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Rhein Affects Arylamine *N*-Acetyltransferase Activity in *Helicobacter pylori* from Peptic Ulcer Patients

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Arylamine *N*-acetyltransferase (NAT) activities with 2-aminofluorene and *p*-aminobenzoic acid were determined in the bacterium *Helicobacter pylori* collected from peptic ulcer patients. Cytosols or suspensions of *H. pylori* with or without specific concentrations of rhein co-treatment showed different percentages of 2-aminofluorene and *p*-aminobenzoic acid acetylation. The data indicate that there was decreased NAT activity associated with increased levels of rhein in *H. pylori* cytosols. Inhibition of growth studies from *H. pylori* demonstrated that rhein elicited dose-dependent bacteriostatic activity in *H. pylori* cultures: i.e. the greater the concentration of rhein, the greater the inhibition of growth to *H. pylori*. For the cytosol and intact bacteria examination, the apparent values of K_m and V_{max} were decreased after co-treatment with 40 μM rhein. This report is the first demonstration of rhein inhibition of arylamine *N*-acetyltransferase activity and rhein inhibition of growth in the bacterium *H. pylori*.

INTRODUCTION

Exposure to environmental and occupational chemicals is thought to be an important cause of chemical carcinogenesis. Arylamine carcinogens require metabolic activation by host enzymes in order to initiate carcinogenesis in specific target organs or tissues.[1,2] *N*-Acetylation, a major metabolic pathway for these arylamine carcinogens, is catalysed by cytosolic arylamine *N*-acetyltransferase (NAT) using acetyl co-enzyme A as an acetyl donor.[3] Arylamine carcinogens, such as 2-aminofluorene (AF) are *N*-acetylated to the 2-acetylaminofluorene (AAF), which can undergo further activation or detoxification reactions. *N*-Acetyltransferase, an enzyme involved in several steps of both arylamine activation and detoxification,[4] is found in many tissues of animals and humans.[5–8] Humans and other mammals exhibit a genetic polymorphism in NAT activity resulting in rapid, slow and some intermediate acetylation phenotypes.[7,9,10] In fact, human epidemiological studies suggested an association between rapid acetylator phenotype in colorectal cancer,[11,12] as well as between slow acetylator phenotype in bladder cancer.[13] The present authors have also found NAT in the nematode,[14] the freshwater shrimp[15] and certain fish.[8]

Warren and Marshall first demonstrated that *Helicobacter pylori* was present in patients' stomachs.[16] It has also been demonstrated that there is a etiological correlation of *H. pylori* with active chronic gastritis.[17] Other investigators have also demonstrated that *H. pylori* is a possible causative factor in patients with gastric cancer.[18] It has been reported that chronic atrophic gastritis is a precursor lesion of gastric cancer, and chronic gastritis might be inducd or exacerbated by *H. pylori*[18–20] because gastritis, peptic ulcer and gastric cancer are related to the presence of this bacterium.[21]

Rhein (4,5-dihydroxy-2-anthraquinone-carboxylic acid) is the active metabolite of diacetylrhein and has been reported to exert several effects both *in vivo* and *in vitro*. Rhein has been shown to exert anti-inflammatory and antirheumatic effects in humans.[22–24] However, a specific antineoplastic activity to Ehlrich ascites tumour cells and human glioma cells has been reported.[25,26] Other investigators also demonstrated that rhein interferes with electron transport, inhibiting mitochondrial oxidation of NAD- and FAD-linked substrates at the dehydrogenase–co-enzyme level.[26] Furthermore, it has been reported that rhein affects energy metabolism by inducing a decrease in ATP production, ultimately leading to an impairment of protein synthesis.[27] It has been shown that rhein inhibits glucose uptake,[28] as well as oxidative phosphorylation,[26] and drastically lowers the adenylate energy charge.[27] It has also been demonstrated that rhein inhibits superoxide production and the activity of lysosomal enzyme.[29,30] Therefore, other investigators have pointed out that rhein may exact direct action on plasma and intracellular membranes.[31]

