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Ursodeoxycholic acid induces glutathione synthesis through activation of PI3K/Akt pathway in HepG2 cells

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ABSTRACT

Ursodeoxycholic acid (UDCA) is widely recognized as an effective compound in the treatment of chronic hepatitis and is known to modulate the redox state of the liver accompanied by an increase of GSH. In the present study, to access the antioxidative effect of UDCA and to clarify the molecular basis of the action on GSH level, we evaluated its effects in HepG2 cells exposed to excessive iron. UDCA inhibited both a decrease in the GSH level and an increase in the reactive oxygen species caused by excessive iron in the cells. UDCA increased the gene expression of the catalytic- and modifier-units of glutamine-cysteine ligase (GCL), which is a key enzyme in GSH synthesis. We further investigated the effect of UDCA on the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and obtained results showing that UDCA-induced increase in the GSH level was prevented by LY294002, a PI3K inhibitor. In addition, Western blot analysis of Akt showed that, while the total Akt level remained unchanged, the phosphorylated Akt level was increased by UDCA, and this increase was also prevented by LY294002. Moreover, UDCA promoted the translocation of a transcription factor, nuclear factor-E2-related factor-2 (Nrf2), into the nucleus, and this action was inhibited by LY294002. From these results, it was indicated that UDCA increased the GSH synthesis through an activation of the PI3K/Akt/Nrf2 pathway. This may be a primary mechanism of antioxidative action of UDCA concerned with its therapeutic effectiveness in chronic hepatitis.

1. Introduction

Ursodeoxycholic acid (UDCA) is a primary bile acid of the bear (*Ursus arctos*, and *Ursus thibetanus*) and a major component of the traditional oriental medicine Fel Ursi (Yutan), which is a dried bear gallbladder, used for stomach pains, diarrhea, jaundice, etc. Today, UDCA is globally used for the treatment of several liver diseases such as primary biliary cirrhosis and

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Abbreviations: ROS, reactive oxygen species; UDCA, ursodeoxycholic acid; CHC, chronic hepatitis C; ALT, alanine aminotransferase; PI3K, phosphatidylinositol 3-kinase; Nrf2, nuclear factor-E2-related factor-2; GCL, glutamine-cysteine ligase; GSHS, GSH synthetase; CDCFH, dichlorodihydrofluorescein; mBCl, monochlorobimane; PCR, polymerase chain reaction; ERK, extracellular signal-regulated protein kinase; MAPK, mitogene-activated protein kinase; GAPDH, glycerine aldehyde dehydrogenase.

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as an optional therapy for hepatitis C when the patient cannot tolerate interferon therapy [1,2].

In chronic hepatitis C (CHC), iron overload has been shown in the liver parenchymal cells [3–6], and sequential phlebotomy markedly ameliorated the alanine aminotransferase (ALT) level in patients unsuccessfully treated with interferon therapy [7,8]. Moreover, combined treatment involving UDCA and phlebotomy additively improved ALT values in many patients [9]. Although the precise mechanisms of the effect of phlebotomy remain unclear, it is assumed that iron mobilization from the liver to hematopoiesis in bone marrow would reduce the oxidative damage caused by iron accumulated in the liver.

UDCA is known to stimulate hepatobiliary secretion and to prevent the retention of toxic hydrophobic bile acids, thereby rendering bile more hydrophilic and less cytotoxic. This action is believed to contribute to the prevention and therapeutics of cholesterolithiasis or primary biliary cirrhosis. Mitsuyoshi et al. [10] reported that UDCA induced GSH levels and prevented hydroperoxide-induced oxidative damage in hepatocytes. Together with the stimulation of hepatobiliary secretion, these actions are thought to be involved in the protection of tissues from reactive oxygen species (ROS) induced by damage to mitochondria caused by hydrophobic bile acids in the liver [2].

GSH consists of three amino acids, glutamate, cysteine, and glycine [11]. GSH is concerned with the detoxification of chemical substances conjugated by the catalytic action of GST and extracellular transport of conjugated compounds, and it is also one of the major cellular antioxidative defence molecules against ROS. Under the condition of ROS overproduction, the reduced form of GSH is converted to the oxidized form of GSSG, thereby removing ROS. The intracellular GSH content is maintained through synthesis by glutamine-cysteine ligase (GCL), a rate-limiting enzyme in GSH synthesis which is regulated by feedback control of GSH. A decrease in the GSH content results in the intracellular redox balance moving toward an oxidized condition.

Concerning GSH synthesis, it has been indicated that activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway [12] and the translocation of nuclear factor-E2-related factor-2 (Nrf2) [13,14] mediate the positive regulatory signal in cells. On the other hand, UDCA has been shown to exhibit an antiapoptotic action in several cell types [15,16]. Moreover, Sola et al. [17] recently indicated that the antiapoptotic effect of UDCA is dependent on the activation of the PI3K signaling pathway.

