RESEARCH LETTER

Anabolic utilization of steroid hormones in *Helicobacter pylori*

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Abstract

In this study, we have demonstrated that *Helicobacter pylori* absorbs a steroid prehormone (pregnenolone) and two androgens (dehydroepiandrosterone and epiandrosterone), glucosylates these steroids, and utilizes glucosyl-steroid hormone compounds as the membrane lipid components. The only common structure among the steroid prehormone and the two androgens is a 3β-OH in the steroid framework. Our results indicate that the 3β-OH in the steroid hormones is a crucial conformation required for steroid glucosylation by *H. pylori*. In addition, we found that *H. pylori* absorbs and holds estrogens possessing 3-OH (estrone and estradiol) into the membrane. The effective absorption of estrogen into the membrane appeared to be controlled by the number of hydroxyl groups modifying the steroid framework. In contrast, *H. pylori* induced neither membrane absorption nor glucosylation of the other steroid hormones possessing 3 = O (progesterone, androstenedione and testosterone) or 3α-OH (androsterone). These results indicate that *H. pylori* selectively absorbs 3β-OH and 3-OH steroid hormones, and utilizes only 3β-OH steroid hormones as the materials for glucosylation.

Introduction

Helicobacter pylori, a pathogen responsible for chronic gastritis and peptic ulcer in humans (Marshall & Warren, 1983; Wyatt & Dixon, 1988; Graham, 1991), aggressively absorbs exogenous cholesterol, glucosylates the cholesterol and thereby produces cholesteryl glucosides (CGs) (Wunder *et al.*, 2006). This bacterium possesses at least three types of CGs: cholesteryl-α-D-glucopyranoside (CGL), cholesteryl-6-*O*-tetradecanoyl-α-D-glucopyranoside (CAG) and cholesteryl-6-*O*-phosphatidyl-α-D-glucopyranoside (CPG) (Hirai *et al.*, 1995). Steryl glucosides (SGs) are universally present in plants and fungi (Bolt & Clarke, 1970; Bush & Grunwald, 1972; Warnecke *et al.*, 1999), but they are unique glycolipids in bacteria (Smith, 1971; Livermore *et al.*, 1978; Patel *et al.*, 1978; Mayberry & Smith, 1983; Haque *et al.*, 1996). Plants and fungi synthesize SGs by attaching a glucose molecule to a 3β-OH of the sterol via β-glucosidic linkage, whereas *H. pylori* synthesizes CGs by attaching a glucose molecule to a 3β-OH of the cholesterol via α-glucosidic linkage. We discovered in previous experiments that *H. pylori* has the potential to glucosylate not only cholesterol but also fungal and phytogenic sterols (ergosterol and campesterol). However, it remains unclear what structure in the sterol is required for *H. pylori* to recognize and glucosylate it (unpublished data).

Steroid hormones such as sex hormones and corticoids are typical sterol analogues in mammals that are derived from cholesterol. A number of investigations have demonstrated that the enzymes involved in the biosynthesis and activation of sex hormones are also expressed in human stomach tissue (Takeyama *et al.*, 2000; Javitt *et al.*, 2001; Turgeon *et al.*, 2001; Miki *et al.*, 2002). In addition, the expression of sex hormone receptors has been found in gastric cancer (Matsuyama *et al.*, 2002; Takano *et al.*, 2002; Kominea *et al.*, 2004). These studies indicate that sex hormones exist in the stomach environment, which *H. pylori* colonizes and inhabits. No earlier studies, however, have investigated the glucosylation of steroid hormones by *H. pylori*. Thus, *H. pylori* may have the potential to glucosylate the steroid hormones. To test this hypothesis, we analyzed the membrane absorption and glucosylation of

steroid hormones using living *H. pylori* cells, and have identified a specific structure in those steroid hormones that is recognized as the substrate for anabolic metabolism by *H. pylori*.