In previous studies, the present authors found that: emodin induced cytotoxicity and DNA damage in

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H. pylori;[32] NAT is present in *H. pylori*;[33] and emodin affects the NAT activity of *H. pylori*.[34] Therefore, the purpose of the present study was to elucidate the possible effects of rhein on the NAT activity in *H. pylori*. Our initial choice of AF and *p*-aminobenzoic acid (PABA) as test substrates was based on previous studies in mice[6] and nematodes[14] and our interest in comparing the metabolism of a carcinogen (AF) and to a non-carcinogen (PABA). The results demonstrated that by using AF and PABA as substrates for NAT activity determination, rhein did decrease *H. pylori* NAT activity in cytosol and in intact bacteria.

EXPERIMENTAL

Chemicals and reagents

Rhein was obtained from Fluka Chemika Co. (Buchs, Switzerland). Ethylenediaminetetraacetic acid (EDTA), *p*-aminobenzoic acid (PABA), acetyl-*p*-aminobenzoic acid (*N*-Ac-PABA),2-aminofluorene (AF), 2-acetyl-aminofluorene (AAF), bovine serum albumin (BSA), phenylmethylsulphonylfluoride (PMSF), TRIS, leupeptin, acetyl carnitine, dithiothreitol (DTT), carnitine acetyltransferase and acetyl co-enzyme A (AcCoA) were obtained from Sigma Chemical Co. (St Louis, MO). Acetic acid, acetonitrile, dimethyl sulfoxide (DMSO), and potassium phosphates were obtained from Merck Co. (Darmstadt, Germany). All chemicals used were reagent grade.

Helicobacter pylori

Helicobacter pylori bacteria were clinically isolated from patients who visited our department (China Medical College Hospital) for endoscopy as described in previous reports.[33,34]

Preparation of bacteria cytosols

About 10×10^{10} colony-forming units (CFU) were washed twice in cold phosphate-buffered saline, placed immediately in 1 ml of lysis buffer (20 mM TRIS-HCl (pH 7.5 at 4°C), 1 mM DTT, 1 mM EDTA, 50 μM PMSF and 10 μM leupeptin), disrupted by a sonicator and centrifuged for 30 min at 10 000 *g*. The supernatant was kept on ice until assayed for NAT activity.

N-Acetyltransferase activity determination

The determination of AcCoA-dependent *N*-acetylation of PABA and AF was performed as described by Andres *et al.*[35] and modified by Mattano and Weber.[5] Incubation mixtures in the assay system consisted of a total volume of 90 μl: tissue cytosol, diluted as required, in 50 μl of lysis buffer (20 mM TRIS-HCl at pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mM acetylcarnitine) and AF or PABA at specific concentrations for the substrate. The reactions were started by the addition of 20 μl of AcCoA. Control reactions had 20 μl of distilled water instead of AcCoA. The final concentration of PABA or AF was 0.1 mM, and that of AcCoA was 0.5 mM. The reaction mixtures were incubated at 37°C for 10 min and stopped with 50 μl of 20% trichloroacetic acid for PABA reactions and with 100 μl of acetonitrile for AF reactions. All the reactions of the experiments and controls were run in triplicate. The amounts of acetylated product and remaining non-acetylated substrate were determined by HPLC.[6,31] An aliquot of the NAT incubation was injected onto a C18 reversed-phase column (Spherisorb 4.6×250 nm) of a Beckman HPLC (pump 168 and detector 126) and eluted at a flow rate of 1.2 ml min^{-1}. For PABA and *N*-Ac-PABA the solvent system was 50 mM acetic acid–CH_3CN (86:14) with detection at 266 nm. The retention time of PABA was 4 min and that of *N*-Ac-PABA was 6.5 min. For AF and AAF, the solvent system was 20 mM KH_2PO_4 (pH 4.5)–CH_3CN (53:47) with detection at 280 nm. The retention time was 6.5 min for AAF and 9 min for AF. All the compounds were quantitated by comparison of the integrated area of the elution peak with that of known amounts of standards. The activity of NAT is expressed as nmol acetylated substrate min^{-1} mg^{-1} cytosolic protein.

