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Antibacterial and anti-atrophic effects of a highly soluble, acid stable UDCA formula in *Helicobacter pylori*-induced gastritis

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ABSTRACT

Helicobacter pylori is one of the main causes of atrophic gastritis and gastric carcinogenesis. Gastritis can also occur in the absence of *H. pylori* as a result of bile reflux suggesting the eradication of *H. pylori* by bile acids. However, the bile salts are unable to eradicate *H. pylori* due to their low solubility and instability at acidic pH. This study examined the effect of a highly soluble and acid stable ursodeoxycholic acid (UDCA) formula on *H. pylori*-induced atrophic gastritis. The *H. pylori* infection decreased the body weight, mitochondrial membrane potential and ATP level *in vivo*. Surprisingly, *H. pylori*-induced expression of malate dehydrogenase (MDH), a key enzyme in the tricarboxylic acid cycle, at both the protein and mRNA levels. However, the UDCA formula repressed MDH expression and increased the membrane potential thereby increasing the ATP level and body weight *in vivo*. Moreover, UDCA scavenged the reactive oxygen species (ROS), increased the membrane potential, and inhibited apoptosis in AGS cells exposed to H_2O_2 *in vitro* through the mitochondria-mediated pathway. Taken together, UDCA decreases the MDH and ROS levels, which can prevent apoptosis in *H. pylori*-induced gastritis.

1. Introduction

H. pylori is a gram-negative bacillus that colonizes the gastric mucosa and produces superoxides and other products such as Vac and Cag, which cause DNA damage and induce cytokines and growth factors that contribute to inflammation and chronic gastritis as well as promote gastric carcinogenesis, the second most common cancer in the world [1]. However, it is unclear how *H. pylori* induces atrophic gastritis.

Bile reflux gastritis occurs in the absence of *H. pylori*, and *H. pylori* only inhabits the areas of the stomach that are not in contact with the soluble bile suggesting the potential antibacterial activity of bile or bile acids against *H. pylori* [2]. Bile acids have been shown to possess antibacterial activity against *H. pylori in vitro* [2,3]. Ursodeoxycholic acid (UDCA), a hydrophilic bile acid, is a major ingredient of bile acids. A treatment with UDCA results in morphologic changes from a primary rodlike shape to a spherical shape with blebs *in vitro*, which is different from the changes caused by amoxicillin *in vitro* [2]. Furthermore, a clinical study demonstrated that UDCA significantly reduces the infiltration of mononuclear inflammation of the gastric antrum but not that of polymorphonuclear inflammation [4]. This indicates that UDCA can be partly useful in the treatment of a *H. pylori* infection.

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However, the UDCA products currently available have low solubility and stability in acidic pH, and do not inhibit the colonization of *H. pylori* [4].

UDCA has been used to treat a variety of liver diseases such as primary biliary cirrhosis, primary sclerosing cholangitis, and cystic fibrosis-related cholestasis [5–7]. UDCA increases the membrane stability and prevents the membrane damage induced by mechanical and chemical stress [8]. In addition, it increases apoptotic threshold in hepatic cells by preventing the mitochondrial release of cytochrome *c*, and the cleavage of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) [9–11]. Furthermore, bile acids activate the thyroid hormone for energy expenditure [12] suggesting that UDCA may affect the generation of ATP in the mitochondria, which is also the main site for reactive oxygen species (ROS) such as O_2^- and H_2O_2 production and the primary target of ROS, thus collaterally activating the mitochondrial cell death pathway [13–17]. However, the mechanism for the action of UDCA on mitochondrial enzymes is unclear.

This paper reports for the first time that *H. pylori* induces malate dehydrogenase (MDH) significantly at both the protein and mRNA levels, and decreases the mitochondrial membrane potential and ATP level *in vivo*. However, these features were reversed by a highly soluble and pH stable UDCA formula *in vivo*. Moreover, the new UDCA formula scavenges ROS and increases mitochondrial membrane potential, which prevents H_2O_2-induced apoptosis through the mitochondria-mediated pathway *in vitro*.

2. Materials and methods

2.1. Animals and treatment

Five-week-old male C57BL/6 mice (Orient Co., Korea) were housed and fed with normal rodent chow and water in the animal room *ad libitum*. The UDCA formula (Yoo solution®) with a solubility of 25 mg/ml (T_{max} 15 min, C_{max} 20.4 μg/ml), which is 50,000 times higher than the commercially available tablets and capsules (T_{max} 83 min, C_{max} 2.9 μg/ml) and is stable from pH 1 to 14 (US patent 6,251,428 with applications for worldwide patents) was provided by PrimePharm Tech. The mice were infected with 10^9 cfu/ml of *H. pylori* every 2 days for 3 times, and kept for 1 week for bacteria colonization followed by the oral administration of 5 mg/mouse of UDCA once per day. Phosphate buffered saline (PBS) was used as the control. As a treatment index for *H. pylori*-induced gastritis; histopathology, body weight, antibacterial effect, myeloperoxidase (MPO) activity, mitochondrial membrane potential, ATP level, gene expressions at the protein and mRNA levels, were examined in the stomach of the *H. pylori*-infected mice.

2.2. Cells and bacteria culture conditions

Human gastric adenocarcinoma AGS cells, which were purchased from ATCC, were cultured in RPMI (Gibco) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (JBI), 2% Penicillin–Streptomycin (Gibco), at 37 °C in 5% CO_2 incubator. The mouse-adapted *H. pylori* strain, Sydney strain 1 (SS1), was cultured as described previously [18]. *H. pylori* lysate was prepared by sonication of the bacterial culture. As an index for *H. pylori*-induced gastritis, the following activities were examined; antibacterial effect, MPO activity, mitochondrial membrane potential, ATP level, and malate dehydrogenase (MDH) and citrate synthase (CS).

2.3. 2-D gel electrophoresis

The *H. pylori*-infected mouse ($n = 3$/group) stomachs were washed thoroughly with PBS to remove all traces of food, and then homogenized in 1 ml of 20 mM Tris–HCl (pH 6.8) in an ice bath. The homogenate was centrifuged at 16,000 rpm for 10 min at 4 °C and the proteins in the supernatant were precipitated by 10% trichloroacetic acid, incubated on ice for 30 min, and centrifuged at 16,000 rpm for 10 min at 4 °C. The pellet was washed by ethanol, air-dried, and resuspended in a lysis buffer (8 M Urea, 4% CHAPS, 40 mM Tris–HCl) for 1 h. The resuspended solution was centrifuged at 16,000 rpm for 10 min at 17 °C, and the protein concentration in the supernatant was determined using the Bradford assay. Three hundred μg of proteins were separated by 2-dimensional gel electrophoresis by isoelectric focusing at pH 4–7 (24 cm, Immobilone dry strip, Amersham-Pharmacia) followed by 12% polyacrylamide gel according to the manufacturer's instructions. The silver stained proteins were scanned and analyzed by PDQUEST (BioRad). The protein spots of interest were destained, and digested with trypsin. The protein spots were arbitrarily numbered, and the intensity of the spot of interest was compared for further analysis. The peptide mass was then determined by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry analysis.

