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Research Paper

In-vitro interaction of L-dopa with bacterial adhesins of *Helicobacter pylori:* an explanation for clinicial differences in bioavailability?

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Abstract

Objectives Recent investigations on the pharmacokinetics of levodopa (L-dopa) indicated that the presence of *Helicobacter pylori* in patients with Parkinson's disease, orally treated with L-dopa, influences the absorption of this compound, which consequently leads to decreased plasma levels. Therefore this work aims to study a potential in-vitro interaction of L-dopa with *H. pylori* and its surface adhesins.

Methods Solutions containing L-dopa of different concentrations were incubated with *H. pylori* at different bacterial densities and time intervals. Free L-dopa was quantified from the incubation supernatants by HPLC. A flow cytometric assay with fluorescence labelled *H. pylori* was used to investigate the influence of L-dopa on the bacterial adhesion of *H. pylori*: FITC-labelled bacteria were pre-incubated with L-dopa, followed by incubation with gastric epithelial cells (AGS cells) and FACS quantification of adhering bacteria.

Key findings Evaluation of time- and concentration-dependent incubation experiments indicated a significant decrease in L-dopa concentrations when coming into contact with *H. pylori*. The reduction in L-dopa concentrations was determined as 47 to 12%, referred to the initial starting concentration, with time-dependency and dependency of the *H. pylori* density. FITC-labelled *H. pylori*, pre-incubated with differing L-dopa concentrations, were shown to have a significantly reduced bacterial adhesion to AGS cells, with a maximum reduction of 22 ± 9%. These results demonstrate a direct interaction of L-dopa with the outer membrane proteins of *H. pylori* responsible for the adhesion to gastric epithelial cells. By this interaction the unbound L-dopa concentration in bacterial suspension was strongly reduced.

Conclusions This study suggests a potential in-vitro interaction of L-dopa with *H. pylori* adhesins, confirming the clinical changes found in pharmacokinetics of L-dopa therapy by *H. pylori*-positive patients with Parkinson's disease.

Keywords adhesion to gastric epithelial cells; *Helicobacter pylori*; interaction; levodopa; Parkinson's disease

Introduction

It has been known for more than a century that bacteria are present in the human stomach. About 25 years ago, Barry Marshall and Robin Warren described the successful isolation and culture of a spiral bacterial species, later known as *Helicobacter pylori*, from the human stomach.[1] *H. pylori* colonises the gastric mucosa and upper duodenum by adhering to, and penetrating into, the mucous layer of the human gastric epithelium.[2,3] For the adherence process to the epithelium the bacterium has developed several outer membrane proteins that can be called adhesins. At least 32 of these proteins have been identified upto now in *H. pylori*, many of which are involved in bacterial adherence.[4] Adhesins interact with either soluble molecules in the direct environment or with a variety of surface receptors and binding proteins of host cells. For instance, *H. pylori* expresses the neuraminyl-lactose-binding hemagglutinin (NLBH), which binds specifically to 3′-sialyllactose and the glycoprotein fetuin,[5,6] or BabA, which mediates the attachment of *H. pylori* to human gastric epithelium by Lewis b, a fucosylated blood group antigen.[7] Besides the well-documented clinical symptoms of gastritis induced by *H. pylori,* nowadays also the changes in the microbial microenvironment within the upper intestinal system have to be considered in more detail. The colonisation of the stomach by *H. pylori* can change the

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mucosal status and therefore also the mucous layer, the epithelium and the respective functionalities of the tissue. Especially, the interference of *H. pylori* with micronutrients and orally administered drugs is becoming a major focus of pharmacokinetic research (for review see references[8]).

It was postulated[9,10] that the presence of *H. pylori* in patients with Parkinson's disease treated orally with levodopa (L-dopa) can affect the plasma levels of L-dopa; the induced changes in the gastric environment affecting the L-dopa absorption can be reversed by eradication of *H. pylori*.[11]

Theoretically, the main site of absorption for L-dopa in the duodenal mucosa[12] can be affected by *H. pylori* in several ways. Inflammative damage of the duodenal mucosa,[13] changes in mucosal pH[14] increase in reactive oxygen species[15,16] capable of inactivating L-dopa,[17] a delayed delivery of L-dopa to the duodenum mucosa,[12] or an increased transformation of L-dopa into dopamine are suggested as potential reasons for the divergent absorption. On the other hand, the typical environment of *H. pylori* is the stomach compartment, while the main absorption of L-dopa occurs in the duodenum. This may be a hint that a direct interaction of L-dopa with *H. pylori* during the transit of the drug through the stomach region leads to a fast inactivation of L-dopa.