Protein concentrations in the cytosols from *H. pylori* were determined by the method of Bradford,[36] with BSA as the standard. All the samples were assayed in triplicate.

Effect of various concentrations of rhein on growth of *H. pylori*

Twenty-two strains of *H. pylori*, cultured individually in *Brucella* anaerobic culture plates in an anaerobic jar for 5 days to obtain growth at the levels of 10^8 bacteria, were placed in individual tubes containing brain heart infusion media with or without different concentrations of rhein (0.04, 0.4, 4, 40 and 400 μM). The culture tubes were incubated at 37°C in a microaerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2) and checked for growth after 5 days. This bacterium grows slowly and usually needs 4–5 days. The determination of the effects of rhein on the *H. pylori* was based on the measurement of the absorbance by an optical density method (OD at 650 nm).[34] The control groups were prepared under the same conditions as the rhein-treated groups except without the rhein.

Effect of various concentrations of rhein on NAT activity of *H. pylori*

Rhein dissolved in DMSO with specific concentrations ranging from 0.04 to 400 μM were prepared. The reaction mixtures consisted of 50 μl of cytosols diluted as required, 20 μl of recycling mixture containing AF or PABA at specific concentrations as substrates and 10 μl of rhein (at a specific concentration). The reactions were started by the addition of AcCoA. The control reactions had 20 μl of distilled water instead of AcCoA. Following the NAT activity determination, the procedure was performed to evaluate the effect of rhein on *H. pylori* NAT activity.

Effect of rhein on kinetic constants of NAT from *H. pylori*

Cytosols of *H. pylori* were co-treated with or without specific concentrations of rhein (400, 40, 4, 0.4 and

0.04 μM) and the NAT activity was determined as described in a previous section. All the reactions were run in triplicate. For the intact bacteria studies, 3×10^9 bacterial cells in brain heart infusion broth were incubated with arylamine substrate (AF) with or without specific concentrations of rhein (400, 40, 4, 0.4 and 0.04 μM) for 96 h at 37°C in a micro-aerobic atmosphere (5% O_2, 10% CO_2 and 85% N_2). At the conclusion of incubation, the cells and media were removed and centrifuged. For the experiments with AF, the supernatant was immediately extracted with ethylacetate–methanol (95:5), the solvent was evaporated and the residue was redissolved in methanol and assayed by HPLC. All the samples were run in triplicate. The kinetic constants were calculated with the Cleland HYPER program,[35] which performs linear regression using a least-squares method. The amounts of acetylated product and remaining non-acetylated substrates were assayed by HPLC as described in the NAT activity determination. The velocity ($1/V$) versus substrate ($1/S$) data were linearized by plotting $1/S$ versus $1/V$.

RESULTS

Effect of various concentrations of rhein on the growth of *H. pylori*

The rationale for the authors' initial studies was based on two observations from other reports. First, cytotoxic effects on cultured cells and bacteria have been associated with emodin.[32,33] Second, emodin and rhein are components of Dahung (a Chinese herb medicine). *Helicobacter pylori* has been reported to contain NAT activity,[33] therefore an examination of the effect of rhein on the growth of *H. pylori* is given in Table 1. *Helicobacter pylori* was inhibited by rhein: i.e. the higher the concentration of rhein in the *H. pylori* culture, the higher the inhibition of *H. pylori*. When the concentration of rhein reached 400 μM, the inhibition reached to 90%. The data from two other bacteria, a Gram-negative *Escherichia coli* and a Gram-positive *Staphylococcus aureus*, indicated that the percentage inhibition was 75% under the same concentrations of rhein as used on *H. pylori*. The most significant effect in both treatment groups was a decrease in the percentage of bacterial cells after rhein compared to control groups (Table 1).