2.4. Reverse-transcriptase (RT)-PCR or real-time RT-PCR

The total RNA was isolated using Trizol® (Invitrogen) according to the manufacturer's instructions, and stored at −70 °C until needed. After a DNase treatment, the cDNA was synthesized by AMV reverse-transcriptase (Promega) and used as a template for the PCR reaction (GeneAmp® PCR

Table 1 – Real-time RT-PCR primer sequences

Gene	Forward primer	Reverse primer	Annealing temperature (°C)
Trap1	ACA AGA TGT GGT AAC AAA GT	ATA AGC CTG AGC AAT ATA AC	50
Prdx5	CGA CAG ACT TAT TAT TGG AT	CTC AGA GTT GAG AGA GGA T	50
Pdia6	GAG ATA ATC AAC GAA GAC AT	CTT CCA AGT AAG AGT TTC TG	50
MDH	TTGGAGTCACTCGTCTTCTT	ACAGCCACTTTAGCATTGTT	50
β-Actin	TGG AAT CCT GTG GCA TCC ATG AAA C	TAA AAC GCA GCT CAG TAA CAG TCC G	50

system 2700-AB) with an annealing temperature of 55 °C. Table 1 shows the primers used in this study. Real-time RT-PCR was carried out using a 2× SYBR I mixture (Qiagen) at 50 °C using a PCR machine (ABI 7900 HT).

2.5. Enzymes and biochemical assays

The MPO activity was determined as described elsewhere [19]. The level of reactive oxygen species including hydrogen peroxide and hydroxyl radicals was determined, as described previously [20]. The ATP content was determined using a protein kinase assay kit (PKLight assay kit, Cambrex Bio Science Rockland, Inc.). The malate dehydrogenase (MDH) and citrate synthase (CS) activities were determined, as described [21,22].

2.6. Measurement of cell membrane depolarization

The level of cell membrane depolarization induced by H_2O_2 or the *H. pylori* lysate was determined using a slight modification of the method described elsewhere [23]. Briefly, 2×10^5 of the AGS cells were seeded in a 24-well plate in complete RPMI media overnight. After washing with PBS, the cells were treated with either 100 μM H_2O_2 or 500 μg/ml of the *H. pylori* lysate in the presence or absence of 2.5 mg/ml of UDCA. At the indicated time, the cells were washed with PBS and stained with 5 μg/ml JC-1 at 37 °C for 10 min. The cells were detached, centrifuged, and resuspended in PBS. The fluorescence signal of JC-1 was measured using a spectrofluorometer (PerkinElmer Life Sciences LS50B) with excitation at 485 nm, and emission at 595 nm and 535 nm, respectively.

2.7. Preparation of mitochondria

The mitochondria were fractionated using an ApoAler® Cell Fractionation kit (BD Biosciences – Clontech Lab.) according to the manufacturer's instructions.

2.8. Mitochondrial cytochrome c release

The AGS cells were treated with 2.5 mg/ml UDCA before being exposed to 4 mM H_2O_2. The mitochondria-enriched fraction of AGS cells was used for Western blot analysis using the anti-cytochrome c antibody, as described previously [24].

2.9. DNA fragmentation

The AGS cells was pretreated with 2.5 mg/ml UDCA for 4 h before being exposed 4 mM H_2O_2 for 0, 20, 40, and 60 min. The cells were harvested, washed with PBS, then resuspended in 200 μl of the PK reaction buffer (20 mM Tris–Cl pH 8.3, 5 mM $MgCl_2$, 100 mM KCl, 1% Tween 20, 1% NP-40) with 250 ng/μl proteinase K. After incubation at 37 °C for 3 h, the proteins were removed by phenol/chloroform extraction followed by DNA precipitation with isopropyl alcohol. The DNA pellet was resuspended in 100 μl of 50 mM Tris–EDTA buffer, pH 8.0, and the DNA solution was treated with 10 mg/ml of RNase for 2 h. Five microgram of the purified DNA was eletrophoresed on a 1.5% agarose gel.

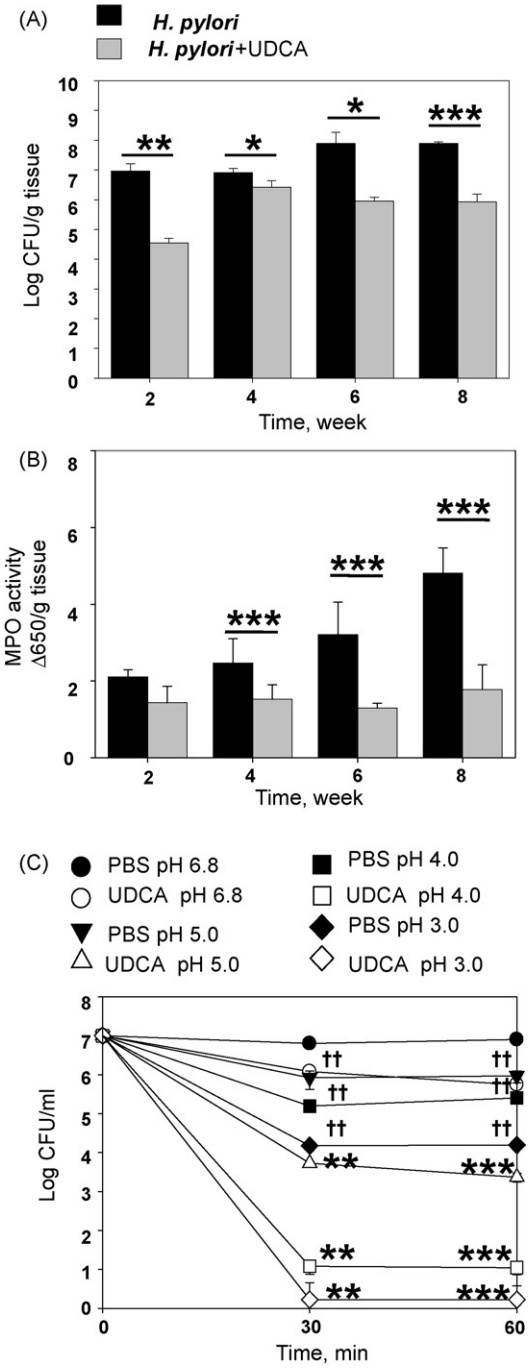

Fig. 1 – Inhibition of *H. pylori* growth and neutrophil recruitment by UDCA. (A and B) After *H. pylori* infection, the mice (n = 5/group) were non-treated (PBS) or treated with UDCA (UDCA group), and sacrificed at the indicated time, and the number of viable cells (A) and MPO activity (B) were determined. (C) Antibacterial activity of UDCA on *H. pylori in vitro* was determined after exposing the *H. pylori* to 2.5 mg/ml of UDCA at various pH, and measuring the viability of the *H. pylori*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the control group. The figure shows the standard deviation from three independent experiments.

