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Mukitake mushroom (*Panellus serotinus*) alleviates nonalcoholic fatty liver disease through the suppression of monocyte chemoattractant protein 1 production in *db*/*db* mice☆

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is emerging as the most common liver disease in industrialized countries. Thus, the discovery of food components that would ameliorate NAFLD is of interest. Various mushrooms have been used in folk medicine for the treatment of lifestyle diseases in eastern countries and several compounds that modulate immune system, lower blood lipid levels, inhibit tumor and viral action have been isolated from them. In this study, we tested whether feeding *Panellus serotinus* (Mukitake) to *db*/*db* mice protects them from hepatic injury. After 4 weeks of feeding, hepatomegaly, hepatic triglyceride accumulation and elevated hepatic injury markers in serum were markedly alleviated in Mukitake-fed *db*/*db* mice compared with control mice. These effects were partly attributable to the enhancement of lipolytic enzyme activity and the suppression of lipogenic enzyme activities due to the Mukitake diet. The severe hyperinsulinemia in control *db*/*db* mice tended to attenuate in Mukitake-fed mice due to an enhanced production of adiponectin, which improves insulin sensitivity. Moreover, production of monocyte chemoattractant protein 1 (MCP1), an inflammatory cytokine, was markedly suppressed by the Mukitake diet. In addition, water-soluble extracts of Mukitake powder showed an inhibitory effect on inhibitor of κB (IκB) kinase (IKK) β, whose activation is required for nuclear factor κB (NFκB)-mediated inflammatory response. We speculate that the development and progression of NAFLD was prevented by the reduction of MCP1 production through the interference in the IKKβ–NFκB signaling pathway in Mukitake-fed db/db mice.

Keywords: Adiponectin; *db*/*db* mouse; Inhibitor κB kinase-β, Metabolic syndrome; Monocyte chemoattractant protein-1; Nonalcoholic fatty liver disease

1. Introduction

The metabolic syndrome which comprises a cluster of metabolic abnormalities such as hyperlipidemia, diabetes mellitus and hypertension, is a widespread and increasingly prevalent disease in industrialized countries and contributes to the increase in cardiovascular morbidity and mortality [1,2]. Nonalcoholic fatty liver disease (NAFLD) is often associated with features of the metabolic syndrome and is emerging as the most common liver disease worldwide [3–6]. NAFLD is the preferred term used to describe the spectrum of liver damage, ranging from hepatic steatosis to steatohepatitis, liver fibrosis and cirrhosis. Most liver-related morbidity and mortality are associated with the development of cirrhosis. Cirrhosis is most likely to occur in individuals who have progressed from hepatic steatosis to steatohepatitis. Although the processes through which steatohepatitis evolves from hepatic steatosis are not fully understood, it is necessary to develop effective therapies for the treatment of NAFLD, and the discovery of nutrients that will reduce the risk of NAFLD would be useful. *db*/*db* Mice suffer from hyperphagia because they have a missense mutation on the leptin receptor gene and develop a syndrome with multiple metabolic and hormonal disorders including NAFLD which shares many features with human metabolic syndrome [7–9].

Diet has been recognized as contributing to the development and prevention of NAFLD [10–13], and various mushrooms have been used in folk medicine for the treatment of lifestyle diseases in eastern countries [14–16]. Several lines of evidence support the nutraceutical effect of edible mushrooms, and many compounds that modulate the immune system, lower blood lipid levels and inhibit tumor and viral action have been isolated from various mushrooms, such as Shiitake and Hatakesimeji [14–19]. *Panellus serotinus* belongs to the same family of mycelia as *Lentinus edodes* (Shiitake) and *Lyophyllum decastes* (Hatakeshimeji), whose fruiting body (Mukitake) is recognized in Japan as one of the most delicious edible mushrooms. The technology for the artificial cultivation of Mukitake in plastic

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greenhouses has recently been developed [20] and has enabled the constant provision of Mukitake mushrooms for the market. In the present study, we evaluated the effect of the Mukitake diet on the development of NAFLD in *db*/*db* mice.

