Hepatoprotective Effects of Whey Protein on d-Galactosamine-Induced Hepatitis and Liver Fibrosis in Rats

Hisae KUME, Keiko OKAZAKI, and Hajime SASAKI

Department of Nutritional Research, Food Science Institute, Meiji Dairies Corporation, 540 Naruda, Odawara 250-0862, Japan

Received January 31, 2006; Accepted March 7, 2006

The hepatoprotective effects of whey protein on two injections of d-galactosamine (300 mg/kg, i.p.) were investigated in rats fed a modified AIN-93M diet formulated with a protein source of casein or whey for 16 d. The whey protein-containing diet clearly suppressed an increase in plasma alanine and aspartate aminotransferase activity, lactate dehydrogenase and bilirubin, which are hepatitis markers, and also hyaluronic acid, a fibrosis marker. In addition, it suppressed histopathological signs of portal fibrosis, bile duct proliferation, and perivenular sclerosis. These results suggest that supplementation with whey protein can help prevent the development of hepatitis and portal fibrosis.

Key words: bile duct proliferation; d-galactosamine; fibrosis; hepatitis; whey protein

Whey protein, a protein complex derived from milk, is known as a functional food with a number of health benefits. It includes \( \beta \)-lactoglobulin, \( \alpha \)-lactalbumine, glycomacropeptide, immunoglobulins, and lactoferrin, and has the ability to act as an antioxidant, antihypertensive, antiinflammatory, immunosuppressive, and antiviral agent in vitro and in vivo.1 It is well-known that lactoferrin inhibits hepatitis B and C viruses in vivo and in vitro.2–4 We have confirmed that lactoferrin protects against the development of hepatitis caused by sensitization of Kupffer cells by lipopolysaccharide.5 Lactoferrin also inhibits production of the inflammatory cytokines tumor necrosis factor (TNF)-\( \alpha \) interleukin (IL)-1\( \beta \) and IL-6 in monocytes.6,7 The inhibition of cytokine production by lactoferrin might be one of the factors that protects against hepatitis. Whey protein supplementation shows variable effects in patients infected with hepatitis B or C virus.8 But, the effect of whey protein is not due to the effect of lactoferrin, since it contains lactoferrin at a concentration of \(<0.01\%\). Therefore, the hepatoprotective effect of whey protein in hepatitis remains to be clearly identified.

In the present study, we investigated the effects of casein and whey-containing diets on blood chemistry, histological findings, and inflammatory cytokines in d-galactosamine (GalN)-induced hepatitis and hepatic fibrosis.

Male Sprague-Dawley rats (Charles River, Yokohama, Japan) 6 weeks old, body weight 200–220 g, were used for the GalN-induced liver injury model. The rats were housed in an air-conditioned room at 21 ± 2°C and 55 ± 15% humidity with lights on from 7:00 to 19:00 h during the experiment. The animals were handled according to protocols approved by the Food Science Institute of Meiji Dairies Corporation, Odawara, Kanagawa, Japan, which follow the Guide for the Care and Use of Laboratory Animals (NRC1996). The rats were given free access to water and experimental diets. The composition of the control diet, modified AIN-93M, was as follows: casein 20 g/100 g, corn starch 56.07 g/100 g, sucrose 10 g/100 g, soy oil 4 g/100 g, cellulose 5 g/100 g, mineral mixture (AIN-93) 3.5 g/100 g, vitamin mixture (AIN-93) 1 g/100 g, l-cysteine 0.18 g/100 g, choline bitartrate 0.25 g/100 g. All materials except for casein were purchased from Oriental Yeast (Tokyo). The rats were divided into two groups (n = 8) according to body weight (day 1), and maintained on a modified control diet (experimental diet) with a protein source of either casein (NZMP, Auckland, New Zealand) or whey protein (Arla Foods Ingredients, Viby J, Denmark). GalN (Sigma, St. Louis, MO) (300 mg/kg body weight) was freshly dissolved in physiological saline and administered at a dose of 300 mg/kg i.p. on days 1 and 14. Blood was withdrawn from the tail vein at days 1, 3, 6, and 15, and from the main abdominal artery at 2 d after the second injection of GalN. The liver was removed for histological analysis after the rats were anesthetized with ether. Blood was centrifuged at 10,000 g for 10 min, and the supernatant used for plasma biochemical analysis. Plasma alanine and aspartate aminotransferase activity (ALT, AST), lactate dehydrogenase (LDH), hyaluronic acid, and total bilirubin
concentration were measured by Fuji Dry Chem (Fuji Film, Tokyo). Part of each liver was fixed in 20% neutral-buffered formalin. Sections of paraffin-embedded livers were stained with hematoxylin and eosin or azan for histological analysis. We evaluated fibrosis and bile duct proliferation according to a five-grade system (0, no fibrosis or bile duct proliferation; 1, low grade; 2, medium grade; 3, high grade; and 4, cirrhosis or extra-high grade). Plasma TNF-α, IL-1β and IL-6 were measured using an ELISA kit (Amersham Bioscience, Tokyo).