Effect of various concentrations of rhein on the activity of *H. pylori* NAT

The possible effects of rhein on the NAT activity in *H. pylori* in cytosol and in intact bacteria were examined by HPLC, assessing the percentage acetylation of AF and PABA. Cytosols of *H. pylori* with or without specific concentrations of rhein co-treatment showed different percentages of AF and PABA acetylation. A comparison of the relative cytosolic NAT activity with or without specific concentrations of rhein is presented in Table 2. Percentage acetylations of AF and PABA by *H. pylori* with or without specific concentrations of rhein co-treatment in intact bacteria are given in Fig. 1.

Table 1. Effect of rhein on the growth of *H. pylori*[a]

	Concentration of rhein (μM)					
Strain	0	0.04	0.4	4	40	400
	Percentage inhibition					
1	0	0	10	70	81	90
2	0	2	10	61	74	82
3	0	0	14	70	80	91
4	0	0	11	68	77	86
5	0	0	14	64	76	88
6	0	0	12	70	79	90
7	0	3	16	80	87	90
8	0	2	10	60	69	84
9	0	0	14	62	70	82
10	0	0	18	70	79	88
11	0	0	10	64	72	86
12	0	4	14	62	78	89
13	0	0	18	76	84	96
14	0	0	10	60	74	84
15	0	0	13	62	76	88
16	0	0	14	60	71	84
17	0	3	16	70	84	94
18	0	0	10	74	83	91
19	0	0	12	66	76	88
20	0	4	16	70	83	96
21	0	0	8	56	72	87
22	0	0	6	47	69	84
23[b]	0	0	11	44	61	72
24[c]	0	0	10	50	60	74

[a] *Helicobacter pylori* was incubated in the presence of various concentrations of rhein (0.04, 0.4, 4, 40 and 400 μM) as described in the Materials and Methods. The percentage inhibition was determined under a spectrophotometer and then calculated. All experiments and controls were run in triplicate.
[b] *E. coli.*
[c] *S. aureus.*

Table 2. Effects of rhein on *H. pylori N*-acetyltransferase activity *in vitro*[a]

Concentration of rhein	AAF (nmol min^{-1} mg^{-1} protein)	*N*-Acetyl-PABA (nmol min^{-1} mg^{-1} protein)
Control	0.86 ± 0.12	0.64 ± 0.08
0.04 μM	0.83 ± 0.14	0.62 ± 0.12
0.4 μM	0.76 ± 0.11[b]	0.55 ± 0.09[b]
4 μM	0.36 ± 0.08[c]	0.29 ± 0.06[c]
40 μM	0.24 ± 0.04[d]	0.11 ± 0.04[d]
400 μM	0.12 ± 0.04[e]	0.06 ± 0.02[e]

[a] *Helicobacter pylori* cytosol was incubated in the presence of various concentrations of rhein (0.04, 0.4, 4, 40 and 400 μM) as described in the Materials and Methods. All experiments and control were run in triplicate. Values are means ± SD ($n = 3$).
[b] Differs between 0.4 μM rhein and control; $P < 0.05$.
[c] Differs between 4 μM rhein and control; $P < 0.02$.
[d] Differs between 40 μM rhein and control; $P < 0.005$.
[e] Differs between 400 μM rhein and control; $P' < 0.001$.