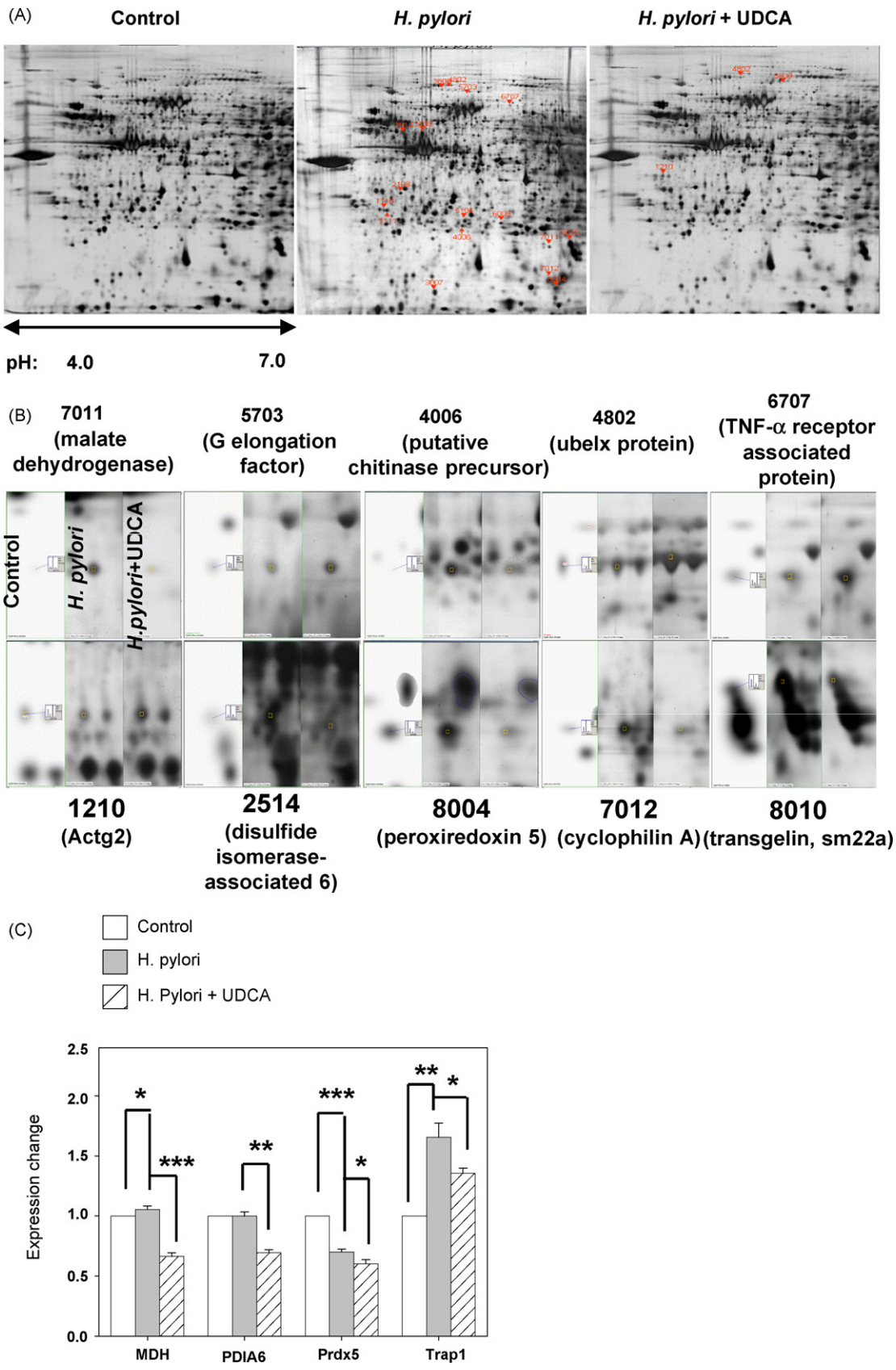

Fig. 2 – Modulation of gene expression *in vivo* by UDCA. (A) Mice were infected with *H. pylori* (*H. pylori*), followed by treatment with UDCA (*H. pylori* + UDCA) for 6 weeks, the mouse stomachs (n = 3/group) were excised, and homogenized. The total proteins were separated on 12% polyacrylamide gel followed by an isocratic pH gradient gel from pH 4 to 7. The protein mass of each spot was analyzed by MADI-TOF. (B) Part of the protein spots whose expression levels changed more than

Table 2 – Gene expression profiles derived from 2-D gel electrophoresis

Spot number	Protein ID	M.W. (D)	Control	*H. pylori*	*H. pylori* + UDCA
1110	Arhgdib protein, GDP dissociation inhibitor	22,894	275	669	1419
1111	HSP 84	83,615	251	1422	1545
1210	Actg2 protein actin, smooth muscles	42,249	1094	443	1660
2108	Serine (cysteine) proteinase inhibitor	44,309	54	1573	1220
2514	Pdia6 (protein disulfide isomerase-associated 6)	49,058	328	1627	400
3007	Chain A	15,833	720	3546	3870
3114	Actin, alpha 2	42,381	585	2991	2203
3508	Serum albumin	70,730	204	3698	1598
3509	Serum albumin	70,730	204	3698	1598
3809	Actin alpha 4	105,368	52	189	403
4006	Putative chitinase precursor	525,230	49	1247	407
4802	Ubelx protein, ubiquitin-activating enzyme E1	118,931	294	194	680
5106	Putative chitinase precursor	52,523	155	1984	783
5703	G elongation factor mitochondrial 2	86,611	86	64	194
6008	Chain B	25,428	151	1275	1085
6707	Trap1 (TNF-α receptor associated protein)	80,370	339	170	776
7011	Malate dehydrogenase soluble	36,659	95	746	38
7012	Peptidyl-prolylisomerase A	18,131	1423	3297	319
8004	Prdx5 (peroxiredoxin 5)	17,175	4709	1761	286
8010	Transgelin, Sm22α	22,618	4606	7471	1768

The average change of 3 mice was shown.

2.10. Statistical analysis

The data was analyzed using a Fisher's analysis of variance (ANOVA) test. All the data is expressed as the mean ± standard deviation of three to five independent experiments. A P value <0.05 was considered significant.

