Effect of sulphide on short chain acyl-CoA metabolism in rat colonocytes

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Abstract

Background—It has been proposed that the diminished n-butyrate oxidation observed in ulcerative colitis may be the result of sulphide induced inhibition of short chain acyl-coenzyme A (acyl-CoA) dehydrogenase activity.

Aim—To examine the acyl-CoA ester profiles in isolated rat colonic epithelial cells treated in vitro with sodium hydrogen sulphide (NaHS).

Methods—Isolated rat colonic epithelial cell suspensions were incubated for 10 minutes in the presence of [1-¹⁴C] n-butyrate (5 mM), with and without NaHS (1.5 mM). Incubations were carried out both in the presence and the absence of exogenous CoA and ATP. Metabolic performance was assessed by ¹⁴CO₂ production and by acyl-CoA ester production measured by HPLC with ultraviolet detection.

Results—Results are given as mean (SEM). For colonocytes incubated in the presence of exogenous CoA and ATP, treatment with NaHS significantly diminished ¹⁴CO₂ production (control 0.97 (0.06) µmol/g dry weight cells/min, treated 0.26 (0.09) µmol/g dry weight cells/min, p=0.0019), was associated with an increase in butyryl-CoA concentrations in the final reaction mixture at 10 minutes (control 2.55 (0.28) µmol/g dry weight cells, treated 3.32 (0.32) µmol/g dry weight cells, p=0.002), and a reduction in crotonyl-CoA concentrations (control 0.274 (0.02) µmol/g dry weight cells, treated 0.120 (0.04) µmol/g dry weight cells, p=0.008). The mean concentration of acetyl-CoA in the reaction mixture at 10 minutes was not significantly different between control and sulphide treated incubations. There were no significant differences in acyl-CoA ester profiles observed when cells were incubated in the absence of exogenous CoA and ATP.

Conclusions—These results support the view that sulphides inhibit n-butyrate oxidation in colonic epithelial cells by inhibiting short chain acyl dehydrogenation of activated fatty acids.

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several authors,^{1 2} but such evidence reflects global outcomes in the beta oxidation pathway. Although detailed assessments of the intermediates of short chain fatty acid oxidation, the short chain acyl-coenzyme A (acyl-CoA) esters, have been undertaken in myocardial, peripheral muscle and hepatic tissues,^{3–5} such studies have not been performed on the colonic epithelium, despite the importance of n-butyrate oxidation in this tissue. Measurements of CoA esters in the epithelium of patients with ulcerative colitis may provide important clues to the mechanism underlying the observed reduction in n-butyrate oxidation in this condition. It has been suggested that luminal sulphides may be of aetiological importance in ulcerative colitis, perhaps by inhibition of colonic mucosal flavoprotein dependent short chain acyl-CoA dehydrogenases through CoA persulphide formation.⁶ The hypothesis that colonic luminal sulphides are aetiological important would be strengthened if an abnormal pattern of acyl-CoA ester concentrations in colonic epithelial cells was to be shared between human ulcerative colitis and an experimental model using mucosal tissues exposed to sulphide. Although colonic mucosal acyl-CoA ester concentrations have not been examined in human ulcerative colitis, we decided to measure acyl-CoA profiles in rat colonic epithelial cell suspensions exposed to sodium hydrogen sulphide (NaHS).

Methods

REAGENTS

All chemicals were of analytic grade and obtained from Ajax Chemical Co (Sydney) with the exception of [1-¹⁴C] n-butyrate (740 Mbq/mmol; New England Nuclear, Boston, MA, USA), bovine serum albumin (BSA, type 5; Sigma Chemical Co, St Louis, MO, USA), and sodium n-butyrate (unlabelled) and sulphosalicylic acid (BDH, Poole, Dorset, UK). Disodium ATP, CoA and acyl-CoA standards (acetyl-, butyryl- and crotonyl-CoA) were obtained from Boehringer Mannheim (Mannheim, Germany). Ultrapure water (MilliQ Plus 185 Ultrapure Water System, conductance >18 MΩcm) was used for all experiments.

