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EFFECT OF 5-AMINOSALICYLIC ACID (5-ASA) AND OTHER SALICYLATES ON SHORT-CHAIN FAT METABOLISM IN THE COLONIC MUCOSA

PHARMACOLOGICAL IMPLICATIONS FOR ULCERATIVE COLITIS

WILLIAM ROEDIGER,* GRAHAM SCHAPEL,† MICHAEL LAWSON,‡ BARBARA RADCLIFFE* and SUSAN NANCE*

*Cell Physiology Laboratory, Queen Elizabeth Hospital and University of Adelaide, Department of ‡Gastroenterology and †Clinical Pharmacology, The Queen Elizabeth Hospital, Woodville, S.A. 5011, Australia

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Abstract—5-Aminosalicylic acid (5-ASA) suppressed nitrite-stimulated oxidation of the fatty acid *n*-butyrate in a dose-dependent manner in isolated human and rat colonic epithelial cells. 4-ASA had one-sixth of the capacity of 5-ASA and sulphapyridine (SP) little of the capacity of 5-ASA to suppress fatty acid oxidation in human colonic epithelial cells. Sulphasalazine (SASP), azodisalicylic acid (ADS), acetyl-5-ASA and acetyl salicylic acid (ASA) did not suppress fatty acid oxidation in rat colonocytes. The suppression index of fatty acid oxidation (SIFO) of respective salicylic acids correlated with the reported clinical effectiveness of each drug against ulcerative colitis (UC). The capacity of 5-ASA to affect nitrite-stimulated oxidation of fat in the colonic mucosa suggests that nitrite ions and control of fatty acid oxidation play a central role in the development and therapy of active UC.

5-Aminosalicylic acid (5-ASA) delivered to the colon is the most active agent in the treatment of ulcerative colitis (UC) [1–4]. The aetiology of UC and the mechanism of action of 5-ASA are variably ascribed but in the main both are unknown. There is an excess production of prostaglandin E_2 in active UC [5], which is inhibited by 5-ASA [6]. Oxidation of arachidonic acid to prostaglandin E_2 is controlled by oxygenases which *in vitro* [7] are inhibited by 5-ASA—a mechanism offered as a central therapeutic action of 5-ASA.

While prostaglandins that mediate inflammatory change in the colon are affected by 5-ASA, no study of the effect of 5-ASA on mucosal metabolism of short chain fatty acids (SCFA) has been undertaken. Unlike other organs, the colonic mucosa in man and animals is richly supplied by SCFAs of bacterial origin [8] and these, even in the presence of amino acids and glucose, account for 70% of the CO_2 production by the mucosa [9]. Oxidation of SCFAs is impaired in acute UC [10] and represents another observable biochemical lesion in the colonic epithelium of UC.

We therefore set out to study the effect of 5-ASA on oxidation of SCFAs in the colonic mucosa by using isolated colonic epithelial cells to establish (*a*) whether fat metabolism was altered by 5-ASA and other salicylate derivatives, (*b*) the nature of altered fat metabolism and metabolites produced, and (*c*) correlation of any observed biochemical changes with the therapeutic efficacy of the respective drug. All studies were performed with isolated cells of the colonic mucosa from undiseased human and rat colon.

MATERIALS AND METHODS

Human tissue and animals. Pathologically uninvolved segments of human colon (10–15 cm) were obtained freshly at operations performed for carcinoma of the colon. The colon was washed with Krebs–Henseleit saline [11], cut open and pinned out so that strips (0.5 × 4 cm) of mucosa could be cut from the underlying muscle for preparation of isolated mucosal cells. Wistar–Lewis rats of both sexes, weight range 150–250 g, were used for all animal experiments. Animals were fed on a commercially prepared diet which on analysis [12] contained no sodium nitrite. To minimise metabolic variability due to a change in diurnal rhythm animals were exposed to artificial light for 12 hr and experiments conducted in the morning on non-fasted animals. That colon defined as proximal and distal colon [13] was used to prepare isolated colonic epithelial cells.

Preparation of isolated epithelial cells. Mucosal strips or everted segments of ligated distended rat colon were incubated at 37° in calcium-free Krebs–Henseleit (K–H) saline containing 0.25% bovine serum albumin and 5 mM EDTA for 30 min in a Dubnoff type shaker in polythene flasks gassed with $O_2 + CO_2$(19:1 v/v). Epithelial cells were disaggregated by vigorous manual stirring with a plastic stirrer and cells separated by centrifugation at 500 *g* for 1 min. Cells were twice washed in oxygenated K–H saline containing calcium and 1 mM DL-dithiothreitol and resuspended in 15 ml of the same saline by being taken up several times into a 10 ml polypropylene pipette. Aliquots of cells were used for

procedures outlined below. Viability of cells was assessed by ability to exclude tetrazolium salts or linearity of lactate production [14]. Bacterial contribution to metabolite function was excluded by incubating cells with and without antibiotics (benzyl penicillin 200 U/ml and streptomycin 200 μg/ml).

