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Ursodeoxycholic acid inhibits endothelin-1 production in human vascular endothelial cells

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Abstract

Endothelin-1 is known to be implicated in the pathogenesis of hepatobiliary diseases such as cirrhosis, especially in portal hypertension. This study aimed to investigate the effects of ursodeoxycholic acid on endothelin-1 production in human endothelial cells. The effects of ursodeoxycholic acid and its conjugates (tauroursodeoxycholic and glycoursodeoxycholic acids) on endothelin-1 production as well as nitric oxide (NO) in human umbilical vein endothelial cells (HUVECs) were examined. The production of endothelin-1 and nitric oxide in culture medium was measured using enzyme-linked immunosorbent assay (ELISA) and the Griess method, respectively. Endothelin-1 and endothelial nitric oxide synthase (eNOS) mRNA expression were investigated by real-time quantitative reverse transcriptase/polymerase chain reaction (RT-PCR). Ursodeoxycholic acid (30–1000 μM) inhibited endothelin-1 production in a concentration-dependent manner, and ursodeoxycholic acid at concentrations higher than 300 μM increased nitric oxide production in culture medium. The conjugates of ursodeoxycholic acid also increased nitric oxide production and decreased endothelin-1 production, which was less effective than ursodeoxycholic acid. *N*-nitro-L-arginine-mythel-ester (L-NAME), a nitric oxide synthase (NOS) inhibitor, suppressed the ursodeoxycholic acid-induced nitric oxide production, but it did not antagonize the inhibitory effects of ursodeoxycholic acid on endothelin-1 production. Ursodeoxycholic acid also induced a concentration-dependent decrease in endothelin-1 mRNA expression without significant changes in eNOS mRNA expression. These results provide novel evidence that ursodeoxycholic acid inhibits endothelin-1 production in human endothelial cells, but nitric oxide is not responsible for the inhibitory effect of ursodeoxycholic acid on endothelin-1. Thus, ursodeoxycholic acid therapy may prevent the development of several pathogenesis such as portal hypertension observed in patients with cirrhosis due to the improvement of endothelial function.

Keywords: Ursodeoxycholic acid; Human endothelial cell; Endothelin-1; Nitric oxide; Real-time RT-PCR

1. Introduction

Endothelin-1, a 21-amino-acid peptide (Yanagisawa et al., 1988), is synthesized by various cells including hepatic and endothelial cells. It has been known to be a potent vasoconstrictor and mitogen for vascular smooth muscle cells in the liver (Gandhi et al., 1990; Serradeil-Le Gal et al., 1991), which is implicated in the pathogenesis of a variety of diseases such as hepatobiliary diseases (Tsai et al., 1995; Alam et al., 2000; Shah, 2001). Endothelin-1 is overexpressed in cirrhotic tissues (Pinzani et al., 1996; Kuddus et al., 2000; Tieche et al., 2001), and endothelin-1 level is elevated in patients or animal models with cirrhosis, especially in portal hypertension (Moller et al., 1995; Bernardi et al., 1996; Bruno et al., 2000). The increased expression of endothelin-1 mRNA and the subsequent increase in production has been

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also reported in the experimental models of liver injury and cirrhotic rats (Leivas et al., 1995; Rockey et al., 1998).

On the other hand, nitric oxide (NO), derived from endothelium or other cells, also plays a vital role in regulating vascular tone (Moncada et al., 1991; Nathan, 1992), and it is another important physiological and pathophysiological mediator in hepatobiliary diseases. Nitric oxide is synthesized from L-arginine by isoforms of nitric oxide synthase (NOS). Nitric oxide released from endothelial nitric oxide synthase (eNOS) is a physiologically potent vasodilator and an inhibitor of vascular smooth muscle cell proliferation, which is considered to have a beneficial role. In fact, diminution in eNOS-derived nitric oxide production in liver has been reported to be involved in development of portal hypertension by increasing intrahepatic resistance (Shah, 2001). And 2-(acetyloxy)benzoic acid 3-(nitrooxymethyl)phenyl ester (NCX-1000), a nitric oxide-releasing derivative of ursodeoxycholic acid, selectively divers nitric oxide to the liver and protects against development of portal hypertension (Fiorucci et al., 2001).