2. Materials and methods

2.1. Animals and diets

All aspects of the experiment were conducted according to the guidelines provided by the ethical committee for experimental animal care at Saga University. Five-week-old male C57BL/6J mice and *db*/*db* mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed individually in plastic cages in a temperature-controlled room (24°C) under a 12-h light/dark cycle. The basal semisynthetic diets were prepared according to recommendations of the American Institute of Nutrition (AIN-76) [21] (Table 1). Mukitake mushrooms were provided by the Forestry Research Institute of the Saga Prefecture, and general components of the samples were routinely determined according to the Association of Official Agricultural Chemists (AOAC) official methods. The *db*/*db* mice were assigned to two groups (six mice each) that were fed one of two diets (Table 1): a semisynthetic AIN-76 diet (Control group) or a semisynthetic AIN-76 diet supplemented with 10% air-dried Mukitake powder at the expense of casein, corn starch and cellulose (Mukitake group). The C57BL/6J mice (n=6), the progenitors of *db*/*db* mice, were fed the same diet as the *db*/*db* mice in the control group. The mice received the diets ad libitum using Rodent CAFE (KBT Oriental, Saga, Japan) for 4 weeks. At the end of the feeding period, the mice were sacrificed by exsanguination from the heart under pentobarbital sodium salt anesthesia after a 9-h starvation period. White adipose tissue (WAT) and livers were excised immediately, and the serum was separated from the blood.

2.2. Measurement of serum parameters

Serum triglyceride and cholesterol levels were measured using commercial enzyme assay kits (Wako Pure Chemicals, Tokyo, Japan). Serum adiponectin, insulin and monocyte chemoattractant protein-1 (MCP-1) levels were measured using commercial mice ELISA kits (Otsuka Pharmaceutical, Tokyo, Japan; Shibayagi, Gunma, Japan; R&D Systems, Minneapolis, MN, USA, respectively). Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in serum were measured using commercial enzyme assay kits (Wako Pure Chemicals).

2.3. Measurement of triglyceride and cholesterol levels in the liver

Liver lipids were extracted according to the method of Folch et al. [22], and the concentrations of triglyceride and cholesterol were measured by the methods of Fletcher [23] and Sperry and Webb [24], respectively.

2.4. Assays of hepatic enzyme activity

The enzyme activities of fatty acid synthase (FAS) [25] and malic enzyme [26] in the cytosomal fraction and carnitine palmitoyltransferase (CPT) [27] in the mitochondrial fraction were determined as described elsewhere. Protein concentration of each fraction was determined according to the method of Lowry et al. [28], with bovine serum albumin used as the standard.

2.5. Analysis of mRNA expression

Total RNA was extracted from 100 mg of perirenal white adipose tissue (WAT), using a RNeasy Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan). A TaqMan Universal polymerase chain reaction (PCR) master mix (Applied Biosystems, Tokyo, Japan); assay-on-demand, gene expression products (Mn00456425_m1 for adiponectin, Mn00441242_m1 for MCP-1, Hs99999901_s1 for 18S RNA; Applied Biosystems) were used for the quantitative real-time reverse transcriptase-PCR analysis of adiponectin, MCP-1 and 18S RNA expression in WAT. Amplification was carried out with a real-time PCR system (ABI Prism 7000 sequence detection system; Applied Biosystems).

Table 1
Composition of experimental diets

Ingredients	Control	Mukitake
	(%)	
Casein	20.0	18.8
Corn starch	15.0	8.9
Cellulose	5.0	3.1
Mineral mixture (AIN-76)	3.5	3.5
Vitamin mixture (AIN-76)	1.0	1.0
DL-Methionine	0.3	0.3
Choline bitartrate	0.2	0.2
Corn oil	5.0	5.0
Mukitake powder[a]	0.0	10.0
Sucrose	50.0	49.2

[a] Carbohydrate: 62.5%, fiber: 19.2%, protein: 12.5%, ash: 3.8%, fat: 1.9%.

Table 2
Effect of Mukitake diet on growth parameters in C57BL/6J and *db*/*db* mice

	C57BL/6J	Control	Mukitake
		db/*db*	
Initial body weight (g)	21.5±0.3	29.4±0.6[a]	29.4±0.6
Final body weight (g)	24.6±0.5	36.8±0.8[a]	33.4±1.4
Food intake (g)	76.8±2.8	121±4[a]	121±2
White adipose tissue weight (g/100 g body weight)	5.97±0.22	19.5±0.2[a]	19.4±0.6

Values are expressed as mean±standard error for six mice.
[a] Significant difference between C57BL/6J mice vs. Control diet-fed db/db mice at P<.05.