In the present study, to clarify the mechanism of the antioxidative action of UDCA, we examined the antioxidative effect of UDCA in HepG2 cells treated with excess iron, and also investigated whether the PI3K/Akt signaling pathway and Nrf2 were involved in the antioxidative effect of UDCA.

2. Materials and methods

2.1. Chemicals

UDCA was kindly supplied by Tanabe-Mitsubishi Pharmaceuticals, Osaka, Japan. LY294002, ferric nitrate nanohydrate, 6-carboxy-2′,7′-dichlorodihydrofluorescein (CDCFH), and monochlorobimane (mBCl) were purchased from Sigma Japan (Tokyo).

2.2. Cell culture

Human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 5% (v/v) heat-inactivated fetal calf serum (BioWest, Nuaillé, France) and 100 U/mL of penicillin (Invitrogen Japan K.K., Tokyo, Japan), 100 μg/mL of streptomycin (Invitrogen), and 0.25 μg/mL of amphotericin B (Invitrogen) in 60-mm plastic dishes in the presence of 5% CO_2 at 37 °C until semi-confluent. Then, varying concentrations of agents were added to the culture medium, and the cells were cultured for designated periods. UDCA and LY294002 were dissolved in DMSO (Sigma) and added to the culture medium. The concentration of DMSO was adjusted to 0.1% (v/v) of culture medium in each group.

2.3. Intracellular ROS assay

The level of ROS in the cells was quantified using CDCFH, as described previously [18–20]. After treatment with chemical substances, cells were harvested from culture dishes using TrypLE Express (Invitrogen) and collected into a glass tube. After centrifugation (1500 rpm × 5 min), cells were resuspended in PBS containing 10 μM CDCFH, which can be oxidized by ROS to a fluorescent molecule, CDCF. After incubation for 20 min at 37 °C, the fluorescence was measured at 480 nm/538 nm.

2.4. Intracellular GSH assay

The level of GSH in the cells was quantified using mBCl, as described previously [21–23]. After treatment with chemical substances, cells were harvested from culture dishes, and suspended in PBS (pH 7.4). mBCl (100 μM) in PBS was added to the suspension and incubated for 20 min at 37 °C. Fluorescence of the GSH-mBCl adduct was measured at 380 nm/470 nm. The results are expressed as the contents (nmol) per mg protein and as the percentage of fluorescence compared with the control.

2.5. Western blotting

For the detection of Akt and phosphorylated Akt, whole cell lysate was prepared according to a previous report [24]. Briefly, HepG2 cells were suspended in a lysis buffer containing 20 mM Tris–HCl (Sigma), pH 7.4, 1 mM EDTA (Sigma), 1 μM dithiothreitol (Sigma), 0.1 mM sodium orthovanadate (Sigma), 0.5 mM phenylmethylsulfonylfluoride (Sigma), 0.05% Protease Inhibitor Cocktail (Sigma), and 0.1% Triton-X 100 (sigma). After mixing for 30 min at 4 °C, the mixtures were centrifuged at 14,000 × *g* for 10 min at 4 °C, and supernatant fractions were used for Western blot analyses of Akt and phosphorylated Akt. The protein concentration was determined using a DC protein assay kit (Bio-Rad Laboratories, Inc.).

For the detection of Nrf2, a nuclear fraction was prepared as follows according to a previous report [25]. Briefly, cells were suspended in a lysis buffer (pH 7.9) containing 10 mM Hepes

(Sigma), 10 mM KCl (Sigma), 0.1 mM EDTA, 0.1 mM EGTA (Sigma), 1 μM dithiothreitol, 0.05% Protease Inhibitor Cocktail (Sigma), and 0.5% Triton-X 100. After mixing for 15 min at 4 °C, the mixtures were centrifuged at 800 × *g* for 10 min at 4 °C. The resulting nuclear pellet was resuspended in 30 μL of ice-cold buffer (pH 7.9) containing 20 mM Hepes, 0.4 M NaCl (Sigma), 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.05% Protease Inhibitor Cocktail) and incubated on ice for 15 min with vortex-mixing for 10–15 s every 2 min. Nuclear extracts were finally obtained by centrifugation at 15,000 × *g* for 5 min at 4 °C and used for Western blot analysis of Nrf2.

Twenty μg of protein of each fraction was applied to SDS-PAGE (10% gel), and transferred onto an Immobilon-P transfer membrane (Millipore Japan, Tokyo, Japan). After blocking with skim milk, the membrane was treated with antibodies specific for Akt (Cell Signaling Technology, MA, USA), phospho-Akt (Cell Signaling) and Nrf2 (C-20, Santa Cruz Biotechnology, CA, USA) as a primary antibody and horseradish-labeled goat anti-rabbit IgG antibody (Cell Signaling) as a secondary antibody. The specific immunoreactive band was detected using Immobilon Western Detection Reagents (Millipore Japan, Tokyo) and a luminescense imager (Light-Capture II, Atto, Tokyo, Japan). Densitometric analysis was performed using Scion Image for Windows supplied by the Scion Corporation, USA.