Materials and methods

Bacterial strains and culture

Helicobacter pylori strains NCTC 11638, ATCC 43504 and A-19 (a clinical isolate) were grown in a pleuropneumonia-like organism (PPLO) broth (Difco Laboratories, Detroit, MI) containing 2,6-di-*O*-methyl-β-cyclodextrin (dMβCD; Sigma-Aldrich Inc., St. Louis, MO), and were cultured by shaking them in an atmosphere of 5% O_2, 10% CO_2 and 85% N_2 at 37 °C (Concept Plus; Ruskinn Technology, Leeds, UK). An appropriate number of the organisms grown on a 0.004% dMβCD–PPLO agar plate was transferred into a 0.004% dMβCD–PPLO broth and cultured for 24 h to prepare the bacterial cell suspension. The organisms grown in the broth were inoculated into the fresh medium at the $OD_{660\,nm}$ of 0.005 and cultured for 24 h. The above subculture was repeated for three generations to obtain preculture bacterial cell suspension. The preculture bacterial cell suspensions were inoculated at a 0.02-fold volume (200 μL) into a fresh medium (10 mL) in the presence or absence of the steroid hormone (50 μM) and incubated for 24 h. After the incubation, the recovered organisms were subjected to the experiments as described below.

Cholesterol and steroid hormones

The chemical structures of the cholesterol and steroid hormones are shown in Fig. 1. The following steroid prehormone, sex hormones and cholesterol were investigated: pregnenolone, estrone, estradiol, estriol, progesterone, dehydroepiandrosterone, epiandrosterone, androsterone, androstenedione, testosterone and free cholesterol (all from Wako Pure Chemical Industries Ltd, Tokyo, Japan, except for epiandrosterone, which is from the Tokyo Chemical Industry Co. Ltd, Tokyo, Japan).

Preparation of the medium-dispersed cholesterol and steroid hormones

First, a powdered form of the specimen (cholesterol or steroid hormones) was pulverized in a mortar and thoroughly mixed in a dMβCD powder by further pulverizing in a mortar. Next, the powdered mixture of dMβCD and the specimen was thoroughly mixed into a PPLO powder in a mortar. Finally, the mixture of the three powders was dissolved in distilled water by way of sonication and autoclaved for 15 min at 121 °C. The concentration of the steroid samples used in this study was 50 μM.