2.6. Inhibitory assay of IKKβ activity in vitro

Water-soluble extracts of Mukitake powder were prepared as follows. One portion of the Mukitake powder was soaked in ×50 volume distilled water at 97°C for 2 h. This was then filtered through Whatman #5 filter paper, and the filtrate was collected and freeze-dried. The freeze-dried sample was crushed into powder and stored at −80°C until use. The effect of Mukitake water extracts on inhibitor of κB (IκB) kinase (IKK) β activity was evaluated using K-LISA IKKβ Inhibitor Screening Kit (Calbiochem, San Diego, CA, USA). The glutathione S-transferase (GST)–IκBα fusion polypeptide substrate (including the Ser^{32} and Ser^{36} IKKβ phosphorylation sites) and IKKβ, His-Tag, human, recombinant *Spodoptera frugiperda* were incubated with or without IKKβ inhibitors in the wells of a glutathione-coated, 96-well plate. The phosphorylated GST-IκBα substrate was detected using an anti-phospho-IκBα (Ser^{32}/Ser^{36}) antibody, followed by the horseradish peroxidase conjugate and color development with 3',3',5,5'-tetramethylbenzidine (TMB) substrate. The absorbance was read at 450 nm using a microplate reader (Model 550, BioRad, Tokyo, Japan). A specific inhibitor for IKKβ, IKK-2 inhibitor IV, was used as positive control.

2.7. Statistical analysis

All values are expressed as means±standard error. The significance of the differences between means for the two groups was determined by Student's t test. Linear regression analysis was used to assess the relationship between MCP-1 levels

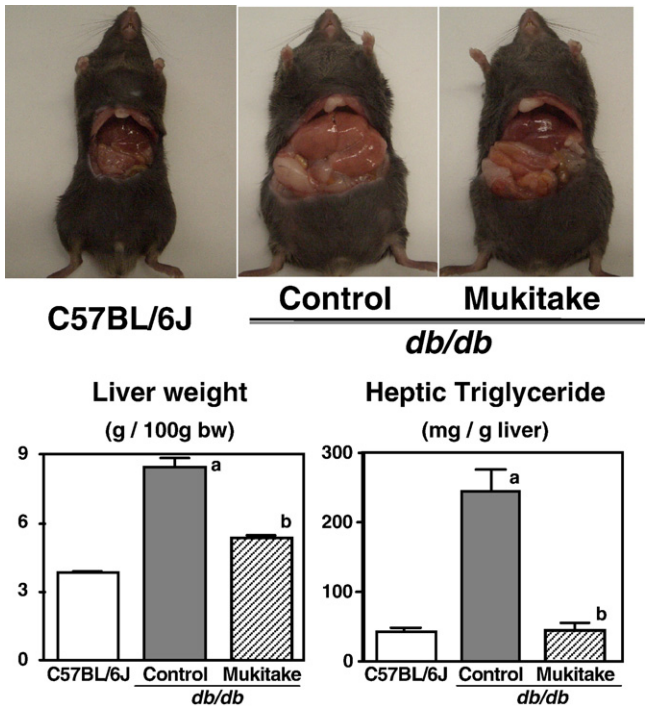

Fig. 1. The relative liver weight and hepatic triglyceride level in C57BL/6J and *db*/*db* mice. Mice were fed the Control diet or Mukitake diet for 4 weeks. Values are expressed as mean±standard error for six mice. See Table 1 for composition of diets. [a]Significant difference at P<.05 between C57BL/6J mice vs. Control diet-fed *db*/*db* mice. [b]Significant difference at P<.05 between Control vs. Mukitake diet in *db*/*db* mice.