3. Results

3.1. Antibactericidal activity and the inhibition of neutrophil recruitment by UDCA in vivo

H. pylori induces chronic inflammation in the gastric mucosa by recruiting neutrophils. Therefore, the antibacterial effect of UDCA *in vivo* was initially investigated. UDCA concentration was determined preliminarily *in vitro* studies which demonstrate dose-dependent antibacterial activity of UDCA (data not shown). Moreover, when AGS cells were treated with various concentrations of UDCA (up to 3200 (g/ml) over a 12 h period, no cytotoxicity was detected (Supplementary Fig. S1). Mice were infected with *H. pylori* and the viability of *H. pylori* in the mouse stomach was determined. After 2, 4, 6, and 8 weeks of treatment with UDCA, the number of viable *H. pylori* cells in the UDCA-treated group decreased significantly to 0.38%, 33%, 1.2%, and 1.1% of the non-treated group, respectively (Fig. 1A). In addition, when *Campylobacter*-like organism test (CLO test®) was used to detect the persistence of *H. pylori* in the stomach, the urease activity of the treated group was lower than that of the non-treated group (data not shown) indicating that UDCA has antibacterial activity *in vivo*.

In order to determine the effect of UDCA on inflammation, the MPO activity, an index of polymorphonuclear cell accumulation, was determined. After the UDCA treatment for 4, 6, and 8 weeks, the MPO activity was decreased significantly down to 60%, 40%, and 37.5% of the non-treated group, respectively (Fig. 1B), indicating that UDCA decreases the viability of *H. pylori*, thereby inhibiting the recruitment of neutrophil *in vivo*.

The antibacterial effect *in vivo* was corroborated by determining the *in vitro* antibacterial effect. After 30 min exposure to UDCA at pH 3, 4, 5, and 6.8, the viability decreased significantly to 1.1×10^{-4}, 7.5×10^{-5}, 6.4×10^{-2}, and 1.9×10^{-1} times that of the non-treated group, respectively, even though viability of *H. pylori* decreased in a pH-dependent manner (Fig. 1C). This shows that UDCA acts as an antibacterial and anti-inflammatory agent in *H. pylori*-induced gastritis.

3.2. Modulation of protein expression by UDCA in vivo

In order to examine the underlying mechanism of UDCA on antibacterial and anti-inflammatory action *in vivo*, proteomic analysis of *H. pylori*-infected mice treated with UDCA for 6 weeks was performed using 2-D gel electrophoresis followed by matrix-assisted laser desorption ionization-time-of-flight (MALDI-TOF) mass spectrometry analysis. Twenty proteins were found to be up- or down-regulated more than 2-fold by either the *H. pylori* infection or *H. pylori* infection followed by UDCA treatment (Fig. 2). The *H. pylori* infection induced 13 genes more than 2-fold but repressed 2 genes (Table 2). After the *H. pylori* infection followed by UDCA treatment, levels of TNF-α receptor associated protein [TRAP], Actg2, ubiquitin-

2-fold after the UDCA-treatment was shown. (C) The mice (n = 3/group) were infected with *H. pylori* (*H. pylori*) or *H. pylori* infected and UDCA treated for 6 weeks (*H. pylori* + UDCA), and the RNA was isolated from the stomachs was used to determine the mRNA levels by real-time RT-PCR. Representative results from duplicate experiments are shown. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to the control group.

activating enzyme E1, G elongation factor, actin α 4, and GDP dissociation inhibitor were up-regulated 456%, 375%, 350%, 303%, 213%, and 212%, respectively, compared with those of the non-treated group. On the other hand, the levels of malate dehydrogenase [MDH], peptidyl-prolylisomerase A, peroxiredoxin 5 (Prdx5), transgelin, protein disulfide isomerase (PDI)-associated 6, putative chitinase precursor, and serum albumin were decreased to 5%, 10%, 16%, 24%, 24%, 33%, and 43% of the non-treated group, respectively (Table 2). These results suggest various activities of UDCA on oxidation (protein disulfide isomerase-associated 6 TRAP, and peroxiredoxin 5), mitochondria (MDH, peptidyl-prolyl isomerase), ubiquitination (Ublex), cell morphology (Actg2, transgelin), and chitinase (putative chitinase precursor).

In order to corroborate the protein expression at the transcription level, the mRNA levels of those genes were determined by real-time reverse-transcriptase PCR. After 6 weeks of UDCA treatment, the mRNA levels of MDH, PDI-