Drugs used. Sulphasalazine (SASP), sulphapyridine (SP), azodisalicylic acid (ADS), acetyl-5-ASA and 5-ASA were obtained from Pharmacia AB (Uppsala, Sweden); acetyl salicylic acid (ASA) from Monsanto (USA) and 4-aminosalicylic acid (4-ASA) as well as DL-dithiothreitol (DDT) were obtained from Sigma Chemical Co. (St. Louis, MO). Purified enzymes and coenzymes for substrate analysis were obtained from Boehringer Corporation (North Ryde, Australia). The concentration of 5-ASA used (1–5 mM) was in the range of those measured in the human colon of patients taking salicylic acid drugs [15].

Incubation and metabolite assay. Isolated colonic epithelial cells, usually 1 ml representing 5–10 mg dry weight, were incubated for 40 min in conical flasks equipped with a glass centre well and stoppered with Suba-seals. Incubations were carried out at 37° in 1 or 2 ml physiological saline containing 2.5% w/v bovine serum albumin, 5 mM DDT and 10 mM sodium nitrite. This concentration of sodium nitrite, which is not a physiological range, was chosen because it was previously found to stimulate *n*-butyrate optimally in isolated colonocytes [16]. Radioactively-labelled [1-^{14}C]-butyrate (New England Nuclear) specific activity 1500 dpm/μmol butyrate, and drugs (1–5 mM) were added together in the incubation mix. Fresh stock solutions of drugs were prepared on the day of experiments either in 0.05 M HCl (5-ASA) or 0.02 M NaOH (all other drugs). Addition of any drug did not alter the final pH of the incubation mix. At the end of each incubation 0.5 ml NaOH was injected into the centre well to collect $^{14}CO_2$ generated by the cells, and immediately afterwards 0.5 ml of 10% $HClO_4$ (v/v) was injected into the cell suspension. After 3 hr samples (0.1 ml) of the alkali solution were taken for counting in a liquid scintillation counter and metabolites measured in the incubation mix after neutralization with 20% KOH. Acetoacetate and β-hydroxybutyrate were measured enzymatically [17]. Sodium nitrite was not found to interfere with any of these estimations.

Calculations. Production of $^{14}CO_2$ and metabolites was calculated [9] and measured as μmol/min/g (dry wt). The percentage suppression of nitrite-stimulated β-oxidation by drugs was derived as follows from rates of $^{14}CO_2$ production measured in aliquots of cells taken from the same batch of isolated epithelial cells:

suppression index of fatty acid oxidation by drug 'a' (SIFO)

$$= 100 \times \frac{\left(\begin{matrix}^{14}CO_2 \text{ production} \\ \text{with } NaNO_2\end{matrix}\right) - \left(\begin{matrix}^{14}CO_2 \text{ production} \\ \text{with } NaNO_2 \\ \text{with drug 'a'}\end{matrix}\right)}{\left(\begin{matrix}^{14}CO_2 \text{ production without drugs} \\ \text{or } NaNO_2\end{matrix}\right)}$$

Statistical analysis. The statistical significance where relevant was evaluated by Student's paired or unpaired *t*-test.

RESULTS

Cellular $^{14}CO_2$ generation and nitrite

The rate of $^{14}CO_2$ production from [1-^{14}C]-butyrate by isolated epithelial cells was linear over 60 min (Fig. 1) and unaltered (results not shown) by bacteria-supressing antibiotics. This indicated that the cells were metabolically viable and unaffected by minor bacterial contamination. Because nitrite is known to stimulate oxidation in the colonic mucosa [16] isolated colonocytes were exposed to 10 mM $NaNO_2$ over 60 min. Slight but not significant loss of linearity of fat metabolism occurred from 40 to 60 min. Experiments with drugs were consequently calculated over 40 min to obviate any effects of poorly viable cells.

Effect of 5-ASA, SASP and SP on fatty acid oxidation

No effect of 5-ASA or SASP was observed on butyrate oxidation when the cells were incubated

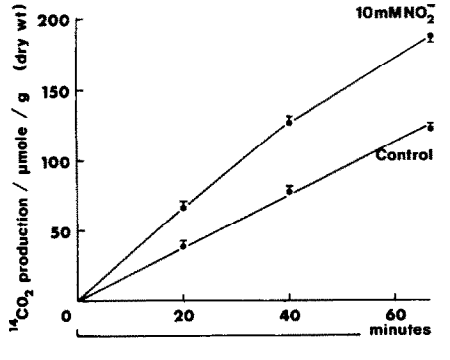

Fig. 1. Time course of $^{14}CO_2$ production from [1-^{14}C]butyrate by colonocytes of the rat with and without 10 mM NO_2^-. Mean ± S.E. of 7 paired experiments. Differences at 20, 40 and 60 min $P < 0.001$ (Student's paired *t* test).