We performed a further experiment to determine the difference between the casein and whey diets without GalN injection. Rats were divided into two groups according to body weight (n = 6) and maintained on the experimental diet for 15 d. Blood was used for biochemical analysis.

All values were expressed as the mean ± SEM and all data were analyzed using Stat View 5.0J (SAS Institute, Cary, NC). The data for IL-1β, IL-6, and the histological evaluations of fibrosis and bile duct proliferation were statistically evaluated by Student’s t-test, and other data by the non-parametric Mann-Whitney test. Differences were considered significant at P < 0.05.

Food intake and body weight gain were almost the same between the casein- and whey-containing diets. Plasma AST and ALT levels without injection of GalN were compared as between the casein and whey diets (Table 1). Plasma AST and ALT levels were 129.0 ± 32.9 and 31.3 ± 5.4 U/l in rats fed the casein diet, and 97.0 ± 28.2 and 24.8 ± 6.5 U/l in those fed the whey diet. Plasma AST and ALT levels were higher on the casein diet, but were not significantly different from normal levels. We examined whether the different proteins influenced the degree of hepatitis and fibrosis after the second injection of GalN in rats that were fed casein or whey protein-containing diets after the first injection of GalN. The results were shown in Fig. 1. After the first injection of 300 mg/kg GalN on day 1, plasma AST and ALT levels became slightly higher on days 3 and 7, although the levels were not significantly different from those on day 1 (Fig. 1). After the second injection of the same amount of GalN on day 14, marked increases were not seen in rats fed the casein diet, but were not significantly different from those on day 1 (Fig. 1). After the second injection of the same amount of GalN on day 14, marked increases in AST and ALT levels were observed on days 15 and 16 in rats fed the casein diet, while such marked increases in AST and ALT levels were observed on the second GalN injection and cytokine levels 1 d after the second GalN injection in rats. Data are expressed as the mean ± SEM (n = 6 for controls, n = 8 for GalN injection). *Significantly different from the casein diet group (P < 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Unit</th>
<th>Casein</th>
<th>Whey</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Control</td>
<td>AST</td>
<td>U/l</td>
<td>129.0 ± 32.9</td>
</tr>
<tr>
<td></td>
<td>ALT</td>
<td>U/l</td>
<td>31.3 ± 5.4</td>
</tr>
<tr>
<td>(B) GalN injection</td>
<td>AST</td>
<td>U/l</td>
<td>1142.4 ± 263.5</td>
</tr>
<tr>
<td></td>
<td>ALT</td>
<td>U/l</td>
<td>1622.3 ± 416.9</td>
</tr>
<tr>
<td></td>
<td>LDH</td>
<td>U/l</td>
<td>1516.8 ± 165.6</td>
</tr>
<tr>
<td></td>
<td>Hyaluronic acid</td>
<td>ng/ml</td>
<td>147.6 ± 31.5</td>
</tr>
<tr>
<td></td>
<td>Bilirubin</td>
<td>ng/ml</td>
<td>1.53 ± 0.64</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>pg/ml</td>
<td>111.5 ± 17.4</td>
</tr>
<tr>
<td></td>
<td>IL-6</td>
<td>pg/ml</td>
<td>178.9 ± 20.7</td>
</tr>
</tbody>
</table>

![Fig. 1. Change in ALT and AST Activity after GalN Injection.](image)

GalN was injected on days 1 and 14, as detailed in Materials and Methods. Data are expressed as the mean ± SEM (n = 8). *P < 0.05.
necrosis in the remaining two (Fig. 2D). As shown by the five-grade analysis shown in Fig. 3, development of fibrosis and bile duct proliferation was significantly suppressed in rats fed the whey diet.

We also investigated plasma TNF-α, IL-1β, and IL-6 levels at 24 h after the second GalN injection (Table 1). TNF-α was not detected, but IL-1β and IL-6 increased in plasma at 24 h after the GalN injection. Plasma levels of both cytokines were lower in rats fed the whey diet as compared with the casein diet. The low level of these cytokines might be one of the factors involved in preventing development of hepatitis and liver fibrosis. These results show that in rats fed the whey-containing diet, the levels of AST, ALT, LDH, and bilirubin, which are markers of hepatitis, were lower, and also that hepatic fibrosis and necrosis, and bile duct development, were all suppressed. Levels of inflammatory cytokines IL-1β and IL-6 were lower in rats fed the whey diet.