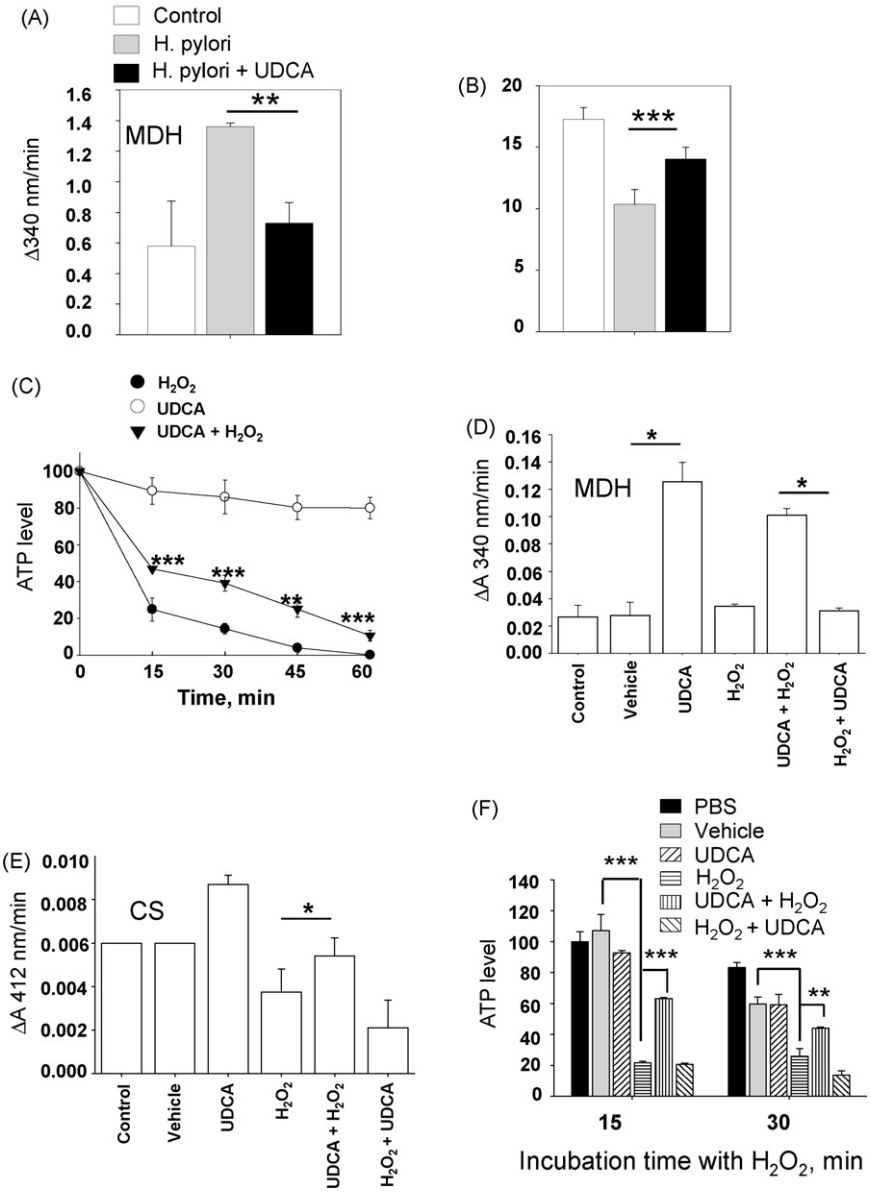

Fig. 3 – Modulation of the MDH activity, and the prevention of ATP depletion *in vivo* and *in vitro* by UDCA. (A and B) The mice (*n* = 3/group) were infected with *H. pylori* (*H. pylori*) and treated with UDCA for 8 weeks (*H. pylori* + UDCA) and the stomachs were homogenized in PBS. The aqueous phase of the homogenate was used to determine the MDH activity (A), or ATP level (B). (C) The ATP level was determined after treating the AGS cells with 2.5 mg/ml UDCA for 4 h before exposing them to 4 mM H_2O_2 for 15, 30, 45, and 60 min. The cell lysate was used to determine the ATP level, and the relative ATP level represents the percentage of ATP level compared with that of the non-treated cells. (D–F) The AGS cells were treated with a 2.5 mg/ml UDCA solution for 4 h before being exposed to 4 mM H_2O_2 for 15 min (UDCA + H_2O_2), or the cells were exposed to 4 mM H_2O_2 for 15 min followed by a treatment with 2.5 mg/ml UDCA for 4 h (H_2O_2 + UDCA). The cell lysate was used to determine the MDH (D) and CS (E) activities, and the ATP level (F). The figure shows the standard deviation from three independent experiments. $^{*}P < 0.05$, $^{***}P < 0.001$.

associated 6, and Prdx 5 were decreased significantly to 67%, 67%, and 87% of the level of the non-treated group, respectively (Fig. 2C), which is consistent with the 2-D gel data. Again reverse-transcriptase (RT)-PCR analyses of the mRNA expression after the 6 or 8 week UDCA treatment in independent experiments showed a similar gene expression pattern to those of the real-time PCR data (Supplementary Table 2).

3.3. *Inhibition of MDH activity and prevention of ATP depletion by UDCA in vivo and in vitro*

Since MDH plays an important role in ATP generation, and UDCA decreases the MDH protein level *in vivo*, UDCA might affect the ATP level and other mitochondria enzyme activities. In order to check this possibility, the mice infected with *H. pylori* were treated with UDCA for 8 weeks, and the activities of MDH and CS, another key enzyme in the tricarboxylic acid (TCA) cycle [25], in addition to the ATP level were determined. After the *H. pylori* infection followed by UDCA treatment, the MDH activity was decreased to 57% of the non-treated group (Fig. 3A) even though the CS activity was unchanged (data not shown). In contrast, the ATP level was increased significantly to 135% of the non-treated group (Fig. 3B). Furthermore, when the AGS cells were pretreated with UDCA for 4 h before exposure to H_2O_2, the UDCA pretreatment significantly increased the relative ATP level by 1.8, 2.7, 6.3, and 53.3 times that of the non-treated group at 15, 30, 45, and 60 min, respectively (Fig. 3C). The post-treatment with UDCA did not prevent the depletion of intracellular ATP (data not shown). This demonstrates that a *H. pylori* infection increases the MDH activity and mRNA level but UDCA decreases both.

In order to corroborate the UDCA effect on atrophic gastritis, mice infected with *H. pylori* were treated with UDCA for 20 weeks, and the body weight and histopathology were examined. The body weight of the *H. pylori*-infected group was consistently lower than that of the UDCA-treated group (Fig. 4A). Although UDCA-treated mice did not show any death during the 20 week period, the non-treated mice started to die at week 16 and by week 20 only 2 mice remained. Therefore, the higher the body weight of the non-treated group compared to that of the treated group, did not reflect the real nature at week 19 and 20. The histopathology of the stomach showed that the mucosal layer of the non-treated *H. pylori*-infected group was markedly thinner than those in the UDCA-treated group as a result of the necrosis of epithelial cells. A medium (grade 2) level of gastric mucosal epithelial cell necrosis as well as necrotic superficial cells and parietal cells with a pyknotic nucleus were observed in the non-treated group (Fig. 4B). However, no specific lesions were found in any of the gastric cell components, superficial mucosal cells, parietal and chief cells after the UDCA treatment for 20 weeks (data not shown), which corroborates the inhibitory effect of UDCA on the depletion of ATP.

The ATP level and MDH and CS expression were determined *in vitro* to confirm effect of UDCA on the mitochondrial enzymes for ATP generation *in vitro*. Although UDCA did not induce MDH and CS mRNA gene expression (data not shown), it increased the *in vitro* activity of MDH (Fig. 3D), which confirmed the stimulatory effect of UDCA on energy expenditure [12]. In addition, UDCA inhibited the decrease in the MDH and CS activities (Fig. 3D and 3E) as well as ATP (Fig. 3F) after exposure to H_2O_2. These results suggest that UDCA has a different effect on TCA cycle enzymes in a normal and gastritis model, i.e., UDCA increases MDH activity in normal AGS cells *in vitro* but decreases MDH activity in *H. pylori*-induced gastritis mice.

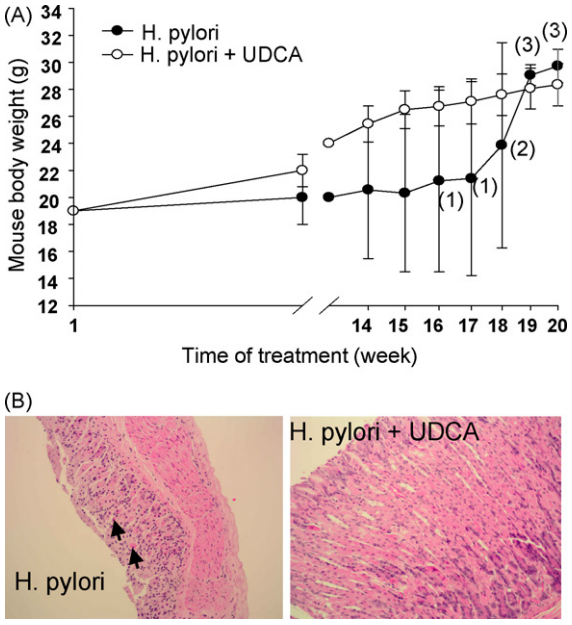

Fig. 4 – Increase in the mouse body weight and histopathological change as a result of the UDCA treatment. (A) The mice (*n* = 5/group) infected with *H. pylori* were treated with UDCA for 20 weeks and the body weight was determined every week from week 14 to 20. The numbers in parenthesis indicate the number of dead mice. (B) At week 20, the mouse stomachs from (A) were taken and stained with hematoxylin–eosin. These slides are from the stomach fundic gland region with the same magnification (×200). Arrow shows a pyknotic nucleus.