2.6. RT-PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. Then, cDNA was prepared by the incubation of 0.5–1.0 μg of the RNA with random primers (12.5 ng, Invitrogen), RNase inhibitor (RNase-OUT, 20 units, Invitrogen), 0.5 mM 5′-deoxynucleotides (dNTPs, Promega, WI, USA), and 100 units of RNA reverse transcriptase (ReverTra Ace, TOYOBO, Tokyo, Japan) in 20 μL of reaction buffer according to the ReverTra Ace data sheet. Following inactivation of the enzyme by incubation at 99 °C for 5 min, the polymerase chain reaction (PCR) was carried out with 0.625 units of G-Taq Taq DNA polymerase (Hokkaido System Science Co., Sapporo, Japan) in 25 μL of PCR solution (1 μL of cDNA solution, 0.4 μM primers, 0.2 mM dNTPs, and 2.5 μL of 10× PCR buffer) in a Thermal Cycler PxE (Thermo Fisher Scientific, MA, USA). PCR primers were synthesized by Invitrogen. The PCR products (10 μL) were electrophoresed on a 1.5% agarose gel and visualized with ultraviolet light after immersion in an ethidium bromide solution (1 μg/mL, Sigma) for 15 min. Images were taken with a digital Coolpix 4500 (Nikon, Tokyo, Japan) equipped with a BPB-60 filter (Fujifilm Japan, Tokyo). Densitometric analysis was performed using Scion Image for Windows.

Primers for RT-PCR were as follows: catalytic subunit of GCL (GCLc): sense: 5′-CTGTTGCAGGAAGGCATTGA-3′, antisense: 5′-CGAAATTCTACTCTCCATCCAATGT-3′ [26]; modifier subunit of GCL (GCLm): sense: 5′-CAGTTGACATGGCCTGTT-CAG-3′, antisense: 5′-TCAAATCTGGTGGCATCACAC-3′ [27]; GSH synthetase: sense: 5′-CAGCGTGCCATAGAGAATGA-3′, antisense: 5′-TTCAGGGCCTGTACCATTTC-3′ [28]; glycerine aldehyde dehydrogenase (GAPDH): sense: 5′-ACCACAGTC-CATGCCATCAC-3′, antisense: 5′-TCCACCACCCTGTTGCTGTA-3′ [29].

2.7. Statistical analysis

Data were expressed as the mean ± S.D. Statistical analyses were performed using Student's *t*-test after a test for equality of variance. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Concentration dependency of the increase in the GSH level induced by UDCA in HepG2 cells

As the mechanism for the hepatoprotective action of UDCA, there are several reports indicating an increase of the cellular GSH level and an antioxidative action [2,10]. Thus, in the present study, we determined the efficacy of UDCA to promote an increase in GSH level in HepG2 cells. As shown in Fig. 1, when the cells were treated for 24 h with 30–300 μM UDCA, the GSH level was significantly increased, and the effect reached a maximum level at 100 μM.

3.2. Effects of UDCA on iron-induced ROS production and the GSH level in HepG2 cells

It is well-known that excess iron deposition in the liver induces hepatotoxicity [30–32]. To evaluate the antioxidative effects of UDCA, we examined the effect on the cellular level of ROS and GSH in the presence of excess iron. By the addition of ferric nitrate (100 μM) to the culture medium, the ROS content tended to increase in both a concentration- and time-dependent manner (Fig. 2A and B), and the cellular GSH content showed a tendency toward reduction (Fig. 2C). In the presence of 100 μM UDCA, the increase in the ROS level by the addition of excess

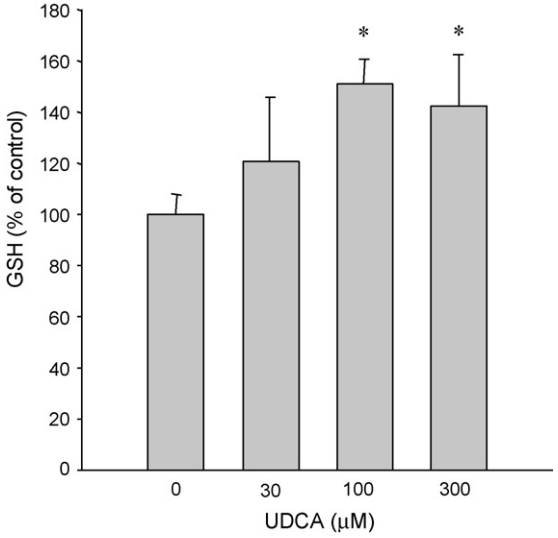

Fig. 1 – Increase of the GSH level in HepG2 cells treated with UDCA. Cells were treated with varying concentrations of UDCA for 24 h, then, intracellular GSH levels were determined using the fluorogenic bimane probe mBCl. Column graph data are expressed as a percentage of control fluorescence and as the mean ± S.D. ($n = 3$). *$P < 0.05$, compared with the control.