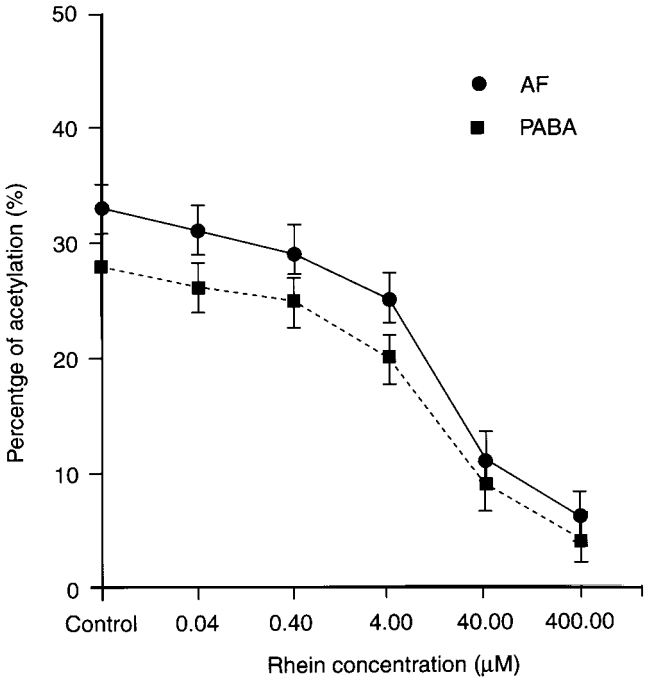

Figure 1. Percentage acetylation of 2-aminofluorene and *p*-aminobenzoic acid by *H. pylori N*-acetyltransferase with or without specific concentrations of rhein. The cytosols were prepared as described in Materials and Methods. The AcCoA concentration was 0.1 mM. Values are the mean ±SD of 1 nmol min^{-1} mg^{-1} protein; $n = 3$ as described in the text.

The data indicate that there was decreased NAT activity associated with increased rhein levels in *H. pylori* cytosols: i.e. the higher the concentration of rhein in the reaction mixtures, the higher the inhibition of NAT activity. Because 400 μM rhein showed inhibition of NAT activity both in cytosol and in intact bacteria (inhibition ratios were 90% and 93%, respectively, for AF and PABA in cytosol and 40% and 37%, respectively, for both substrates in intact bacteria), the inhibition of rhein to the acetylation of PABA was slightly higher than that seen for AF.

Effect of various concentrations of rhein on the kinetic constants of *H. pylori* NAT

The kinetic constants determined for *H. pylori* NAT using AF and PABA as substrates with or without 40 μM rhein are shown in Figs 2 and 3. For the cytosol examination, the apparent values of K_m and V_{max} were 3.25 ± 0.66 mM and 17.26 ± 4.86 nmol min^{-1} mg^{-1} protein, respectively, for 2-AF (Table 3) and 2.60 ± 0.44 mM and 11.90 ± 2.28 nmol min^{-1} mg^{-1} protein, respectively, for PABA (Table 4). However, when rhein was added to the reaction mixtures, the apparent values of K_m and V_{max} were 2.54 ± 0.5 mM and 10.48 ± 3.06 nmol min^{-1} mg^{-1} protein, respectively, for 2-AF (Table 3) and 2.26 ± 0.36 mM and 6.72 ± 0.70 nmol min^{-1} mg^{-1} protein, respectively, for PABA (Table 4). For the intact bacteria examination, the apparent values of K_m and V_{max} were 3.18 ± 0.76 mM and 16.02 ± 3.48 nmol min^{-1} $(10 \times 10^{10}$ colony forming units (CFU)$)^{-1}$, respectively, for 2-AF (Table 3) and 3.26 ± 0.64 mM and 18.10 ± 4.18 nmol min^{-1} $(10 \times 10^{10}$ CFU$)^{-1}$, respectively, for PABA (Table 4). However, when rhein was added to the reaction mixture, the apparent values of K_m and V_{max} were 2.67 ± 0.18 mM and 9.76 ± 0.92 nmol min^{-1} $(10 \times 10^{10}$ CFU$)^{-1}$, respectively, for AF (Table 3) and 2.16 ± 0.18 mM and 6.72 ± 0.70 nmol min^{-1} $(10 \times 10^{10}$ CFU$)^{-1}$, respectively, for PABA (Table 4).

