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The Inhibitory Effect of Eupatilin on *Helicobacter Pylori*-Induced Release of Leukotriene D_4 in the Human Neutrophils and Gastric Mucosal Cells

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In this report, the inhibitory action of eupatilin was investgated by using leukotriene D_4 in the human neutrophils and Kato III cells (Gastric adenoma cells as a substitute for gastric mucosal cells) stimulated by *Helicobacter pylori*. Leukotriene D_4 (LTD_4) was released from both neutrophils and Kato III cells when these cells were incubated with *H. pylori*. The release of LTD_4 increased time-dependently and the maximum release of LTD_4 was 2.3~2.5 pmol. But in the presence of eupatilin, the release of LTD_4 from these cells was inhibited in a dose-dependent manner. In the neutrophils, the release of LTD_4 was suppressed to 70% and 50% of the control levels when neutrophils was incubated with 0.01 and 0.1 mM of eupatilin. In the Kato III cells, the release of LTD_4 was suppressed to 59% and 27% of the control levels by adding 0.01 and 0.1 mM of eupatilin. We estimated the intracellular Ca^{2+} levels when Kato III cells and neutrophils were stimulated by *H. pylori* using ^{45}Ca. But the suppressive effect of eupatilin on Ca^{2+} influx into these cells was not significant. We also obtained the results that *H. pylori* induced Ca^{2+} influx into these cells by confocal microscopy, however there was no differences in the dose level of eupatilin. These results were confirmed by ^1H Nuclear Magnetic Resonance(NMR) spectroscopy. The NMR patterns of eupatilin in the absence of Ca^{2+} was changed compare with when Ca^{2+} was present, but its effect was not strong.

Key Words: *Helicobacter pylori*, Leukotriene D_4, Eupatilin, Confocal microscopy

INTRODUCTION

The presence of the gram-negative bacterium *Helicobacter pylori* in the human stomach is closely associated with chronic gastric inflammatory diseases (Andersen et al, 1987; Goodwin et al, 1986; Marshall & Warren, 1984). *H. pylori* in infection induces active inflammation with neutrophilic infiltration and also elicits chronic inflammation with infiltration of lymphocytes, macrophage/monocytes (Correa, 1980; 1988; Price, 1991). *H. pylori* stimulates neutrophils to induce secreation of inflammatory mediators such as leukotrienes (LTs) which disturbs circulation of gastric tissue resulting in inflammed state of this tissue (Lehr et al, 1991; Sidebotham & Baron, 1990). LTs might play an important role in several pathological processes such as allergic reactions, endotoxemias, tissue injury, and inflammatory responses (Barnes & et al, 1984; Feuerstein & Hallenbeck, 1987; Samuelsson, 1983).

Eupatilin isolated from *Artemisia asiatica* nakai are being currently used as a therapeutics for gastritis and peptic ulcer, but their mechanisms of action have not been known clearly yet.

On the other hand, LTs are known to 5-lipoxygenase metabolites of arachidonic acid and 5-lipoxygenase activity is highly Ca^{2+} dependent (Rouzer et al, 1990). Furthermore, it is also reported that LTs

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can be produced by both immune and nonimmune stimuli, for example IgE, hormones, Ca^{2+} ionophore (Dixon et al, 1990; Goldman et al, 1990; Rouzer et al, 1990). In the present study, we investigated the effect of eupatilin on leukotriene D_4 release from human neutrophils and Kato III cells stimulated by *H. pylori* or calcium ionophore A23187. We also investigated whether *H. pylori* induce Ca^{2+} influx in these human cells and Ca^{2+} influx could be prevented by eupatilin.

