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THE EFFECT OF DIETARY CALCIUM SUPPLEMENTATION ON INTESTINAL LIPID METABOLISM

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Summary—Population studies in man and experimental animal work support the contention that dietary supplementation with calcium may prevent the development of colorectal cancer. The mechanism of action is postulated to be bile acid chelation in the small-bowed forming non-toxic calcium soap compounds but such substances have yet to be isolated and quantified. In this 2-part study faecal concentrations of acidic lipids and neutral sterols were measured in 93 Sprague–Dawley rats whose calcium intake was modulated by enriching the chow and adding calcium lactate (24 g/l) to the drinking water. In study-1 (dietary calcium intake doubled from 0.4–0.8%) small bowel resection was used to manipulate colonic lipid concentration for comparison with control rats who had undergone transection with immediate restoration of bowel continuity at an equivalent point. Faecal concentrations of free bile acids were 53–67% less in animals receiving added calcium [1.76 ± 1.33 vs 0.82 ± 0.65 mg/g (transection); 2.74 ± 3.73 vs 1.03 ± 1.27 mg/g (small bowel resection): $P < 0.001$]. In study-2 (dietary calcium intake trebled to 1.21%) faecal bile acid concentration was reduced by 32% (1.86 ± 0.57 vs 1.27 ± 0.34 mg/g: NS) whereas long chain fatty acid concentrations were increased by 117% (6.77 ± 2.39 vs 14.67 ± 4.82 mg/g: $P < 0.001$) in animals receiving added calcium. Serum calcium levels remained unchanged in these animals. Calcium soaps of the bile acids were not detected in faeces and therefore contrary to popular theory these results indicate that conditions within the intestinal lumen favour calcium chelation of long chain fatty acids rather than bile acids.

INTRODUCTION

The risk of colorectal cancer is increased by Western-style diets, rich in red meat and fat, although a specific colonic carcinogen has yet to be isolated [1, 2]. A wealth of evidence supports the contention that luminal bile acids form an important intermediary between diet and colorectal cancer [3–9].

Experimental colonic carcinogenesis is strongly enhanced by small bowel resection (or bypass) with a concomitant adaptive response manifest as increased crypt cell production rate (CCPR) [10]. In man, extensive small bowel resection leads to considerable diarrhoea caused by bile acid catharsis [11] and 80% jejunoileal bypass for morbid obesity raises rectal CCPR by 104% [12]. It is conceivable that elevated colonic levels of bile acids, lost from the enterohepatic circulation of bile after small bowel shortening, are partly responsible for the observed mechanical and cytokinetic changes.

Newmark [13] proposed that bile acid and fatty acid binding ions may reduce exposure of the colonic mucosa to damaging free bile acids (FBA) and fats by forming harmless, insoluble soaps which themselves become unavailable due to precipitation. In support, if calcium lactate is added to rats' drinking water, the number of axoxymethane-induced colonic neoplasms is reduced by over 50% [14] (with a corresponding fall in CCPR [15]); calcium also reverses the promotional effect of a high-fat diet [16]. Both fatty acids and deoxycholic acid are less toxic to rectal epithelium if administered to animals given supplemental calcium gluconate orally [17, 18]. Using extensive (80%) small bowel resection to manipulate colonic levels of FBA, and by varying dietary concentration of calcium, the present study was designed to investigate Newmark's

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hypothesis. Faecal concentrations of bile acids, fatty acids, sterols and calcium compounds (including soaps) were recorded.

EXPERIMENTAL

Ninety-three male Sprague–Dawley rats were allocated to a 2-part study on faecal fats and steroids. During the experiments the animals were caged and had access to standard laboratory chow (containing 0.4% calcium and 12% fat by weight) and drinking water *ad libitum*.