Ursodeoxycholic acid, a hydrophilic tertiary bile acid, has been wildly used to treat patients with chronic cholestatic liver diseases (Luketic and Sanyal, 1994) and has benefits for the treatment in patients with various liver diseases such as primary biliary cirrhosis and chronic viral hepatitis (Cirillo and Zwas, 1994; Makino and Tanaka, 1998). Ursodeoxycholic acid also inhibits progression of chronic hepatic disorders with special reference to increases in blood flow and limits the development of portal hypertension induced by bile duct ligation (Poo et al., 1992; Poo et al., 1995). The basic mechanism has not yet been identified, but a wide range of cellular actions of ursodeoxycholic acid, i.e., anti-inflammatory and immunomodulating effects (Makino and Tanaka, 1998; Ma et al., 2003), has been proposed. It also protects hepatocytes against oxidant injury via induction of antioxidants (Mitsuyoshi et al., 1999). In human vascular endothelial cells, we have reported that bile acids such as chenodeoxycholic acids increase nitric oxide production by increasing intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ (Nakajima et al., 2000; Chisaki et al., 2001), but the effects of ursodeoxycholic acid on endothelin-1 production have not been investigated.

In the present study, the effects of ursodeoxycholic acid on endothelin-1 production as well as nitric oxide in human endothelial cells were investigated. Here, we provided novel evidence that ursodeoxycholic acid inhibits endothelin-1 production in human umbilical vein endothelial cells (HUVECs), but nitric oxide is not responsible for ursodeoxycholic acid effects on endothelin-1.

2. Materials and methods

2.1. Materials

HUVECs were purchased from BioWhittaker and cultured in endothelial growth medium (EGM) supplemented with 0.1% human epithelial growth factor, 0.1% hydrocortisone, 0.1% Gentamicin sulfate Amphotericin (GA1000), 0.4% bovine brain extract, 2.0% fetal bovine serum in an atmosphere of 5% CO_2, and 95% air at 37 °C in 25-cm^2 flasks. At confluence, cells were split 1:3 after they were detached using 0.25% trypsin in 0.02% EDTA. Media were changed twice weekly. The confluent cells were used within 3 weeks of establishing primary cultures and at the third to fifth passage.

Ursodeoxycholic acid (Na salt), tauroursodeoxycholic acid (Na salt), and glycoursodeoxycholic acid (Na salt) were kindly provided by Mitsubishi Pharma (Osaka, Japan). *N*-nitro-L-arginine-mythel-ester (L-NAME) was purchased from Sigma (St. Louis, MO).

2.2. Determination of nitric oxide

Nitric oxide released from HUVECs was determined by measuring the concentration of NO_2^-, a stable metabolite of nitric oxide, in culture medium, using the Griess method, as described previously (Nakajima et al., 2000; Ma et al., 2003). Confluent monolayers cultured in 35-mm dishes were washed twice with phosphate buffered saline (pH 7.4), and then, 2 ml of the EGM supplemented with or without various concentrations of ursodeoxycholic acid or the conjugated bile acids (tauroursodeoxycholic and glycoursodeoxycholic acids) were added. Two hours later, 1.5-ml aliquots of cultured medium were collected and centrifuged for 2 min at 12,000 $\times g$. One milliliter of Griess reagent (0.5% naphthylethylenediamine dihydrochloride and 5% sulphanilamide in 25% H_3PO_4) was added to 1 ml supernatant, and the mixture was incubated at room temperature for 10 min. The absorbance at 540 nm was measured in a Beckman DU-70 spectrophotometer (Fullerton, CA). The concentration of NO_2^- was calculated by comparison with the absorbance at 540 nm of standard solutions of 0–150 μM $NaNO_2$ prepared in the EGM.