EXPERIMENTAL PROTOCOL

The experimental protocol for this study was approved by the Ethics of Animal Research Committee of The Queen Elizabeth Hospital. All experiments were performed in the morning

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Evidence for diminished n-butyrate oxidation in ulcerative colitis has been presented by

with animals in the fed state. Six animals were used for each experiment to ensure sufficient cell harvest (greater than 10 mg/ml dry weight cell suspension). Animals were sacrificed by stunning/cervical dislocation and their colons rapidly harvested, rinsed clear of faecal material with normal saline, and isolated colonocyte suspensions prepared and viability assessed as described previously.⁷ Cell suspensions (1 ml) were incubated in duplicate in the presence of 5 mM [$1\text{-}^{14}\text{C}$] labelled *n*-butyrate in a final incubation volume of 2.0 ml (made up with ordinary Krebs–Henseleit (KH) saline) with or without NaHS (1.5 mM). Additional incubations were carried out as above but with the addition of disodium ATP (4.5 mM) and free CoA (0.2 mM). Cells were incubated at 37°C in stoppered 25 ml flasks in a shaking water bath. After 10 minutes, 0.1 ml 5% sulphosalicylic acid (SSA) containing dithioerythritol (DTE; 62.5 μM) was added to halt incubation.⁸ $^{14}\text{CO}_2$ was collected in 0.5 ml 1.0 M NaOH (after shaking on ice for 90 minutes) and $^{14}\text{CO}_2$ production calculated as described previously.⁷ The SSA/DTE quenched incubation mixture was homogenised using a glass Teflon homogeniser (Braun type 853204) on maximum power setting for 20 seconds. The resulting homogenate was centrifuged (2500 *g*, 10 minutes, 4°C) and 80 μl supernatant used for injecting onto the HPLC. A Waters HPLC system (Millipore Australia, Sydney) was used for HPLC analysis, comprising a 510 HPLC pump, 715 Ultra Wisp sampler and Baseline 810 software. The analytical column was a LiChroCART 250 $\times$ 4 mm column (Merck #50839, Kilsyth, Victoria, Australia) equipped with a LiChroCART 4-4 (4 mm) guard column (Merck #1.50963, Darmstadt, Germany), and the packing material was LiChrospher 60 RP-select B (5 micron particle size) (Merck Australia). Acyl-CoA ester elution was achieved using a variable flow rate and mobile phase concentrations as modified from Corkey and Deeney.⁹ Detection was by ultraviolet absorbance at 260 nm using a Waters 486 UV/visible detector (Waters, Sydney, Australia). Standard solutions of CoA esters (acetyl-, butyryl- and crotonyl-CoA) and free CoA were prepared as described by DeBuysere and Olson¹⁰ as 5 mM stock solutions in 1 M KH_2PO_4 (pH 4.0) and stored in aliquots at -80°C . The concentration of acyl-CoA ester in the reaction mixture was calculated by relating peak area to a standard of known concentration, prepared for each run by diluting stock standard in SSA/DTE (20 μl) and KH saline containing 2.5% BSA (380 μl). Samples were run in duplicate and the mean value used for calculations.

Peak identity was confirmed by co-elution of standard and when alkaline hydrolysis of sample supernatant caused the observed peak to disappear as would be expected of a CoA ester. Alkaline hydrolysis was performed on 100 μl sample supernatant to which 7.5 μl 5 M KOH was added. This mixture was incubated (63°C, 30 minutes) and then acidified with an equivalent amount of SSA/DTE to ensure pH <3.0 and assayed as above.

TIME COURSE STUDIES

Duplicate incubations of isolated rat colonic epithelial cell suspensions were stopped at one, three, five, and 10 minutes in the presence of ATP (4.5 mM) and CoA (0.2 mM), and acyl-CoA ester concentrations were measured.

