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Effects of red meat and fiber in high fat diet on activities of sphingomyelinase, ceramidase and caspase-3 in rat colonic mucosa

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Abstract

Red meat and fiber rich foods are the dietary factors most consistently related to colon carcinogenesis. Although several components in these dietary sources may contribute, the biochemical mechanism by which red meat and fiber affect colorectal carcinogenesis has not yet been established. Sphingomyelin metabolism is a novel signal transduction pathway that may have an impact on colonic tumorigenesis. The present study investigated the activity changes of sphingomyelinase (SMase), ceramidase and caspase-3 in colonic mucosa of rats fed on a high fat control diet, the control diet with beef and the control diet with fiber (cellulose). After a three week feeding period the colonic mucosa were scraped and homogenized and enzyme activities were determined. The fiber diet significantly increased the activities of neutral and acid SMases but had no effect on those of alkaline SMase and neutral ceramidase. The beef diet, on the other hand, significantly reduced neutral ceramidase activity, but had no effect on the activities of any SMase. In addition, the beef diet significantly reduced and the fiber diet increased caspase-3 activity in the colonic mucosa when compared with the control diet. The changes of caspase-3 activities were abolished by preincubating the samples with caspase-3 inhibitor. No significant changes of intestinal alkaline phosphatase could be found among the three dietary groups. In conclusion, fiber and red meat in the high fat diet affected in an opposite way the enzymes responsible for sphingomyelin metabolism and apoptosis in the colon. The effects may have implications in colorectal tumorigenesis.

Keywords: Sphingomyelinase; Ceramidase; Caspase-3; Beef; Fiber; Colon; Rat

1. Introduction

Epidemiological, case-control and experimental studies in rodents all suggest that red meat and fiber rich foods are the dietary factors most consistently related to colon carcinogenesis [1,2]. Although several factors such as heterocyclic amines, calcium, bile salts, and short chain fatty acids have been suspected to be involved, the biochemical mechanism by which red meat and fiber affect tumorigenesis in the colon have not yet been established.

Sphingomyelin (SM) metabolism triggered by sphingomyelinase (SMase) represents a novel signal transduction pathway that generates multiple lipid messengers such as ceramide and sphingosine [3]. Ceramide may inhibit cell proliferation, induce cell differentiation and apoptosis by activation of ceramide-activated protein kinase (CAPK), ceramide activated protein phosphatase (CAPP) and *Raf* kinase [4,5]. The levels of ceramide are mainly determined by SMase and ceramidase; the latter can catalyze both hydrolysis and biosynthesis of ceramide [6]. Previous studies have indicated that the SM pathway may have implications in colon cancer development [7,8]. Chemical carcinogen caused an accumulation of SM in the colonic mucosa associated with a reduction of neutral SMase activity [9]. Supplement of SM or ceramide in the diet inhibited colonic tumorigenesis in animal studies [10–12]. In the tissues of human colorectal adenoma, carcinoma and familial adenomatous polyposis, we found that the activities of SMases were significantly decreased [13,14]. The present study aims to investigate whether red meat (beef) and fiber (cellulose) in a high fat western type diet has any effect on activities of colonic SMase and ceramidase. To further identify a down-stream mechanism, the activity of caspase-3, a key enzyme in the execution phase of apoptosis [15] was also determined.

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2. Materials and methods

2.1. Materials

Female Sprague-Dawley rats weighing about 200 g were obtained from Möllegård (Ry, Denmark) and housed in a temperature-controlled room under a 12 h light and dark cycle with free access to water and semisynthetic diets. Phenylmethylsulfonylfluoride (PMSF), benzamidine, taurocholate (TC), 3-[(3-cholamidopropyl)dimethylammoniol]-1-propanesulfonate (CHAPS), *p*-nitrophenyl phosphate (*p*NPP), p-nitrophenyl (*p*NP), *p*-nitroaniline (*p*NA), Triton X100 (TX100) and dithiothreitol (DTT) were purchased from Sigma Co. (St. Louis, MO, USA). The purified SM was choline-labeled with [N-^{14}C-CH_3] by the methods of Stoffel [16] and the specific activity of labeled SM [^{14}C-SM] was 56 μCi/mg. The ^{14}C-palmitoyl labeled ceramide [^{14}C-Cer] was prepared by Peter Ström at Astra-Drago (Lund, Sweden) with the specific activity being 45 mCi/mmole [17]. The caspase-3 substrate, N-acetyl-Asp-Glu-Val-Asp-*p*NA (Ac-DEVD-*p*NA), and caspase-3 inhibitor (DEVD-CHO) were purchased from Calbiochem (Stockholm, Sweden).