METHODS

Helicobacter pylori

H. pylori was cultured by the method of Han et al (1995). *H. pylori* was incubated in media consisting of Muller-Hinton and brucella broth (1 : 1) enriched with 0.1% dimethyl-β-cyclodextrin. The medium was placed in an anaerobic jar. The atmosphere of an anaerobic jar was changed with microaerophilic condition (N_2 85%, CO_2 10%, and O_2 5%) and then gently shaked (200 rpm) at 37°C for 15~18 h. After washing with Hank's Balanced Salt solution (HBSS), cells were resuspended to 2.5×10^8 cells/ml.

Kato III cells

Human stomach adenoma (Kato III) cells obtained from the Seoul National University Cell Bank (Seoul, Korea) were cultured in RPMI 1640 media (Gibco) supplemented with 10% fetal bovine serum and antibiotics (100 IU ml^{-1} penicillin and 100 μl ml^{-1} streptomycin). The cells were incubated in air with 5% CO_2 and 95% humidity at 37°C and then cells were collected and resuspended to 1×10^6 cells/ml.

Neutrophils

Human neutrophils were purified by the method of Grisham et al (1985). Whole blood (20 ml) from adult volunteers was collected in 0.1 ml of heparin and mixed with 13 ml of 2% (w/v) dextran and allowed to settle for 20~30 min at room temperature. The supernatant was mixed with 1/4 volumes of ficoll and centrifuged at 1450 rpm for 30 min. The pellet was suspended to 50 ml of 0.2% NaCl and mixed briefly, and then resuspended to 50 ml of 1.6% NaCl. The suspension was centrifuged at 1050 rpm for 5 min and then cells (neutrophils) were collected, diluted with Ca^{2+}-free KRP (Krebs-Ringer-Phosphate) buffer, counted and resuspended to 1×10^6 neutrophils/ml.

Sample preparation for leukotriene (LT) assay

LTD_4 was generated by incubating cells with either *H. pylori* or calcium ionophore A23187 (1 μM) and arachidonic acid (10 μg) for 30 min after preincubation of the cells with eupatilin for 10 min. Control was prepared by treating with DMSO (0.05%). After incubation, the reaction mixture was centrifuged at 10,000 rpm for 10 min at 4°C and supernatant was saved at −20°C till LTD_4 assay.

Leukotrinen D_4 assay

LTD_4 was measured by Radioimmunoassay (RIA) method. LTD_4 antibody (No.333, a gift from Dr. Rober Krell) was diluted 1000 fold in Phosphate Buffered Saline (PBS) containing 0.1% gelatin, and then 50 μl of diluted antibody solution was added to 100 μl of the supernatant above along with 50 μl of ^{3}H-LTD_4 (39 Ci/mmol). The reaction mixture was mixed and incubted for 2 hours at 4°C. The reaction was terminated by adding 0.5 ml dextran-coated charcoal. After 5 min, the mixture was centrifuged, and 0.1 ml of the supernatant was mixed with Aquasol (NEN). LTD_4 was counted in Liquid Scintillation Analyzer (Packard 1600 CA) and quantitated by using a standard curve of the known amount of LTD_4 ranging from 4.5×10^{-10} to 1×10^7 mol/l. The detection limit was 0.045 pmol.

Measurement of Ca^{2+} influx by neutrophils

Kato III cells were washed several times and suspended in HBSS. The cells were pretreated with 2 mM $CaCl_2$ plus 1 Ci ^{45}Ca or 2 mM $CaCl_2$ plus 1 Ci ^{45}Ca+eupatilin for 10 min and then *H.pylori* was added to induce Ca^{2+} uptake by Kato III cells. Control was prepared by substituting DMSO (0.05%) for eupatilin. After 30 min reaction, the mixture was filtrated through Millipore filter (HAWP 13 mm, average pore size of 0.45 μm). Cells were collected from the filter paper and suspended in 1 ml HBSS. Ten milliliters of Liquid Scintillation Cocktail was

added and radioactivity was measured by Liquid Scintillation Analyzer (Packard 1600 CA). The Ca^{2+} influx was estimated from the standard curve of known amount of ^{45}Ca.