In rats, administration of a single dose of GalN induced liver injury similar to that seen in human viral hepatitis, and acute, self-limiting hepatitis with necrosis, inflammation, and regeneration, resembling a drug-induced disease in humans. The mechanism underlying the hepatotoxic action of GalN has been studied extensively. Within 30 min of GalN administration in rats, there is a large accumulation of UDP-GalN derivatives in the liver, leading to depletion of hepatic UTP. This results in a cessation of macromo-
lecular biosynthesis (RNA, proteins, glycoproteins, and glycogen). These alterations lead to eventual cell damage and death, which damage, at later stages, can be identified by an increase in liver enzymes in the blood and by histopathological changes. Repeated injections of GalN in rats result in progressive liver disease, characterized by fibrosis and, ultimately, cirrhosis. These pathological alterations bear some resemblance to the morphological changes in chronic biliary disease in humans.

We used a rat model in which GalN was injected twice intraperitoneally. We observed an increase in liver enzymes in the blood and pathological alterations, such as hepatic fibrosis, focal necrosis, and bile duct proliferation, but, there was no sign of cirrhosis. These histological alterations were very similar to those seen following multiple injections of GalN. Whey protein prevented development not only of necrosis, but also of fibrosis and bile duct proliferation. The mechanism of GalN-induced hepatitis has been established, but the mechanisms involved in the development of hepatic fibrosis and bile duct proliferation have not been clarified. In relation to hepatic fibrosis, connective tissue proliferates in conjunction with hepatocyte proliferation in the restoration of tissue necrosis and tissue lost by phagocytosis of Kupffer cells. It has been reported that hepatic stellate cells produce mainly type I collagen, which causes liver fibrosis. Cytokines (e.g., TNF-α and IL-1β) and growth factors (e.g., transforming growth factor-α and epidermal growth factor) are involved in hepatic fibrosis.

Just how the development of hepatitis and liver fibrosis are suppressed in rats fed whey protein-containing diets is still not clearly established. But, we suggest that the alterations here show that whey protein can aid in recovery from hepatitis and/or suppress its development after a first injection of GalN, and prevent liver damage after a second injection of GalN. Hepatotoxins, including GalN, have been reported to be associated with inflammatory cytokines, which can induce a variety of pathophysiologic responses. TNF-α and IL-1β are recognized as critical early mediators of organ injury, and might play an important role in GalN-induced hepatic injury. Shito et al. investigated the time course of inflammatory cytokines in blood and liver tissue after 1.4 g/kg GalN injection. TNF-α and IL-1β were not detected in blood, while the TNF-α level was highest at 1 h, and was still elevated 36 h after GalN injection into the liver. The concentration of IL-1β was greatest from 6 to 12 h after GalN injection, and higher still at 24 and 36 h. Histological changes, especially focal necrosis, were also observed as early as 6 h after the first injection of GalN. At 12 h after injection, hepatocellular necrosis with acute inflammatory response was visible throughout many lobules, and after 24 h, there was further necrosis and neutrophil infiltration in the lobular and portal areas. Lozano et al. also reported that TNF-α, IL-1α, and IL-6 in serum were increased by 1 g/kg GalN injection. The concentration of TNF-α was higher than normal, but still very low. According to our results, plasma TNF-α was not detected, but, IL-1β and IL-6 were higher than normal at 24 h after the second injection of GalN. These levels were lower in rats fed the whey diet. We have found that whey protein inhibited TNF-α and IL-6 production on lipopolysaccharide-induced inflammation in THP-1 and RAW264.7 cells, and that lipopolysaccharide causes hepatitis in rodents when it is injected in combination with GalN. We suggest that after GalN is injected, internal endotoxins such as lipopolysaccharide, might induce cytokine production such as TNF-α and IL-6 from macrophages, including Kupffer cells. Whey protein might inhibit such production, and as a result the liver would be protected from hepatitis and fibrosis, although it remains unclear how whey protein inhibits cytokine production. Further studies are now in progress using DNA microarrays.

Our results indicate that whey protein supplementation prevents the development of GalN-induced hepatitis and liver fibrosis in rats. Furthermore, whey protein also inhibits production of the inflammatory cytokines IL-1β and IL-6, inhibition of which might have an important hepatoprotective effect.

Acknowledgments

The authors are grateful to Dr. Mutsunori Fujiwara (Japanese Red Cross Medical Center) and Hiroshi Tsuboi on helpful suggestions and comments for evaluation of the pathological alterations.

References

7) Mattsby-Baltzer, I., Roseau, A., Motas, C., Elverfors,