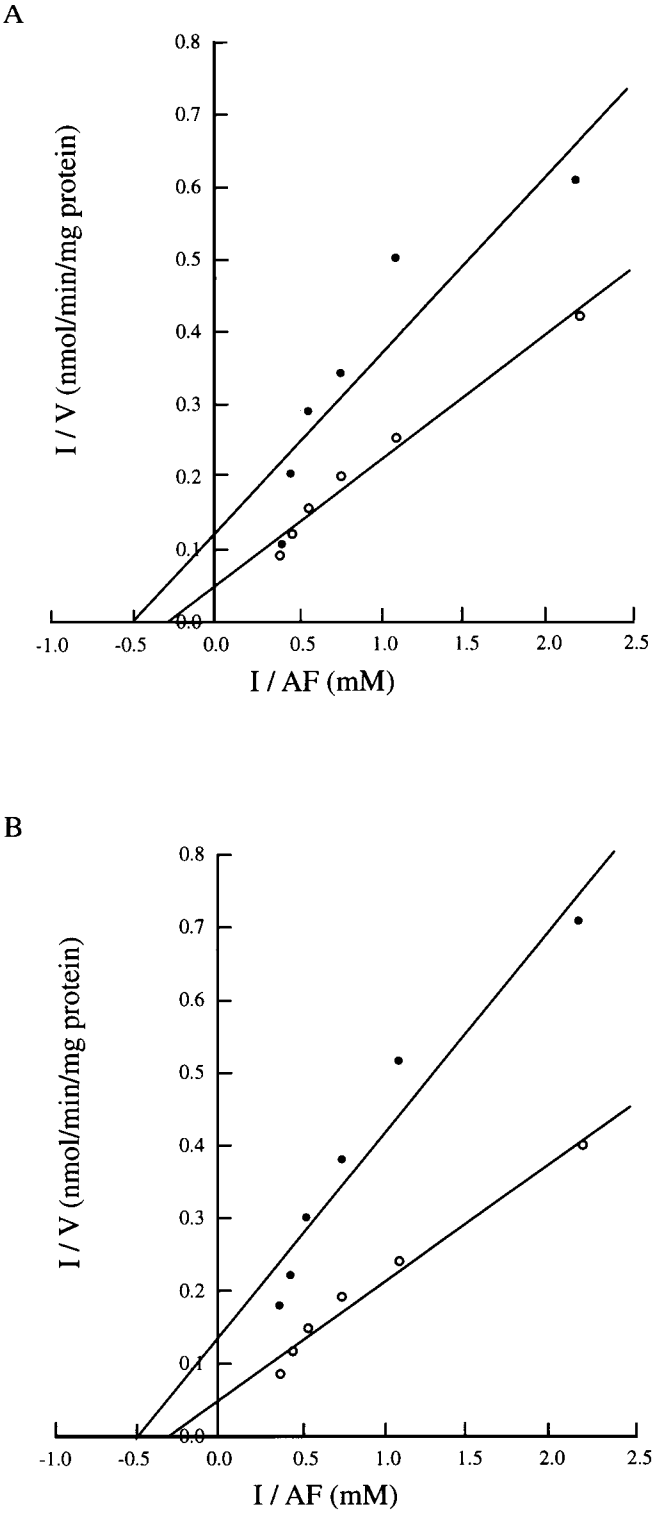

Figure 2. Lineweaver–Burk double reciprocal plot of *H. pylori N*-acetyltransferase activity as a function of 2-aminofluorene concentration in cytosol (A) and in intact bacteria (B). The cytosols and suspensions were prepared as described in Materials and Methods. The AcCoA concentration was 0.1 mM. Values are the mean ± SD of 1 nmol min^{-1} mg^{-1} protein; $n = 3$ as described in the text.