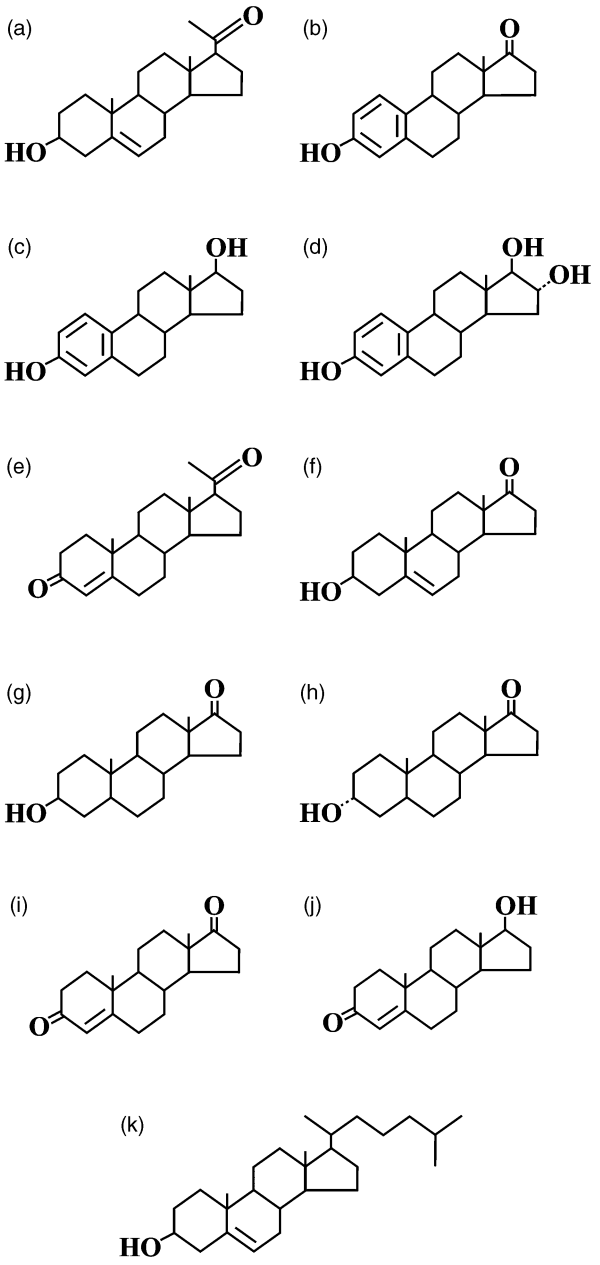

Fig. 1. Chemical structures of steroid prehormone, sex hormones and cholesterol. The chemical structures of pregnenolone (a), estrone (b), estradiol (c), estriol (d), progesterone (e), dehydroepiandrosterone (f), epiandrosterone (g), androsterone (h), androstenedione (i), testosterone (j) and free cholesterol (k). The hydroxyl (OH) group at carbon position 3 in pregnenolone, dehydroepiandrosterone, epiandrosterone and free cholesterol shows a β-configuration (3β-OH). The OH group at carbon position 3 in estrone, estradiol and estriol shows a flat configuration (3-OH). The OH group at carbon position 3 in androsterone shows an α-configuration (3α-OH).

Proliferation assay

The preculture bacterial cell suspension was inoculated at a 0.02-fold volume (200 μL) into a 0.004% dMβCD–PPLO broth (10 mL) and grown for 24 h in the presence or absence of the steroid hormone (50 μM) under microaerobic conditions. After the incubation, the cells in the suspension (1 mL) were harvested using centrifugation, resuspended in distilled saline (1 mL) at a volume equal to the initial medium volume and the ODs of the cell suspensions were measured at a $OD_{660\,nm}$ using a spectrophotometer (Ultrospec; LKB Biochrom Ltd, Cambridge, UK).

Purification and analysis of membrane lipids

The membrane lipids were extracted and purified following the method of Folch *et al*. (1957). In brief, the organisms were cultured for 24 h in the presence of various steroid hormones. The cell pellets were washed three times in distilled saline, sonicated with chloroform : methanol (2 : 1), and then the insoluble materials in the sonicated cell lysates were removed via centrifugation to obtain the supernatant. After adding a 0.2-fold volume of 0.9% KCl to the supernatant, the mixture was vigorously shaken and incubated at room temperature to separate the water phase from the chloroform phase. The chloroform phase was recovered and evaporated to obtain purified membrane lipids. The purified lipids were subjected to thin-layer chromatography (TLC) with chloroform : methanol : water (70 : 30 : 5), and glycolipids (glucosyl-steroid hormones and CGs) and total lipids were detected using the reagent orcinol–sulfuric acid (0.2% orcinol in 2 N H_2SO_4) and 60% sulfuric acid, respectively.

Hydrophobicity analysis of estrogens

The estrogens estrone, estradiol and estriol used in this study were dissolved in chloroform : methanol (2 : 1) at a concentration of 1 mg mL^{-1}, spotted on a silica gel plate at 50 μg per lane, developed with a solvent of chloroform : methanol : water (70 : 30 : 5) and detected using a solution of 60% sulfuric acid. The R_f values of the three estrogens were calculated according to the distance that each estrogen migrated on the silica gel plate.

Membrane affinity assay of estrogens

The organisms (10^9 CFU mL^{-1}) were autoclaved for 15 min at 121 °C in saline to prepare the heat-killed bacterial cells. Subsequently, the heat-killed cells were suspended in a 0.004% dMβCD–PPLO broth containing estrone, estradiol or estriol at a concentration of 50 μM, incubated overnight at 37 °C, washed three times with distilled saline and then recovered via centrifugation. The membrane lipids were analyzed as described in the previous section.