Based on this hypothesis this study aims to define more clearly the in-vitro interaction of L-dopa with *H. pylori* and, especially, its influence on the microbial adhesins.

Materials and Methods

Materials

3′-Sialyllactose (NeuAc$\alpha_{2\text{-}3}$Gal$\beta_{1\text{-}4}$Glc), 3,4-dihydroxy-L-phenylalanine (L-dopa) and fluoresceinisothiocyanate isomer I (FITC) were purchased from Sigma Chemicals (St Louis, MO, USA). Other chemicals and reagents were purchased from VWR (Darmstadt, Germany) in analytical quality. Fresh okra fruits extract (okra FE) was prepared as described by Lengsfeld *et al*.[18]

Helicobacter pylori

Helicobacter pylori J99 (ATCC 700824) was incubated for 48 h under microaerophilic conditions at 37°C on tryptic soy agar (Becton Dickinson, Germany), supplemented with 5% defibrinated sheep blood (Oxoid, UK).

Cell culture

Human gastric epithelial cells (AGS cells) were kindly provided by Prof. Winfried Beil (Medizinische Hochschule Hannover, Germany). The cells were grown in RPMI 1640 with L-glutamine (PAA, Germany), supplemented with 10% fetal calf serum (FCS), in tissue culture flasks (75 cm^2; Sarstedt, Newton, USA) and 6-well-plates (Sarstedt) in 5% CO_2.

HPLC

HPLC was carried out using a Varian pump 9012 and Varian UV detector 9050; the conditions were: λmax 280 nm, stationary phase Kromasil C18 (Phenomenex, Torrance, CA, USA), mobile phase Millipore water with 0.1% trifluoroacetic acid (A), methanol 85 : 15 (B), isocratic, flow 1.0 ml/min, injection volume 20 μl. External calibration was carried out against L-dopa reference standard (5–300 μg/ml). Validation data: linearity and range: five concentrations (5–300 μg/ml) showed linearity (y = 3462.4–5571, r^2 = 0.9999). Precision: the intra-assay precision (repeatability) at 100 μg/ml L-dopa level was ± 7%, determined under the same conditions performed with double injections of four independent sample preparations. Stability of L-dopa in phosphate-buffered saline (PBS) solution (100 μg/ml) was verified for 3 h, being analysed in 10-min intervals (data not shown).

Interaction studies

Interaction studies[19,20] between *H. pylori* and L-dopa were performed by HPLC determination of unbound L-dopa after incubation with *H. pylori*: L-dopa solutions were prepared in sterile PBS (pH 6.0). Agar-grown *H. pylori* were harvested and resuspended in sterile PBS. Bacterial suspensions with different titres, characterized by the optical density (OD, at 550 nm, corresponding to 2.0×10^7 *H. pylori* cells/ml), were incubated with L-dopa-containing solutions with defined concentration for 5, 15, 45 and 120 min. Bacteria were pelleted (3150*g*, 5 min) and the supernatant taken for the HPLC quantification of free L-dopa.

Labelling of bacteria

Agar-grown *H. pylori* were harvested and resuspended in sterile carbonate buffer (pH 9.0). The OD of the suspension was measured photometrically at 550 nm and 1.0×10^8 bacteria were resuspended in 1 ml of sterile buffer.[21] Ten microlitres of an FITC solution (1% in dimethyl sulfoxide (DMSO)) were added and incubated with the bacteria for 45 min. Fluorescent labelling was terminated by pelleting the bacteria (3150*g*, 5 min). Bacteria were washed twice in PBS to remove excess FITC and were gently resuspended for further use.

Preliminary studies were performed to determine labelling conditions and to prove that the fluorescence was maintained during the course of co-incubation experiments with epithelial cells.

Quantitative flow cytometric adhesion assay

The quantitative flow cytometric (FACS) adhesion assay was performed to quantify effects of L-dopa on *H. pylori* binding to AGS cells. Briefly, labelled *H. pylori* were statically co-incubated with AGS cells (ratio 10 : 1) in 6-well plates at 37°C for 1 h. After vigorous washing (2 ml PBS/well, three times) AGS cells were detached from the wells by incubation with trypsin–EDTA (0.05% in D-PBS, 3 min) and then suspended in RPMI (+10% FCS). After pelleting (600*g* for 5 min) the cells were resuspended in 1 ml RPMI (+10% FCS) and analysed by flow cytometry (Becton Dickinson, FACS Calibur). Flow cytometry instrument settings: FSC (Detector): E-1 (Voltage), 3.00 (Amp Gain), Lin (Mode); SSC: 352, 1.00, Lin; FL1: 360, 1.00, Log.