2.2. Animal diets

Three types of semisynthetic diets were prepared as described previously [18] and they were based on the AIN-93 G semisynthetic standard diet [19]. The high fat (40% energy as fat) control diet contained casein as a protein source. The fat used in the diets was a mixture of butter, rape seed oil, and sunflower seed oil providing the intake of saturated, monounsaturated and polyunsaturated fatty acid in the ratio 3:2:1. It corresponded to the intake of these fatty acids in the Western type diet. The beef use was a type of low fat beef (*M. longissimus dorsi*) and was minced and freeze-dried before addition to the fat diet. The freeze-dried beef contained about 20% of fat and 73% of protein. The amount of beef added to the beef diet was calculated to replace casein as a protein source. The third diet was prepared by adding 10% (w/w) fiber (cellulose) in high fat control diet. All these diets were composed in a way that the nutrient intake as percentage energy was kept constant in all diets. All these diets were stored at −20°C and given to animal daily. The composition of the diets is shown in Table 1.

Table 1
Composition of the experimental diets (%)[a]

Ingredient	Control	Control + beef	Control + fiber
Casein	23.6	—	21.3
Dextrose	47.9	47.5	43.1
Beef	—	24.0	—
Butter	14.9	14.9	13.4
Sunflower oil	1.3	1.3	1.2
Rapeseed oil	6.2	6.2	5.6
Cellulose	—	—	10.0
Mineral mixture	4.2	4.2	3.7
Vitamin mixture	1.2	1.2	1.1
L-cystine	0.36	0.36	0.32
Choline chloride	0.36	0.36	0.32
BHQ	0.0014	0.0014	0.0014

[a] Casein was obtained from Kainuun Osuusmeijeri (Sotkamo, Finland), dextrose from Six Oy (Helsinki, Finland), mineral and vitamin mix from Harlan Teklad (Madison, WI), cellulose, L-cystine, choline chloride and tertiary butylhydroxyquinone (BHQ) from Yliopiston Apteekki (Helsinki, Finland). Butter, sunflower oil, and rapeseed oil were from a local market.

2.3. Animal treatment and sample preparation

Rats were randomly divided into three groups and each group contained nine animals. The rats were fed one of the three diets for 3 weeks. The body weight was measured weekly. At the end of the experiment, the rats were anesthetized by diethyl ether. The colon was removed, cut open longitudinally, and rinsed with ice-cold 0.15 M NaCl containing 1 mM benzamidine. The colonic mucosa was gently scraped and homogenized in 0.25 M sucrose buffer containing 5 mM $MgCL_2$, 0.15 M KCl, 50 mM K_2HPO_4, 1 mM PMSF, 1 mM benzamidine and 6 mM TC, pH 7.4, followed by centrifugation at 5000 rpm at 4°C for 20 min [20]. The supernatant was used for enzyme activity assay and protein determination.

2.4. Assay of SMase activity

SMase activity was determined according to Duan and Nilsson [21]. For alkaline SMase assay, 5 μl sample was added to 75 μl of 50 mM Tris-HCl buffer containing 0.15 M NaCl, 2 mM EDTA and 6 mM TC, pH 9.0. EDTA in the buffer serves as an inhibitor of Mg^{2+}-dependent neutral SMase. The reaction was started by addition of 20 μl of [^{14}C-SM] (80 pmole, 8000 dpm) in the assay buffer. After incubation at 37°C for 30 min, the reaction was terminated by addition of 0.4 ml of 2:1 chloroform/methanol, followed by centrifugation at 10 000 rpm for 5 sec. An aliquot (100 μl) of the upper phase containing the cleaved phosphocholine was taken, and the radioactivity was counted by liquid scintillation.