Confocal microscopy analysis

Fluo-3 acetoxymethylester (Fluo-3-Am) (Kao et al, 1989; Merritt et al, 1990) was used as Ca^{2+}- sensitive probe. Kato III cells were washed in HBSS and resuspended at the appropriate concentration. Ten microliters of Fluo-3 was added and incubated for 30 min. Pretreatment of the cells was done by incubating with either 2 mM $CaCl_2$ or 2 mM $CaCl_2$ with eupatilin for 10 min. After the pretreated cells were incubated with *H. pylori* for 30 min, total reaction mixture was washed in PBS and then diluted in Mounting buffer (Research Genetics). The diluted solution was fixed on the slide glass and dried completely. The samples were analyzed by Confocal Microscope Bio-Rad MRC 1024 with 480 nm excitation filter and 522/30 nm emission filter.

Nuclear Magnetic Resonance (NMR) Assay

The reaction mixture of eupatilin (13.64 mg) and $CaCl_2$ (6.66 mg) in DMSO-d6 (10 ml) was stirred for 2 hours. NMR study on the eupatilin-Ca^{2+} complex was investigated using ^1H NMR spectroscopy (500 MHz FT-NMR, Bruker).

RESULTS

The release of LTD_4 from H. pylori-stimulated neutrophils and Kato III cells

As shown in Fig.1, when *H. pylori* 2.5×10^8 cells/ml was added into the medium containing the neutrophils or Kato III cells. The production of LTD_4 by neutrophils and Kato III cells was measured by RIA. LTD_4 markedly increased with time dependently. The maximum level of LTD_4 from these cells was ranged 2.3 to 2.5 pmol after 30 min.

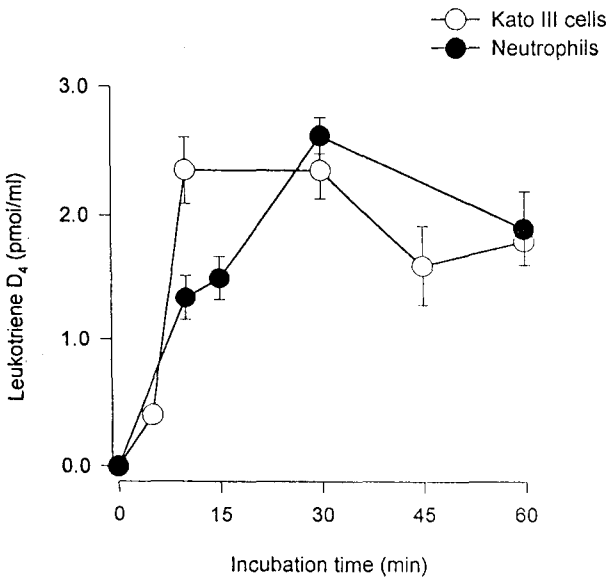

Fig. 1. Incubation time-dependent effect of *H. pylori* on the LTD_4 released from neutrophils and Kato III cells. LTD_4 induced by incubating neutrophils (1×10^6 cells/ml) or Kato III cells (1×10^6 cells/ml) with *H. pylori* (2.5×10^8 cells/ml) up to 1h. LTD_4 was measured by RIA method. The minimum value of LTD_4 that can be detected was 0.045 pmol. The data are expressed as means±SEM of four measurements.