Study-1

Twenty nine rats weighing 445 ± 29 g underwent (and survived) 80% mid-small bowel resection (SBR) and 28 had a jejunal transection at an equivalent point. Half of the animals in each of these groups received calcium lactate (24 g/l) added to the drinking water from the first postoperative day until the conclusion of the experiment (7 weeks later) in order to double their daily intake of calcium. Faeces from the distal half of the opened colon were collected from all rats at the time of sacrifice.

Study-2

Adult rats ($n = 36$) weighing 472 ± 39 g either acted as controls ($n = 19$) and received standard chow and tap water, or they had calcium lactate (24 g/l) added to the drinking water and ate a calcium-enriched chow (calcium 0.8% w/w) ($n = 17$) so trebling their total daily calcium intake to 1.21% (w/w). Two weeks later all animals were sacrificed. A blood specimen was taken by cardiac puncture for measurement of serum calcium; faeces were collected from the distal half of the large bowel.

Faecal specimens from both studies were stored at −40°C until analysis. Specimens from both studies were analysed for FBA and neutral sterols. Specimens from study-2 were also analysed for calcium soaps of bile acids, long-chain fatty acids (LCFA) and calcium.

Faecal steroid analysis

The measurement of total and individual steroids was conducted by the method of Owen *et al.* [19] with some modifications. Faecal samples were ground to a fine homogeneous powder and then extracted with chloroform–methanol (1:1) for 10 h in a Soxhlet apparatus. The dried extract was resuspended in 72% ethanol and fractionated by anion-exchange column-chromatography on diethylamino-hydroxypropyl-sephadex (DEAP-LH-20) into (1) neutral sterols, (2) free bile acids, and (3) glycine and taurine amidated bile acids and sulphated steroids.

The neutral sterol fraction was taken up in 5 ml of diethyl ether and 5 ml of methanol containing 5 mg of 4-androsten-3,17-dione as internal standard, and quantified by gas-liquid chromatography (GLC) on a Pye 304 gas chromatograph fitted with a flame ionization detector (FID) and a 2 m × 2 mm silanized glass column filled with 3% OV-1. Individual steroids were separated using a temperature gradient from 160–260°C at 4° per min. The nitrogen flow rate through the column was 30 ml/min.

Free bile acids were methylated with ethereal diazomethane and redissolved in 2 ml methanol containing 1 mg methyl nor-deoxycholate as internal standard. Methyl bile acids were then silylated, in 1 ml of a cocktail containing pyridine–hexamethyldisilazane–chlorotrimethylsilane (3:2:1) at 60°C for 30 min. The silylating reagent was evaporated under a stream of nitrogen and the derivatized bile acids were dissolved in hexane, filtered, dried, and resuspended in 250 μl hexane. Methyl trimethyl silyl (TMS) ether derivatives of the bile acids were prepared immediately prior to capillary gas chromatography (CGC) which was conducted on a Carlo Erba gas chromatograph (HRGC) fitted with a FID and a 25 m BP1 capillary column (i.d. 0.25 μ). Ethers were separated using a temperature gradient from 200–270°C at 4°C/min maintaining the temperature at 270°C for 45 min. A 1 μl sample in hexane was injected onto the column using the split mode with a split ratio of the carrier gas (helium; 2 ml/min) of 20:1. The injection port temperature was 250°C, the FID temperature was 270°C. Bile acids were identified by reference to retention times of known standards. Integration of peak areas was by means of a Pye-Unicam PU4800 video chromatography control centre. FID responses were corrected against the internal standard methyl TMS nor-deoxycholate.

The bile acid amidate and sulphated steroid fraction was dried and desulphated by solvolysis [11]. The sample was deconjugated in 1 ml 20% potassium hydroxide dissolved in ethylene glycol at 120°C for 2 h. 1 ml methanol and 9 ml 20% aqueous sodium chloride were added followed by 6 N hydrochloric acid (600 μl) to acidify the sample. Two extractions were performed with 10 ml diethyl ether. After removing the solvent, dried samples were resuspended in 1.0 ml meth-

anol, methylated with ethereal diazomethane and dissolved in 0.5 ml methanol containing 1.25 mg methyl hyodeoxycholate as internal standard.