The activities of acid and neutral SMases were measured by the same procedure described above except the buffers. Acid SMase activity was assayed in 50 mM Tris-Maleate buffer (pH 5.0) containing 0.15 M NaCl and 6 mM TC, whereas neutral SMase activity was determined in 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M NaCl, 2 mM $MgCl_2$, and 0.12% TX100. TX100 at this concentration fully activates neutral SMase, but abolished the activity of intestinal alkaline SMase [20].

2.5. Assay of neutral ceramidase activity

The assay of ceramidase activity was a modification of a method reported previously [21]. [^{14}C-Cer] (10 pmole with

18000 dpm) was dried under nitrogen and mixed with 95 μl of 50 mM Tris-Maleate buffer containing 10 mM TC, pH 7.0, followed by sonication for 2 min. Samples were added in the buffer and incubated at 37°C for 1 h. The reaction was terminated by addition of 0.6 ml of methanol/chloroform/heptane (28/25/20, v/v/v) and 0.2 ml of 0.05 M (K_2CO_3+$K_2B_2O_4$)-KOH, pH 10 according to Belfrage and Vaughan [22]. After centrifugation, 200 μl of the upper phase was taken and the radioactivities were measured by liquid scintillation counting. The hydrolysis of ceramide was calculated and ceramidase activity was expressed as the released fatty acids per hour per mg of sample proteins.

2.6. Assay of caspase-3 activity

The caspase-3 activity in colonic mucosa was determined as described previously [23]. Sample in 20 μl was added to 100 μl of 50 mM HEPES buffer containing 0.1 M NaCl, 0.1% CHAPS, 10 mM DTT, 0.1 mM EDTA, 10% glycerol, pH 7.4 in a microplate. The reaction was started by adding 10μl of Ac-DEVD-*p*NA to a final concentration of 200 μM, and the absorbance was assayed every 5 min for a period of 30 min at 405 nm with a microplate reader (Bio-Rad, Hercules, CA). The slope of the reaction was determined and caspase-3 activity was calculated.

To verify the activity of caspase-3, in another set of assay, 20 μl of the samples were preincubated with 10 μl (500 nM) of DEVD-CHO, caspase-3 inhibitor, in the assay buffer for 10 min. The remaining activity was determined by the same method as described.

2.7. Assay of alkaline phosphatase activity and protein

Alkaline phosphatase activity was determined with *p*NPP as substrate. In 200 μl of 50 mM glycine buffer, pH 10.0, 25 μl of 100 mM *p*NPP and 20 μl of sample were added and mixed. After incubation at 37°C for 30 min, the reaction was terminated by addition of 1 ml of 0.2 M NaOH. The optical density was determined as 405 nm by a Bio-Rad Microplate Reader. The proteins were determined using a kit from Bio-Rad, with bovine serum albumin as a standard.

2.8. Statistical analysis

The results are expressed as mean ± standard error of the mean (SEM). The statistical analysis was performed by unpaired Students t-test and $p < 0.05$ was considered significant.

3. Results

3.1. Changes of SMase activity

After feeding, no significant difference of the body weight was identified among the rats fed the three different diets. The final body weights were 251 ± 12.1 g, 253 ± 15.8 g, and 248 ± 16.0 g for control, beef and fiber groups, respectively. When the activities of three different SMase were determined, the fiber diet increased the activity of neutral SMase by about 200% ($p < 0.01$) and that of acid SMase by 60% ($p < 0.01$), but had no significant effect on that of alkaline SMase (Fig. 1). Red meat had no effect on any of the SMase analyzed.