Results

Growth of *H. pylori* in the presence of steroid hormones

dMβCD is known to support the growth of *H. pylori* (Marchini *et al*., 1995), and a 0.1–0.2% concentration of dMβCD is widely used as a standard component in serum-free culturing of the organisms. However, in our preliminary experiments, a 0.2% concentration of dMβCD entirely prevented the absorption of steroid hormones in *H. pylori* from the medium (data not shown). Previous investigations by others have also shown that steroid hormones treated with a high concentration of β-cyclodextrins were not absorbed into the membrane of *H. pylori* (Trampenau & Müller, 2003). We therefore decided to carry out our experiments using the *H. pylori* acclimatized to a low concentration of dMβCD. First, we estimated the growth of *H. pylori* strain NCTC 11638 in the presence or absence of various steroid hormones in a 0.004% dMβCD–PPLO broth (Fig. 2). When the cell suspension was incubated with each steroid hormone or with cholesterol, the $OD_{660\,nm}$ values of the cell suspensions were all higher than the initial $OD_{660\,nm}$ (lane i) before the incubation, but they were somewhat lower than the $OD_{660\,nm}$ (lane 1) of the cell suspension incubated without steroids. The $OD_{660\,nm}$ values of cell suspensions treated with estradiol (lane 5), progesterone (lane 7) and androstenedione (lane 11) were especially low compared with the $OD_{660\,nm}$ of the untreated cell suspension (lane 1). These results indicate that all steroid hormones examined in this study conferred a weak inhibitory effect on the growth of *H. pylori* acclimatized to the low concentration of dMβCD, but these levels of growth inhibition had a negligible influence on the analysis of the anabolic metabolism of steroid hormones in the organisms.

Glucosylation of steroid hormones in *H. pylori*

Based on the above results, we proceeded with further experiments on the glucosylation of steroid hormones using *H. pylori* grown in 0.004% dMβCD–PPLO broth. First, we examined the glucosylation of 3β-OH steroid hormones (pregnenolone, dehydroepiandrosterone and epiandrosterone) in *H. pylori*. When *H. pylori* strain NCTC 11638 was cultured with pregnenolone, the organisms glucosylated pregnenolone in a manner similar to that observed with cholesterol, and three spots of the glucosyl-pregnenolones equivalent to CG spots were detected in the membrane lipid compositions in the TLC analysis (Fig. 3a). As with pregnenolone, *H. pylori* glucosylated dehydroepiandrosterone and epiandrosterone; two kinds of glucosyl-dehydroepiandrosterones and two kinds of glucosyl-epiandrosterones, the glucosylated androgens equivalent to CAG and CPG, were detected in the membrane lipids of the organisms cultured