GLC of the solvolyzed and deconjugated fractions and LCFA (methyl esters) was conducted in the same manner as for the neutral steroids except that the internal standard used for the former was methyl hyodeoxycholate and for the latter methyl nor-deoxycholate. FID responses were corrected against the internal standards as necessary.

Calcium analysis

Freeze-dried rat faeces (50 mg) from study-2 were analysed for soluble calcium and total calcium. Soluble calcium was extracted with distilled water (10 ml) overnight. Total calcium was extracted with concentrated HNO_3 (1 ml) and concentrated H_2SO_4 (0.1 ml) by heating at 120°C for 3 h. The extract was cooled and diluted with 10 ml 1 M HCl. Both soluble and total calcium were assayed by atomic absorption spectrophotometry.

Calcium soaps

After Soxhlet extraction of the steroids from the rat faeces the residues were treated in the same way as for total calcium analysis. Acidic extracts were partitioned with diethyl ether (2 × 10 ml) and the combined ethereal extracts were filtered and dried under a stream of nitrogen. Duplicate tubes without added faeces were also assayed in the same way to serve as negative controls. All extracts were methylated, silylated and assayed by CGC as described above.

Statistical analysis

Levels of the different compound measured were analysed by the Kruskal–Wallis test and subsequently compared by the Mann–Whitney U test.

RESULTS

Serum calcium

The 3-fold difference in calcium intake had no appreciable effect on serum calcium concentration. The level was 2.90 ± 0.21 mmol/l in controls and 2.79 ± 0.15 mmol/l in rats with the enriched diet.

Faecal steroids, fatty acids and calcium

Study-1. Faecal concentration of FBA was 26–57% higher in rats with 80% SBR than

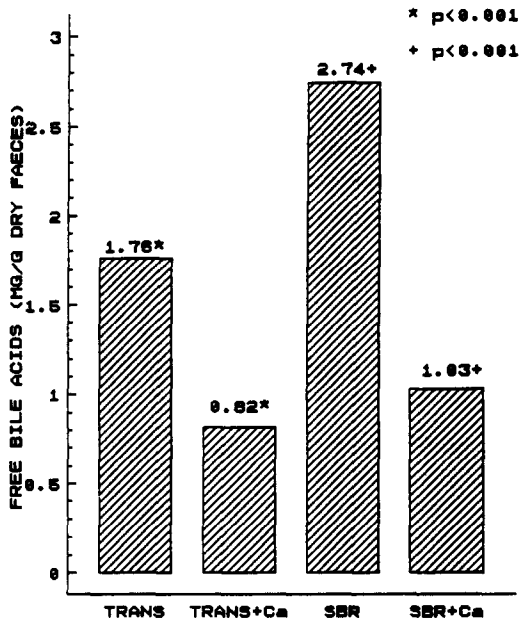

Fig. 1. Faecal free bile acids in the study groups. TRANS = transection; TRANS + Ca = transection plus calcium; SBR = small bowel resection; and SBR + Ca = small bowel resection plus calcium.

in control rats with jejunal transection, ($P < 0.001$). Doubling the intake of dietary calcium reduced the faecal concentration of total FBA from 2.74 ± 3.73 to 1.03 ± 1.27 mg/g dry faeces (a fall of 62%; $P < 0.001$) in the SBR group and from 1.76 ± 1.33 to 0.82 ± 0.65 mg/g dry faeces (a fall of 53%; $P < 0.001$) in the transection group. (Fig. 1). Deoxycholic acid (DCA) and hyodeoxycholic acid (HDCA) were the principal bile acids found. There were minor amounts of lithocholic acid (LCA) and the muricholic acids (MCA α-muricholic, β-muricholic and Ω-muricholic acids). The changes in the profiles of the individual bile acids mainly reflected the overall trend. SBR and dietary calcium supplementation had little effect upon the faecal concentration of total sterols and cholesterol (Table 1).