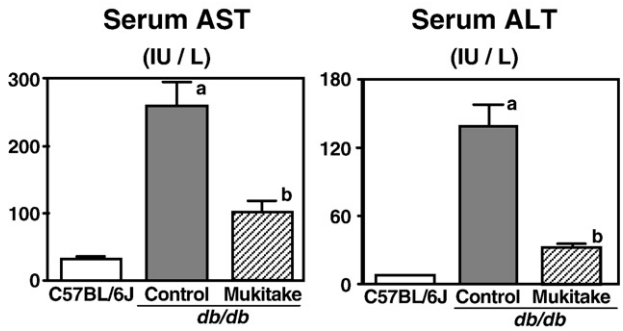

Fig. 2. Hepatic injury marker activities in C57BL/6J and *db*/*db* mice. Mice were fed the Control diet or Mukitake diet for 4 weeks. Values are expressed as mean±standard error for six mice. See Table 1 for composition of diets. [a]Significant difference at $P<.05$ between C57BL/6J mice vs. Control diet-fed *db*/*db* mice. [b]Significant difference at $P<.05$ between Control vs. Mukitake diet in *db*/*db* mice.

and hepatic injury marker levels in serum. Differences were considered to be significant at $P<.05$.

3. Results

After the 4-week feeding period, the food intake and final body weight was significantly higher in the control diet-fed *db*/*db* mice than in the C57BL/6J mice (Table 2). Additionally, it was apparent that the *db*/*db* mice developed marked abdominal obesity and severe NAFLD after being fed the control diets for 4 weeks (Table 2, Fig. 1). The two groups of *db*/*db* mice did not differ in initial body weight, final body weight, food intake or total white adipose tissue weight (Table 2). In contrast, the relative liver weight and hepatic triglyceride concentration differed between *db*/*db* mice fed the Control and Mukitake diets. The relative liver weight was 37% less in Mukitake-fed *db*/*db* mice, and this was associated with a marked reduction (81%) in the triglyceride accumulation in the liver (Fig. 1). Additionally, the hepatic cholesterol level had decreased significantly (by 21%) in the Mukitake-fed *db*/*db* mice (Control, 3.50±0.23; Mukitake, 2.78±0.12 mg/g liver weight, $P<.05$). Consistent with the alleviation of hepatomegaly and hepatic steatosis by the Mukitake diet, the activities of hepatic injury markers such as AST and ALT were markedly reduced (by 61% and 77%, respectively) in the serum of Mukitake-fed *db*/*db* mice compared to those in Control-fed *db*/*db* mice (Fig. 2). These results indicate that Mukitake diet protects *db*/*db* mice from the development of NAFLD.

To further examine the effect of the Mukitake diet on the liver, hepatic enzymes related to triglyceride metabolism were analyzed (Fig. 3). The activities of FAS and malic enzyme, which are lipogenic enzymes related to fatty acid de novo synthesis, were significantly reduced in the Mukitake-fed *db*/*db* mice. Additionally, the activity of CPT, a key enzyme in fatty acid β-oxidation, was significantly greater in the Mukitake group as compared with the Control group in *db*/*db* mice. These results suggest that the alleviation of NAFLD by the Mukitake diet was partially attributable to the suppression of lipogenic enzyme activities and the enhancement of lipolytic enzyme activity.

After a 4-week feeding period, the *db*/*db* mice fed the control diet were suffering from severe hyperinsulinemia. As shown in Fig. 4, serum insulin level tended to decrease (by 34%) in the Mukitake group as compared with the Control group. Additionally, serum levels of adiponectin, which enhances insulin sensitivity, were markedly reduced in the Control-fed *db*/*db* mice as compared with the C57BL/6J mice and significantly raised in the Mukitake group as compared with the Control group in *db*/*db* mice (Fig. 4). These results suggest that the Mukitake diet improves insulin resistance by increasing serum adiponectin levels through the enhancement of mRNA expression in WAT (Fig. 5). In the present study, serum levels of MCP-1, which induce inflammatory responses, were markedly increased in the Control-fed *db*/*db* mice as compared with the C57BL/6J mice and drastically reduced in the Mukitake group as compared with the Control group in *db*/*db* mice (Fig. 4). Additionally, these alterations were consistent with the alterations in MCP-1 mRNA expression levels in WAT (Fig. 5). Because there was a highly positive correlation between serum MCP-1 levels and levels of hepatic injury markers (vs. AST, $r=0.805$, $P<.0001$, vs. ALT, $r=0.867$, $P<.0001$, $n=18$), we consider that the suppression of MCP-1 production contributes to the prevention of development and progression of NAFLD in *db*/*db* mice.