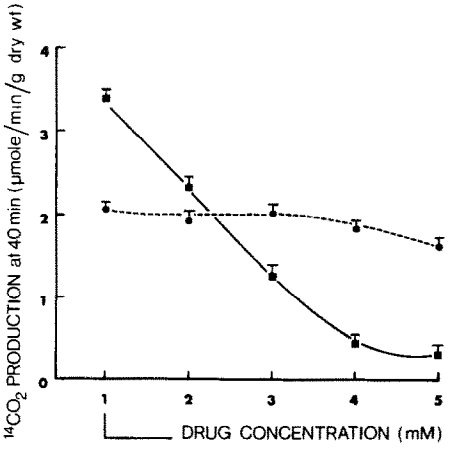

Fig. 2. Dose–response curve of $^{14}CO_2$ production from [1-^{14}C]-butyrate by rat colonocytes in the presence of 5-ASA with (■—■) and without NO_2^- (●– –●).

Table 1. Rate of $^{14}CO_2$ and acetoacetate production from [1-^{14}C]-butyrate (10 mM) and the effect of salicylic acid drugs: mean ± S.E. of paired rat experiments carried out over 40 min

Condition	Metabolite produced (μmol/min/g dry wt) $^{14}CO_2$	Acetoacetate
Control	2.08 ± 0.05	2.85 ± 0.23
+ NO_2^- (10 mM)	3.42 ± 0.17*	0.44 ± 0.07†
NO_2^- (10 mM) + SASP (2 mM)	3.49 ± 0.20	0.47 ± 0.2
NO_2^- (10 mM) + SP (2 mM)	3.36 ± 0.16	1.52 ± 0.21‡
NO_2^- (10 mM) + 5-ASA (2 mM)	1.98 ± 0.1*	2.40 ± 0.80†‡

* $P < 0.001$ (Student's paired t test).
† $P < 0.025$ (paired t test).
‡ NS (paired t test).

without nitrite (Fig. 2). In the presence of nitrite 5-ASA added to rat colonocytes markedly suppressed $^{14}CO_2$ production and enhanced the appearance of acetoacetate sixfold (Table 1). SASP and SP had no effect on suppressing fatty acid oxidation although SP promoted ketogenesis. The fat-suppressing effect of 5-ASA on colonocytes was dose-dependent (Fig. 2). In human colonocytes the rates of $^{14}CO_2$ production were less than that in rat colonocytes (Table 2). Suppression of butyrate oxidation and enhancement of ketogenesis again were observed with 5-ASA both of which depended on drug concentration (2 and 4 mM) (Table 2).

Suppression index of fatty acid oxidation and clinical efficacy of drugs

The ability to suppress oxidation of fatty acid was expressed as an index—suppression index of fatty acid oxidation or SIFO—and calculated for seven drugs in paired experiments with rat and human colonocytes (Table 3). The SIFO was correlated with the clinical evaluation by rectal enema of each drug as reported by others [1–4, 18–22]. The highest SIFO score correlated with the clinically most efficacious drug (5-ASA) and the lowest SIFO score correlated with drugs that were clinically ineffective (ASA and SP). High concentrations of 4-ASA (6 mM) had moderate ability to suppress fatty acid oxidation in parallel with clinical experience that high doses (2 or 4 g rather than 1 g enemas) were needed to bring about a therapeutic effect [21, 22].

DISCUSSION

The present observations indicate that 5-ASA effectively suppresses colonic mucosal oxidation of SCFAs in the presence of high concentrations of nitrite anions. While this effect was shown in healthy mucosal cells, the marked correlation of the observation with clinical efficacy of the drug suggests that the observation has strong implications in the therapeutic action of these drugs in UC.

The concentration of 5-ASA reached in the colonic lumen may be as high as 14 mM [15] and our concentrations of 5-ASA were within this range. An action of 5-ASA has been observed on inflammatory cells [23] and prostaglandin production by the inflamed colon [6] though some reservation has been cast on a direct interaction between prostaglandins

Table 2. The effect of NO_2^- and salicylic acid derivatives on β-oxidation and ketogenesis in human colonocytes

Condition	$^{14}CO_2$	Metabolite production [μmol/min/g (dry wt)] βOH-butyrate	Acetoacetate
Control	1.0 ± 0.09(7)	0.48 ± 0.04(7)	3.9 ± 0.48(7)
+ NO_2^- (10 mM)	1.79 ± 0.16(7)	0.48 ± 0.04(7)	0.85 ± 0.12(7)
+NO_2^- (10 mM) + 5-ASA (2 mM)	1.03 ± 0.19(7)	ND	1.79 ± 0.32(7)
+ NO_2^- (10 mM) + 5-ASA (4 mM)	0.25 ± 0.06(5)	ND	1.50 ± 0.33(5)
+ NO_2^- (10 mM) + SP (2 mM)	1.76 ± 0.16(7)	ND	1.76 ± 0.17(7)
+ NO_2^- (10 mM) + 4-ASA (6 mM)	1.4 ± 0.11(7)	ND	1.94 ± 0.3 (7)

Cells were prepared as described in Methods and values are mean ± S.E. of the number of colectomy specimens in brackets and represent paired experiments.

