal content, liver and muscle

Triglycerides and cholesterol were measured in the liver and muscle as previously described for blood samples following an extraction with chloroform-methanol according to Folch et al. [34]. To determine fecal fat, feces was dried at 70 °C and then extracted according to the Folch method. Organic material was dried, weighed, and assigned as total lipid [35]. Dry residues were resuspended in isopropanol and triglycerides, NEFA and cholesterol were then measured as previously described for blood samples. Protein concentration was measured by the Bradford method using bovine serum albumin as standard [36].

2.5. Fat histochemical detection

A fraction of the muscle or the main liver lobe was fixed-frozen in Tissue-tek in liquid nitrogen-cold isopentane. For the detection of neutral lipids, frozen sections were sliced and stained with the oil red O, using 0.5% oil red O dissolved in propylene glycol for 10 min at 60 °C. The sliced sections were then counterstained [27].

2.6. Real-time quantitative PCR

Total RNAs from liver and adipose tissues were prepared using TriPure reagent (Roche Diagnostics, Basel, Switzerland). PCRs were performed using an ABIPrism 5700 Sequence Detection System instrument and software (Applied Biosystems, Foster City, CA, USA) as described before [37]. Data were normalized to RPL19 mRNA (ΔΔCT analysis). Primer sequences for the targeted mouse genes are available on request (audrey.neyrinck@uclouvain.be).
Fig. 1. Body weight evolution (A), body weight gain (B), feed efficiency (C), adipose tissue weights (D), cecal tissue weight (E) and cecal content weight (F) of mice fed a high fat (HF) diet or a HF diet supplemented with 5% fungal chitosan for 10 weeks. * \( P<0.05 \), Student’s \( t \) test.

Fig. 2. Fat staining (with red oil) of liver (A,B) and hind limb muscle (C,D) sections. Mice were fed a high fat (HF) diet (A, C) or a HF diet supplemented with 5% fungal chitosan (B, D) for 10 weeks. Bar = 100 µm.
2.7. Statistical analysis

Results are presented as mean±SEM. Statistical significance of differences was analyzed using Student’s t test. Correlations between parameters were assessed by Pearson’s correlation test (GraphPad Software, San Diego, CA, USA; www.graphpad.com). p<0.05 was considered as statistically significant.

3. Results

3.1. Supplementation with fungal chitosan lowers body weight gain, feed efficiency and fat mass development whereas it induces caecal enlargement

Body weight gain was significantly lower when chitosan was added in the HF diet (Fig. 1A and B). Food intake, taking into account spillage, was recorded twice a week. Total caloric intake was obtained by multiplying total food intake (g) for 4 mice per cage (n = 2) by the caloric value of the diets, i.e. 6 kcal and 5.7 kcal for HF and HF+chitosan, respectively; the total energy intake for 4 mice was then 4868±203 kcal and 4473±25 kcal for HF and HF+chitosan, respectively. The feed efficiency (weight gain divided by calories consumed during the whole treatment) was significantly lower in HF+chitosan treated-mice (Fig. 1C). No modification of liver weight was observed (data not shown). Interestingly, the weight of three WAT (epididymal, visceral and subcutaneous) was systematically lower in the group treated with chitosan (Fig. 1D). We have calculated the index of adiposity as follows: we have summed the weight of the three WAT, multiplied by 100, and divided by the body weight. This index was nearly two fold lower in the liver, the muscle and the serum of HF+chitosan mice, compared to the HF group (Table 1). Lipid contents, mostly triglyceride concentrations decreased in those organs but also in the caecal tissue and caecal content were increased by the addition of chitosan to HF diet (Fig. 1E and F).

3.2. Supplementation with fungal chitosan reduces lipid accumulation in the liver, the muscle and the serum

Fat staining of the tissue showed that lipid accumulation was reduced both in the liver and the muscle after chitosan treatment (Fig. 2). Indeed, triglyceride concentrations decreased in those organs but also in the serum of HF+chitosan mice, compared to the HF group (Table 1). Furthermore, cholesterol concentrations were lower in both the serum and the muscle of chitosan-fed mice (Table 1). Lipid contents, mostly NEFA, were higher in the caecal content (Table 1). We measured [β]-hydroxybutyrate as an index of hepatic [β]-oxidation to explore the shift in lipid metabolism. We demonstrated that adding chitosan to the HF diet increased [β]-hydroxybutyrate in the serum after dietary treatment (Fig. 3A) whereas NEFA were unchanged in postprandial state (Fig. 3B) or after 6 h fasting (Table 1). The level of mRNA coding key enzymes involved in hepatic [β]-oxidation, such as PPARα and carnitine palmitoyl transferase-1 (CPT-I) were unchanged in the liver 10 weeks after chitosan supplementation (data not shown). Furthermore, PPARγ mRNA level in adipose tissue was unaffected following chitosan treatment (data not shown). Interestingly, FIAF mRNA levels in the visceral adipose tissue was decreased by two fold after chitosan treatment (Fig. 4A). FIAF regulates lipoprotein lipase activity, thereby influencing the level of circulating triglycerides. Indeed, in our study, triglyceridaemia was

![Figure 4](image-url)
positively correlated with FIAF gene expression in visceral adipose tissue (Fig. 4B).

