Production of Diacylglycerol, an Activator of Protein Kinase C, by Human Intestinal Microflora

Masami Morotomi, Jose G. Guillem, Paul LoGerfo, and I. Bernard Weinstein

ABSTRACT

Although dietary lipids have been implicated in colon cancer causation, the underlying mechanisms are not known. This paper indicates that when bacteria obtained from normal human feces are incubated with ^14C-labeled phosphatidylcholine there is appreciable production of diacylglycerol (DAG), monacylglycerol, and free fatty acid. Curiously, the production of DAG and monacylglycerol, but not fatty acid, is strictly dependent on addition of certain bile acids to the incubation system. Among the bile acids tested deoxycholic acid is the most active. Assays of fecal specimens from 10 normal individuals demonstrate a 27-fold interindividual variation in the production of DAG in the in vitro assay system, and also in the absolute levels of DAG present in the same fecal samples. On the other hand, both parameters of DAG are quite constant in repeated fecal samples obtained from the same individual over a period of about 4 months. DAG is a normal physiological activator of protein kinase C, an enzyme that plays a key role in growth control and tumor promotion. We speculate, therefore, that DAG produced by the intestinal microflora might stimulate growth of colonic epithelial cells. Thus an interaction between dietary lipids, bile acids, and specific bacteria in the intestinal lumen could contribute to the risk of colon cancer development in humans.

INTRODUCTION

Colon cancer is the second most frequent cancer in the United States. Over 6% of Americans living today are expected to develop colon cancer at some time during their lives, of whom about one-half will die from this disease. Its etiology, like that of most human neoplasms, is probably multifactorial. Hereditary factors certainly play a predominant role in the development of familial polyposis coli and familial nonpolyposis colon cancer. However, in the majority of colon cancer patients, dietary factors probably provide the overriding impetus for tumor development (1). Evidence obtained from epidemiological and experimental studies suggests that high fat diets increase the risk of cancer of the colon (1-3). At the same time, there is an increasing awareness that specific bacteria in the intestinal lumen may also metabolize dietary lipids to DAG. The DAG produced could then enter the colonic epithelium and activate PKC, thus stimulating cell proliferation inappropriately. This phenomenon would cause a chronic state of increased cell proliferation of the colonic epithelium, which is seen in populations at high risk of colon cancer (7, 8), and would explain how both dietary lipid and the intestinal bacteria might influence colon cancer risk.

It is well known that certain intestinal bacterial species have phospholipase C activity (12). They are, therefore, capable of producing DAG from phospholipids. To our knowledge, however, this has not been directly demonstrated with samples of the human intestinal microflora. In this paper, we provide the first evidence that bacteria in human fecal specimens can produce DAG, and that this activity is markedly enhanced by specific bile acids, and that there is considerable interindividual variation in this activity.

MATERIALS AND METHODS

Assay of DAG Production. For the standard assay of DAG production from PC, a stock solution of labeled PC was prepared as follows; an aliquot of L-3-phosphatidylcholine, 1,2-dipalmitoyl (10 mg/ml of chloroform solution) was mixed with labeled PC (L-3-phosphatidylcholine, 1,2-di[l-14C]palmitoyl, 110 mCi/mmol; Amersham) and DC (100 mM methanolic solution), and taken to dryness at room temperature under nitrogen. To the dried residue distilled water was added and was followed by sonication for 1 min in a Bransonic bath sonicator. For the experiments shown in Fig. 4, CA, CDCA, TNC, or Triton X-100 was added to the reaction mixture instead of DCA. Fifty μl of the sonicated substrate were incubated with 25 μl of a fecal homogenate in a shaking water bath at 37°C. The final concentration of PC in the reaction mixture was 1.6 mM and 10^6 cpm/assay. The incubation was terminated by adding 670 μl of chloroform:methanol (1:2, v/v) and 100 μl of 2 M KCl. After mixing, 220 μl of chloroform and 220 μl of 2 M KCl were added and mixed, and the phases were separated by centrifugation. The organic phase was dried under nitrogen. The dried lipid residue was dissolved in a small amount of chloroform:methanol (2:1, v/v), chromatographed on silica gel G plates by developing in hexane/diethylether/acetic acid (50:50:3 by volume) and subjected to AR. Under the TLC-AR conditions, degradation of PC (Rf = 0) and formation of free FA (Rf = 0.37), DAG (Rf = 0.29) and MAG (Rf = 0.08) could be readily resolved.
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detected. The corresponding radioactive spots were cut out and, after the addition of Hydrofluor, were counted in a scintillation counter. All assays were performed in duplicate, and each point represents the mean value.