2.3. Measurement of endothelin-1 concentration

The amount of endothelin-1 produced by HUVECs was determined by measuring the concentration of endothelin-1 in cultured medium, using enzyme-linked immunosorbent assay (ELISA) Kit (TECHNE, Minneapolis, MN). Diluted conjugate (100 μl; antibody to endothelin-1 conjugated to horseradish peroxidase) was added to each endothelin-1 antibody-precoated well. Then 100 μl supernatant (above-mentioned in nitric oxide determination) or standard solution was added to each well with sufficient force to ensure mixing and was incubated at room temperature for 30 min. Afterwards, the content from each well was aspirated and washed with wash buffer (buffered surfactant). Substrate (100 μl; tetramethylbenzidine) was added to each well and was incubated at room temperature for 30 min. Then, 100 μl stop solution (acid solution) was added to each well. The optical density of each well was determined using

a biolumin 960 microplate reader (Molecular Dynamics Japan, Tokyo) set at 450 nm, with the correction wavelength set at 620 nm. The concentration of endothelin-1 was calculated by comparison with standard solutions containing 0–150 pg/ml endothelin-1 prepared in the EGM.

2.4. Protein assay

After HUVECs proliferated to confluence in 35-mm dishes, the cells were washed three times in a HEPES buffered saline solution and then lysed in 0.5 M NaOH. The protein content of the cytolysate of the total cells was measured by the Bradford protein assay.

2.5. RNA extraction and real-time quantitative reverse transcriptase/polymerase chain reaction (RT-PCR)

Total RNAs were isolated as described above (Jo et al., 2004) and then treated with DNase I. They were then converted to cDNAs, using a Super Script first-strand synthesis system (Invitrogen). Quantitative RT-PCR was performed with the use of real-time Taq-Man technology and a sequence detector (model 7700, Applied Biosystems, Foster City, CA). Gene-specific primers and Taq-Man probes (endothelin-1, accession no. NM 001955; eNOS, accession no. NM 000603) were used to analyze the transcript abundance. The 18 S ribosomal RNA was analyzed as an internal control and was used to normalize the values for the transcript abundance.

2.6. Statistical analysis

Statistical comparison was carried out with three or more groups using one-way analysis of variance and Dunnett's test. Data are expressed as the mean±S.E.M., and values of $p<0.05$ were considered statistically significant.

3. Results

3.1. Effects of ursodeoxycholic acid on endothelin-1 and nitric oxide production

Fig. 1 shows the effects of ursodeoxycholic acid on the production of endothelin-1. The amount of endothelin-1 (pg/ml) released from HUVECs for 2 h in the culture medium under the control medium and in the presence of various concentrations of ursodeoxycholic acid (30–1000 μM) is shown in Fig. 1. Compared with the control solution, ursodeoxycholic acid decreased the production of endothelin-1 in the culture medium in a concentration-dependent manner. Significant inhibitory effects of ursodeoxycholic acid were observed at concentrations above 30 μM. The basal endothelin-1 release for 2 h was 52±4 (n=6) and 245±24 pg/mg (n=6). The addition of ursodeoxycholic acid in the culture medium decreased the endothelin-1 production (46±2 and 214±20 pg/mg ($p<0.05$, n=6) at 100 μM; and 41±3 and 196±11 pg/mg ($p<0.01$, n=6) at 300 μM; and 35±4.7 and 181±16 pg/mg ($p<0.01$, n=6) at 500 μM).

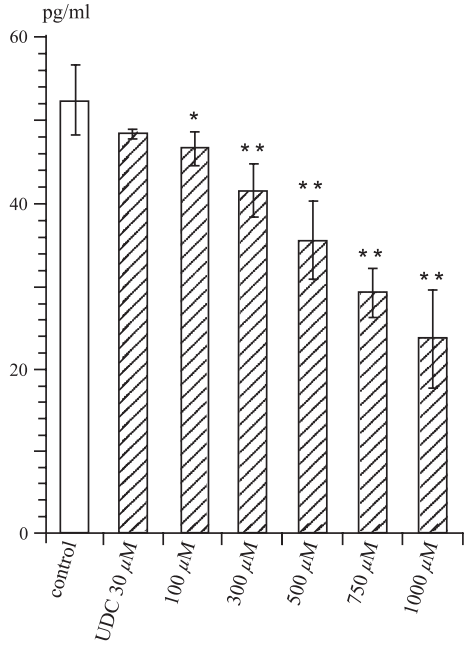

Fig. 1. Effects of ursodeoxycholic acid on the production of endothelin-1 in human umbilical vein endothelial cells (HUVECs). The cells were treated with or without various concentrations of ursodeoxycholic acid (UDC) for 2 h. The amount of endothelin-1 (pg/ml) in the culture medium released from HUVECs was measured and plotted against each concentration of UDC (30–1000 μM). Note that UDC inhibits endothelin-1 production in a concentration-dependent manner. The data represent mean±S.E.M of six different experiments. *$p<0.05$, **$p<0.01$ vs. control.