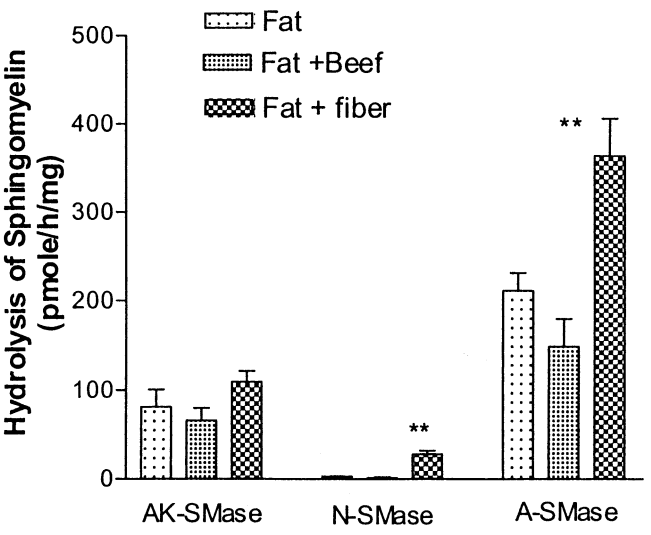

Fig. 1. Changes of SMase activity in colonic mucosa of rats fed the high fat control diet, the control diet plus beef, or the control diet plus fiber for 3 weeks. The colonic mucosa were scraped, homogenized and centrifuged. The enzyme activities in the supernatant were determined using ^{14}C-choline labeled sphingomyelin as substrate. Results are mean ± SE, $^{**}P < 0.01$ compared with the control diet. N = 9.

3.2. Changes of colonic ceramidase activity

As shown in Fig. 2, the beef diet significantly ($p < 0.001$) decreased neutral ceramidase activity by 80%. Fiber in the diet slightly increased (24%) neutral ceramidase activity, but the change was not statistically significant.

3.3. Changes of alkaline phosphatase activity

To address the question whether these changes in SMase and ceramidase are caused by a general alteration of the brush border, the activity of intestinal alkaline phosphatase was determined, because alkaline phosphatase, intestinal alkaline SMase and neutral ceramidase are similarly located in the brush border of the intestinal tract [20,24]. As shown in Fig. 3, there were no significant changes of colonic alkaline phosphatase activity among the three dietary groups. The results indicate the changes in SMase and ceramidase shown above are specifically related to the differences in diet composition.

3.4. Changes of caspase-3 activity

Caspase-3 is an important enzyme in the execution phase of apoptosis. Compared with the control fat diet, the beef

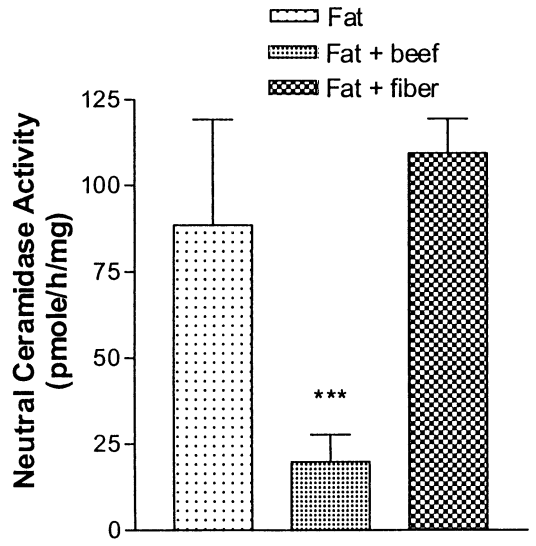

Fig. 2. The changes of neutral ceramidase activity in colonic mucosa of rats fed the high fat control diet, the control diet plus beef, or the control diet plus fiber for 3 weeks. The colonic mucosa were scraped, homogenized and centrifuged. The ceramidase activity in the supernatant was determined using ^{14}C-fatty acid labeled ceramide as substrate. Results are mean ± SE. ***$P < 0.001$, compared with the control diet. N = 9.

diet reduced caspase-3 activity by 57% ($p < 0.001$) whereas the fiber diet increased the activity by 59% ($p < 0.001$) (Fig. 4). To verify that the activities determined were caspase-3, the samples were pretreated with caspase-3 inhibitor for 10 min and the remaining activities of caspase-3 were determined. As shown in the Fig. 4, caspase-3 inhibitor almost abolished the activities in samples from the control and beef groups, and significantly reduced the activity in rats fed with the fiber diet.