Statistical analysis

Statistical tests were performed by using SPSS. The experimental results were expressed as the mean ± SD. Data were assessed by analysis of variance. If this analysis indicated significant differences between group means, then each group

was compared by using Dunnett T (2-sided), and $P < 0.05$ was considered to be statistically significant.

Results

To investigate a direct interaction of L-dopa with *H. pylori*, suspensions of bacteria with different cell densities were incubated with L-dopa (0.51 mM, 100 μg/ml). After 5, 15, 45 and 120 min, L-dopa concentrations were quantified by HPLC-UV from the supernatant (Figure 1). Already after 5 min a significant decrease in L-dopa concentration was observed at higher cell densities. This interaction of L-dopa with *H. pylori* did not increase further over the incubation time, indicating a typical saturation phenomenon with an equilibrium reached after 5 min. Such saturation curves can be seen as indicators for adsorption or absorption phenomena on or into the bacterial surface.

Within dose-time-dependent incubation assays at a constant bacterial density (1.0×10^7/ml) and with different L-dopa concentrations (0.51 and 2.54 mM), again interactions were obvious between *H. pylori* and L-dopa (Figure 2). Calculating the relative values to an absolute mass balance, the loss of L-dopa in the assay after 120 min incubation time corresponded for the 0.51 mM group to 47.5 ± 1.3 μg and for the 2.54 mM group to 81.5 ± 3.5 μg L-dopa.

Because *H. pylori* is an organism with strong adhesive properties (for review see references[22,23]), the observed interaction of L-dopa could be assumed to occur with outer membrane proteins (OPMs) belonging to the class of bacterial adhesins. In principle, interaction with such adhesins can result in a blocking of these proteins leading to a decreased adhesion of *H. pylori* to epithelial cells. This adhesion can be quantified easily by different methods of cell biology.[18,24] Therefore the influence of L-dopa on the adhesion of *H. pylori* to gastric epithelial cells (AGS cell line) was analysed quantitatively by a flow cytometric assay. FITC-labelled *H. pylori* were co-incubated with a monolayer of AGS cells. Non-adhering *H. pylori* were washed out. Cells from the monolayer with adhering *H. pylori* were trypsinized. The individualized cells with adhering and labelled bacteria were quantified by FACS. Measured values were related to the maximal adhesion determined by an untreated control. 3′-Sialyllactose, a known adhesion blocker that interacts with the bacterial neuraminyl-lactose-binding hemagglutinin (NLBH),[5,6] was used as a positive control, leading to a 30% reduction of adhesion. Additionally fresh extract from immature okra fruits from *Abelmoschus esculentus* was used as positive control.[18] This strong adhesion blocker of *H. pylori* is a glycoprotein–polysaccharide mixture, interacting with multiple targets on the bacterial surface.

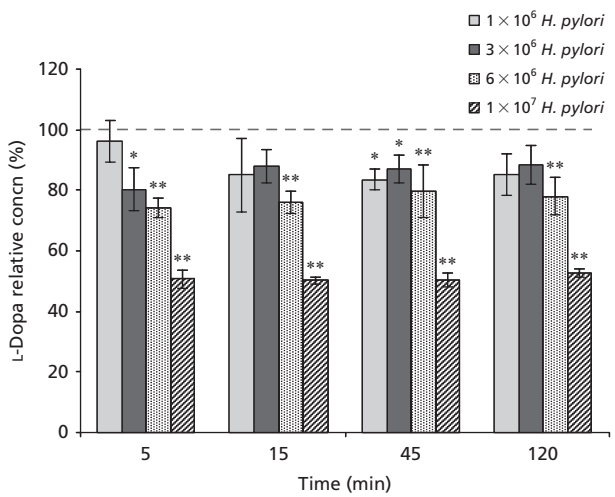

Figure 1 L-Dopa concentrations after time-dependent incubation of L-dopa with *H. pylori*-containing suspensions with different cell densities and quantification of L-dopa with HPLC-UV. Data are related to the untreated L-dopa control solution (0.51 mM = μg/ml). Data are mean ± SD, $n = 4$; $^*P < 0.05$, $^{**}P < 0.01$.