Fig. 2 illustrates the effects of ursodeoxycholic acid for 2 h on nitric oxide production. The concentration of NO_2^- in the culture medium was measured in the control and the presence of various concentrations of ursodeoxycholic acid (30–1000 μM). Ursodeoxycholic acid at concentrations more than 300 μM significantly increased nitric oxide production. The production of NO_2^- under the control medium for 2 h was 0.55±0.06 μM (n=6) and 2.5±0.3 μM/mg (n=6). The addition of ursodeoxycholic acid in the culture medium increased the NO_2^- production (0.67±0.03 μM, 3.23±0.2 μM/mg ($p<0.05$, n=6) at 300 μM, and 0.82±0.05 μM, 4.01±0.4 μM/mg (n=6, $p<0.01$) at 500 μM).

Figs. 3 and 4 compared the effects of ursodeoxycholic acid and the conjugated bile acids on endothelin-1 (Fig. 3) and nitric oxide (Fig. 4) production. Tauroursodeoxycholic and glycoursodeoxycholic acids as well as ursodeoxycholic acid (500 and 750 μM) increased nitric oxide production in a similar manner (Fig. 4), but they inhibited endothelin-1 production less than ursodeoxycholic acid (Fig. 3).

3.2. Effects of ursodeoxycholic acid on endothelin-1 and eNOS mRNA expression

Fig. 5 shows the effects of ursodeoxycholic acid on endothelin-1 (Fig. 5A) and eNOS (Fig. 5B) mRNA

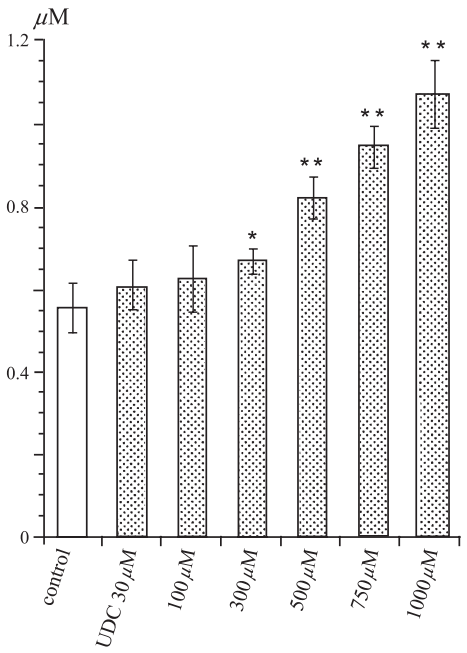

Fig. 2. Effects of ursodeoxycholic acid on the production of nitric oxide in HUVECs. The cells were treated with or without various concentrations of ursodeoxycholic acid (UDC) for 2 h. The concentration of NO_2^- (μM) in the culture medium released from HUVECs was plotted against each concentration of UDC (30–1000 μM). Note that UDC at concentrations higher than 300 μM increases the nitric oxide production. Each column represents the mean±S.E.M of six different experiments. *p<0.05, **p<0.01 vs. control.