Study-2. Rats fed on calcium-enriched diets had 33% less total FBA in their faeces compared to controls. LCA excretion was reduced by 6%, DCA by 32%, HDCA by 41% and MCA by 52%. Excretion of bile acid amidates and sulphates was reduced by 44% and total bile acid excretion was reduced by 32% (Table 2). After organic extraction of bile acids from the faecal samples, bile acid soap complexes could not be detected in the residues.

The concentration of faecal LCFA was 117% higher in the group with dietary calcium supplementation and this was due to increased excretion of both saturated (110%; $P < 0.001$) and

Table 1. Faecal free bile acid and neutral steroid concentration (study-1)

	Trans	Trans + Ca	SBR	SBR + Ca
LCA	ND	ND	0.08 ± 0.12	0.02 ± 0.03
DCA	0.57 ± 0.33	0.26 ± 0.31	0.87 ± 1.08	0.24 ± 0.31
MCA	0.38 ± 0.62	0.09 ± 0.10	0.78 ± 1.21	0.23 ± 0.36
HDCA	0.56 ± 0.44	0.18 ± 0.26	0.35 ± 0.38	0.12 ± 0.17
OBA	0.25 ± 0.23	0.29 ± 1.19	0.65 ± 1.17	0.43 ± 0.49
Total FBA	1.76 ± 1.33*	0.82 ± 0.65	2.74 ± 3.73*,**	1.03 ± 1.27
XOL	1.01 ± 0.39	1.15 ± 0.81	1.33 ± 0.38	1.39 ± 0.65
TAS	1.49 ± 0.58	1.79 ± 1.34	2.20 ± 0.79	2.38 ± 1.27
Total	4.49 ± 1.70	5.33 ± 3.84	6.45 ± 2.81	6.96 ± 4.74

Results expressed in mg/g ± SD dry faeces, *$P < 0.001$ vs equivalent group with added calcium; **$P < 0.001$ vs equivalent group with transection. Ca = calcium; DCA = deoxycholic acid; FBA = free bile acids; HDCA = hyodeoxycholic acid; LCA = lithocholic acid; MCA = muricholic acid; OBA = other bile acids; SBR = small bowel resection; TAS = total animal sterols; Trans = transection; XOL = cholesterol; ND = not detected.

unsaturated LCFA (131%; $P < 0.001$). Total faecal calcium excretion was increased by 14% in rats taking dietary calcium supplementation and a greater fraction of this was present as bound calcium than in controls ($P < 0.05$). By contrast, calcium supplementation had little effect upon the faecal excretion of total sterols and cholesterol (Table 2).

DISCUSSION

Dietary supplementation with calcium increases the faecal excretion of fats whilst lowering faecal concentration of FBA. The formation of calcium soaps probably explains both our observations on fats and the inhibition of dietary-fat-induced colon carcinogenesis in rats given supplemented dietary calcium [16]. Calcium soaps of fatty acids precipitate readily in the upper small intestine, are relatively insoluble and are non-toxic to the colorectal mucosa [13]. Although calcium is thought to bind fats even more strongly than to the chelator EDTA [13], we have shown that calcium–LCFA complexes were relatively weak because of the significant amounts of LCFA released by Soxhlet extraction using chloroform–methanol and the failure to detect LCFA in faecal residues after HNO_3 hydrolysis.