3.3. Supplementation with fungal chitosan decreases circulating adipocytokines; a phenomenon related to its anti-obesity properties

Besides having an effect on FIAF mRNA in adipose tissue, chitosan lowered all other analysed plasma adipocytokines (IL-6, MCP-1, PAI-1, resistin and leptin). Its effects were significant for the proinflammatory cytokine IL-6 and two other adipocytokines, namely resistin and leptin (Fig. 5). We performed multiple correlations analyses between those factors (IL-6, MCP-1, PAI-1, resistin and leptin) and adiposity index (Fig. 6) to identify whether the secretion of adipocytokines was related to adiposity. Among adipocytokines tested, leptin and resistin levels significantly and positively correlated with adiposity index. Correlation between the adiposity index and the IL-6 level after chitosan treatment was less straightforward although data showed that both parameters decreased after chitosan treatment.

3.4. Supplementation with fungal chitosan decreased hyperinsulinaemia without improving of hyperglycaemia upon fasting

A chitosan-enriched HF diet did not improve the glucose tolerance compared to HF mice (1789 ± 64 mM 120 min and 1822 ± 83 mM 120 min, respectively; *P* < 0.05, Student’s *t* test). In addition, chitosan did not improve the insulin resistance index occurring upon HF feeding (9605 ± 1700 and 10620 ± 1800 for HF + chitosan and HF mice, respectively; *P* > 0.05, Student’s *t* test). After 10 weeks of treatment, the

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**Fig. 5.** Plasma IL-6 (A), monocyte chemoattractant protein (MCP)-1 (B), leptin (C), resistin (D), plasminogen activator inhibitor (PAI)-1 (E) concentrations in mice fed a high fat (HF) diet or a HF diet supplemented with 5% fungal chitosan for 10 weeks. *P* < 0.05, Student’s *t* test.

**Fig. 6.** Correlation between adiposity index and plasma IL-6 (A), monocyte chemoattractant protein (MCP)-1 (B), leptin (C), resistin (D), plasminogen activator inhibitor (PAI)-1 (E) concentrations; insert corresponds to Pearson’s *r* correlation and *P* value (*n* = 14).
HF-fed mice exhibit a higher fasting glycaemia (>10 mM), this alteration being maintained upon chitosan supplementation (Fig. 7A). However, fasting hyperinsulinaemia was significantly lower in chitosan-fed group (Fig. 7B). Interestingly, fasting insulinaemia was positively correlated with adiposity index (Fig. 7C).

4. Discussion

Several studies reported that chitosan had hypocholesterolaemic effects in different animal models [21,24,38,39]. Sumiyoshi and Kimura also showed that the oral gavage with chitosan (300 mg/kg, twice daily) prevented the increase in body weight, and lowered liver lipids (by 23% and 66% for cholesterol and triglycerides, respectively) induced by a HF diet in mice over a 20-week period [24]. Our results confirm that chitosan from mushrooms — when added in the diet — can limit HF-induced body weight gain. This treatment also significantly decreases the adiposity index. Although anti-obesity effect of chitosan may be partly attributed to the lower caloric intake, the feed efficiency was lower in HF + chitosan mice, i.e. mice gained lower weight for the same calories consumed. Furthermore, we demonstrated that dietary supplementation with chitosan counteracts triglyceride accumulation in the liver (decrease of triglyceride content by 39%) and in the muscle (decrease of triglyceride content by 66%). Fungal chitosan can therefore reduce the ectopic accumulated of lipids (triglycerides) both in the liver and in the muscle tissue. As a matter of fact, the accumulation of intrahepatic and intramuscular triglycerides and a low capacity to oxidise fatty acids have been shown to be correlated to the presence of insulin resistance in those tissues [10–12]. Although we did not observe any improvement of insulin resistance index or glucose tolerance upon oral glucose tolerance test, further studies could aim to investigate insulin-sensitive glucose use and production specifically in the liver and muscle together with assessment of hepatic insulin signalling at the molecular level (Akt, IRS-1, IRS-2, etc).