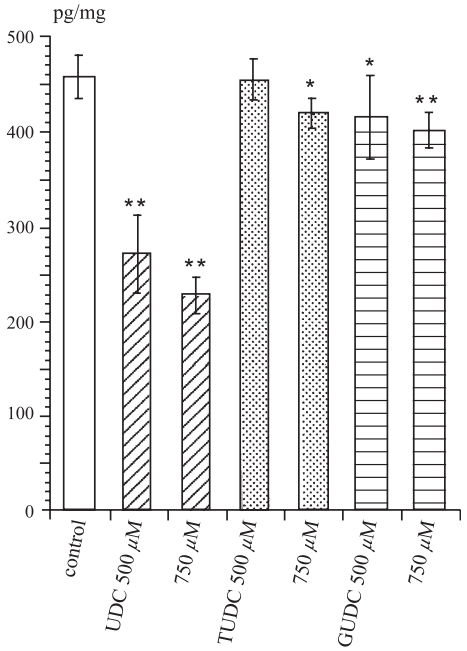

Fig. 3. Effects of ursodeoxycholic acid and the conjugated bile acids on endothelin-1 production. The cells were treated with ursodeoxycholic acid (UDC) or the conjugated bile acids [tauroursodeoxycholic acid (TUDC) and glycoursodeoxycholic acid (GUDC)] for 2 h, and the amount of endothelin-1 released from the cells (pg/mg) was measured and plotted against each concentration of these agents. Each column represents the mean±S.E.M of six different experiments. *p<0.05, **p<0.01 vs. control.

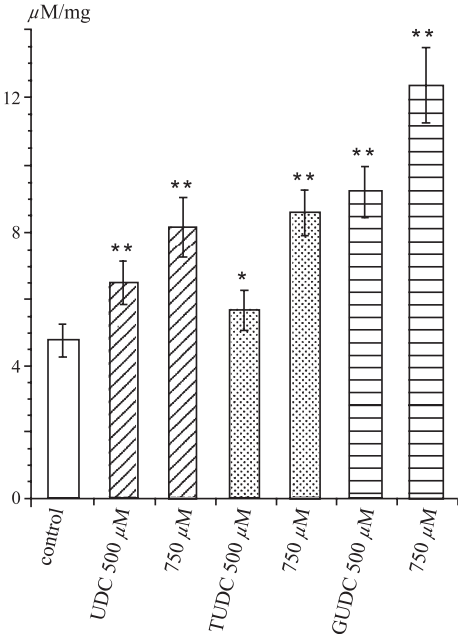

Fig. 4. Effects of ursodeoxycholic acid and the conjugated bile acids on nitric oxide production. The cells were treated with ursodeoxycholic acid (UDC) or the conjugated bile acids [tauroursodeoxycholic acid (TUDC) and glycoursodeoxycholic acid (GUDC)] for 2 h, and the amount of nitric oxide production under these conditions is plotted against each concentration of these drugs. Each column represents the mean±S.E.M of six different experiments. *p<0.05, **p<0.01 vs. control.

expression. The expression of mRNA was compared in cells treated with or without ursodeoxycholic acid (100–750 μM) for 4 h. Ursodeoxycholic acid induced a significant decrease in endothelin-1 mRNA expression. The mean % decrease was 32% of the control at 100 μM, 53% at 300 μM, 91% at 500 μM, and 91% at 750 μM, respectively. On the other hand, ursodeoxycholic acid (100–750 μM) did not modify the iNOS mRNA expression significantly. Similarly, treatment of cells with 500 μM ursodeoxycholic acid for 2 h decreased endothelin-1 mRNA expression to approximately 20% of the control.

3.3. Effects of N-nitro-L-arginine-mythel-ester (L-NAME) on the inhibitory effects of ursodeoxycholic acid on endothelin-1 production

Fig. 6A shows the effects of L-NAME, an inhibitor of nitric oxide synthase, on endothelin-1. The endothelin-1 production was 318±61 pg/mg (n=6) in control, and it was 158±44.7 pg/mg (n=6, p<0.01) at ursodeoxycholic acid (500 μM). The inhibitory effect of ursodeoxycholic acid on endothelin-1 production was not blocked by L-NAME (Fig. 6A, 1 and 2 mM, n=6). The statistical significance was not observed in between ursodeoxycholic acid in the absence of L-NAME and ursodeoxycholic acid in the presence of L-NAME (1 and 2 mM, n=6).