Table 2. Faecal bile acid, long-chain fatty acid, neutral steroid and calcium concentration (study-2)

	Control	Added calcium	% Change
LCA	0.18 ± 0.06	0.17 ± 0.06	−6
DCA	0.67 ± 0.25	0.46 ± 0.23	−32
HDCA	0.66 ± 0.27	0.39 ± 0.17	−41
MCA	0.23 ± 0.18	0.11 ± 0.11	−52
OBA	0.01 ± 0.02	0.03 ± 0.05	
Total FBA	1.70 ± 0.53	1.15 ± 0.34	−33
C + S	0.16 ± 0.19	0.09 ± 0.06	−44
Total BA	1.86 ± 0.57	1.27 ± 0.34	−32
SAT LCFA	3.09 ± 1.45	6.50 ± 2.47*	+110
UNSAT LCFA	2.63 ± 0.93	6.07 ± 2.38*	+131
Other LCFA	1.04 ± 0.34	2.09 ± 0.88	+101
Total LCFA	6.77 ± 2.39	14.67 ± 4.82	+117
Sol Ca	6.71 ± 2.30	5.01 ± 1.74	
Bound Ca	17.56 ± 2.90	22.89 ± 2.89**	+27
Total Ca	24.27 ± 3.32	27.62 ± 2.40	+14
XOL	1.09 ± 0.35	1.08 ± 0.36	
Total sterols	4.89 ± 1.32	5.29 ± 1.04	

Results expressed in mg/g ± SD dry faeces, *$P < 0.001$; **$P < 0.05$. BA = bile acids; C + S = conjugated and sulphated bile acids; LCFA = long chain fatty acids; SAT = saturated; SOL = soluble; UNSAT = unsaturated, for other abbreviations see legend to Table 1.

There was no evidence of bile acid chelation by supplemented calcium as we were unable to detect calcium soaps of bile acids in faecal residues. The finding that total LCFA excretion was more than doubled by tripling dietary calcium intake indicates that calcium binds LFCA preferentially to FBA. With LCFA thus relatively unavailable to form mixed micelles in the small intestine (because of chelations with calcium), feedback inhibition of bile acid synthesis in the liver could lead to a smaller biliary bile acid pool. Fewer bile acids would then escape from the enterohepatic circulation into the large bowel resulting in reduced levels of faecal FBA. Alternatively, it is also possible that chelation of bile acids by calcium in the intestinal lumen does occur and this increases the efficiency of enterohepatic conservation by improved absorption of these complexes across the intestinal epithelium thereby resulting in reduced faecal bile acid concentration. Nevertheless our data are consistent with a recent report [20] in which the promotional effect of cholic acid on colorectal neoplasia in carcinogen-treated rats was unaffected by tripling dietary calcium.

The rise in faecal FBA excretion after 80% enterectomy lends support to the hypothesis that in patients with extensive SBR or bypass, diarrhoea is due to colonic irritation by bile acids that enter the colon in increased amounts because of defective ileal absorption [11].

The considerable increase (26%) in FBA after enterectomy in the calcium groups was not significant probably because of generally low FBA levels in these rats. It is interesting to note that tripling dietary calcium intake had no more

effect than doubling calcium intake on faecal FBA concentration. Presumably feedback inhibition of hepatic bile acid synthesis mediated by LCFA chelation is limited so that complete inhibition would be unphysiological.

The faecal concentration of total neutral sterols, cholesterol, and coprostanol was little affected by increasing dietary calcium intake. Neither case–control studies in man nor animal experiments have shown a clear association between cholesterol or its metabolism and the incidence of colorectal cancer (or adenoma). It seems unlikely that the action of intraluminal calcium is mediated via cholesterol.

In addition to its intraluminal "indirect" effect, evidence is now substantial for a "direct" antitropic role of calcium on colorectal mucosa. The growth rate of many types of cultured cells is inhibited by increasing the concentration of calcium in the medium [21]. Autoradiographic studies on colorectal biopsies from "high-risk" subjects demonstrate that high labelling indices (usually found in these patients) are reduced to normal when the concentration of calcium in the culture medium is doubled [22]. Higher concentrations of calcium (and vitamin D_3) also lead to increased differentiation in a cell line derived from a human colorectal carcinoma [23]. It thus seems that both "direct" and "indirect" effects of intraluminal calcium on colonic adaptation and carcinogenesis are important, but the relative contribution of each is unknown.

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