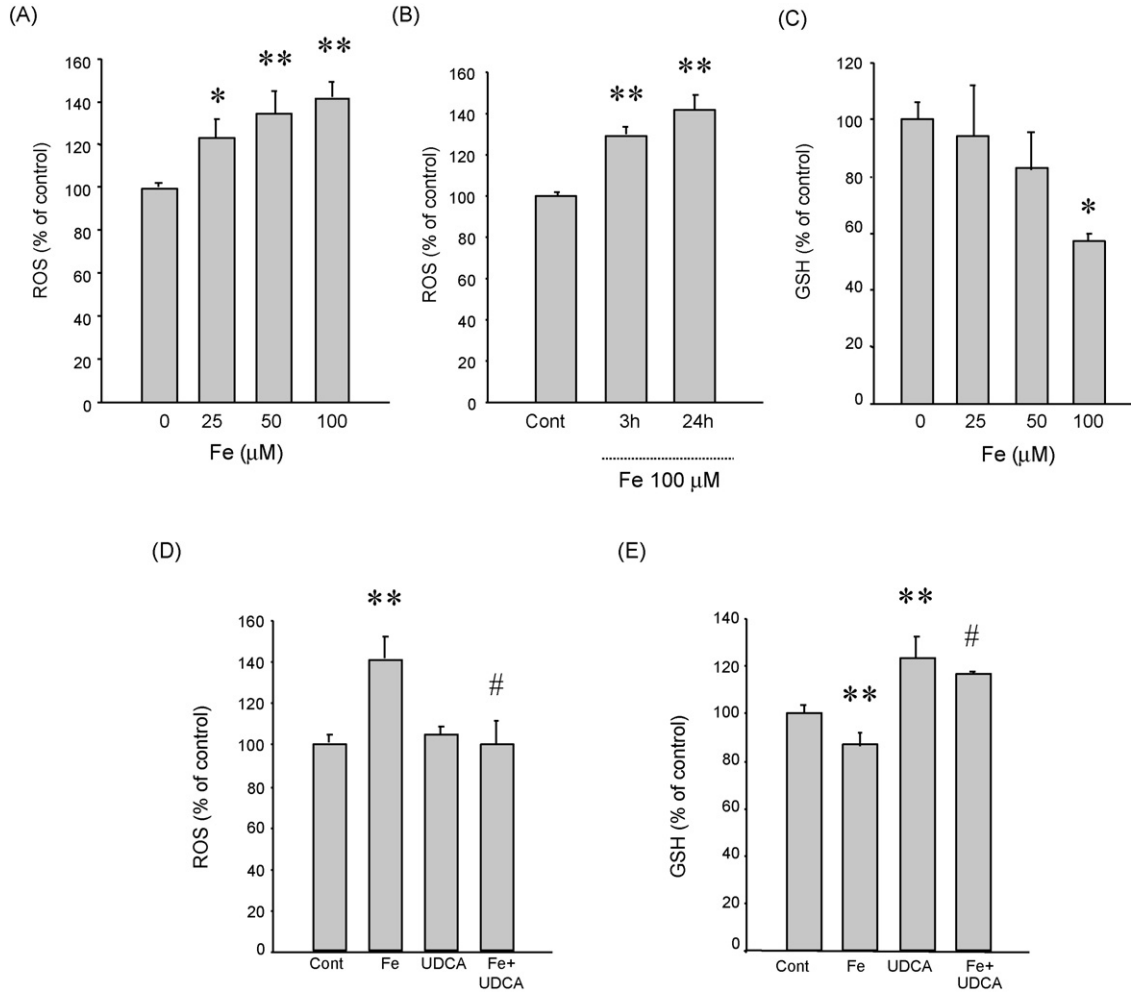

Fig. 2 – Effect of UDCA on the levels of ROS and GSH in HepG2 cells treated with UDCA. (A, C): After the cultivation of cells for 24 h in the presence or absence of varying concentrations of ferric nitrate (Fe), the ROS level was determined using the fluorogenic dye CDCFH, and the GSH level was determined using mBCI. (B): After the cultivation of cells with 100 μM ferric nitrate (Fe) for 3 or 24 h, intracellular ROS were determined. (D, E): After the cultivation of cells in the presence or absence of 100 μM ferric nitrate (Fe) and/or 100 μM UDCA, intracellular ROS and GSH were determined. Column graph data are expressed as the mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control. $^{\#}P < 0.01$, compared with the ferric nitrate (Fe) group.

iron was inhibited almost completely (Fig. 2D). The GSH level was increased by UDCA even in the presence of excess iron (Fig. 2E). From these results, it was suggested that UDCA is effective for the prevention of oxidative damage induced by excess iron, and the inhibitory action of UDCA was dependent on the increase of the GSH level.