3.4. *Increase of membrane potential in vivo and in vitro by UDCA*

Although MDH expression was decreased by UDCA, the ATP level was much higher than the non-treated group *in vivo*. Since UDCA has been reported to stabilize the membrane [8], this incongruity can be explained by the membrane instability in the *H. pylori*-infected group. UDCA was administered for 8 weeks to check the possibility of membrane stabilization by UDCA. The mitochondria membrane fraction was enriched and the membrane potential was determined. The purity of the mitochondria-enriched fraction prepared from the stomach was checked by measuring the level of the mitochondrial marker cytochrome *c* oxidase 4 (COX4) by Western blotting. The mitochondrial protein was detected mainly (80%) in the mitochondrial fraction but a small amount was also detected in the cytosol fraction (Fig. 5A). The subsequent determination of the mitochondria membrane potential showed a significant increase in the mitochondria membrane potential (0.59 ± 0.02) in the UDCA-treated group compared

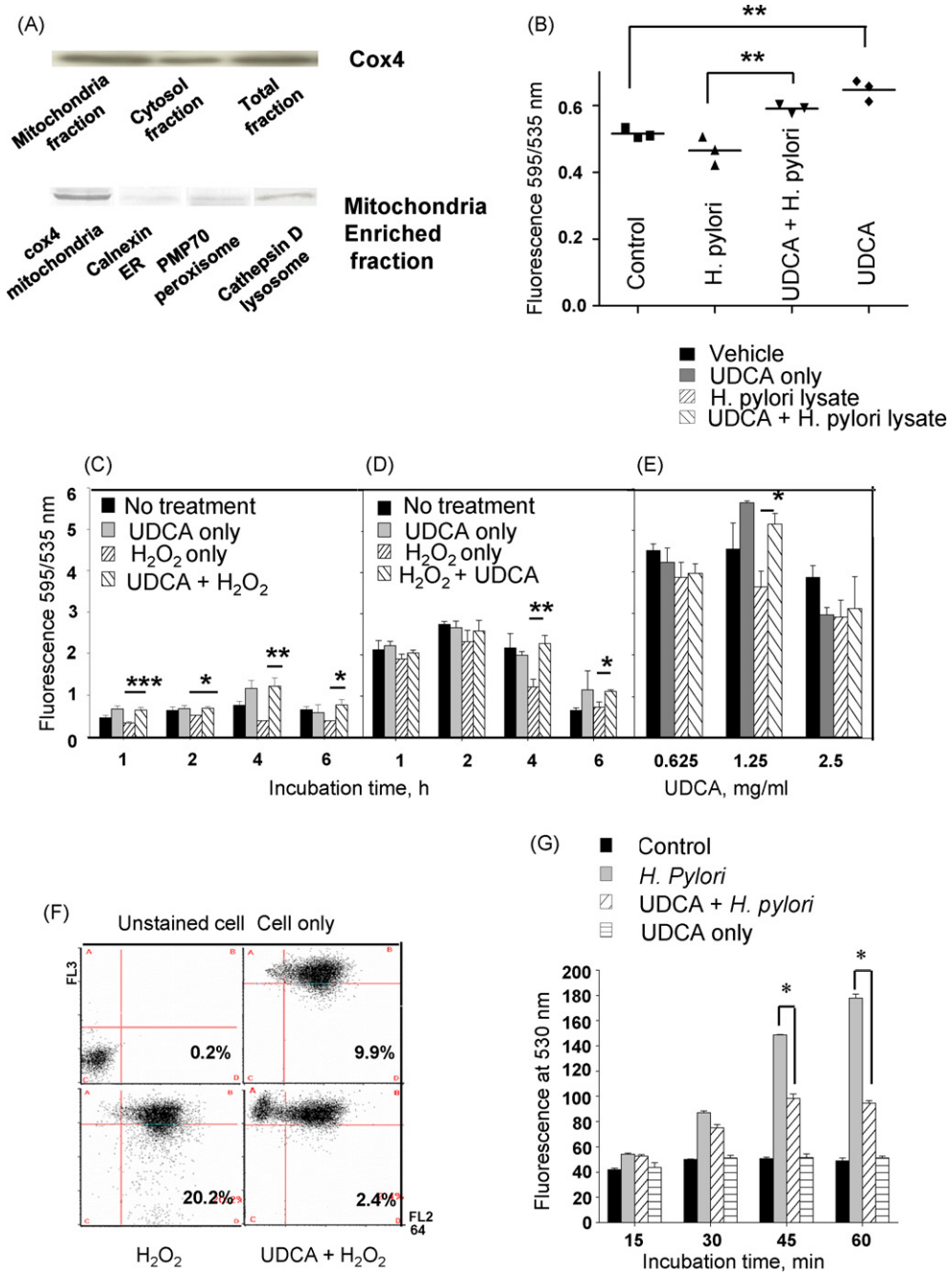

Fig. 5 – Increase in the membrane potential by UDCA *in vivo* and *in vitro*. (A) The mice (n = 3/group) were treated with 5 mg/mouse/day of UDCA for 8 weeks, and the mitochondrial fraction was then purified. The purity of the mitochondrial fraction was checked by Western blotting. (B) The purified mitochondrial fraction from (A) was incubated with a JC-1 dye. (C–F) The AGS cells were pretreated with UDCA for 4 h before being exposed to H_2O_2 for 1, 2, 4, and 6 h. The cells were then stained with the JC-1 dye (C). The AGS cells were exposed to H_2O_2 for 4 h followed by a treatment with UDCA for 1, 2, 4, and 6 h (D). The AGS cells were pretreated with UDCA for 4 h before being exposed to the *H. pylori* lysate for 4 h (E). The AGS cells were pretreated with UDCA for 4 h then exposed to H_2O_2 for 2 h. The cells were stained with JC-1 dye followed by flow cytometry (F). (G) AGS cells were pretreated with 2.5 mg/ml of UDCA 4 h prior to infection with *H. pylori* (MOI 100:1). At the indicated time, the cells were stained with JC1 dye, and the intracellular fluorescence levels were determined by spectrofluorometer. The results show the standard deviation from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

with that in the *H. pylori*-infected group (0.46 ± 0.04). In addition, UDCA itself could increase the mitochondria membrane potential (0.64 ± 0.03) compared with the control (0.51 ± 0.02, Fig. 5B).