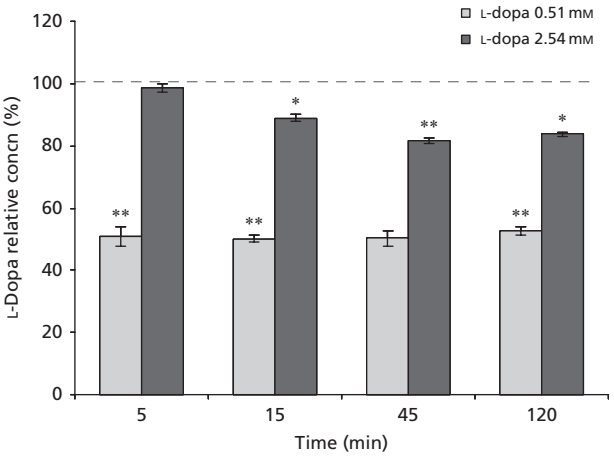

Figure 2 L-Dopa concentrations after time-dependent incubation with 1×10^7 *H. pylori* and quantification with HPLC-UV. Data are always related to the correspondent untreated L-dopa control solutions. L-dopa concentrations: 0.51 mM = 100 μg/ml; 2.54 mM = 500 μg/ml. Data are mean ± SD, $n = 4$; $^*P < 0.05$, $^{**}P < 0.01$.

L-Dopa showed significant anti-adhesive effects at both investigated concentrations, 2.54 mM ($P < 0.05$) and 5.1 mM ($P < 0.01$), reducing the adhesion of *H. pylori* to AGS cells by 19 ± 10% and 22 ± 9% (Figure 3). The L-dopa-mediated inhibition of adhesion was similar to that observed for 3′-sialyllactose (10 ± 5%), but less than the 90% inhibition obtained with okra FE (98 ± 2%). From these data it becomes obvious that L-dopa interacts with bacterial adhesins, similarly to 3′-sialyllactose, but L-dopa seems to have no multi-target affinity to adhesins.

Because reduced bacterial adhesion can also be due to a direct cytotoxic effect of L-dopa on *H. pylori*, toxicity of this compound against *H. pylori* was excluded by an agar diffusion test at 0.5 and 1 mg/ml L-dopa for 48 h incubation time. Evaluation indicated no sensitivity of *H. pylori* towards L-dopa and no signs of bacteriostatic or bactericidal effects were observed (data not shown).

Discussion

Pierantozzi *et al.*[8–10] already postulated a correlation of reduced L-dopa plasma levels in Parkinson's disease patients with the presence of *H. pylori*, being normalized after *H. pylori*

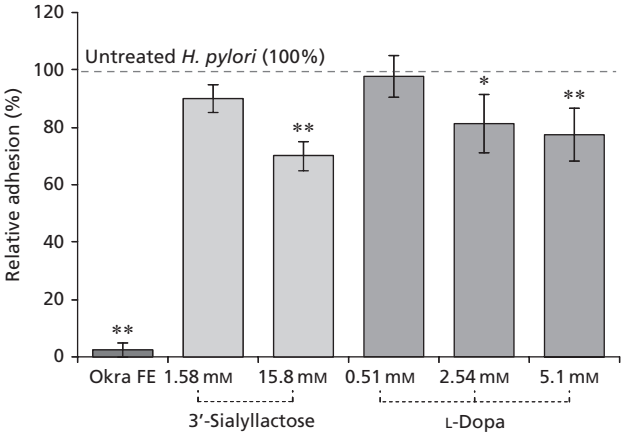

Figure 3 Mean adhesion of FITC-labelled *H. pylori* to AGS cells, pre-incubated for 2 h at room temperature, pH 7 with okra fruit extract, 3′-Sialyllactose and L-dopa. Test compounds were removed by washing (3×) with PBS-Tween 20. Data are related to the untreated control of *H. pylori* (= 100%). 3′-Sialyllactose 1.58 mM = 1 mg/ml, 15.8 mM = 10 mg/ml; L-dopa 0.51 mM = 100 μg/ml, 2.54 mM = 500 μg/ml; 5.1 mM = 1 mg/ml. Okra FE, okra fruit extract. Data are mean ± SD, $n = 5$; *$P < 0.05$, **$P < 0.01$.

