BACTERIAL VIRULENCE

The effects of unsaturated fatty acids on *Helicobacter pylori* in vitro

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Summary. The effects of three unsaturated free fatty acids on *Helicobacter pylori* growth in vitro was determined. Growth of *H. pylori* in Brucella broth was inhibited in a dose-dependent manner by arachidonic, linoleic and oleic acids. The degree of inhibition at any one concentration was related to the degree of fatty acid unsaturation. Triolein, a triacylglycerol ester of oleic acid did not inhibit growth. Inhibition of *H. pylori* growth was associated with disruption of cell membranes. Incubation with ¹⁴C linoleic acid and ¹⁴C oleic acid showed incorporation of these fatty acids into *H. pylori* cell mass and phospholipids leading to alteration of the phospholipid composition of the organism. Incorporation was greater with linoleic than oleic acid and this was associated with a greater inhibition of growth. These findings indicate that *H. pylori* is sensitive to unsaturated free fatty acids through their incorporation into phospholipids and membrane destruction. This may have therapeutic implications.

Introduction

The importance of *Helicobacter pylori* in the pathogenesis of duodenal ulceration is well established.^{1–3} Colonisation of both gastric antrum and duodenum are essential prerequisites to ulceration^{4,5} and eradication of *H. pylori* results in cure of duodenal ulcer disease.^{1,6}

Dietary polyunsaturated fatty acids have been implicated in the prevention of peptic ulcers. This is based on epidemiological⁷ and clinical studies,⁸ but the mechanism of protection remains unclear. The antibacterial effect of fatty acids has long been recognised^{9–11} and is believed to be an important factor regulating the distribution and density of colonisation in the lung,¹² on the skin¹³ and in the gastrointestinal tract.^{14,15}

In-vitro studies have examined the sensitivity of different bacteria to fatty acids in relation to the physicochemical properties of both the substances and the organisms.^{10,16–19} Generally, gram-positive bacteria are more susceptible to the damaging effects of fatty acids than gram-negative bacteria.²⁰ Both the composition and permeability of the bacterial outer membrane are important factors in this difference.²¹

Unsaturated fatty acids tend to be more inhibitory than saturated ones and *cis* unsaturated isomers are more damaging than *trans* isomers.^{17,22} The concentration of unsaturated fatty acids also determines their inhibitory effect on bacteria; generally, low concentrations have either growth stimulating or bacteriostatic effects, whereas high concentrations have toxic effects on susceptible organisms.^{20,23}

A proportion of bacterial fatty acids are unsaturated whereas others have a cyclic structure, a feature not normally present in eukaryotic cells. Changes in the environment alter the proportions of membrane fatty acids,^{24–26} suggesting that it may be possible to manipulate fatty acid composition and, thus, membrane characteristics.

Arachidonic, linoleic and oleic acids, present in cell membranes as phospholipid components, are the three commonest fatty acids present in biological systems. They have different physicochemical properties and are either part of dietary intake or synthesised by the human body. This study reports the results of investigations into the effects of these three unsaturated fatty acids on *H. pylori* in vitro.

Materials and methods

Growth in liquid culture

H. pylori strains from human endoscopic biopsy

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samples were grown in Brucella Broth (Oxoid) supplemented with horse serum 5% and vancomycin, trimethoprim, cefsulodin and amphotericin pH 7.4, at 37°C in micro-aerophilic conditions (O₂ 5%, CO₂ 7%, H₂ 8%, N₂ 80%; Oxoid gas generating kit and gas jar, Unipath)²⁷ in thin-layer, non-shaking containers for 2–3 days. Only those cultures yielding heavy, pure growths were used; bacteria from poorly growing cultures tended to give variable results and were discarded. Three free fatty acids—oleic (C18; Δ 9, *Cis*) linoleic (C18; Δ 9, 12, *Cis*) and arachidonic acid (C20; Δ 5, 8, 11, 14, *Cis*)—at 0.05, 0.1, 0.5, 1.0, 2.0 and 5 mM concentrations, and the triacylglycerol of oleic acid, triolein, at 2 and 5 mM were studied. Slightly opalescent suspensions of these substances in Brucella broth were added to separate cultures of the same strain, containing *c.* 5×10^7 organisms/ml in mid-log growth and were incubated for 48 h. Optical density changes of the cultures at 540 nm (SP6-450 UV/VIS spectrophotometer; Pye Unicam) and viable count measurements²⁸ were used to assess numbers of organisms at 24 and 48 h. All incubations with fatty acids were repeated with six different strains to ensure that the results were reproducible. At 48 h, *H. pylori* cells were harvested by centrifugation at 25000 *g* for 20 min and were washed twice in Brucella broth. They were then re-incubated in fresh Brucella broth for a further 24 h to determine whether any fatty acid-induced effect on growth was reversible.