3.3. *Induction by UDCA of the expression of GCL and GSHS genes in HepG2 cells*

Previously, Mitsuyoshi et al. indicated that UDCA increased the cellular content of GSH and induction of GCL [10]. Therefore, we examined the effect of UDCA on the expression of enzymes catalyzing GSH synthesis. GSH is synthesized by two enzymes: GCL and GSHS. GCL catalyzes the first and rate-limiting step [11] of the reaction producing γ-glutamylcysteine. GCL consists of two components: a catalytic subunit (GCLc) and modifier subunit (GCLm). GSHS synthesizes GSH from γ-glutamylcysteine and glycine. As shown in Fig. 3A–C, when we examined the effects of UDCA on the mRNA levels of GCLc, GCLm, and GSHS after treatment for 24 h, UDCA increased the expression of these genes in a concentration dependent manner. In the presence of excess iron, expressions of GCLc, GCLm, and GSHS genes were decreased. However, UDCA prevented the reduction of the mRNA levels caused by excess iron (Fig. 3D–F). From these results, it was indicated that UDCA increased the GSH level through upregulation of the gene expression of GCL and GSHS under the oxidative condition in the cells, and that UDCA contributes to the protection of cells from oxidative damage.

3.4. *Inhibition by LY294002 of the increases in the GSH level and gene expressions in HepG2 cells exposed to UDCA*

Previously, several reports indicated that PI3K and extracellular signal-regulated protein kinase (ERK) are involved in

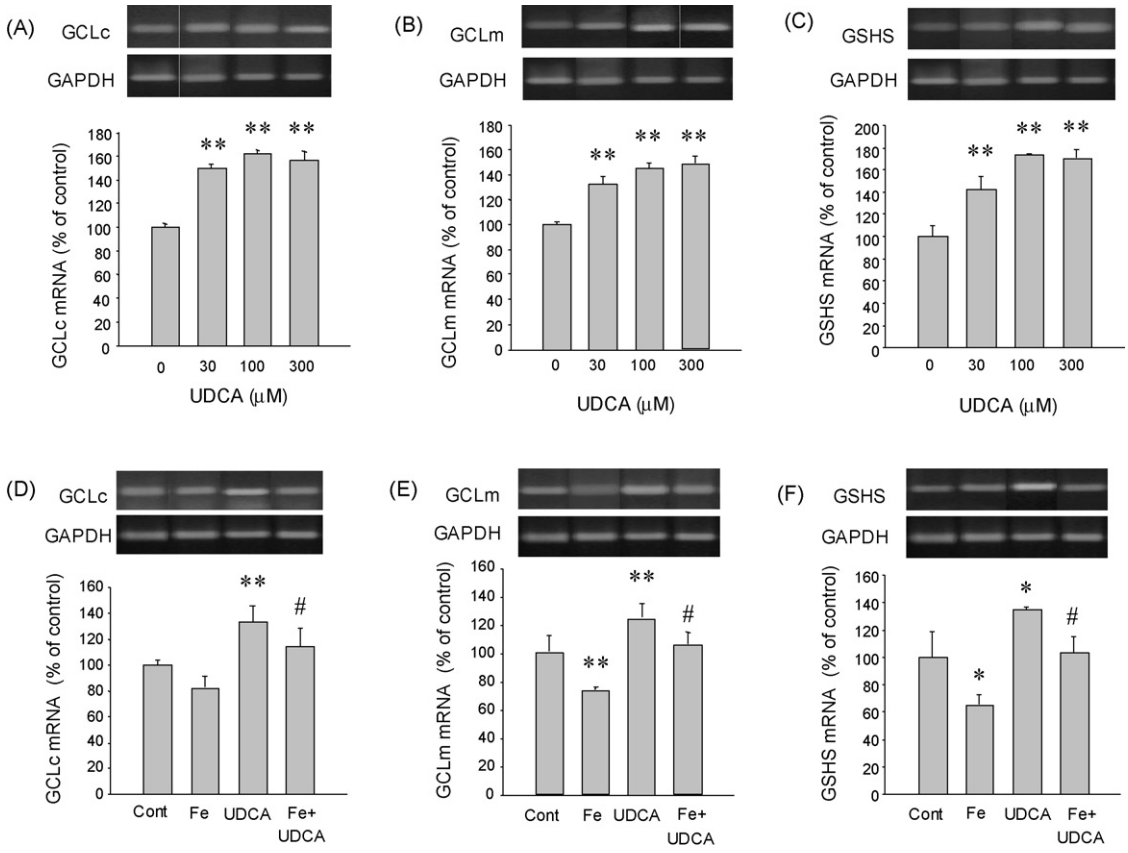

Fig. 3 – Concentration dependency of the induction of gene expression involved in GSH synthesis in HepG2 cells treated with UDCA. (A, B, C): After treatment with varying concentrations of UDCA for 24 h, the mRNA levels of GCLc, GCLm, and GSHS genes were determined by semi-quantitative RT-PCR. (D, E, F): After the cultivation of cells in the presence or absence of excess ferric nitrate (Fe; 100 μM) and/or UDCA (100 μM), the mRNA levels of three genes were determined. The photograph is typical of three independent experiments. Column graph data represent the relative level against GAPDH and are expressed as the mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control. $^{\#}P < 0.05$, compared with the ferric nitrate (Fe) group.