eradication. The data from the present in-vitro investigations clearly indicate a dose- and bacteria-dependent interaction of L-dopa with *H. pylori*, leading to a decreased concentration of L-dopa in the incubation supernatant. This reduction in L-dopa concentrations can have different reasons: for instance, metabolism of L-dopa by the bacteria (as is known for other neutral amino acids), degradation by reactive oxygen species from *H. pylori* or an interaction with OMPs could play a role. Since the concentration of L-dopa rapidly decreased during the incubation experiments within a short time followed by a typical saturation, metabolism of L-dopa does not seem to be likely. In this case a more or less linear concentration reduction over the time is to be expected. Degradation of L-dopa, especially to dopamine, was also unlikely because no additional peaks were detected by HPLC over the time of incubation. Also, stability experiments over the period of 3 h in the absence of bacteria showed no significant reduction of the initial L-dopa concentration.

Therefore adsorption of L-dopa to the bacterial surface is possible. Because many surface proteins of *H. pylori* are associated with adhesion to the host tissue, specific binding to OMPs should result in diminished bacterial adhesion. This was unambiguously proven by flow cytometric analysis. The significant ($P < 0.01$) reduction of adhesion by about 22% indicates an influence of L-dopa on the adherence process of *H. pylori*, possibly by interacting with OMPs responsible for this mechanism (adhesins). Toxic effects of L-dopa on *H. pylori* can be excluded.

Comparing the anti-adhesive capacity of L-dopa with known anti-adhesives, such as 3′-sialyllactose, which interacts specifically with one adhesin of *H. pylori* J99, the effect were in a similar range. Aqueous extract of *Abelmoschus esculentus* (okra) fruits completely blocked the bacterial adhesion, due to the interaction of this complex glycoprotein and polysaccharide mixture to a variety of different *H. pylori* adhesins. Because of the moderate anti-adhesive effect of L-dopa we assume that this drug interacts only with a very limited amount of bacterial adhesins. As far as it is possible, relevant adhesins involved in this interaction with L-dopa should be identified by different immunoassays in future investigations. Specific interactions with known receptors for several adhesins and *H. pylori* deletion mutants are intended to be used for the development and evaluation of the assays.

Data provided here are from in-vitro experiments under conditions not completely comparable to the situation in the human stomach, especially concerning the pH. The cultivation of *H. pylori* under acidic assay conditions at pH 2 was not possible during the laboratory experiments because of strong cytotoxic effects on *H. pylori*. On the other hand, it must be taken into account that *H. pylori* in the human stomach is not located in the free stomach solution but is mainly found embedded and attached in the mucin layer at pH 4–6.5. This again is exactly the pH range of our adhesion assays. Considering that L-dopa distributes well into the stomach mucus, an interaction with *H. pylori* may be very probable. Direct proof of the interaction should also be gained by using two of the rare animal infection models for *H. pylori.* One model involves the use of rhesus monkeys with videogastroscopy investigations, during which gastric biopsy specimens can be taken for quantitative cultures and histology.[25] Another animal model is based on guinea-pigs that are intragastrically inoculated with *H. pylori*. This model can be used to investigate the role of selected OMPs in colonization of the guinea-pig stomach.[26]

The suggested change in the dosage regime shouldn't cause any further side effects because this improved dosage should only compensate for the amount lacking due to possible interaction with *H. pylori*. By this means, blood plasma concentrations of L-dopa should not get much higher than with oral treatment in those patients with Parkinson's disease who have an *H. pylori*-negative status.

The main absorption site of L-dopa is the duodenum but it also has to be considered that *H. pylori* is also found in a higher amount in regions of the upper duodenum,[3] a fact that will also increase contact time of both reacting partners. Additionally Parkinson's disease patients themselves show a significantly delayed gastric emptying in comparison with healthy age-matched individuals,[27] which again increases contact time and the possibility of a stronger interaction. From these points the possibility of interaction within the upper gastrointestinal tract and gastric antrum seems to be very likely.

Conclusions

From the data obtained by this investigation we assume that a strong interaction of L-dopa with *H. pylori* takes place by adsorption of the drug to the bacterial surface adhesins. These results prove the clinical changes found in the pharmacokinetics of L-dopa during therapy for Parkinson's disease in *H. pylori*-positive patients. Change of dosage regimes during Parkinson's disease therapy needs to be considered.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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