Table 3. Percentage suppression of fat oxidation (SIFO) in rat and human colonocytes compared with reported clinical efficacy of drug for treatment in acute UC

Pharmacological agent and concentration (mM)		SIFO Rat colonocytes	SIFO Human colonocytes	Clinical efficacy
SASP	(2)	0	(0)*	Split molecule mainly [1–3]
ADS	(2)	0	—	? split molecule
5-ASA	(2)	(42.8 ± 7)*	77.4 ± 14.7‡	Active molecule [1–4]
	(4)	134.0 ± 6.3†	150.0 ± 10.9	
SP	(2)	11 ± 5.4	2.7 ± 2.0‡	Inactive molecule [1–3]
	(4)	27 ± 4.5†	—	
Acetyl-5-ASA	(4)	0	—	Inactive [18]/active [19]
ASA	(2)	0	—	inactive [20]
4-ASA	(4)	16 ± 4.2	—	Inactive (1 g) [21]
	(6)	—	38.7 ± 6.4	active (2–4 g) [22]

* Tested separately and not part of paired experiment.
† $P < 0.001$ (Student's paired t test).
‡ $P < 0.01$ (Student's paired t test).

and 5-ASA [24]. Other biochemical actions of 5-ASA are on a variety of oxidation-reduction enzymes [25], including lipoxygenase activity of soybeans [7] and we now add suppression of stimulated butyrate oxidation to this list. The action of 5-ASA is thus both on long and short chain fatty acid oxidation in the colonic mucosa.

Drug action in terms of the SIFO correlated with the clinical efficacy of the respective drugs. The value of acetyl-5-ASA as a therapeutic agent is controversial [18, 19] and our *in vitro* results and the SIFO would favour the study of Binder *et al.* [18] which indicated that acetyl-5-ASA was not clinically useful. If indeed the biochemical findings and the SIFO now reported correlate with the clinical efficacy of drugs then the colonic epithelial cell system may aid assessment of new drugs for clinical evaluation in the management of UC. One limitation of the proposed system is that carrier molecules such as are used in SASP and ADS may mask the active therapeutic radicle which in 5-ASA appears to be the 5 amino group [26].

Nitrites are widely assumed to have a pathological role especially in colonic carcinogenesis [27] while a physiological role for these anions in the colon has been relegated to one of unimportance. Nitrite is measurable in the colon [28, 29] and stimulates mucosal oxidation of fatty acids in the colonic mucosa [16]. Whether nitrite and 5-ASA combine to form nitroso-5-ASA [30] in the mucosa or whether 5-ASA acts secondarily on a metabolic action of nitrite in the colonic mucosa needs to be evaluated further. Nitrites in many organs are oxidized by catalase [31] an enzyme which is also found in the colonic mucosa and could possibly be one site of action for 5-ASA.

The inhibitory action of 5-ASA on oxidation of fatty acids in the presence of nitrite has implications both for the pathogenesis of UC and for the therapeutic action of 5-ASA. Biochemical studies revealed a diminished rate of CoA-dependent oxidation of SCFAs to CO_2 and ketone bodies in the colonic epithelium involved with UC [10]. This observation may be the result of a prolonged hyperstimulatory effect of nitrite ions on colonic epithelial metabolism of fatty acids, now known to occur [16], leading to an exhaustion state of β-oxidation in the colonic epithelium. In the light of this hypothesis the action of 5-ASA would be to prevent excessive stimulation of fatty acid metabolism and the development of the biochemical lesion as outlined above. Such a proposal for the action of 5-ASA is in line with the clinical observation that 5-ASA is most useful in decreasing the frequency of acute attacks of UC [32], which we propose are diminished in frequency because hyper-stimulation of fatty acid oxidation does not occur in the presence of 5-ASA.

In conclusion, the present results indicate that 5-ASA, in the presence of nitrite, inhibits β-oxidation in isolated colonic epithelial cells, an action which varies according to the clinical effectiveness of each individual salicylate derivative tested.

Proposals concerning a new therapeutic action for 5-ASA in UC are hypothetical but provide a framework to verify current interpretations.

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