STATISTICAL ANALYSIS

$^{14}\text{CO}_2$ values are expressed as $\mu\text{mol/g}$ dry weight cells/min and acyl-CoA values as $\mu\text{mol/g}$ dry weight cells in the final reaction mixture at 10 minutes. Linearity of CoA ester concentrations over time was assessed using the Spearman correlation coefficient. Differences in variance between groups was assessed using Bartlett's test. Comparison of means of continuous variables ($^{14}\text{CO}_2$, CoA ester production) between control and sulphide treated groups was by paired *t* test. Significance was set at the 5% level.

Results

Mean cell harvest for these studies was 13.6 mg, with viability, as assessed by using the trypan blue exclusion test, greater than 90% in all experiments. The delay between the sacrifice of the first rat and incubation of the six colonic sacs in calcium free KH/EDTA was approximately eight minutes – that is, incubations were carried out well within the time frame that $^{14}\text{CO}_2$ production is known to be linear.⁷ Figure 1 shows the typical chromatograms derived from a Co-A ester standard and a colonic epithelial cell sample.

Figure 1: Chromatograms demonstrating detection of acyl-CoA esters in (A) standard (5 mM) solution (see text for details) and (B) SSA/DTE quenched rat colonic epithelial cell sample (incubated in the presence of exogenous CoA (0.2 mM) and ATP (4.5 mM)). Peak identity: a=free CoA; b=acetyl-CoA; c=acetoacetyl-CoA; d=crotonyl-CoA; e=butyryl-CoA.

Figure 2: Time course of acyl-CoA ester concentrations over 10 minute incubations. Each point represents the mean (SEM) of three experiments.

TIME COURSE EXPERIMENTS

The appearance of crotonyl-CoA and butyryl-CoA after incubation with exogenous ATP and CoA was linear over the 10 minute time course studied (fig 2; butyryl-CoA, $r=0.99$, $p<0.0001$; crotonyl-CoA, $r=0.99$, $p<0.0001$). The appearance of acetyl-CoA followed a much more rapid and non-linear time course over the 10 minute incubation period.

INCUBATION IN THE ABSENCE OF EXOGENOUS ATP/CoA

A metabolic effect of sulphide treatment on isolated rat colonic epithelial cells was demonstrated by the reduction in mean (SEM) control $^{14}\text{CO}_2$ production from 0.84 (0.07) to 0.18 (0.05) $\mu\text{mol/g}$ dry weight cells/min after the addition of 1.5 mM NaHS ($t=12.85$, $p=0.0002$). Table 1 summarises values for acyl-CoA ester for experiments carried out in the absence of exogenous CoA and ATP. In the absence of exogenous ATP and CoA, the final concentration of butyryl-CoA in the reaction mixture was not significantly different between control and treated groups (control 0.025 $\mu\text{mol/g}$ dry weight cells, treated 0.021 $\mu\text{mol/g}$ dry weight cells, $t=0.76$, $p=0.49$, paired t test).

TABLE 1 $^{14}\text{CO}_2$ and acyl-CoA ester values for incubations carried out in the absence of exogenous ATP and CoA

	Control	Treated
$^{14}\text{CO}_2$	0.84 (0.07)	0.19 (0.02)***
Free CoA†	0.211 (0.05)	0.245 (0.04)*
Butyryl-CoA†	0.025 (0.004)	0.021 (0.006)‡
Crotonyl-CoA†	0.016 (0.005)	0.011 (0.005)‡
Acetyl-CoA†	0.134 (0.016)	0.137 (0.019)‡

Values are mean (SEM) expressed as $\mu\text{mol/g}$ dry weight cells/min or $\dagger\mu\text{mol/g}$ dry weight cells in the final reaction mixture at 10 minutes' incubation.

* $p<0.05$, *** $p<0.001$; ‡ $p=NS$, treated versus control value, paired t test.