Because preliminary experiments had shown that changes in pH, incubation temperature and inoculum size all independently altered the sensitivity of *H. pylori* to unsaturated fatty acids, these factors were kept constant in all experiments. The identity of the organisms was confirmed at each stage by Gram's stain, urease and catalase tests. The morphology of the organism and the integrity of the membrane were assessed by light microscopy (Leitz Dialux 20 EB) and by transmission electronmicroscopy (Zeiss EM 900), respectively.

Incorporation of radiolabelled fatty acids

¹⁴C linoleic acid, oleic acid and triolein (fatty acid labelled) were added to separate cultures containing organisms in mid-log growth. After incubation for 24 and 48 h, *H. pylori* cells were separated from the culture medium by centrifugation at 25000 *g* for 20 min and washed twice with saline. The total incorporation of radiolabelled fatty acid was measured by scintillation counting of the washed cells. *H. pylori* lipids were isolated by the method of Folch *et al.*²⁹ and then separated by thin layer chromatography (TLC) on silica gel G plates (20 × 20, 250 μ m, Anachem) with a solvent mixture of chloroform:hexane:methanol:acetic acid:water (12:7:4:3:0.3).³⁰ Free fatty acid and phospholipid bands on chromatograms were visualised by iodine staining and quantified by scintillation counting of the bands collected by scraping.

Fatty acid methyl ester profiles

The phospholipids extracted from *H. pylori* by the Folch method,²⁹ and separated by TLC as described above, were dried under nitrogen and boiled in boron trifluoride 14% in methanol.³¹ Fatty acid methyl esters were then extracted in hexane, dried under nitrogen and analysed by gas liquid chromatography (GLC) with a DB-23 Megabore 30-m column, with 0.5 μ m film thickness (J and W Scientific, CA, USA) in a GC-6000 GLC machine (Vega series 2, ICU 600, Carlo Erba Instruments). Peaks were identified by comparison with the retention times of known standards and the area under the curve was measured to determine the percentage of each fatty acid in the sample.

Results

Growth in liquid media

The optical density of liquid cultures related closely to viable counts on chocolate agar plates ($r = 0.94$, $p < 0.001$) thus validating the use of optical density in the measurement of numbers of viable organisms in liquid cultures (fig. 1).

Exponential growth of *H. pylori* in control conditions is shown in fig. 2. In the presence of unsaturated fatty acids growth was inhibited compared to control growth in a dose-dependent manner at 24 and 48 h (fig. 3). The inhibitory effect of the three fatty acids, at a given concentration, was related to their degree of unsaturation: arachidonic > linoleic > oleic acid. Esterification of oleic acid resulted in the complete loss of its inhibitory effects on *H. pylori* growth.

The diminution in the growth rate of *H. pylori* in the presence of the highest concentration of oleic acid (5 mM) persisted after incubation for 48 h in the absence of oleic acid. By contrast, cultures in lower concentrations (0.05–1 mM), produced a small and insignificant inhibition of growth at 24 h, but the growth rate reverted completely to normal by 48 h. Interestingly, growth inhibition by 2 mM oleic acid was

Fig. 1. Relationship between OD₅₄₀ of cultures and viable counts; $r = 0.94$, $p < 0.001$.

Fig. 2. Growth of *H. pylori* in liquid culture under control conditions.

Fig. 3. Relative growth of *H. pylori* to that of control after (a) 24 h and (b) 48 h incubation with arachidonic acid (□), linoleic acid (●) and oleic acid (○); * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus control.

reversible at 48 h, with the growth rate returning to that of the control within a further 24 h. Growth inhibited by 0.05 and 0.1 mM linoleic acid and arachidonic acid was similarly reversible. However, growth inhibition by all higher concentrations of these two fatty acids was not reversible.

Morphology of *H. pylori*

Light microscopy of cultures grown under control conditions and of those with the higher (> 90% of control) growth in the presence of fatty acids showed *H. pylori* cells with normal curved or S-shaped morphology; < 10% of cells had coccoid forms. Increased proportions of coccoid forms were formed during growth in inhibitory concentrations of all three fatty acids after 24 and 48 h until, at < 50% of the control growth, both spiral and coccoid forms of *H. pylori* were sparse and difficult to find.