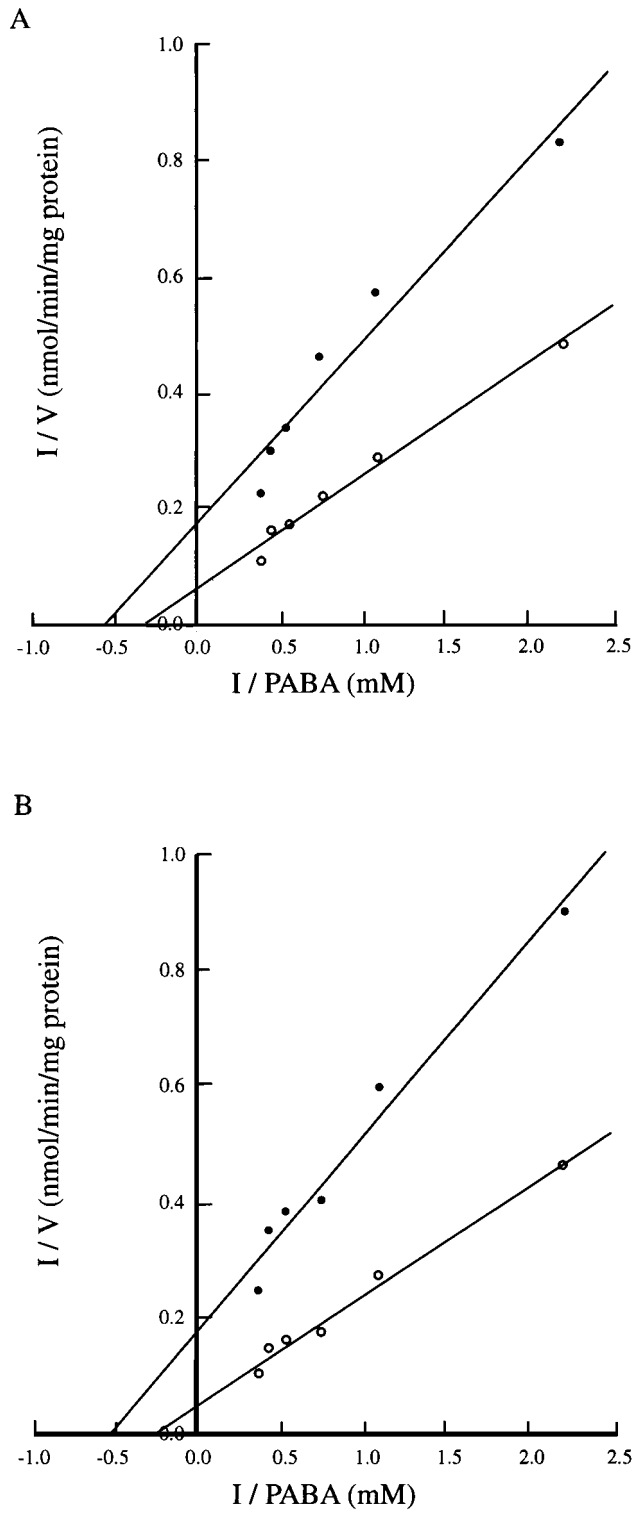

Figure 3. Lineweaver–Burk double reciprocal plot of *H. pylori* *N*-acetyltransferase activity as a function of *p*-aminobenzoic acid concentration in cytosol (A) and intact bacteria (B). The cytosols and suspensions were prepared as described in Materials and Methods. The AcCoA concentration was 0.1 mM. Values are the mean ± SD of 1 nmol min^{-1} mg^{-1} protein; $n = 3$ as described in the text.

Table 3. Kinetic data for acetylator of 2-aminofluorene in *H. pylori*[a]

	In cytosol		In intact bacteria	
	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (nmol $(10 \times 10^{10}CFU)^{-1}$)
Control	3.25 ± 0.66	17.26 ± 4.86	3.18 ± 1.76	16.02 ± 3.48
Emodin	2.54 ± 0.48[b]	10.48 ± 2.56[c]	2.67 ± 0.18[d]	9.76 ± 0.92[e]

[a] Values are means ± SD ($n = 3$). The acetyl CoA and rhein concentrations were 0.1 mM and 40 μM, and the kinetic constants were calculated from the modified HYPER Program of Cleland. All experiments and controls were run in triplicate.
[b] Differs between 40 μM rhein and control; $P < 0.05$.
[c] Differs between 40 μM rhein and control; $P < 0.001$.
[d] Differs between 40 μM rhein and control; $P < 0.005$.
[e] Differs between 40 μM rhein and control; $P < 0.001$.