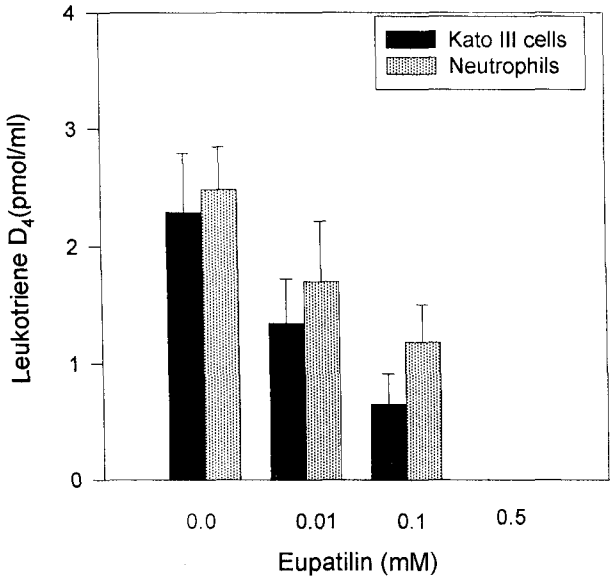

Fig. 2. Effect of eupatilin on the LTD_4 released from neutrophils and Kato III cells stimulated by *H. pylori*. Neutrophils or kato III cells were preincubated for 10min with eupatilin and then reacted with *H. pylori* for 30min. LTD_4 antibody (No. 333, a gift from Dr. Rober Krell) was diluted 1000 fold in PBS. LTD_4 was quantitated by Scintillation analyzer. The results are expressed as means ±SEM of five measurement.

Effect of eupatilin on LTD_4 release from neutrophils and Kato III cells stimulated by H. pylori or calcium ionophore A23187

In the presence of eupatilin, the release of LTD_4 from neutophils and Kato III cells stimulated by H. ppylori was inhibited in a dose dependent manner (Fig. 2). In the neutrophils, LTD_4 was suppressed to 70% and 50% of the control levels when neurtophils was incubated with 0.01 and 0.1 mM of eupatilin, respectively. In the Kato III cells, the release of LTD_4 was suppressed to 59% and 27% of the control levels by adding 0.01 and 0.1 mM of eupatilin, respectively. The release of LTD_4 was completely inhibited by adding 0.5 mM eupatilin in the both neutrophils and kato III cells.

Furthermore, the release of LTD_4 from these cells was induced by calcium ionophore A23187 and arachidonic acid. As shown in Fig. 3, eupatilin suppressed the release of LTD_4, dose dependently. The release of LTD_4 was suppressed to 31% and 40% of the control levels by adding 0.5 mM eupatilin in both neutrophils and kato III cells, respectively. The release of LTD_4 was also completely inhibited by Kato III cells.

Effect of eupatilin on H. pylori-induced Ca^{2+} influx into neutrophils and Kato III cells

After we confirmed the inhibitory action of eupatilin on LTD_4 release, we futher investigated the mechanism of its inhibitory action. We measured the level of the intracellular Ca^{2+} when neutrophils and Kato III cells were stimulated by *H. pylori* using ^{45}Ca (Fig. 4). *H. pylori* induced Ca^{2+} influx into these cells and this influx was inhibited by eupatilin.

Eupatilin decreased the level of the intracellular Ca^{2+}, dose-dependently, but its effect was not strong. The level of Ca^{2+} was reduced to only 61% of *H. pylori*-induced Ca^{2+} influx even at 3 mM. In the case