By electronmicroscopy, cultures with minimal growth inhibition contained predominantly curved or S-shaped organisms. A few had a more rod-like appearance (fig. 4a). The cells from cultures with reduced growth (< 50% of the control) showed total loss of the normal curved morphology, fragmentation of the bacterial cell membranes, disruption of proto-plasmic cylinders and cell lysis (fig. 4b).

Incorporation of radiolabelled fatty acids

Incorporation of ^{14}C linoleic and oleic acids into *H. pylori* cells was detected at 2 and 5 mM; however, ^{14}C -triolein was not incorporated. At both concentrations there was greater incorporation of linoleic than oleic acid (fig. 5). Folch extraction of *H. pylori* lipids and subsequent TLC separation showed that a proportion of the fatty acids taken up was incorporated into phospholipid. More linoleic acid than oleic acid was incorporated into it (fig. 6).

Fatty acid methyl ester profiles

H. pylori grown under control conditions showed a distinctive fatty acid methyl ester profile (table). Myristic and cyclopropano-nonadecanoic acid were the major component fatty acids and comprised 32% and 26% of the total, respectively. Palmitic, stearic, oleic and linoleic acids were also present in significant amounts, each comprising 6–10% of the fatty acid content of *H. pylori* phospholipid. Smaller contributions ($\leq 2\%$ each) came from lauric, hydroxy-

Fig. 4. Thin sections of *H. pylori* incubated with linoleic acid: (a) 0.05 mM for 24 h, showing normal organism morphology; (b) 2 mM for 24 h, showing distortion of the protoplasmic cylinder, and disruption and fragmentation of the cell membranes.

palmitic, linolenic, arachidic, arachidonic and clupanodonic acids, and a further 3% was in the unidentified fractions. Addition of free linoleic acid or oleic acid (2 mM) to the culture medium resulted in a greater than two-fold increase in the amount of these fatty acids in *H. pylori* phospholipid, compared to the control (table), but a decrease in its content of myristic and cyclopropano-nonadecanoic acids.

Discussion

H. pylori growth can be inhibited *in vitro* by unsaturated fatty acids and the extent of inhibition is related to the degree of unsaturation of the fatty acid. The greatest inhibition was produced by arachidonic acid with four double bonds, followed by linoleic acid with two. The least inhibitory was oleic acid with a

Fig. 5. Incorporation of (a) linoleic acid and (b) oleic acid into *H. pylori* cell mass at 24 h (▨) and 48 h (■).

Fig. 6. Proportion of (a) linoleic acid and (b) oleic acid converted to phospholipid; ▨, fatty acid; ■, phospholipid.

single double bond. Significant inhibition of growth was associated with loss of the normal morphology of the organism, fragmentation and disruption of cell membranes and cell lysis. More pronounced growth inhibition and membrane destruction, as given by linoleic acid rather than oleic acid, was associated with greater incorporation of linoleic acid into *H. pylori*. The incorporation of both linoleic and oleic acids resulted in enhancement of the peaks produced by their respective methyl esters on GLC, indicating that these fatty acids were taken up and utilised in phospholipid synthesis by the organism. This incorporation was also accompanied by a reduction in the levels of other fatty acid constituents. Esterification of

oleic acid inhibited its incorporation into *H. pylori* and negated its growth inhibitory effects. These findings suggest that alteration in phospholipid composition as a result of free fatty acid uptake may affect the physicochemical characteristics of *H. pylori* membranes resulting in disturbance of both their structure and function.

An alternative explanation is that the inhibitory effect of fatty acids is related to their chain length, as arachidonic acid has 20 carbon atoms while the other two fatty acids have only 18. However, this does not explain the difference in effects between linoleic acid and oleic acid. *H. pylori* grown *in vitro* is sensitive to changes in pH, and fatty acids may produce their

Table. Fatty acid composition of *H. pylori* under control conditions and comparative data from cultures incubated with oleic and linoleic acids