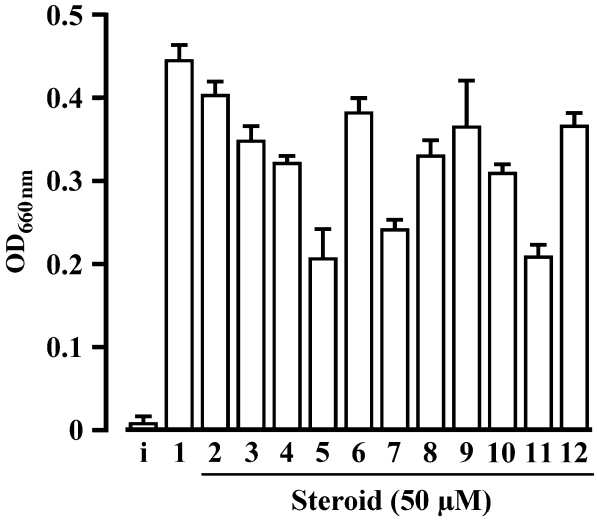

Fig. 2. Growth of *Helicobacter pylori* acclimatized to a 0.004% dMβCD–PPLO broth in the presence or absence of steroid hormones. The preculture bacterial cell suspension of *H. pylori* strain NCTC 11638 was inoculated at the $OD_{660\,nm}$ value of 0.005 into the fresh medium and grown for 24 h in the presence or absence of each steroid hormone (50 μM). After 24 h, the cells were recovered by centrifugation, resuspended in saline at a volume equal to the initial medium volume, and then the ODs of the cell suspensions were measured at a $OD_{660\,nm}$. Lane i indicates the $OD_{660\,nm}$ immediately after inoculation of the preculture bacterial cell suspension. Lane 1 shows the $OD_{660\,nm}$ of the cell suspension without steroid hormones. Lanes 2 and 3 show the $OD_{660\,nm}$ of the cell suspension incubated in the presence of cholesterol and pregnenolone, respectively. Lanes 4, 5, 6 and 7 show the $OD_{660\,nm}$ of the cell suspension incubated in the presence of estrone, estradiol, estriol and progesterone, respectively. Lanes 8, 9, 10, 11 and 12 show the $OD_{660\,nm}$ of the cell suspension incubated in the presence of dehydroepiandrosterone, epiandrosterone, androsterone, androstenedione and testosterone, respectively. The results are shown as the mean $OD_{660\,nm} \pm$ SD obtained from three independent experiments.

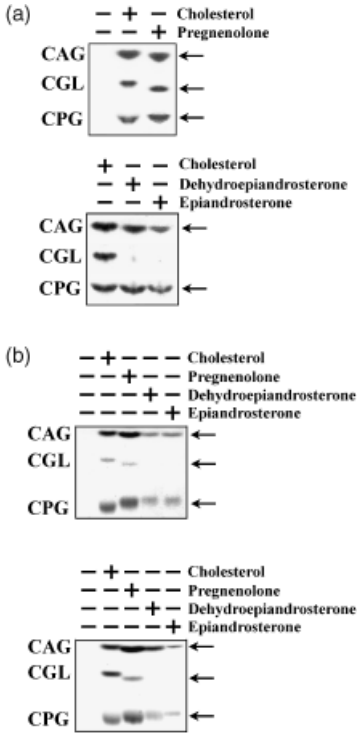

Fig. 3. Detection of glucosyl-steroid hormones in *Helicobacter pylori*. (a) *Helicobacter pylori* strain NCTC 11638 was cultured for 24 h with cholesterol (50 μM) or each steroid hormone (50 μM), and thereafter harvested using centrifugation. Membrane lipids were purified using the Folch method and subjected to TLC analysis, after which glycolipids were detected with the reagent orcinol–sulfuric acid. Purified membrane lipids were spotted at the 400 μg per lane (steroid hormone-treated specimens) and the 200 μg per lane (cholesterol-treated specimen) as the total lipid on the silica gel plate. The results shown are representative of the findings obtained from three independent experiments. (b) TLC profiles of the glucosyl-steroid hormones in the membrane lipids obtained from *H. pylori* strain ATCC 43504 (10^8 CFU mL^{-1}; upper panel) and strain A-19 (10^8 CFU mL^{-1}; lower panel) cultured with steroid prehormone and each androgen at a concentration of 50 μM. The results shown are representative of the findings obtained from two independent experiments. The arrows indicate the glucosyl-steroid hormone spots.