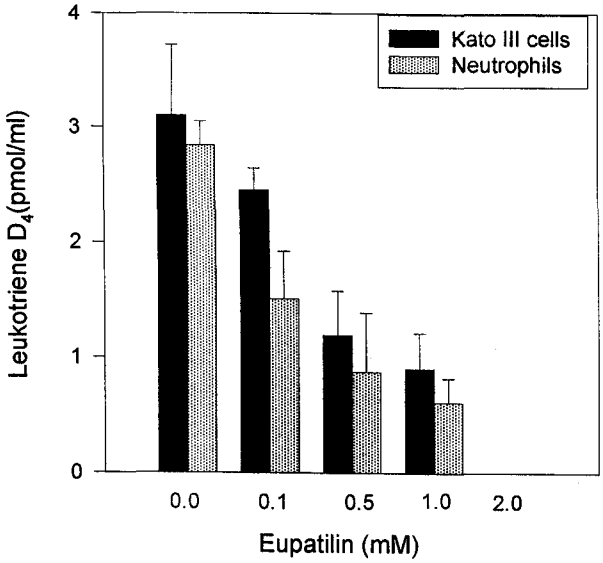

Fig. 3. Effect of eupatilin on the LTD_4 released from neutrophils and Kato III cells stimulated by A23187 (1uM). LTD_4 was generated by incubating cells with calcium ionophore A23187 for 30 min after preincubation of the cells with eupatilin for 10min. LTD_4 was quntitated by Scintillation Analyzer by using a standard curve of the known amount of LTD_4 ranging from 4.5×10^{-10} to 1×10^{7} mol/l. the data are expressed as means ± SEM of five measurements.

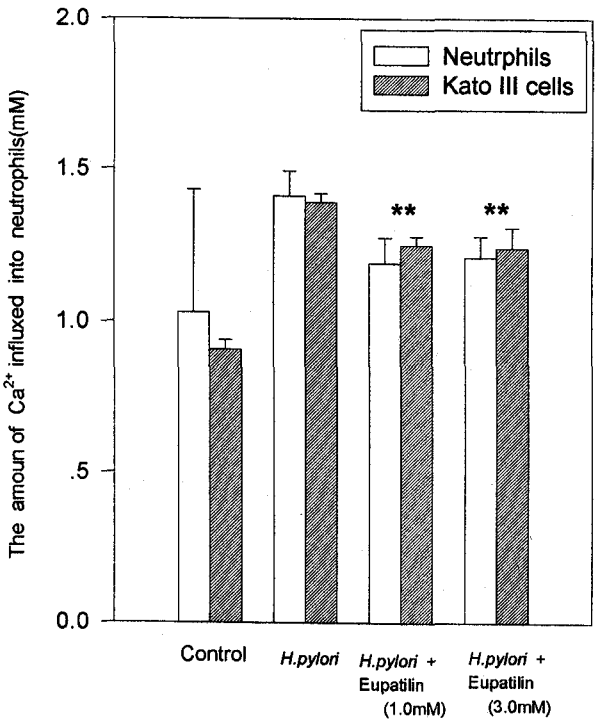

Fig. 4. Effect of eupatilin on *H. pylori*-induced Ca^{2+} influx into Kato III cells and neutrophils. The cells were preincubated with either 2 mM $CaCl_2$ and $1Ci^{45}Ca$ or 2 mM $CaCl_2$,$1Ci^{45}Ca$,and eupatilin for 10min and then *H. pylori* (2.5×10^{8} cells/ml) was added to induce Ca^{2+} uptake by Kato III cells and neutrophils. The Ca^{2+} influx was estimated from the standard curve of known amount of ^{45}Ca by Liquid Scintillation Analyzer. **indicates a significant difference from *H. pylori* only ($p<0.01$).