Fig. 6B shows the effects of L-NAME on nitric oxide production. Under the control medium, L-NAME

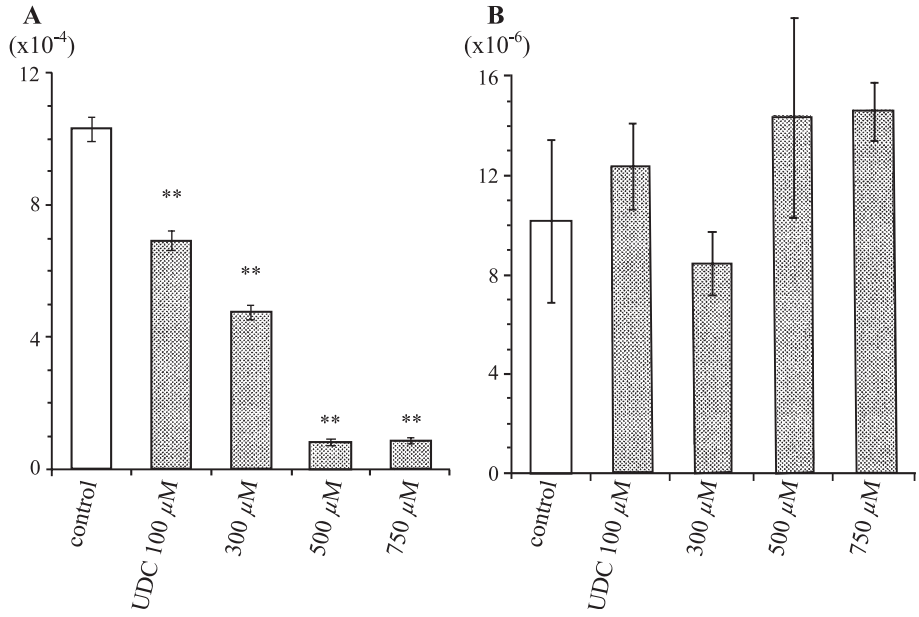

Fig. 5. Effects of ursodeoxycholic acid on endothelin-1 and eNOS mRNA expression. The cells were treated with various concentrations of ursodeoxycholic acid (UDC) for 4 h, and the total RNA was isolated from cells. The expression levels of endothelin-1 (A) and eNOS (B) mRNA were normalized to those of the 18 S ribosomal RNA levels. Data are means±S.E.M from six different samples. **p<0.01 vs. control.

decreased nitric oxide production from 3.06±0.5 μM/mg (n=6) in the control to 1.90±0.26 μM/mg (n=6, p<0.01) at 1 mM L-NAME, and 2.2±0.17 μM/mg (n=6, p<0.01) at 2 mM L-NAME. L-NAME also significantly inhibited the production of NO_2^- induced by ursodeoxycholic acid (Fig. 6B).