The phosphorylation of IKKβ induces the expression of MCP-1 through the activation of nuclear factor κB (NFκB). In the present study, water-soluble extracts of Mukitake powder decreased IKKβ activity in a dose-response fashion (Fig. 6). Fifty-percent inhibition (IC_{50}) was calculated to be 0.449 mg/ml, and was similar to the inhibitory effect of 0.1 μM IKK-2 inhibitor IV.

4. Discussion

We evaluated the effect of the Mukitake diet on the development of NAFLD in *db*/*db* mice. The results suggested that the development of NAFLD was prevented by the reduction of MCP1 production through the interference in the IKKβ–NFκB signaling pathway in Mukitake-fed *db*/*db* mice.

NAFLD is common in Type 2 diabetic and obese patients. Although the mechanisms responsible for the development of NAFLD are unclear, it has been suggested that hepatic steatosis

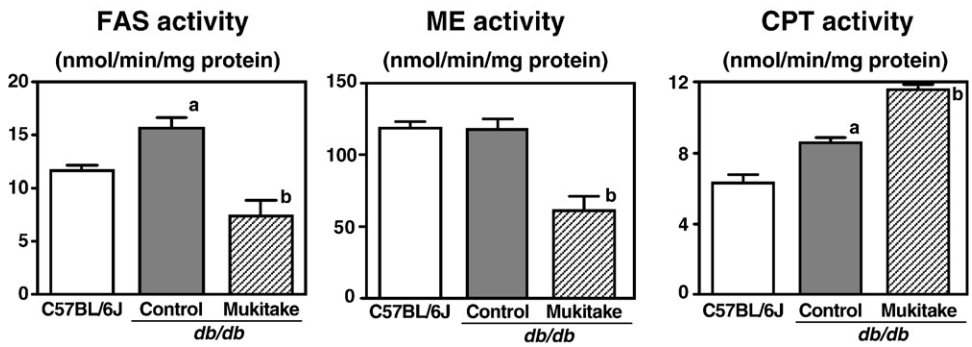

Fig. 3. Activities of hepatic enzymes related to triglyceride metabolism in C57BL/6J and *db*/*db* mice. Mice were fed the Control diet or Mukitake diet for 4 weeks. Values are expressed as mean±standard error for six mice. See Table 1 for composition of diets. [a]Significant difference at $P<.05$ between C57BL/6J mice vs. Control diet-fed *db*/*db* mice. [b]Significant difference at $P<.05$ between Control vs. Mukitake diet in *db*/*db* mice.

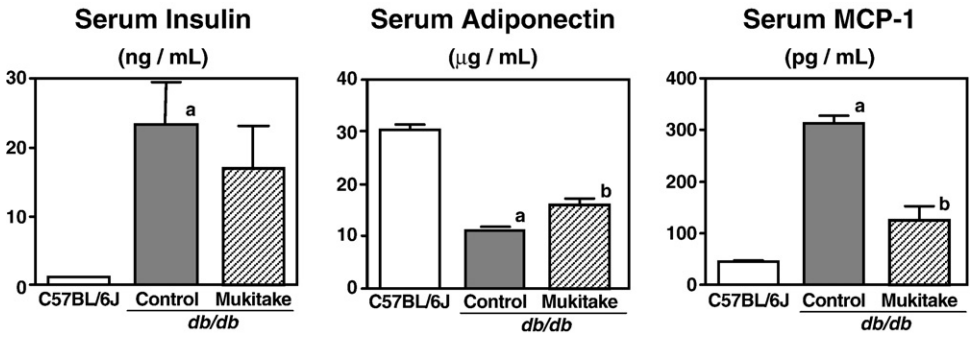

Fig. 4. Serum insulin, adiponectin and MCP-1 levels in C57BL/6J and *db*/*db* mice. Mice were fed the Control diet or Mukitake diet for 4 weeks. Values are expressed as mean±standard error for six mice. See Table 1 for composition of diets. [a]Significant difference at $P<.05$ between C57BL/6J mice vs. Control diet-fed *db*/*db* mice. [b]Significant difference at $P<.05$ between Control vs. Mukitake diet in *db*/*db* mice.