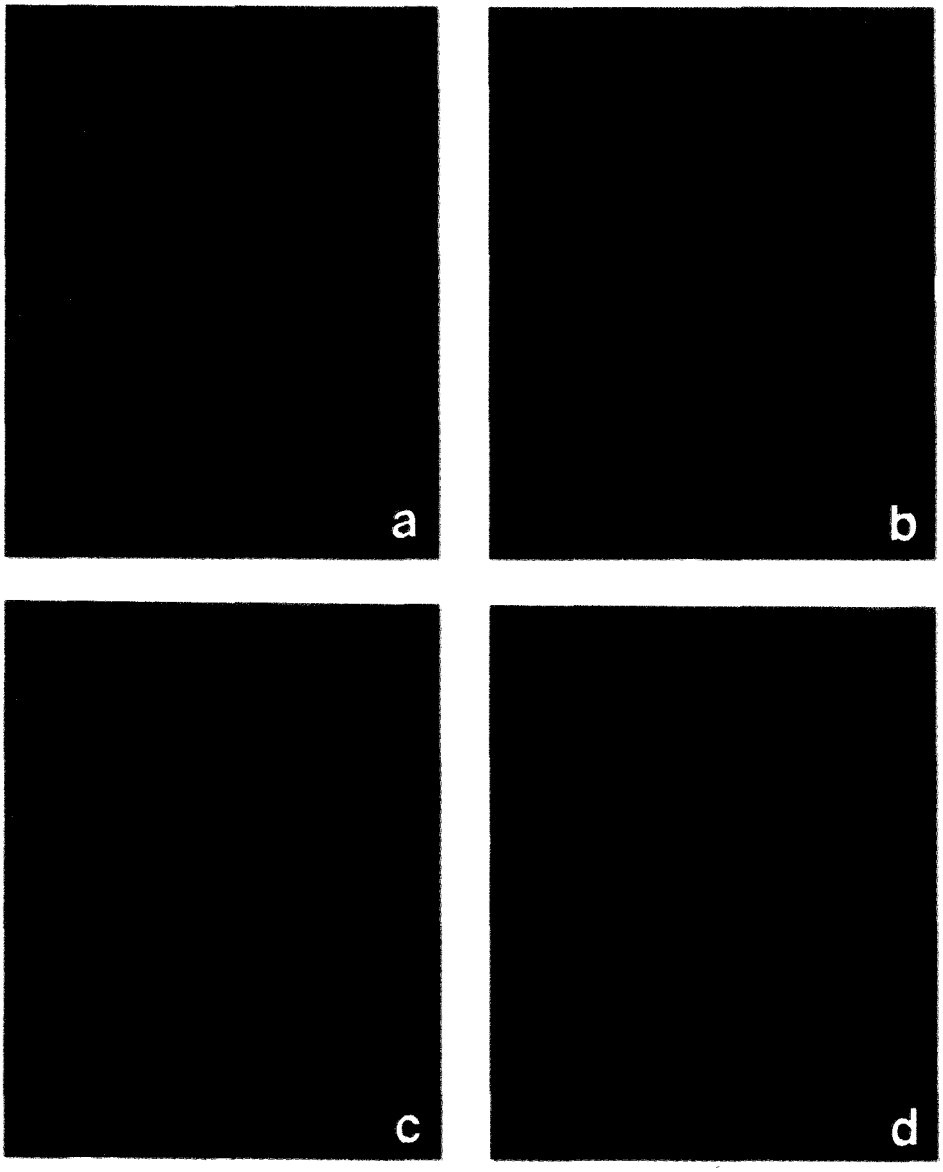

Fig. 5. The preventive effect of eupatilin on *H. pylori*- induced Ca^{2+} influx into kato III cells. The effect of eupatilin against Ca^{2+} influx in *H. pylori*-stimulated Kato III cells was visualized by Confocal laser-scanning microscopy. The cells were labelled with Fluo-3-acetoxymethylester (Fluo-3-Am). (a) control cells, (b) treatment with *H. pylori*, (c) (d) the same as (b) but in the presence of 2 mM and 4 mM eupatilin.

of LTD_4, eupatilin completely suppressed the release of LTD_4 from these cells stimulated by *H. pylori* (at 0.5 mM eupatilin) or calcilum ionophore A23187 (at 2.0 mM eupatilin).

Fig. 5 shows the results with confocal laser-scanning examination of Kato III cells. Cells were labelled with Ca^{2+}-sensitive probe, Fluo-3-acetoxymethyl ester (Fluo-3-Am). When neutrophils were treated with *H. pylori* (Fig 5b), the fluorescence intensity much more strong than that observed in the control (Fig. 5a). But the effect of eupatilin in the fluorescence intensity was not significant, and the suppression at 2 mM of eupatilin (Fig. 5c) was almost comparable to that at 4 mM of eupatilin (Fig. 5d). The fluorescence data showed that maxium intensity for the control, H. pylori, 2 mM of eupatilin, and 4 mM of eupatilin was