Table 4. Kinetic data for acetylator of *p*-aminobenzoic acid in *H. pylori*[a]

	In cytosol		In intact bacteria	
	K_m (mM)	V_{max} (nmol min^{-1} mg^{-1})	K_m (mM)	V_{max} (nmol $(10 \times 10^{10}CFU)^{-1}$)
Control	2.60 ± 0.44	11.90 ± 2.28	3.26 ± 0.66	18.10 ± 4.18
Emodin	2.26 ± 0.36[b]	6.72 ± 0.70[c]	2.16 ± 0.18[d]	6.72 ± 0.70[e]

[a] Values are means ± SD ($n = 3$). The acetyl CoA and rhein concentrations were 0.1 mM and 40 μM, and the kinetic constants were calculated from the modified HYPER Program of Cleland. All experiments and controls were run in triplicate.
[b] Differs between 40 μM rhein and control; $P < 0.05$.
[c] Differs between 40 μM rhein and control; $P < 0.005$.
[d] Differs between 40 μM rhein and control; $P < 0.005$.
[e] Differs between 40 μM rhein and control; $P < 0.0001$.

DISCUSSION

There are many events that are likely to be prerequisites for the observed effects of rhein on NAT activity:

(i) The AcCoA-dependent arylamine NAT enzyme has been reported to be present in many kinds of experimental animals, including humans,[3,4] and NAT has been shown to be involved in some chemical carcinogenesis.[38,39]

(ii) Rapid and slow acetylation has been demonstrated as a predisposing factor for the sensitivity of individuals to the toxicity during exposure to many arylamines.[11,12] Therefore, the genetically mediated variations in NAT activities within target tissues or organs for arylamine-induced neoplasm may indicate differential risks among the human population.

(iii) Some enzymes of enteric bacteria are known to contribute to the metabolic activation of chemical carcinogens in animal studies.[40,41]

(iv) The present authors' previous studies already showed that emodin induced the inhibition of growth and DNA damage in *H. pylori*, and emodin decreased NAT activity in *H. pylori*.[32,33]

(v) According to the present authors' preliminary studies, many kinds of enteric bacteria such as *Klebsiella pneumoniae*, *Salmonella* group B and *E. coli*, exhibit NAT activity (manuscript in preparation). It was found that *H. pylori* cytosols

contained NAT activity. Therefore, the present studies were focused on the effect of rhein on the NAT activity of *H. pylori*.

Thus, the data presented in this report clearly demonstrated that rhein did affect *H. pylori* NAT activity and growth. The results clearly indicated that rhein, in concentrations of 0.4–400 μm for cytosol tests, decreased the acetylated product of AF and PABA by *H. pylori*. The results also show that when rhein decreased the NAT activity in *H. pylori*, it was a dose-dependent effect: i.e. the higher the concentration of rhein, the higher the inhibition of NAT activity. The data presented from the intact bacteria tests also showed that rhein decreased the percentage of acetylated products of AF and PABA. Rhein also induced a dose-dependent effect on the NAT activity in *H. pylori*. The data also demonstrated that rhein induced inhibition of the growth effect on the *H. pylori* culture: i.e. the higher the concentration of rhein, the higher the inhibition of *H. pylori* growth. In other words, rhein can be used as a bactericide to *H. pylori*. This is the first report to show that rhein could act as an antimicrobial agent.

Because rhein does inhibit the NAT activity of *H. pylori*, the kinetic constants were also affected. For cytosol examination, the apparent values of K_m and V_{max} decreased by 0.23- and 0.30-fold for acetylation of AF and by 0.58- and 0.16-fold for acetylation of PABA. For the intact bacteria examination, the apparent values of K_m and V_{max} decreased by 0.39- and 0.33-fold for acetylation of AF and by 0.36- and 0.54-fold for acetylation of PABA. Based on the kinetic constant decreases, it was suggested that rhein may act as an uncompetitive inhibitor. This needs further investigation. The cytosol and intact bacteria data showed different degrees of rhein inhibition on the NAT enzyme. Therefore, this finding is very important to the possibility of decreasing arylamine carcinogens in induced carcinogenesis, because other reports have demonstrated that elevated levels of NAT activity may be associated with increased sensitivity to the mutagenic effects of many arylamines[42] and attenuation of NAT activity is associated with several disease process.[3,4]

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