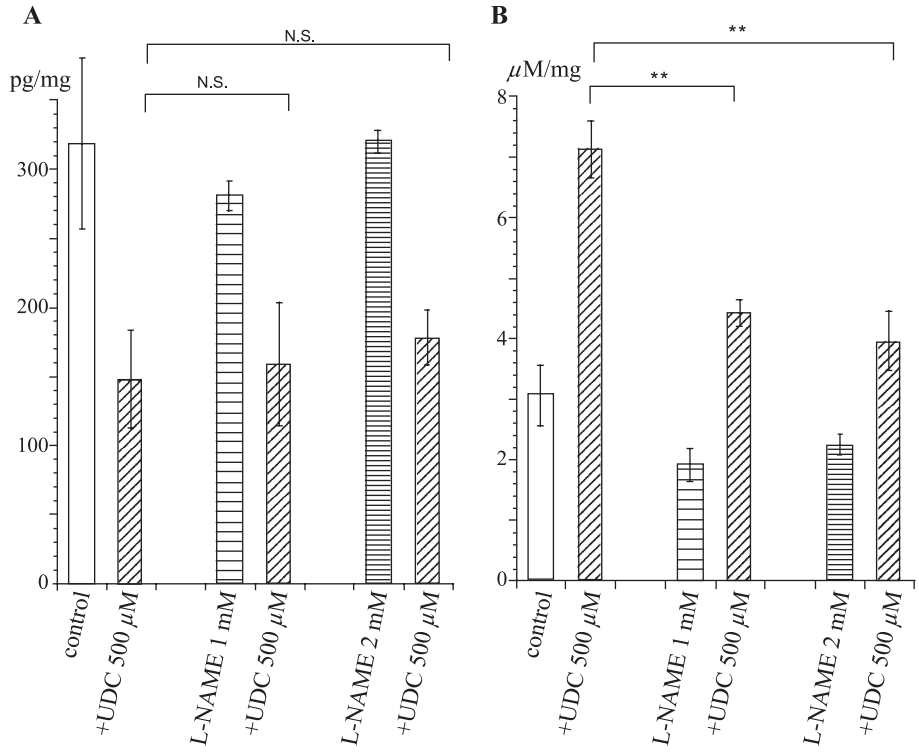

Fig. 6. Effects of L-NAME on the inhibitory effects of ursodeoxycholic acid on endothelin-1 production and the stimulatory effects of ursodeoxycholic acid on nitric oxide production. The cells were treated with or without ursodeoxycholic acid (UDC, 500 μM) in the absence or presence of L-NAME (1 and 2 mM). The amount of endothelin-1 released from the cells for 2 h (pg/mg) were measured and plotted in panel (A). In panel (B), the amount of nitric oxide production under these conditions is plotted. Note that L-NAME inhibits nitric oxide production induced by UDC, but it fails to suppress the inhibitory effects of UDC on endothelin-1 production. Each column represents the mean±S.E.M of six different experiments. **p<0.01 vs. UDC in the absence of l-NAME.

4. Discussion

The present study showed novel evidence that ursodeoxycholic acid significantly decreased endothelin-1 mRNA expression and endothelin-1 production in human endothelial cells. Ursodeoxycholic acid at concentrations higher than 300 μM increased nitric oxide production, but the inhibitory effects of ursodeoxycholic acid on endothelin-1 production was not inhibited by a nitric oxide synthase inhibitor, L-NAME. These results suggest that ursodeoxycholic acid inhibits endothelin-1 production in human endothelial cells, but nitric oxide is not responsible for the inhibitory effect of ursodeoxycholic acid on endothelin-1 production.

Several mechanisms may be proposed in the inhibitory effects of ursodeoxycholic acid on endothelin-1 production. The bile acids are known to induce cell damage because of their detergent property. However, as reported previously in human endothelial cells (Garner et al., 1991), the concentration of ursodeoxycholic acid used in the present study did not affect the morphology of HUVECs, and the viability measured by trypan blue excursion was not different in between control cells and cells treated with ursodeoxycholic acid. Thus, it is unlikely that the detergent effects are involved in ursodeoxycholic acid effects on endothelin-1. In addition, we have reported that bile acids such as chenodeoxycholic acid increase intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$; Nakajima et al., 2000). But, ursodeoxycholic acid at concentrations below 300 μM did not significantly increase $[Ca^{2+}]_i$ (Chisaki et al., 2001). Therefore, it also seems unlikely that $[Ca^{2+}]_i$ is involved in the inhibitory effects of ursodeoxycholic acid on endothelin-1 production.

It has been reported that nitric oxide reduces endothelin-1 production in endothelial cells mediated by cGMP-dependent pathway (Cao et al., 1994; Mitsutomi et al., 1999). Bile acids such as chenodeoxycholic acid increase nitric oxide production in HUVECs by increasing $[Ca^{2+}]_i$ (Nakajima et al., 2000). In the present study, ursodeoxycholic acid at concentrations higher than 300 μM increased nitric oxide production without significant changes in eNOS mRNA expression (Fig. 4). High concentrations of ursodeoxycholic acid increase $[Ca^{2+}]_i$ (Chisaki et al., 2001), which may be involved in ursodeoxycholic acid-induced nitric oxide increase. But the inhibitory effects of ursodeoxycholic acid on endothelin-1 were observed at the lower concentration, suggesting that the basic mechanism underlying these effects is different. In addition, tauroursodeoxycholic and glycoursodeoxycholic acids as well as ursodeoxycholic acid increased nitric oxide production in a similar manner, while the effects of these conjugates on endothelin-1 were less potent than those of ursodeoxycholic acid. Furthermore, to investigate the role of nitric oxide on ursodeoxycholic acid actions, the effects of L-NAME were investigated. L-NAME significantly inhibited the production of nitric oxide but failed to suppress the inhibitory effects of ursodeoxycholic acid on endothelin-1 production. These findings suggest that ursodeoxycholic acid inhibits endothelin-1 production in HUVECs via nitric oxide-independent mechanism.