We also demonstrated that supplementation with fungal chitosan decreased the atherogenic lipid profiles of diet-induced obese mice. Hypocholesterolaemic effects of chitosan have been described by several authors using different animal models (normo- or hypercholesterolaemic rats or mice, apolipoprotein E-deficient mouse model of atherosclerosis) [20,21,38–40]. It is noteworthy that this is the first study showing a significant effect on fasting triglyceridaemia due to chitosan supplementation. We observed a reduction of blood triglycerides by approximately 32% with fungal chitosan in HF diet-induced obese mice. It is known that chitosan has the capacity to counteract dietary lipid absorption [39]. In our study, the analysis of lipids in caecal content, suggests that chitosan lessens lipid absorption in the gastrointestinal tract. This could be linked to its capacity to bind fatty acids [41].

In addition to those observations, we demonstrated an effect of chitosan on ketone body production. The higher level of β-hydroxybutyrate observed in the serum of chitosan-treated mice could suggest a higher hepatic fat β-oxidation. However, the hepatic expression of key enzymes/peptides regulating β-oxidation (CPT-1 mRNA and PPARγ) was not modified when chitosan was added to the diet. Interestingly, we have identified that the mRNA content of FIAF significantly decreased in the visceral adipose tissue of chitosan-treated mice. This effect could not be related to any modification of PPARγ expression, which is sometimes considered as a driver of FIAF expression [5]. Interestingly, a positive correlation between FIAF mRNA and triglyceridaemia could be shown. Considering the significant reduction of WAT weight due to chitosan supplementation, our results suggest a strong activation of LPL activity due to lower FIAF expression in WAT leading to the higher clearance of circulating lipoprotein (VLDL). Indeed, Desai et al. [42] demonstrated that both genetic and antibody inhibition of FIAF resulted in lower triglyceride levels, due to increased VLDL clearance, decreased VLDL production, and slightly lower cholesterol levels. Gut microbiota is an environmental factor that regulates fat storage through modulation of FIAF expression and/or activity [43,44]. Gut fermentation is also translated in mice or rats, by a trophic effect on the caecum; this effect appears a phenomenon of adaptation of the gut microbiota (number of bacteria, production of short chain fatty acids), that contribute to the regulation of host metabolism [45,46]. Therefore, the caecal enlargement (tissue and content) due to supplementation with fungal chitosan, may be an important mechanism explaining modulation of FIAF expression in WAT. This hypothesis deserves further investigation.

In this study, we have shown that several circulating adipocytokines, such as IL-6, resistin and leptin, were decreased after supplementation with fungal chitosan in the HF diet and that the lower resistin and leptin levels were related to lower fat mass development (adiposity index). IL-6 is an important component of obesity-related insulin resistance in the liver, namely through impairment of insulin signalling [47,48]. In rodents, IL-6 may also contribute to hypertriglyceridaemia by stimulating hepatic secretion of triglycerides [49]. However, we did not observe any relationship between triglyceridaemia and serum level of IL-6. Furthermore, the lower leptin concentration observed in the serum of chitosan-treated mice could not be related to the improvement of metabolic alterations, such as the lipid-lowering effect of chitosan. Indeed, leptin is generally believed to have an insulin sensitizing effect. In addition, leptin also contributes to preventing excess lipid accumulation [10]. Finally, resistin is another obesity-related adipocytokine that decreased upon chitosan treatment in HF-fed mice. Despite the controversy regarding the role of resistin in the development of insulin resistance, studies have demonstrated that resistin overexpression — or chronic increase in resistin serum level — in rodents is associated with impaired insulin signalling in skeletal muscle [10]. Supplementation with fungal chitosan counteracts HF-induced fasting hyperinsulinaemia without modifying fasting hyperglycaemia or the insulin resistance index upon OGTT. This would suggest that there is a better insulin response upon fasting, but that chitosan has no effect upon exogenous glucose challenge. Lower hyperinsulinaemia, related to the anti-obesity properties of chitosan (significant relationship between insulinemia and the adiposity index), may help to counteract metabolic...
disorders associated with obesity. Indeed, it has been demonstrated that chronic physiological hyperinsulinaemia (1) induces peripheral but not hepatic insulin resistance with respect to glucose metabolism; (2) causes blood pressure elevation; (3) increases lipogenesis; and (4) is generally accepted as an independent risk factor for atherosclerosis, though it does not cause an elevation in VLDL-triglyceride or a reduction in HDL-cholesterol [29,50].

In conclusion, our results suggest that, besides its action on dietary lipid absorption, supplementation with chitosan extracted from white mushrooms is effective in reducing adipocytokine secretion in the serum, a phenomenon that could be linked to lowered fat mass and could contribute to the reduction of ectopic fat deposition in the liver and the muscles. We postulate that a new mechanism, namely the lower iR activity in WAT, could be involved in the hypotriglyceridaemic effect of fungal chitosan.

5. Conflict of interest

The authors have declared no conflict of interest.

Acknowledgment

P.D. Cani is postdoctoral researcher from the FNRS (Fonds de la Recherche Scientifique, Belgium); L.B. Bindels is PhD student from the FNRS and the techniques were developed under a FNRS grant (1.5.2313.06).

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