with those steroids (Fig. 3a). Next, we analyzed the glucosylation of 3α-OH (androsterone), 3-oxo (3 = O; androstenedione, testosterone and progesterone) and 3-OH (estrone, estradiol and estriol) steroid hormones. The spots of glucosyl-steroid hormone compounds equivalent to CG spots were not detectable in the membrane lipid compositions of the organisms cultured with those steroid hormones in the TLC analysis (data not shown). These results indicate that *H. pylori* specifically glucosylates the steroid hormones possessing 3β-OH independently of the other functional groups modifying the steroid framework. Next, we analyzed the glucosylation of the 3β-OH steroid hormones in other *H. pylori* strains. The two *H. pylori* strains (ATCC 43504 and a clinical isolate, A-19) induced the glucosylation of pregnenolone, dehydroepiandrosterone and epiandrosterone, although it appeared that there was difference among the *H. pylori* strains in their ability to glucosylate the steroid hormones. In the TLC analysis, three kinds of glucosyl-pregnenolones, two kinds of glucosyl-dehydroepiandrosterones and two kinds of glucosyl-epiandrosterones were detected in the membrane lipids obtained from the two *H. pylori* strains cultured with those 3β-OH steroid hormones (Fig. 3b). These findings entirely corresponded to those for the *H. pylori* strain NCTC 11638. Thus, these results indicate that glucosylation of 3β-OH steroid hormones is universally observed in *H. pylori*.

Membrane adsorption of steroid hormones in *H. pylori*

As described above, the steroid hormones other than pregnenolone, dehydroepiandrosterone and epiandrosterone that have a 3β-OH structure were not glucosylated by

the *H. pylori*. This raises the question whether the steroid hormones with a 3α-OH, 3 = O or 3-OH structure adsorb to *H. pylori* membrane in the first instance. To test this, we examined the adsorption of steroid hormones that were not glucosylated into the *H. pylori* membrane. When *H. pylori* strain NCTC 11638 was cultured with estrone, a spot of estrone (E1), the estrogen identified with authentic estrone in TLC analysis, was detected at a tremendously high density in the membrane lipid compositions of the organisms (Fig. 4a). In addition, the estradiol (E2) spot was detected at scanty levels in the membrane lipid component of the organisms cultured with its estrogen, whereas estriol and progesterone were undetectable in the membrane lipids of the organisms cultured with those steroids. As with estriol and progesterone, androsterone, androstenedione and testosterone were all undetectable in the membrane lipid compositions of the organisms. The membrane adsorption of E1 was also observed in *H. pylori* strains ATCC 43504 and A-19 cultured with its estrogen (data not shown). These results indicate that *H. pylori* sufficiently absorbs estrone with a 3-OH structure into the membrane but fails to utilize its estrogen as a material for glucosylation, whereas the 3α-OH and 3 = O steroid hormones hardly adsorb to the surface of the *H. pylori* cells.