the regulation of GSH homeostasis and other antioxidative systems [33–39]. To investigate the involvement of the PI3K/Akt signaling pathway in the induction of GSH expression by UDCA, we studied the effect of an inhibitor of PI3K, LY294002, on both the GSH level and the mRNA levels of GCLc, GCLm, and GSHS genes. As shown in Fig. 4A–C, LY294002 significantly suppressed the expression of GCLc, GCLm, and GSHS genes, and it completely inhibited the induction of these genes by UDCA. Regarding the GSH level, although LY294002 did not affect the basal level of GSH, it completely inhibited the increase in the cellular GSH level induced by UDCA (Fig. 4D). From this evidence, it was suggested that GSH synthesis is dependent on the PI3K/Akt pathway, and UDCA increased the GSH concentration through the activation of PI3K/Akt pathway-dependent GSH synthesis in HepG2 cells.

3.5. *UDCA induced Akt phosphorylation, which was inhibited by LY294002 in HepG2 cells*

To obtain direct evidence regarding the activation of Akt, we conducted Western blot analysis of Akt and phosphorylated Akt in HepG2 cells after the treatment with 100 μM UDCA for 24 h. As shown in Fig. 5, while UDCA did not affect the level of Akt, it significantly increased the level of phosphorylated Akt. In addition, LY294002 decreased the phosphorylated Akt level in the presence or absence of UDCA. Thus, the phosphorylation of Akt seemed to be dependent on the activation of PI3K by UDCA.

3.6. *UDCA induced the translocation of Nrf2 into the nucleus, and this translocation was inhibited by LY294002 in HepG2 cells*

Nrf2 is a transcription factor which serves as a sensor for oxidative stress and coordinates the expression of GCL [36] and other antioxidative stress genes in response to oxidative stimulations. Recently, Okada et al. [40] showed that UDCA stimulated the translocation of Nrf2 and induced GCL gene expression in both the liver of mice and Wistar rat liver-derived RL34 cells. In the present study, we also showed that UDCA (100 μM) increased the level of Nrf2 in the nucleus of HepG2 cells. In addition, LY294002 antagonized the effect of UDCA (Fig. 6). From this evidence, it was indicated that UDCA