In endothelial cells and aortic smooth muscle cells, the half-life of endothelin-1 mRNA is very short (approximately 20 min; Horie et al., 1991; Marsden and Brenner, 1992; Hu et al., 1992). This short lifespan of mRNA has been attributed to the presence of three AUUA sequence in the 3′ -untransated region that is thought to mediate selective mRNA (Inoue et al., 1989). Endothelin-1 mRNA is short lived and endothelin-1 is not stored in secretary granule, suggesting that endothelin-1 biosynthesis is mainly regulated at the levels of transcription (Rubanyi and Polokoff, 1994). Therefore, it is likely that the inhibitory effects of ursodeoxycholic acid on endothelin-1 production are due to the inhibition of the transcriptional level. In fact, the present study clearly showed that ursodeoxycholic acid significantly inhibited endothelin-1 mRNA expression.

The inhibitory effects of ursodeoxycholic acid on endothelin-1 production were observed at concentrations above 30 μM in the present study, and the significant inhibition was evident at 100 μM. The serum ursodeoxycholic acid concentration in patients with primary biliary cirrhosis receiving ursodeoxycholic acid therapy has been reported to vary between 10 and 100 μM (Stiehl et al., 1990; Kita et al., 1999), and it reached to approximately 50–100 μM in patients with liver cirrhosis, receiving ursodeoxycholic acid therapy (Chisaki et al., 2001), suggesting that physiological concentrations of ursodeoxycholic acid inhibit endothelin-1 production in human endothelial cells.

Endothelin-1 has been proposed to be involved in augmenting intrahepatic vascular resistance and subsequently contributing to portal hypertension in patients with hepatobiliary diseases (Rockey and Weisiger, 1996; Sogni et al., 1998; Reichen et al., 1998; Shah, 2001). Taken into account that hepatic endothelin-1 is derived from sinusoidal endothelial and stellate cells and modulates an intrahepatic vascular resistance in a paracrine or autocrine manner, endothelin-1 overproduction in the injured liver may increase portal pressure. Actually, endogenous endothelin-1 increases portal pressure in vivo as well as isolated perfused liver (Isales et al., 1993). A number of studies showed that plasma and hepatic endothelin-1 concentration were elevated in human and experimental liver cirrhosis and obstructive jaundice (Moller et al., 1995; Bernardi et al., 1996; Bruno et al., 2000). Thus, it is very likely that endothelin-1 may play a key role in various pathophysiological conditions such as cirrhotic portal hypertension. It has been reported that endothelin-1 receptor antagonists decrease portal pressure in the experimental cirrhotic rat (Rockey and Weisiger, 1996; Sogni et al., 1998; Reichen et al., 1998). Our findings showing that ursodeoxycholic acid inhibits production of endothelin-1 in human endothelial cells suggest that ursodeoxycholic acid therapy may prevent the development of several pathogenesis such as portal hypertension observed in patients with cirrhosis. In fact, ursodeoxycholic acid has

been reported to limit liver histological and portal hypertension induced by bile duct ligation in the rat (Poo et al., 1992). In addition, the increased circulating plasma endothelin-1 also contributes in part to renal dysfunction in patients with cirrhosis (Tsai et al., 1995), suggesting that ursodeoxycholic acid may provide the additional protective effects by inhibiting endothelin-1 production.

In conclusion, the present study provides novel evidence that ursodeoxycholic acid inhibits endothelin-1 production in human endothelial cells, but nitric oxide is not responsible for ursodeoxycholic acid effects on endothelin-1. Ursodeoxycholic acid therapy may prevent the development of several pathogenesis such as portal hypertension observed in patients with cirrhosis due to the improvement of endothelial function.

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