Preparation of Fecal Homogenates. For the preparation of fecal homogenates, fresh fecal specimens from healthy adults were homogenized with 4 volumes (w/v) of 0.1 M potassium phosphate buffer (pH 7.0) by shaking vigorously with glass beads. Dietary debris was removed by centrifugation at 500 × g for 1 min.

Preparation of [14C]DAG. Radiolabeled DAG was prepared from L-3-phosphatidylcholine, 1,2-di[1-14C]palmitoyl by incubation with phospholipase C (from Clostridium perfringens, type IX, Sigma) as described previously (13). [14C]DAG was purified by TLC on silica gel plates developed under the same conditions as described above.

Quantitation of Total DAG in Fecal Specimens. Lipids were extracted by the method of Bligh and Dyer (14) from portions of the same fecal specimens (5 and 10 mg equivalent to wet weight) used for the assays of DAG-producing activity shown in Fig. 5. The total amount of DAG in the fecal extract was analyzed by using Escherichia coli DAG kinase (Lipex; Middleton, WI), using a previously described procedure (15, 16) and expressed as nmol per g wet weight of original feces. Control studies indicated that the extraction procedure for DAG was quantitative and that the enzymatic assay was linear.

RESULTS

To test the hypothesis that DAG might be produced from phospholipids by the human intestinal microflora, 14C-labeled dipalmitoyl phosphatidylcholine (labeled in the palmitoyl residues) was incubated with fecal homogenates containing intact bacteria, and the production of DAG was monitored by TLC-AR. In our initial studies, we found that accumulation of DAG in the reaction mixture was observed, but only in the presence of the bile acid DCA (Fig. 1), although the release from PC of free FA occurred in the absence of DCA (data not shown here). DCA produced a concentration-dependent stimulation of DAG production when tested at 2 to 10 mM and the reaction continued for at least 2.5 h (Fig. 1). The effects of varying the pH from 5 to 8 on degradation of PC and the formation of DAG were also examined, as a function of DCA concentration (Fig. 2). The extent of PC degradation increased with increasing pH and was maximum at 5 mM DCA. Curiously, increasing the DCA concentration from 5 to 10 mM resulted in a significant decrease in PC degradation (Fig. 2a). On the other hand, the formation of DAG was highest at the 10 mM concentration of DCA and at a pH of about 6 (Fig. 2b). Fig. 3 compares the time course of degradation of PC to that of the formation of FA, DAG, and MAG, when the incubation was done in the presence of 10 mM DCA at pH 7. The degradation of PC and the accumulation of FA, the major product of PC degradation, were found to be approximately linear over a 2-h period (Fig. 3, a and b). During this time period about 22% of the PC was converted to FA. The accumulation of MAG was also approximately linear during this time period. On the other hand, the accumulation of DAG was curvilinear and tended to plateau with time (Fig. 3c; see also Fig. 1). The time course results obtained in Figs. 1 and 3 suggested that with time some of the DAG that is synthesized is subsequently degraded to MAG and free FA. Indeed, when [14C]DAG (1.6 mM, 2 × 10^5 cpm) was incubated in the same reaction system containing fecal bacteria and 10 mM DCA, 16.1% of the added DAG was converted to MAG (1.0%) and free FA (15.1%) after a 120-min incubation period.

No differences were seen when the incubations were performed under aerobic or anaerobic conditions in terms of PC degradation and the formation of DAG, FA, and MAG. In addition, more than 90% of all the above metabolic activities were found in the sedimentable fraction of the fecal homogenate obtained after low-speed centrifugation, indicating that they reflected the activity of intact bacteria rather than soluble enzymes (data not shown).