In order to confirm the effect of UDCA on the membrane potential *in vitro*, the AGS cells were pretreated with UDCA for 4 h before being exposed to H_2O_2, and the membrane potential was determined. UDCA prevented the decrease in the

membrane potential induced by the H_2O_2 treatment at all time points. Four hours after the H_2O_2 treatment, the membrane potential in UDCA-treated cells was increased 3-fold compared with the cells treated with H_2O_2 only (Fig. 5C). These results were confirmed by flow cytometry analysis. The H_2O_2 treatment depolarized 20.2% of the AGS cells. However, a pretreatment with UDCA reduced the level of depolarization to only 2.4% (Fig. 5F). In addition, when the AGS cells were treated with UDCA for 4 and 6 h after exposure to H_2O_2, UDCA significantly increased the membrane potential by 185% and 153%, respectively, of the H_2O_2-treated group (Fig. 5D). Moreover, when a *H. pylori* lysate was used to induce membrane depolarization, a pretreatment with 1.25 mg/ml UDCA increased the membrane potential to 141% of that observed in the *H. pylori* lysate treated group (Fig. 5E). Furthermore, when AGS cells were co-incubated with live *H. pylori*, ROS levels of the UDCA pretreated group after 45 and 60 min of infection were 66% and 55%, respectively. Lower than those of the non-treated group although UDCA itself did not affect the ROS level (Fig. 5G). Also, flow cytometry analysis showed that pretreatment with UDCA suppressed increases of ROS from *H. pylori* infection (data not shown). This show that UDCA increases the mitochondria membrane potential both *in vivo* and *in vitro*, thereby preventing the depletion of ATP from the mitochondria in a *H. pylori*-infected mouse stomach.

3.5. *Decrease of ROS level by UDCA in H_2O_2- and H. pylori-induced apoptosis*

UDCA can protect against indomethacin-induced oxidative stress [26], and modulate the expression of oxidoreductases (TRAP1, Prx5, and PDI-associated 6) as well as increase the membrane potential. Therefore, UDCA was further examined to determine if it could scavenge ROS and reduce apoptosis. In order to check this possibility in *H. pylori*-induced gastritis, the AGS cells were pretreated with UDCA for 4 h before being exposed to H_2O_2, and the ROS levels were determined using a cell permeate probe H2HCFDA dye, which is non-fluorescent but becomes fluorescent in the presence of ROS. The ROS level increased gradually after exposure to H_2O_2 (Fig. 6A). However, a pretreatment with UDCA significantly reduced the ROS level, and after 30, 45, and 60 min exposure to H_2O_2, the ROS levels in the UDCA pretreated group were 70%, 57% and 62% lower than those in the non-treated group. On the other hand, UDCA itself did not affect the ROS level (Fig. 6A). Flow cytometry analysis also showed that a pretreatment with UDCA (mean value: 121) suppressed the increase in ROS induction by H_2O_2 (mean value: 144) but a post-treatment with UDCA did not have any effect (Fig. 6B) highlighting the ROS scavenging effect of UDCA.

UDCA inhibits the deoxycholate-induced apoptosis of liver cells by inhibiting mitochondrial membrane depolarization [11]. Therefore, in order to check whether UDCA can inhibit the apoptosis of gastric cells, the AGS cells were preincubated with UDCA for 4 h before being exposed to H_2O_2 for 20, 40, and 60 min. The purity of the mitochondria-enriched fraction was checked by Western blotting using the anti-COX4 antibody (Fig. 6C). No COX4 mitochondria marker band was detected from the cysotol fraction indicating that the cytosolic fraction did not contain any mitochondrial impurities. In order to examine the release of cytochrome *c* from the mitochondria, fractions of AGS cells pretreated with UDCA and exposed to H_2O_2 were probed with the anti-cytochrome *c* antibody by Western blotting (Fig. 6D). H_2O_2 exposure triggered the release of a large amount of cytochrome *c* from the mitochondria to the cytosol. On the other hand, the UDCA pretreatment inhibited the release of cytochrome *c* significantly (Fig. 6D).

In order to corroborate the anti-apoptotic effect of UDCA, the level of DNA fragmentation was determined after pretreating the AGS cells with UDCA for 4 h and incubating them with H_2O_2 for either 6 or 12 h. The genomic DNA was extracted and subjected to agarose gel electrophoresis. The exposure of AGS cells to H_2O_2 resulted in DNA fragmentation of the AGS cells but the UDCA pretreatment prevented the DNA fragmentation significantly (Fig. 6E) demonstrating a protective effect against H_2O_2-induced apoptosis. To check a possibility that decreases of ROS by UDCA treatment could generate less apoptosis, AGS cells were treated with UDCA prior to *H. pylori* infection. Results showed that UDCA pretreatment inhibited the DNA fragmentation induced by *H. pylori* infection (Fig. 6F).

To check the anti-apoptotic effects of UDCA *in vivo*, cell apoptosis was identified by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling using the stomach of mice infected with *H. pylori* and treated with UDCA for 8 weeks. But no difference was found between UDCA-treated and non-treated groups (data not shown). Although after an 8 week period results did not reveal any significant difference in apoptosis, it does not exclude possibilities of differences in apoptotic cell numbers between UDCA-treated and non-treated groups at short time treatment since *in vitro* data demonstrates a significant difference between UDCA-treated and non-treated groups.

4. Discussion

Multi-antibiotics therapies for the eradication of *H. pylori* still have many disadvantages and require an advanced, shortened dose, as well as non-antibiotics to eradicate *H. pylori*. However, due to the instability of UDCA in the stomach, only limited antibacterial activity of bile acids against *H. pylori* has been reported. This study demonstrated for the first time that the highly soluble and acid stable UDCA formula has antibacterial activity against *H. pylori in vivo* and prevents the initial accumulation of polymorphonuclear neutrophils in an infected stomach, which can help prevent the atrophic gastritis and subsequent gastric carcinogenesis.