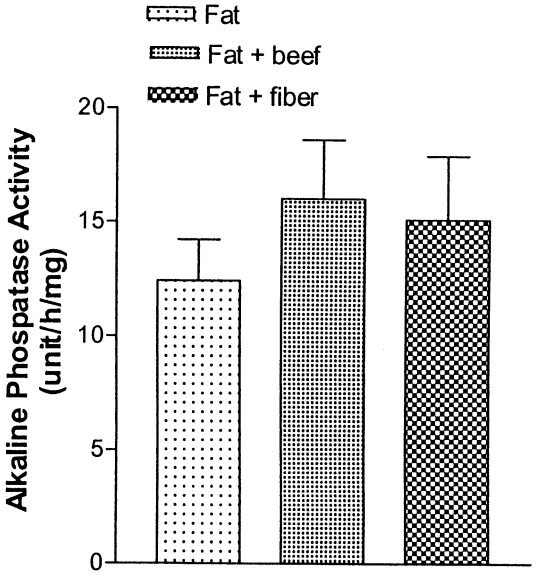

Fig. 3. The alkaline phosphatase activity in colonic mucosa of rats fed the high fat control diet, the control diet plus beef, or the control diet plus fiber for 3 weeks. The colonic mucosa were scraped, homogenized and centrifuged. The alkaline phosphatase activity in the supernatant was determined using pNPP as substrate. Results are mean ± SE. N = 9.

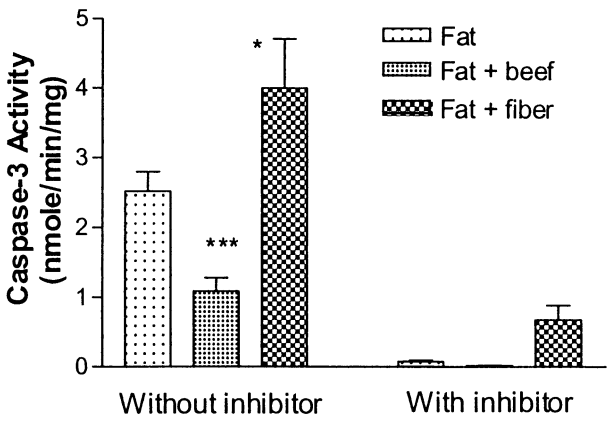

Fig. 4. The caspase-3 activity in colonic mucosa of rats fed the high fat control diet, the control diet plus beef, or the control diet plus fiber for 3 weeks. The colonic mucosa were scraped, homogenized and centrifuged. The caspase-3 activity in the supernatant was determined using ac-DEVD-pNA as substrate. To verify the activity, the same samples were preincubated with DEVD-CHO, the caspase-3 inhibitor for 10 min, followed by determination of the remaining activity. Results are mean ± SE. *$P < 0.05$, ***$P < 0.001$ compared with the control diet. N = 9.

4. Discussion

The present study demonstrated that fiber and red meat in a high fat diet affect the activities of several enzymes that are responsible for metabolism of SM in the colon. Fiber increased the activities of neutral and acid SMases, but had no significant effect on that of alkaline SMase. Furthermore, it also increased significantly caspase-3 activity, indicating that several favorable effects were obtained in cellular mechanism inside the colon during the fiber diet. Red meat, on the other hand, had no effects on SMase activities, but decreased both ceramidase and caspase-3-activities. These results cast a light on the biochemical mechanism by which these dietary factors affect the risk of colorectal cancer.

There are evidence showing that fiber in the diet may inhibit fat-induced carcinogenesis in the colon [25]. Several hypotheses have been postulated to explain the biochemical mechanisms such as the changes of the concentration of calcium, bile salt, and short chain fatty acids in the colon [26]. In this study, we found that cellulose in high fat diet increased neutral and acid SMase activities but had no effect on neutral ceramidase activity. These effects could result in an elevated level of ceramide in the cells. Ceramide is an important lipid messenger that has multiple downstream targets including CAPK, CAPP, *Raf* kinase and caspases [27,28]. In human mammary epithelial cells and dermal fibroblasts cells, ceramide may stimulate cell proliferation by upregulation of cyclooxygenase-2 expression [29,30]. However, in human colonic cells, ceramide appears to have antiproliferative effect. Incubation of HT29 colon cancer

cells with C_2-ceramide would induce apoptosis of the cells [31], and with purified rat alkaline SMase inhibited cell proliferation and DNA synthesis [32]. Dietary SM has been shown to inhibit carcinogenesis caused by chemical carcinogens [10], and the levels of ceramide in the colonic tissues is proportional to the amount of SM intake [33].