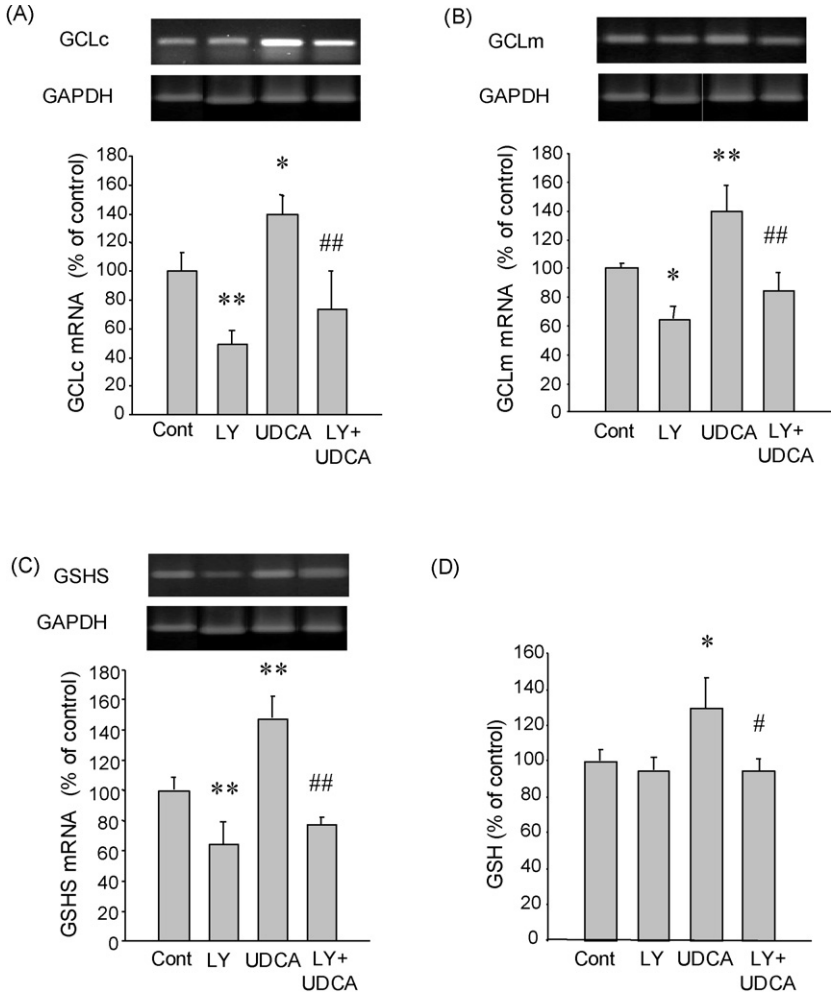

Fig. 4 – Inhibitory effect of LY294002 (LY) on the increases in the GSH level and the expression of genes concerned with GSH synthesis induced by UDCA. (A, B, C): After the cultivation of cells for 24 h in the presence or absence of 20 μM LY294002 (LY) and/or 100 μM UDCA, the mRNA levels of GCLc, GCLm, and GSHS genes were determined by semi-quantitative RT-PCR and were expressed as relative levels against GAPDH. The photograph is typical of three independent experiments. (D): After the cultivation of cells as well, the GSH content was determined using mBCl. Column graph data are expressed as the mean ± S.D. ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, compared with the UDCA group.

promoted the translocation of Nrf2 through activation of the PI3K/Akt pathway.

4. Discussion

The hepatoprotective action of UDCA has been suggested to be partially dependent on its antioxidative effect, because UDCA increased the intracellular GSH content and gene expression of GCL, which is a rate-limiting enzyme of GSH synthesis, in rat hepatocytes [10].

In the present study, we demonstrated that UDCA led to a reduction of the intracellular ROS content and an increase of the GSH content accompanied by the induction of the expression of GCL and GSHS genes catalyzing GSH synthesis even in the presence of excessive iron, which induced ROS production and slight GSH consumption. Our present results not only supported the findings of Mitsuyoshi et al. [10], but also indicated the availability of UDCA for a protection of oxidative stress caused by iron overload, although the direct antioxidant action of UDCA may be involved in these effects, as Lapenna et al. [41] indicated that UDCA molecules scavenged hydroxyl radicals and suppressed lipoperoxidation induced by the co-existence of Fe^{3+}, H_2O_2, and ascorbate.

It has been suggested that hepatic iron accumulation in CHC patients is associated with advanced hepatic fibrosis [3–6]. In CHC patients, it is also suggested that hepatic GSH is reduced [42,43] and ROS correlate with disease activity [44]. Hayashi et al. [7] previously reported that phlebotomy to remove accumulated body iron improved serum ALT values in CHC patients, and its efficacy was verified in a multicenter trial in Japan [8]. Our previous study [9] indicated that UDCA combined with phlebotomy additively ameliorates ALT values in CHC patients. Regarding the combined effect, the present study showed that UDCA actually increased GSH even under the condition of iron overload. The effect of UDCA on the

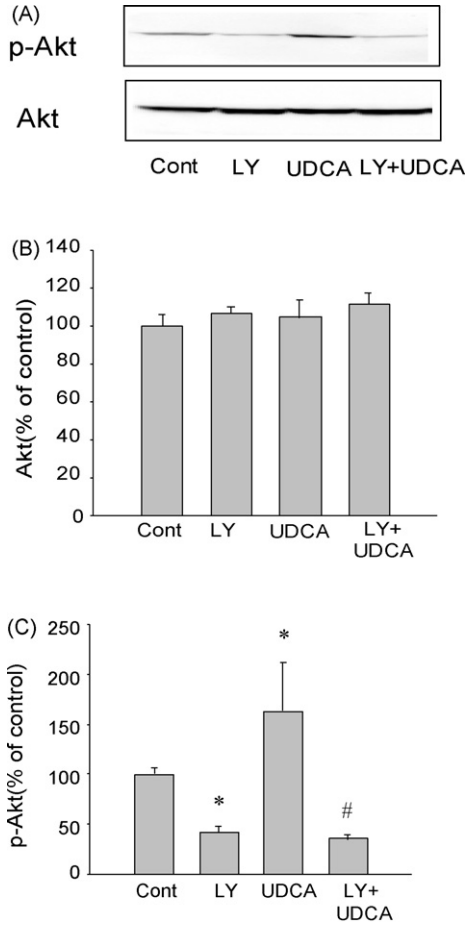

Fig. 5 – UDCA-induced Akt phosphorylation. HepG2 cells were exposed to 100 μM UDCA in the presence or absence of 20 μM LY294002 for 24 h. Then, whole cell lysates were used for Western blot analysis of Akt and phosphorylated Akt (P-Akt). (A): The photograph is typical of three independent experiments. (B, C): Column graph data are expressed as the mean ± S.D. ($n = 3$). $^{*}P < 0.05$, compared with the control. $^{\#}P < 0.01$, compared with the UDCA group.