Under the assay conditions described, the concentration of crotonyl-CoA was not affected by treatment with 1.5 mM NaHS (control 0.016 $\mu\text{mol/g}$ dry weight cells, treated 0.011 $\mu\text{mol/g}$ dry weight cells, $t=1.33$, $p=0.26$, paired t test). Similarly, acetyl-CoA concentrations in the final reaction mixture did not differ between the groups (table 2) but free CoA concentrations were slightly but significantly raised in the treated group compared with controls (control 0.211 $\mu\text{mol/g}$ dry weight cells, treated 0.245 $\mu\text{mol/g}$ dry weight cells, $t=3.22$, $p=0.03$, paired t test).

INCUBATION IN THE PRESENCE OF EXOGENOUS ATP/CoA

Table 2 summarises the results. A similar and highly significant reduction in $^{14}\text{CO}_2$ production was observed after the addition of 1.5 mM NaHS to isolated cell suspensions incubated in the presence of exogenous ATP and CoA (control 0.97 (0.06) $\mu\text{mol/g}$ dry weight cells/min, treated 0.26 (0.09) $\mu\text{mol/g}$ dry weight cells/min, $t=7.30$, $p=0.0019$, paired t test). This reduction was associated with a significant increase in the concentration of butyryl-CoA in the final reaction mixture (control 2.55 $\mu\text{mol/g}$ dry weight cells, treated 3.32 $\mu\text{mol/g}$ dry weight cells, $t=7.17$, $p=0.002$). Concurrently, the concentration of crotonyl-CoA in the final reaction mixture in NaHS treated incubations was significantly reduced compared with controls (control 0.274 $\mu\text{mol/g}$ dry weight cells, treated 0.120 $\mu\text{mol/g}$ dry weight cells, $t=4.90$, $p=0.008$). The mean concentration of acetyl-CoA in the reaction mixture at 10 minutes was not significantly different between control and NaHS treated incubations (control 6.94 $\mu\text{mol/g}$ dry weight cells, treated 6.52 $\mu\text{mol/g}$ dry weight cells, $t=1.42$, $p=0.22$).

Discussion

That flavoprotein dependent dehydrogenation by colonic epithelial cells is inhibited by sulphides has been suggested indirectly by the observation that in sulphide treated isolated colonic epithelial cell suspensions, ketogenesis derived from crotonate (the enoyl derivative of n-butyrate) was less affected than that observed from n-butyrate.^{6 11} Our results provide direct evidence that such FAD dependent acyl-CoA dehydrogenation is one mechanism by which the observed effect of sulphide on colonic epithelial metabolism may be produced. Alterations in acyl-CoA profiles could only be shown when cells were incubated in the presence of exogenous ATP and CoA. Similar findings have been reported previously in which detection of beta oxidation intermediates depended on co-incubation with CoA and ATP in rat liver and brown fat peroxisomes.^{12 13} The finding in controls that the rate of crotonyl-CoA production was significantly lower than that of butyryl-CoA (274 v 2552 $\mu\text{mol/g}$ dry weight cells/min) supports the suggestion that short chain acyl-CoA dehydrogenation is an important rate limiting step in

TABLE 2 $^{14}\text{CO}_2$ and acyl-CoA ester values after incubation of rat colonocytes in the presence of exogenous ATP and CoA for 10 minutes

	Control	Treated
$^{14}\text{CO}_2$ ($\mu\text{mol/g}$ dry weight cells/min)	0.97 (0.06)	0.26 (0.05)†
Butyryl CoA ($\mu\text{mol/g}$ dry weight cells)	2.55 (0.28)	3.32 (0.31)†
Crotonyl-CoA ($\mu\text{mol/g}$ dry weight cells)	0.274 (0.023)	0.120 (0.041)**
Acetyl-CoA ($\mu\text{mol/g}$ dry weight cells)	6.94 (1.31)	6.52 (1.06)‡

Results expressed as mean (SEM).