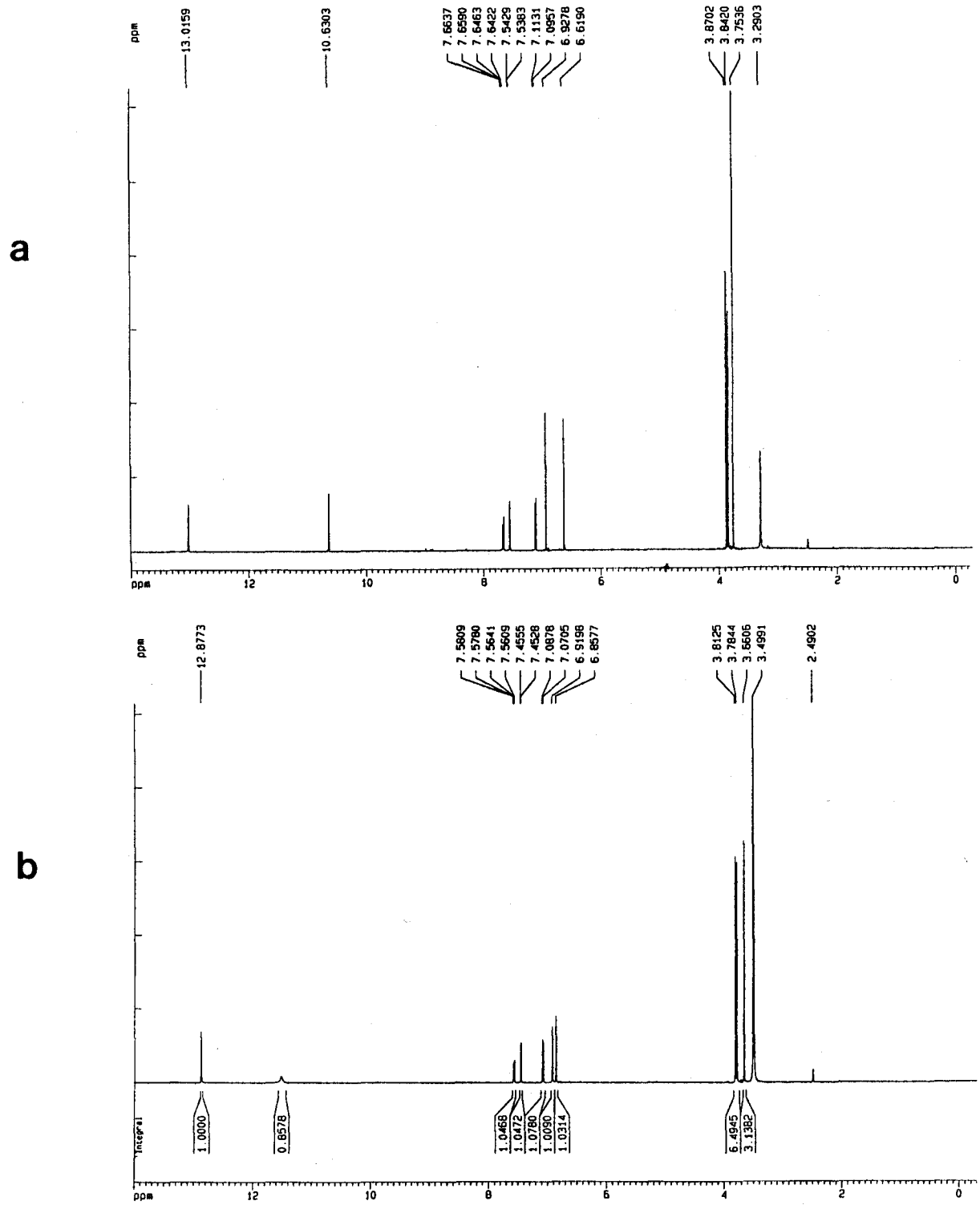

Fig. 6. ^{1}H NMR analysis of eupatilin in the absence and presence of Ca^{2+}. (a) eupatilin (4 mM), (b) eupatilin (4 mM) with $CaCl_2$ (6 mM). The reaction mixture of eupatilin and $CaCl_2$ in DMSO-d6 (10ml) was stirred for 2 hours. NMR study on eupatilin-Ca^{2+} complex was investigated using 1H NMR spectroscopy (500MHz FT-NMR, Bruker).