We also examined the effects of different types of bile acids, and of the detergent Triton X-100, on the conversion of PC to its various metabolites in the above system. PC degradation and the formation of FA, the major product of PC degradation, were enhanced by the addition of several bile acids; CA showed the highest enhancing activity, producing an 8-fold increase (Fig. 4, a and b). On the other hand, DCA showed the highest enhancing activity with respect of the formation of both DAG and MAG (Fig. 4, c and d). In all of these assays Triton X-100 had only a modest enhancing effect (Fig. 4).

Fig. 1. Effect of DCA on production of DAG from PC by a fecal homogenate. Fifty μl of the sonicated substrate was incubated with 25 μl of a fecal homogenate in a shaking water bath at 37°C. After incubation lipids were extracted, chromatographed on TLC plates and subjected to AR. The corresponding radioactive spots were counted in a scintillation counter. All assays were performed in duplicate; points, mean value. The SD of duplicate samples were always less than 10%. DCA concentrations: 0 mM ( ), 0.5 mM ( ), 2 mM ( ), 5 mM ( ), 10 mM ( ).

Fig. 2. Effects of pH on degradation of PC and formation of DAG. Potassium phosphate buffer (0.1 M; pH 5, 6, 7, and 8) was used for the preparation of fecal homogenates. Incubations were terminated after 60 min; (a) degradation of PC; (b) production of DAG.
Fig. 3. Time-course study of degradation of PC. The assays were performed at pH 7 with 10 mM DCA. Mean values and SD of the four assays are shown; (a) percentage of PC remaining; (b), (c), and (d), percentage of conversion to FA, DAG, and MAG, respectively.

Fig. 4. Effects of different bile acids and Triton X-100 on degradation of PC. The conditions of the assay were similar to those described in Fig. 1. Fecal homogenates from 3 healthy volunteers were incubated individually with PC in the presence of the indicated bile acid (10 mM) or Triton X-100 (TX; 0.3 or 3 mg/ml) for 60 min at pH 7.0. Significance of the data was analyzed by Student's t test. *, significantly different from control (no addition), P < 0.05; **, significantly different from control (no addition), P < 0.01. bars, range of SD values obtained with the three different samples, (a) percentage of PC degradation; (b), (c), and (d), percentage of conversion to FA, DAG and MAG, respectively.

Fig. 5 shows the DAG-producing activity of 10 fecal specimens obtained from different normal individuals, as well as day-to-day variations in one individual, when assayed by the method described in Fig. 1 in the presence of 10 mM DCA. Fig. 6 shows the absolute amounts of 1,2-sn-DAG present in the same fecal specimens, when assayed by the 1,2-sn-DAG-specific DAG kinase method. The donors of the specimens were healthy male volunteers (age, 33.1 ± 6.6 (SD); range, 23–45) working in research laboratories at this university. They are from China, Columbia, Ecuador, Japan, India, and the United States. Their diets were quite variable and this aspect was not controlled or monitored in this pilot study. We found that both the DAG-producing activity of fecal bacteria and the absolute amount of fecal DAG differed considerably (over 27-fold) among individuals. Replicate assays of the same sample, however, showed little variation (less than 10%) and a time course study on one individual extending over a period of 115 days displayed less than a 4-fold variation for both activities (Figs. 5 and 6). There
Fig. 6. Quantitation of total DAG in fecal specimens from 10 individuals and the day-to-day variation in one individual. The assay conditions were; pH 7, 10 mM DCA, and incubation time, 60 min; (a) mean values of duplicate assays of 12 stool specimens collected from 10 healthy volunteers. The same symbols represent specimens from the same subject; (b) day-to-day variation of DAG producing activity in one individual.

was no correlation, however, between a given individual’s DAG-producing activity and the absolute level of DAG present in the same fecal specimen (P > 0.05). This may reflect the fact that multiple factors influence the steady state level of DAG, as discussed above.