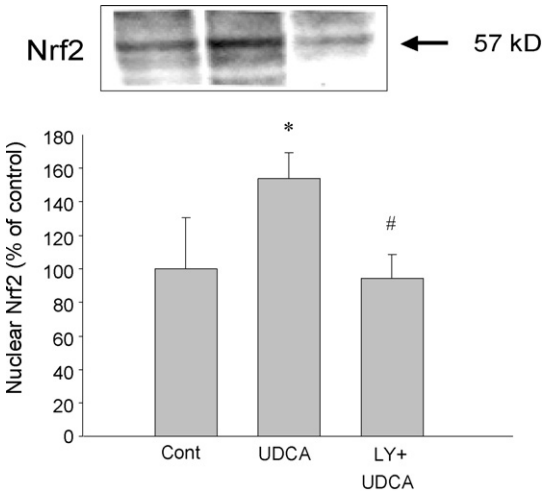

Fig. 6 – UDCA-induced translocation of Nrf2 into the nucleus. HepG2 cells were exposed to 100 μM UDCA in the presence or absence of 20 μM LY294002 for 24 h. Then, the nuclear fraction was prepared and used for Western blot analysis of Nrf2. The photograph is typical of three independent experiments. Column graph data are expressed as the mean ± S.D. ($n = 3$). $^{*}P < 0.05$, compared with the control. $^{\#}P < 0.05$, compared with the UDCA group.

improvement of biochemical parameters would primarily depend on the increase in the GSH content, although there may be an involvement of other mechanisms, such as direct an antioxidative action [41] and antiapoptotic action of UDCA [15,16].

Concerning the increase in GSH synthesis induced by UDCA, a detailed mechanism has not been reported up until the present study, indicating the activation of the PI3K/Akt pathway. Several studies have been reported in recent years regarding antiapoptotic activity of UDCA. For example, in biliary epithelial cells in primary biliary cirrhosis, enhanced DNA fragmentation and Bcl-2 expression were suppressed by UDCA [45]. In addition, the activation of p38, extracellular signal-regulated protein kinase, mitogene-activated protein kinase (MAPK), and PI3K pathways [35], and the activation of Akt [11] were reported. Moreover, Rajesh et al. [46] showed that UDCA activated phosphorylation of Akt and Bad, a proapoptotic Bcl-2 family member, and that the phosphorylation of Bad prevented its translocation into mitochondria, thereby preventing the down-regulation of Bcl-2 expression, resulting in the prevention of apoptosis. From these reports and the evidence showing the involvement of the Akt pathway in GSH synthesis [12], we supposed that UDCA activated the Akt pathway and caused a GSH increase, as well as exhibited an antiapoptotic action, as described next.

Recently, it was shown that PI3K regulates the translocation of Nrf2 in the nucleus [13]. Based on this report and our results on the GSH level (Figs. 1 and 2), we studied whether GSH synthesis was regulated by the PI3K/Akt/Nrf2 signaling pathway, and if UDCA upregulated the GSH concentration through stimulation of this signal pathway in HepG2 cells. As was shown, UDCA stimulated the phosphorylation of Akt, and the effect was inhibited by LY294002 (Fig. 5), indicating the involvement of the activation of the PI3K/Akt pathway in the increase of the GSH level by UDCA. Moreover, Nrf2 was translocated on treatment with UDCA (Fig. 6), as recently shown by Okada et al. [40], and mRNA levels of GCLc, GCLm, and GSHS genes were increased by UDCA (Fig. 3). All these effects were inhibited by LY294002 (Fig. 4). Together with these results, it was concluded that GSH synthesis was positively regulated by the PI3K/Akt/Nrf2 signaling pathway, and that UDCA upregulated the GSH level through the activation of this system in HepG2 cells.

Okouchi et al. [47] recently reported that GCL activity was stimulated by the activation of PI3K/Akt/mTOR signaling accompanied by the nuclear translocation of Nrf2 and upregulation of Nrf2-dependent GCLc subunit expression in brain endothelial cells. In addition, it was reported that in human retinal pigment epithelium cells, the PI3K/Akt

pathway plays key roles in regulating Nrf2 and antioxidant response element-dependent protection against oxidative stress by increasing gene expressions of GCL and glutathione S-transferase and the intracellular GSH content [13]. Other than this PI3K/Akt pathway, there are several studies indicating the involvement of ERK and p38 MAP kinase in the promotion of Nrf2 translocation and GCL gene expression [12,24,39]. Furthermore it was shown that UDCA activated p38 and ERK [33–35]. However, despite this evidence, considering the present evidence indicating the complete inhibition by LY294002 of the UDCA-induced increase of GSH, GSH synthesis induced by UDCA may be primarily dependent on activation of the PI3K/Akt pathway.

In summary, it was revealed that UDCA decreased the intracellular ROS level accompanied by an increase in the GSH content even under oxidative stress caused by excess iron. Moreover, it was suggested that the effect of UDCA was dependent on stimulation of the PI3K/Akt pathway followed by the nuclear translocation of Nrf2 and the activation of GSH synthesis. This may be a primary mechanism of antioxidative action of UDCA.

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