81, 209, 192, and 163, respectively.

For the Nuclear Magnetic Resonance (NMR) assay, for the reaction mixture of eupatilin (4 mM) and $CaCl_2$ (6 mM), we used ^{1}H-NMR. As shown in Fig. 6a, we obtained NMR peaks of δ 2.47 (S, 3H, CH3), 3.29~3.87 (m, 3H, CH_3), 6.61~7.66 (m, 8H, ArH), 10.63 (S, 1H, OH), 13.01 (S, 1H, OH) correspond to eupatilin. After treatment of Ca^{2+}, we also obtained NMR peaks of δ 2.50 (S, 3H, CH3), 3.49~3.81 (m, 3H, CH_3), 6.85~7.58 (m, 8H, ArH), 11.52 (S, aH, OH), 12.87 (S, 1H, OH) correspond to eupatilin. Compare with these results, NMR patterns of eupatilin with and without Ca^{2+} were not significantly different. It is presumed that eupatilin-Ca^{2+} complex was not formed.

DISCUSSION

In the previous study, we found that eupatilin isolated from *Artemisia asiatic* a nakai protects the damage of gastric mucosal cells induced by ischemia-reperfusion (IR) injury (Park, unpublished data). In addition to IR-injury, *H. pylori* infection is an important factor causing gastric cell damage.

A characteristic feature of chronic gastritis is the abundant inflammatory response in close association with *Helicobacter pylori* (Nielsen & Andersen, 1992). A novel group of mediators of inflammation are called leukotrienes (LTs), that is better reflected by studies involving tissue injury, inflammatory precesses and shock (Denzlinger et al, 1985; Lefer, 1986).

It was the aim of this study to investigate not only the inhibitory effect of eupatilin on the release of LTD_4 from neutrophils and Kato III cells stimulated with *H. pylori* but also its mechanism of action. One possibility is that its preventive effect is ascribed to interfering with the *H. pylori*-induced release of LTD_4 from these cells.

LTD_4 was released from both neutrophils and Kato III cells when these cells were incubated with either *H. pylori* or calcium ionophore A23187. The release of LTD_4 was suppressed time dependently, and reached after 30 min. This results is supported by the reports that the metabolism of leukotrienes (LTs) is highly Ca^{2+} dependent (Rouzer et al, 1990). The release of LTD_4 was inhibited by eupatilin in these cells induced by both *H. pylori* and calcium ionophore A23187.

With regard to the inhibitory mechanism, we obtained an important finding suggesting that the inhibitory action of eupatilin is due to the fact that *H. pylori* induce Ca^{2+} influx in the both neutrophils and Kato III cells, and eupatilin inhibits the Ca^{2+} influx. The finding is that the release of LTD_4 was induced by calcium ionophore A23187 and the A23187-induced release was also inhibited by eupatilin. But in the presence of eupatilin, the release of LTD_4 from these cels stimulated by *H. pylori* or calcium ionophore A23187 was inhibited, whereas Ca^{2+} influx into the cells was not affected. It was confirmed by confocal laser-scanning microscopy and supported by the NMR study showing the insignificant variation of the chemical shift value caused by the formation of eupatilin-Ca^{2+} complex. This result was in contrast with that of rebamipide known as antiulcer drug which inhibited both. It seems that the suppressive action of eupatilin, therefore, is not through its interaction with the level of intracellular Ca^{2+}.

Finally, we think that it is necessary to investigate the possible other mechanism about the effect of eupatilin on *H. pylori*-induced release of LTD_4 in the human neutrophhils and Kato III cells.

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