** $p<0.01$, † $p<0.005$, ‡ $p=NS$ versus controls (paired t test).

the oxidation of short chain fatty acids.¹⁴ Although the acetyl-CoA concentrations in the final reaction mixture after incubation in the presence of exogenous CoA and ATP was reduced in NaHS treated cells, this reduction was not statistically significant. The importance of this observation is difficult to assess given the findings of the time course studies, which suggest that these values reflect a steady state situation, and given the many alternative sources and metabolic pathways using acetyl-CoA within colonic epithelial cells. The reduction observed, although not statistically significant, amounts to a 7.5% reduction compared with control values. Whether such a reduction is physiologically relevant cannot be stated, although the reduction in ¹⁴CO₂ values indirectly argues that it may be important. The effect of sulphide on electron transport, which undoubtedly contributes to this, has not been tackled directly however. It has been suggested that CoA persulphides may have an important role in the physiological regulation of acyl-CoA dehydrogenation¹⁵⁻¹⁷ which is thought to be an important regulatory step in the beta oxidation pathway.¹⁴ The absorption spectra of long and medium chain fatty acid dehydrogenases has a pattern that corresponds to that of the FAD prosthetic group.¹⁸ Native short chain acyl-CoA dehydrogenases (SCAD) exhibit a similar pattern but also produce a band at approximately 700 nm which is responsible for the characteristic green colour of these enzymes.^{18 19} It has been shown that this is because of a charge transfer reaction between the FAD prosthetic group of SCAD and a CoA persulphide.¹⁶ Such a persulphide could result from the combination of free sulphide and the thiol group of free CoA. It has been shown that ox mitochondrial butyryl-CoA dehydrogenase can be "degreened" (losing its CoA persulphide/FAD absorption peak) by incubation in the presence of excess substrate (butyryl-CoA) and that CoA persulphide saturated enzyme (assessed by absorption spectral changes) exhibits only 10% of the activity of unliganded enzyme.¹⁷ The same study suggested that enzyme extracted from healthy ox liver mitochondria was 60% liganded as a SCAD-CoA persulphide complex and proposed that CoA persulphides may be important regulators of mitochondrial beta oxidation. The strong substrate affinity of SCAD (K_m approximately 1–2 μM¹⁴), suggests that substrate (butyryl-CoA) concentrations in mitochondria are unlikely to reach the excess concentrations required for the CoA persulphide displacement described by Shaw and Engel¹⁷ to occur and that such a displacement mechanism in the physiological control of beta oxidation is unlikely. This does not, however, rule out a role for pathophysiological inhibition of SCAD activity in the presence of raised concentrations of agents capable of forming CoA persulphides. The presence of high concentrations of reduced forms of sulphur, including sulphide, in the colonic lumen may provide an appropriate pathway for the generation of pathological concentrations

of colonic epithelial mitochondrial SCAD-CoA persulphide complex, capable of inhibiting fatty acid oxidation. The results of incubations in the presence of exogenous ATP and CoA are consistent with such a mechanism with higher butyryl-CoA and lower crotonyl-CoA values observed after treatment of cell suspensions with sulphide. The effects of persulphide formation may be expected to be reflected in antioxidant defences within the colonic mucosa such as the ratio of reduced to oxidised glutathione. Recent reports²⁰ have demonstrated a significant reduction in reduced glutathione in colonic mucosal extracts from carrageenan fed rats, an animal model known to be associated with raised colonic luminal sulphide concentrations.²¹

An abnormal pattern of acyl-CoA ester concentrations has been established in rat colonic epithelial cells treated with physiologically relevant concentrations of sulphide. An important step in determining whether this is of relevance to the epithelial cell metabolic abnormality observed in ulcerative colitis will be to show a similar abnormality in colonic epithelial cells isolated from colitic tissue. Such studies are currently being undertaken to explore this possibility.

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