DISCUSSION

Epidemiological data and experimental animal studies have shown a positive association among dietary lipid intake, fecal bile acid excretion, and the development of colon cancer (for review see Refs. 2 and 3), although the significance of this association in terms of causal mechanisms is not known. The results of the present study suggest that dietary lipid and bile acids might act together to influence the production of DAG by bacteria present in the lumen of the colon, since we were able to demonstrate this type of effect with homogenates of human fecal specimens. Among the bile acids examined in the present study, DCA showed the highest activity, and CDCA the next highest activity with respect to stimulating DAG formation from PC (Fig. 4e). DCA and CDCA have an unconjugated carboxylic head group and a hydrophobic domain containing two hydroxyl groups, which are not present in CA and TDCA. Thus, steric features of the hydrophobic domain and the amphiphilic character of DCA and CDCA may play important roles in this enhancing effect. Triton X-100 showed little effect on the bacterial conversion of PC to DAG (Fig. 4), even though this detergent and all of the bile acids tested readily solubilized the PC present in the reaction medium, since the medium became clear with the addition of these agents. Our results could be of biological significance since bile acids are present in the lumen of the colon at mM concentrations (17-19), i.e., the concentration range in which we find that they enhance DAG formation by fecal bacteria. In previous studies we reported that at mM concentrations specific bile acids can also act directly to enhance or inhibit PKC activity (20).

The present findings are consistent with a recent report (21) that DAGs are present at relatively high levels in human feces, although these authors did not identify the precise sources of the DAGs and the factors that might influence their levels. It is of interest that these authors also demonstrated that DAGs induce DNA synthesis in colon adenoma and colon carcinoma cell cultures, but do not have this effect in normal primary colon epithelial cell cultures (21). The authors suggested that DAG is generated from the partial breakdown of dietary triglycerides in the intestine (21). Experimental studies indicate that PKC is specifically stimulated by DAGs that have the 1,2-en configuration (22). However, DAGs derived from triglycerol by the action of digestive lipases such as pancreatic lipase and liver lysosomal lipase do not show stereospecificity (23-25).

Furthermore, DAGs derived from triacylglycerol by the action of a heparin-releasable hepatic lipase or a lipoprotein lipase have a 2,3-en configuration (23-25). It seems more likely, therefore, that the 1,2-en-DAGs produced by the phospholipase C enzyme present in specific intestinal bacteria would stimulate PKC activity in colonic epithelial cells. We should emphasize, however, that it remains to be determined whether DAGs produced by bacteria in the lumen of the colon are actually taken up by the colonic epithelium and influence the PKC activity and proliferation of these cells. This does not seem unlikely in view of the evidence that exogenously added DAGs stimulate PKC activity when added to cultured human epithelial carcinoma cells, human colon adenoma or carcinoma cells, fibroblasts, and platelets (10, 21). Furthermore, Craven et al. (26) found that intracolonic instillation of 5 mM 1-oleoyl-2-acetylglycerol activated colonic epithelial PKC and induced ornithine decarboxylase and [3H]thymidine incorporation in rats. We should also emphasize that in the present studies we used 14C-labeled dipalmitoyl PC as a convenient substrate, but we assume that other phospholipids present in the lumen of the intestine also serve as a source of DAG. Indeed, in recent studies we have found that phosphatidyl inositol and phosphatidyl ethanolamine also serve as substrates for the production of DAG by human fecal bacteria. Studies are in progress to determine the effects of using substrates that contain various types of fatty acids. It is known that in humans a high fat diet increases fecal excretion of phospholipids to levels as high as 400 mg/day. The components include PC, phosphatidyl glycerol, phosphatidyl ethanolamine, and phosphatidyl inositol (27). They are derived from dietary sources and also from synthesis by the intestinal bacteria. In view of this diversity of potential substrates, it is not unreasonable to assume that some of the DAGs produced would have structures that favor cell uptake and activation of PKC. Studies are in progress to verify this assumption. It will also be of interest to isolate the specific strains of fecal bacteria that are the most active in producing DAG. We are currently attempting to identify which of the
large number of bacterial species in human feces produce DAG. This is a difficult task because of the large number of species (over 200) and the difficulty in growing them. Using purified ATCC type strains we have found that Clostridium perfringens does show DAG-producing activity in our assay, but we have not yet established which strain or strains of bacteria are responsible for the activity we detect in human fecal samples.

The results obtained in the present study suggest that the levels of specific DAGs in the lumen of the colon are a function of multiple factors, including the dietary intake of specific lipids; the lipid metabolizing activity of specific bacteria in the intestinal microflora, including the relative levels of DAG-producing and DAG-degrading enzymes; and the levels of specific bile acids, which are also a function of dietary lipid. If the present hypothetical framework is correct, then the interplay between these variables could play an important role in colon cancer causation and the design of new approaches to its prevention.

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