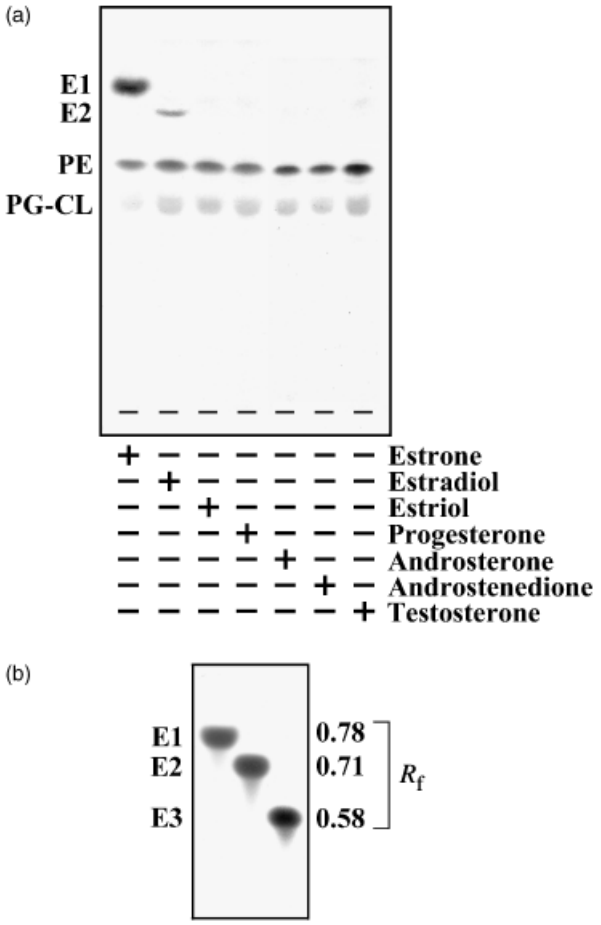

Fig. 4. Membrane lipid profiles in *Helicobacter pylori* cultured with sex hormones. (a) *Helicobacter pylori* strain NCTC 11638 was cultured for 24 h in the presence of each sex hormone (50 μM) and recovered using centrifugation. The membrane lipids were purified using the Folch method, spotted at the 100 μg per lane as the total lipid on a silica gel plate for the TLC analysis, and detected by visualizing using a solution of 60% sulfuric acid. E1, E2, PE and PG-CL denote estrone, estradiol, phosphatidylethanolamine and phosphatidylglycerol-cardiolipin, respectively. The results shown are representative of the findings obtained from three independent experiments. (b) Each estrogen was spotted at the 50 μg per lane on a silica gel plate, developed with a solvent of chloroform : methanol : water (70 : 30 : 5) and detected using a solution of 60% sulfuric acid. E3, estriol.

To better understand the membrane adsorption of the estrogens, we examined hydrophobic intensities of the three estrogens (estrone, estradiol and estriol) using TLC analysis (Fig. 4b). Estrone (E1) exhibited the highest R_f value of the three estrogens, and estradiol (E2) the second highest R_f value. The R_f of estriol (E3) was the lowest value of the three estrogens. Thus, the ranking of the hydrophobic intensities of the three estrogens was, in descending order, E1, E2 and E3. Next, we analyzed the membrane adsorption of the estrogens using the heat-killed bacterial cells (data not shown). In the TLC analysis of membrane lipids obtained from the heat-killed cells incubated with each estrogen, an enormous estrone spot was detected in the membrane lipid component, whereas estradiol was only detected at a negligible level. In contrast, estriol was undetectable in the membrane lipids. These results entirely corresponded to the results observed in the living *H. pylori* cells, as shown in Fig. 4a. We therefore suggest that the hydrophobic intensities of estrogens (or the number of hydroxyl group modifying its steroid framework) play an important role in the interaction between the estrogens and the *H. pylori* membrane lipids.

Discussion

Our results from this study confirmed that *H. pylori* glucosylates pregnenolone, dehydroepiandrosterone and epiandrosterone. Recent studies have identified the enzyme responsible for the synthesis of CG as cholesterol α-glucosyltransferase, encoded by the HP0421 gene in *H. pylori* (Lebrun *et al.*, 2006; Lee *et al.*, 2008). The recombinant HP0421 expressed in *Escherichia coli* has also been shown to catalyze α-glucosylation of various sterols including phytogenic and fungal sterols (Lebrun *et al.*, 2006). In addition, *H. pylori* lacks the gene that encodes sterol β-glucosyltransferase in plants and fungi (Lebrun *et al.*, 2006). Thus, the three kinds of glucosyl-pregnenolones, two kinds of glucosyl-dehydroepiandrosterones and two kinds of glucosyl-epiandrosterones observed in this study are likely to be α-glucosyl-steroid hormones. In the TLC analysis, the spot