Fatty acid	Structure	Percentage composition of <i>H. pylori</i> grown with		
		control broth	oleic acid	linoleic acid
Lauric	C12	1.3	1.2	1.5
Myristic	C14	32.0	29.1	23.5
Palmitic	C16	6.4	7.2	7.1
Hydroxy-palmitic	C16, OH	1.4	1.2	1.3
Stearic	C18	8.5	8.6	8.1
Oleic	C18, Δ 9	8.1	17.3	10.3
Linoleic	C18, Δ 9, 12	6.9	6.8	18.7
α -Linolenic and γ -linolenic	C18, Δ 9, 12, 15 } C18, Δ 6, 9, 12 }	1.2	1.4	2.2
Cyclopropano-nonadecanoic	C19, cyclic	25.9	19.6	18.8
Arachidic	C20	1.2	1.1	0.8
Arachidonic	C20, Δ 5, 8, 11, 14	1.9	2.1	1.8
Clupanodonic	C22, Δ 7, 10, 13, 16, 19	1.8	1.6	2.8
Unidentified		3.4	2.8	3.1

inhibitory effects through a lowering of pH. However, strict regulation of conditions in all incubations make this unlikely.

It has been shown that micro-organisms meet their fatty acid requirements by different mechanisms, including uptake from their environment. Increased incorporation of long chain fatty acid results in alteration and damage to bacterial membranes and may also affect metabolic pathways, resulting in bacterial destruction.³² The growth inhibitory effect of unsaturated fatty acids on other organisms has long been recognised and the role of physicochemical properties, including isomerism and the degree of unsaturation, has been demonstrated in susceptible bacteria.^{17,33} Furthermore, studies have shown the importance of a free carboxyl group in the damaging effects of fatty acids and the negation of this by esterification with cholesteryl or methyl groups.^{17,33}

Incorporation of linoleic acid resulted in reduction in the level of cyclopropano-nonadecanoic acid in *H. pylori* phospholipid. The synthesis of cyclic fatty acids and the activity of "desaturase"—which is involved in their production—are stimulated by hydrogen ions and inhibited by hydroxyl ions in other organisms,³⁴ suggesting that this class of fatty acids may play a part in stabilising membranes against adverse pH levels. Cyclopropano-nonadecanoic acid is a significant constituent of *H. pylori* membranes and may be important in protecting the organism in an acidic environment. It is possible that factors that reduce the level of this fatty acid *in vivo* will render *H. pylori* more susceptible to damage by gastric acid.

The growth inhibitory effects of low concentrations of all three free fatty acids in this study were reversible. This indicates that, at these concentrations, they produced a bacteriostatic effect. Other studies have also shown that, in low concentrations, fatty acids produce a reversible inhibition of micro-

organisms.^{33,35} Lecithin, cholesterol, haemoglobin and horse serum are effective in reversing fatty acid inhibition. In this study the presence of horse serum in the fresh Brucella broth probably played a role in reversing the bacteriostatic effect. However, with the higher concentrations of fatty acids, inhibition of *H. pylori* growth was irreversible because of disruption of cell membranes and cell lysis.

The finding that oleic acid at low concentrations inhibited *H. pylori* growth within 24 h, but by 48 h the bacteria had recovered and the growth rate had returned to normal, has been observed with other bacteria and is believed to be related to biotin metabolism.³³

The inhibitory effects of unsaturated fatty acids may arise by direct chemical action on bacterial metabolism. The formation of short chain aldehydes through oxidation of fatty acid double bonds has a toxic effect in a pH-dependent manner. However, the concentrations of aldehydes required to produce damage are far greater than those detected in the presence of even the high concentrations of unsaturated fatty acids used, suggesting that this mechanism may not be important. An alternative theory, which is supported by our findings, is based on the physicochemical properties of the molecules. Long-chain unsaturated fatty acids are lipophilic to an extent, which is related to the *cis/trans* configuration as well as the degree of unsaturation. Adsorption of a monolayer of fatty acid to the bacterial cell surface may lead to incorporation of the fatty acid into the cytoplasmic membrane, resulting in alteration of membrane permeability. Low concentrations of fatty acids may reduce permeability. This will decrease the entry of essential nutrients and produce a bacteriostatic effect. Incorporation of larger amounts of fatty acid may produce an opposite effect and increase permeability leading to osmotic cell lysis and a bactericidal effect.

In conclusion, this study demonstrated that unsaturated fatty acids were inhibitory to *H. pylori* growth *in vitro*. Inhibition was related to the concentration and degree of unsaturation of the fatty acid and was irreversible at high concentrations. The degree of growth inhibition was related to the incorporation of fatty acids into *H. pylori* phospholipid and was

accompanied by morphological changes and membrane disruption. This study has identified an area of *H. pylori* metabolism that is susceptible to manipulation and may, therefore, provide a focus for therapy.

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