results from the increased lipogenesis and decreased lipolysis in addition to accelerated mobilization of fat from expanded visceral WAT to the liver [4,5]. In the present study, Mukitake diet counteracted both the hepatomegaly and the hepatic steatosis that occur in control *db*/*db* mice. The alleviation of NAFLD by the Mukitake diet was partially attributable to the suppression of lipogenic enzyme activities and the enhancement of lipolytic enzyme activity. Although we did not carry out a hepatic histological evaluation, these data indicate that the Mukitake diet protects *db*/*db* mice from the development of NAFLD.

Insulin resistance is the essential first pathologic step in the development of NAFLD [29–31]. In fact, hepatic steatosis is now proposed to be a feature of the insulin resistance syndrome along with Type 2 diabetes, visceral obesity and hyperlipidemia [29–31]. Recently, it has been recognized that adipose tissue not only stores excess energy in the form of fat but also secretes physiologically active substances called adipocytokines [32]. Among these, adiponectin is one of the most abundant secretory proteins produced by adipose tissue in rodents and humans [32]. Because several reports indicate that adiponectin can lead to enhanced insulin action in vitro and in vivo, it has been strongly suggested that adiponectin plays a protective role in insulin resistance [32]. Moreover, previous studies indicated that adiponectin has a protective effect against NAFLD [33–35]. In the present study, serum adiponectin levels were markedly reduced in the Control-fed *db*/*db* mice as compared with the C57BL/6J mice and significantly raised in the Mukitake group as compared with the Control group in *db*/*db* mice (Fig. 3). These results suggest that the Mukitake diet improves insulin resistance and protects from NAFLD by increasing serum adiponectin levels through the enhancement of mRNA expression in WAT. Given the previous report showing that synthesized thiazolidine derivatives (insulin-sensitizing drugs) relieve insulin resistance with increasing adiponectin production in *db*/*db* mice [36], we suppose that the Mukitake diet might act as an insulin sensitizer. Moreover, several reports indicate that adiponectin enhances fatty acid oxidation by activating adenocine monophosphate (AMP)-activated protein kinase and peroxisome proliferator-activated receptor (PPAR)-α in the liver and muscle [37,38]. Thus, we consider that the increase in serum adiponectin levels also contributes to the enhancement of hepatic fatty acid β-oxidation in Mukitake-fed *db*/*db* mice.

The pathogenesis of steatohepatitis, the more advanced form of NAFLD, has yet to be clearly defined, but the recently proposed major theory is the "two-hit" hypothesis [39]. The first "hit" is triglyceride accumulation within the liver. It was proposed that lipid-laden hepatocytes are more susceptible to a second "hit," i.e., injury by oxidative stress and inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and MCP-1. MCP-1 is a member of the CC chemokine family, induces inflammatory responses through the recruitment of inflammatory cells and is up-regulated by inflammatory stimuli such as TNF-α [40,41]. Interestingly, recent findings also show that transgenic mice expressing MCP-1 exhibit insulin resistance and hepatic steatosis, whereas in MCP-1 knockout mice and after acute

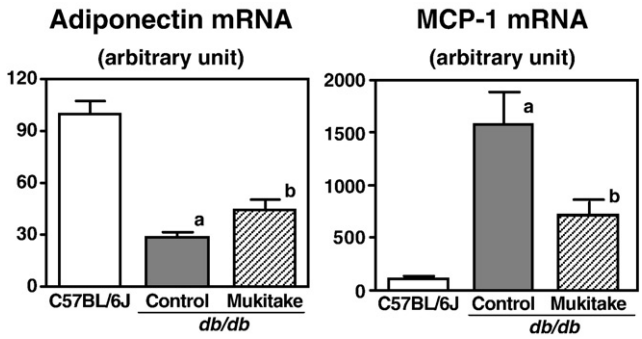

Fig. 5. mRNA Expressions of inflammatory genes in perirenal white adipose tissues of C57BL/6J and *db*/*db* mice. Mice were fed the Control diet or Mukitake diet for 4 weeks. Values are expressed as mean±standard error for six mice. See Table 1 for composition of diets. [a]Significant difference at $P<.05$ between C57BL/6J mice vs. Control diet-fed *db*/*db* mice. [b]Significant difference at $P<.05$ between Control vs. Mukitake diet in *db*/*db* mice.