positions of three glucosyl-pregnenolones corresponded roughly to the spot positions of CGL, CAG and CPG, respectively. The functional group to which a glucose molecule can be attached via the catalytic action of HP0421 is the only 3β-OH in the framework of the pregnenolone. Thus, the one spot of the three glucosyl-pregnenolones that is equivalent to CGL spot must be 3β-(α-D-glucosyl)-pregnenolone, a basic structure of glucosyl-pregnenolones. Our previous study demonstrated that CGL, a basic structure among CGs, is converted to CAG when modified by a fatty acid ($C_{14:0}$) and CPG when modified by a phosphatidyl group, and that the CGL level in the membrane lipid compositions remarkably decreases when it is converted to CAG and CPG in *H. pylori*, undergoing morphological changes (Shimomura *et al.*, 2004). Thus, it is highly possible that the two spots of the three glucosyl-pregnenolones that corresponded roughly to the CAG and CPG spots are the 3β-(α-D-glucosyl)-pregnenolones modified by a fatty acid and by a phosphatidyl group, respectively. The TLC profiles of the glucosyl-androgens detected the two spots of glucosyl-dehydroepiandrosterones and glucosyl-epiandrosterones equivalent to CAG and CPG spots, respectively, but did not detect the spots of glucosyl-dehydroepiandrosterone and glucosyl-epiandrosterone equivalent to CGL spot in the membrane lipid compositions of *H. pylori*. These results indicate that *H. pylori* promptly converts the 3β-(α-D-glucosyl)-androgens, which are basic structures of glucosyl-androgens, to those modified by a fatty acid or a phosphatidyl group. The transferases that attach a fatty acid or a phosphatidyl group to the CGL molecule have still not been identified in *H. pylori*. Investigations into the CGL acyltransferase and CGL phosphatidyltransferase are required to elucidate the anabolic pathway in CGs and glucosyl-steroid hormones.

As was the case with estrone, when the heat-killed *H. pylori* cells were incubated with cholesterol and pregnenolone, spots of cholesterol and pregnenolone were also detected at a tremendously high density in the membrane lipid compositions of the heat-killed cells in the TLC analysis (data not shown). Thus, cholesterol and pregnenolone adsorbed to the cell surface of the organisms in the same way as estrone and estradiol, even when the membrane structure was significantly altered by the heat-killing treatment. In contrast, dehydroepiandrosterone and epiandrosterone did not adsorb to the membrane of the heat-killed *H. pylori* (data not shown). These results indicate that *H. pylori* absorbs cholesterol, pregnenolone and the estrogens estrone and estradiol via a passive mechanism, whereas dehydroepiandrosterone and epiandrosterone are incorporated into the *H. pylori* membrane via another mechanism. Further investigation is necessary to clarify the mechanism involved in the uptake of dehydroepiandrosterone and epiandrosterone in *H. pylori*.

Epidemiological studies and animal models have suggested that female hormones, particularly estrogens, play a protective role in gastric cancer (Ketkar *et al.*, 1978; Furukawa *et al.*, 1982; Campbell-Thompson *et al.*, 1999; Sipponen & Correa, 2002; Freedman *et al.*, 2007). *Helicobacter pylori* colonizes the human gastric epithelium and causes chronic gastritis and peptic ulcers. Over longer periods, it also has been found to contribute to the development of gastric cancer (Forman & The Eurogast Study Group, 1993). A recent study has demonstrated that a 17β-estradiol has a protective effect against the development of *H. pylori*-induced gastric cancer in a mouse model (Ohtani *et al.*, 2007). Unfortunately, the above investigation did not examine the protective effect of estrone in *H. pylori*-induced gastric cancer. In this study, we found that estrone effectively adsorbs to the surface of *H. pylori* cells, better than estradiol. Our results concerning estrogens may partially explain the protective role of estrogens in the development of *H. pylori*-induced gastric cancer: *H. pylori* cells to which estrogens have adsorbed may be blocked from adhering to gastric epithelial cells. Further research is necessary to determine the inhibitory effect of estrogens on the adhesion of *H. pylori* into the epithelial cells.

This study is the first to describe the glucosylation of steroid hormones by *H. pylori*, a unique bacterial species. A recent study has demonstrated that CGs play an important role in evading the immunoresponse of the hosts; *H. pylori* manifesting CGs resists phagocytosis in macrophages and does not stimulate the activation of antigen-specific T cells (Wunder *et al.*, 2006). In this study, we found that *H. pylori* does glucosylate steroid hormones. These results suggest that the resulting glucosyl-steroid hormone compounds are also involved in the immune evasion or may have a novel biological activity in the host cells. Further investigations are necessary to analyze the structures of glucosyl-steroid hormone compounds in detail and to estimate the biological activity of glucosyl-steroid hormones upon the host cells and the link to the pathogenicity of *H. pylori*.

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