Of interesting is the finding that fiber in this study had no effect on the activity of alkaline SMase, which was previously shown to be sharply decreased to an extent greater than those of acid and neutral SMase in colonic adenoma and carcinoma [13,14]. There are many types of fiber and in this study, we only tested the role of cellulose, which is a type of non starch polysaccharides and is water insoluble. Although cellulose has been shown to have anticarcinogenic effect on colonic mucosa [34], other types of fiber may have different effects on colon cancer development [24,35]. Soluble fiber such as pectin has recently been shown to induce apoptosis in colonic tissues [36], and soluble fiber psyllium is more effective than cellulose in term of inhibition of colonic carcinogenesis [34]. Whether pectin and psyllium may increase alkaline SMase activity remains elusive.

Evidence is fairly strong to support the fact that red meat increases the risk of colon cancer. As red meat diets are usually also rich in fat, whether the carcinogenic effect of red meat is contributed by the fat has not been clearly established. In this study, replacing casein in the high fat (40%) diet with low fat beef reduced neutral ceramidase activity significantly but had no effect on the activities of any types of SMase. The results are likely caused by the fundamental change of protein source from casein to beef, not by small increase of fat (less than 5%) derived from the low fat beef. It may indicate that non-fat component of beef may affect ceramide metabolism by modulating ceramidase activity. Intestinal ceramidase is a brush border enzyme [24] and is distributed in parallel to alkaline SMase [37]. The enzyme is able to catalyze both hydrolysis and biosynthesis of ceramide and appears more effective in catalyzing ceramide hydrolysis than ceramide synthesis [37]. In addition, negative effect of red meat on SMase activity is a confirmation to a recent study of Pajari et al [38].

Apoptosis is an important process in preventing tumorigenesis. Apoptosis can be induced by both receptor- and non receptor-mediated pathways and both pathways result in activation of caspase-3, a key enzyme in execution phase of apoptosis [39]. Our study demonstrated that the activity of caspase-3 was decreased by beef and increased by fiber in the fat diet, indicating that these dietary factors have profound effects on apoptosis. Under physiological conditions, the spontaneous apoptosis mainly occurs in the luminal colonocyte of the crypt [40]. In the present study caspase-3 was measured only in the total colonic mucosa, which did not provide information about which colonic type was more sensitive to dietary beef and fiber. However, Avivi-Green et al [36] previously demonstrated that pectin, a water soluble fiber, induced upregulation of caspase-3 in all colonocyte population in both normal rats and rats treated with 1,2-dimethylhydrazine. Put together, both insoluble and soluble fibers seem to affect caspase-3 activity in the colon.

The biochemical mechanism by which fiber and beef affect caspase-3 activity in this study remains unknown. Ceramide has been considered a lipid messenger that can induce apoptosis by activation of caspase-3 [27,28]. Recently Rodringuez-Lafrasse et al [41] confirmed the key role of ceramide in apoptosis and found that lacking of ceramide formation failed to induce apoptosis even in the presence of caspase-8 activation. In our study, a significant increase in acid and neutral SMase activity by fiber and a decrease in ceramidase activity by beef may alter ceramide levels in the cells, affecting caspase-3 activity. However, other factors can not be excluded, particularly bile acids and short chain fatty acids in the colon. The levels of the two factors are significantly altered by dietary fat and fiber, and they have been shown to have an impact on caspase cascade [26,42]. In addition, it is important to point out that the present study was performed on a basis of Western type of high fat diet. The effects of fat, fatty acids and cholesterol on the enzymes responsible for SM metabolism is a target for further study.

Acknowledgments

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