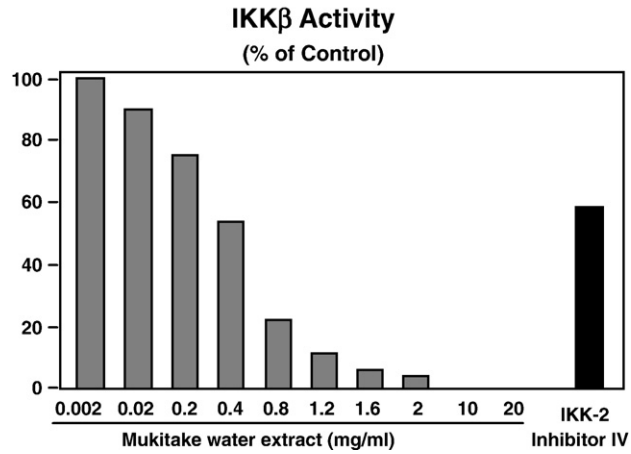

Fig. 6. Inhibition assay for IKKβ activity by Mukitake water extracts and IKKβ specific inhibitor. IKKβ, His-tag, human, recombinant *S. frugiperda* (1.5 ng) was incubated in the presence of increasing concentrations of water-soluble extracts of Mukitake powder and 0.1 μM of IKK2 inhibitor IV. Data were expressed as a percentage of recombinant IKKβ activity in the absence of inhibitors.

inhibition of MCP-1 by expression of a dominant-negative mutant in mice, there is an improvement in insulin resistance and hepatic steatosis [42]. In the present study, serum MCP-1 levels were markedly increased in the Control-fed *db*/*db* mice as compared with the C57BL/6J mice and drastically reduced in the Mukitake group as compared with the Control group in *db*/*db* mice (Fig. 4). The highly positive correlation between serum MCP-1 levels and levels of hepatic injury markers suggested that the suppression of MCP-1 production contributed to the prevention of development and progression of NAFLD in Mukitake-fed *db*/*db* mice. Moreover, the present data led us to speculate that there is mutual suppression of adiponectin and MCP-1 and also mutual antagonism of their actions in their target tissues in addition to antagonism between adiponectin and TNF-α [43], as previous studies have shown that the serum adiponectin level was significantly increased in MCP-1 knockout mice compared with in wild-type controls [42].

NFκB is a transcriptional factor that regulates a wide range of proinflammatory genes, including MCP-1 [44]. In its inactive state, NFκB, a heterodimer of p50 and p65, is retained in the cytoplasm by interaction with an IκB. NFκB activation is regulated by the IKK complex (IKKα, IKKβ and IKKγ). Phosphorylation of IκB by the IKK complex leads to the release of NFκB from its inhibitor, and then, NFκB can translocate to the nucleus. It has been suggested that, of the three subunits, the phosphorylation of IKKβ triggers the activation of NFκB in response to proinflammatory stimuli [44]. Given the fact that salicylate treatment (a known inhibitor of IKKβ) prevented insulin resistance in diabetic mice [45], the inhibition of NFκB signaling through the inactivation of IKKβ is a potent therapeutic target during the prevention and alleviation of inflammatory diseases, including metabolic syndrome. In the present study, water-soluble extracts of Mukitake powder showed an inhibitory effect on IKKβ activity (Fig. 6). There are several reports indicating that bioactive compounds, such as polysaccharides, nucleosides, polysaccharo-peptide, from fungal species could modulate NF-κB activity [46]. Although further investigation will be necessary to characterize the active components from water-soluble extracts of Mukitake powder, the results suggest that the Mukitake diet reduces MCP1 production through the interference in the IKKβ–NFκB signaling pathway in *db*/*db* mice.

In conclusion, our present study provides the first evidence that Mukitake mushroom is a promising source of naturally occurring IKKβinhibitors and suggest that a diet which includes Mukitake extracts will alleviate NAFLD through its action as an insulin sensitizer and an anti-inflammatory agent in metabolic syndrome.

Acknowledgments

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