A fever, i.e., energy expenditure, usually accompanies microbial infection [27,28]. However, it is unclear how energy mobilization is controlled during inflammation at the ATP level, which is generated in the mitochondria through the TCA cycle. In the TCA cycle, MDH plays a key role in the transport of NADH from the cytosol to the mitochondria, and the NADH shuttle system is essential for coupling the glycolytic metabolism with the generation of mitochondrial energy. An increase in MDH activity accelerates the transfer of NADH, and increases ATP production in the mitochondria. Therefore, an increase of MDH activity is believed to reflect the energy metabolic conditions in tissues. Moreover, the Semliki forest

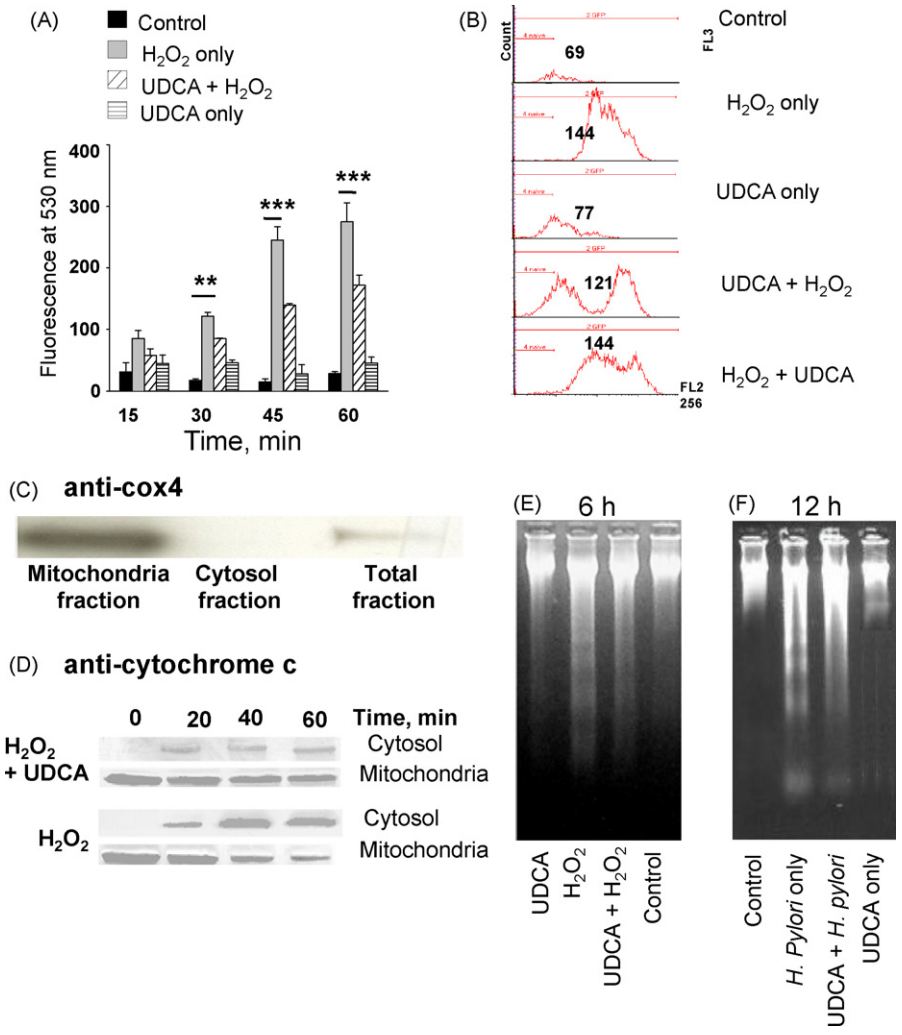

Fig. 6 – ROS scavenging by UDCA in H_2O_2- and *H. pylori*-induced apoptosis. (A and B) The AGS cells were pretreated with 2.5 mg/ml UDCA for 4 h before being exposed to 4 mM H_2O_2. At the indicated time, the cells were stained with the H2DCFDA dye, and the intracellular fluorescence was determined (A). The AGS cells were pretreated with 2.5 mg/ml of UDCA for 4 h before being exposed to 4 mM H_2O_2 for 1 h (UDCA + H_2O_2), or exposed to 4 mM H_2O_2 for 1 h followed by a treatment with 2.5 mg/ml of UDCA (H_2O_2 + UDCA). The fluorescence signal was analyzed by flow cytometry (B). (C) The AGS cells were pretreated with 2.5 mg/ml UDCA for 4 h and exposed to 4 mM H_2O_2 for 20 min. The cells were harvested, fractionated and analyzed by Western blotting. (D) The AGS cells were either non-treated or pretreated with UDCA for 4 h, and then exposed to H_2O_2 for 20, 40, and 60 min. The mitochondria-enriched fraction was used for Western blot analyses. (E and F) The AGS cells were pretreated with UDCA for 4 h and exposed to H_2O_2 for 6 h (E) or infected with *H. pylori* (MOI 100:1) for 12 h (F). The genomic DNA was visualized by agarose gel electrophoresis. The results show the standard deviation from three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$.

virus and a *Trypanosoma* infection were reported to increase the MDH activity [29,30]. Here, *H. pylori*-induced gastritis increases the MDH mRNA and protein levels. Therefore, MDH might be a promising candidate as a biomarker for *H. pylori*-induced gastritis. Attempts directed to determine the feasibility of MDH as a biomarker in *H. pylori*-induced gastritis patients are currently underway.

In contrast to the increase in the MDH activity in *H. pylori*-induced gastritis, the ATP level was lower than in the normal control. This phenomenon has also been observed in acute gastritis patients [31]. The low ATP level in *H. pylori*-induced gastritis might be due to a microvascular dysfunction and membrane damage, which are caused by the virulence factors CagA and VacA [32,33] as well as by ROS. ROS are produced in the mitochondria during an infection to kill microbes, but could decrease the ATP level (Fig. 3C) and decrease the membrane potential *in vitro* (Fig. 5C). Moreover, the mice with *H. pylori*-induced gastritis showed a lower membrane potential *in vivo* (Fig. 5B) and a higher level of neutrophil infiltration (Fig. 1B), which can promote the further leakage of MDH and ATP and subsequently lower the ATP level. However, UDCA increased both the mitochondrial membrane potential and membrane integrity in *H. pylori*-infected gastritis by quenching the ROS by repressing MDH

expression. Therefore, the UDCA treatment inhibits the *H. pylori*-induced energy expenditure and helps preserve the intracellular ATP level.

H. pylori infection caused a decrease of body weight but UDCA treatment prevented loss of body weight. Moreover, *H. pylori* infection increased MDH activity, but not intracellular ATP level. These results suggest that *H. pylori* infection induces MDH activity and energy exhaustion leading to body weight decrease. However, UDCA could reverse this process by preventing membrane damage and apoptosis from ROS, and by maintaining membrane integrity resulting in the prevention of weight loss.

Although UDCA repressed body weight decreases in the *H. pylori*-infected mice, it did not promote carcinogenesis. Since substantial evidences demonstrated that UDCA acts as an anti-carcinogenic agent; UDCA is a chemopreventive agent in the azoxymethane (AOM) model of experimental colonic carcinogenesis [34,35], and represses development of tumors with Ras mutations and blocked oncogene Ras activation. Furthermore, it inhibited Cox-2 induction by Ras-dependent and independent mechanisms [36]. Thus, body weight gain and hyperplasia in UDCA-treated groups did not seem to represent carcinogenic status.

In conclusion, UDCA not only represses the induction of MDH in *H. pylori*-infected gastritis *in vivo*, but also prevents H_2O_2-induced apoptosis *in vitro* by potentially modulating MDH expression, and preventing energy depletion and a mitochondria membrane dysfunction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.03.008.

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