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Short communication

Ursodeoxycholic acid inhibits the induction of nitric oxide synthase

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Abstract

Ursodeoxycholic acid was recently recognized as an effective agent in the treatment of primary biliary cirrhosis. Since the beneficial effect of ursodeoxycholic acid therapy appears to be mediated in part by an immune mechanism, we evaluated the effects of ursodeoxycholic acid on the synthesis of nitric oxide (NO), elevated production of which could be important in the pathogenesis of autoimmunity. Ursodeoxycholic acid (0.1–1000 μM) inhibited NO production by bacterial lipopolysaccharide-activated J774 macrophages in a concentration-dependent fashion, but the cytotoxicity was also evident at higher concentrations (250 and 1000 μM). Ursodeoxycholic acid did not have any effect on the activity of NO synthase that had already been induced. Treatment with lipopolysaccharide led to a significant expression of NO synthase mRNA that was significantly reduced by ursodeoxycholic acid. Findings indicated that ursodeoxycholic acid inhibited NO synthesis by inhibiting the induction of NO synthase, rather than its catalytic activity. Ursodeoxycholic acid therapy may exert a beneficial effect, in part, by attenuating the production of NO.

Keywords: Ursodeoxycholic acid; Nitric oxide (NO) synthase; Lipopolysaccharide; J774 macrophage

1. Introduction

Ursodeoxycholic acid therapy leads to major improvements in patients with primary biliary cirrhosis. Recently, it was proven that long-term ursodeoxycholic acid therapy slows the progression of primary biliary cirrhosis and reduces the need for liver transplantation (Poupon et al., 1994). Although its mechanism of action is unclear, the immunomodulatory effects of ursodeoxycholic acid may work beneficially for primary biliary cirrhosis (Yoshikawa et al., 1992; Lacaille and Paradis, 1993). Recent studies suggest that elevated nitric oxide (NO) production could be important in the pathogenesis of autoimmunity and that treatments to block the production of NO or block its effects might be valuable therapeutically (Kolb and Kolb-Bachofen, 1992; Weinberg et al., 1994). It is therefore possible that ursodeoxycholic acid exerts a beneficial effect in patients with autoimmune-mediated disease, in part, by affecting the production of NO. Immunostimulants induce an isoform of NO synthase in a variety of cells which produce large quantities of NO (Nathan, 1992); this process may be responsible for immune-mediated inflammation and/or destruction of the affected tissue. Indeed, enhanced NO synthase expression and increased NO production have been demonstrated in some autoimmune animal models and humans with autoimmune diseases (Kolb and Kolb-Bachofen, 1992; Weinberg et al., 1994; Farrell et al., 1992; Sakurai et al., 1995). In the present study, we examined the effects of ursodeoxycholic acid on the induction of NO synthesis and inducible NO synthase gene expression in J774 macrophages.

2. Materials and methods

2.1. Cell culture

The mouse macrophage cell line J774 was cultured and prepared as described previously (Hattori et al., 1995). J774 macrophages were seeded into 96-well plates for nitrite assay and cytotoxic assay and into 10-cm dishes for RNA preparation.

2.2. Nitrite production

Nitrite production, an indicator of NO synthesis, was measured in the supernatant of J774 macrophages, as

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previously described (Hattori et al., 1995). Briefly, the cells were cultured in 96-well plates with 200 μl of culture medium until they attained confluence. To induce NO synthase in the cells, fresh culture medium containing *Escherichia coli* lipopolysaccharide was added at a concentration of 1 μg/ml. Nitrite accumulation in the cell culture medium was measured after 24 h. To assess the effects of ursodeoxycholic acid (0.1–1000 μM), the agent was added to the cells simultaneously with lipopolysaccharide. Nitrite was measured by adding 100 μl of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid) to 100-μl samples of cell culture medium. Absorbance at 550 nm was determined with a microplate reader. Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium. Because ursodeoxycholic acid was dissolved in dimethylsulfoxide (DMSO), control nitrite production in response to lipopolysaccharide was determined in the presence of an equivalent volume of DMSO.

2.3. Cell respiration

Cell respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan (Mosmann, 1983). To examine the cytotoxic effect of ursodeoxycholic acid, the cells were incubated (37°C) with MTT (0.4 mg/ml) for a further 60 min after a 24-h incubation for nitrite assay. Culture medium was removed by aspiration and the cells were solubilized in DMSO. The extent of reduction of MTT to formazan within cells was quantified by the measurement of OD_{550}. Formazan production is expressed as a percentage of the values obtained from the control cells (no ursodeoxycholic acid).

2.4. Slot blot analysis of iNOS mRNA

Reverse transcriptase-mediated polymerase chain reaction (RT-PCR) for inducible NO synthase (iNOS) was performed by standard methods as previously reported (Hattori and Gross, 1993). Subcloning and dideoxynucleotide sequencing of the PCR product revealed a sequence that was identical to the cloned cDNA from cytokine-activated mouse macrophages (Lyons et al., 1992). The PCR product labeled with [α-^{32}P]dCTP by random priming was used as a probe for RNA slot blot analysis which was performed using a filtration manifold (Bio-Dot Microfiltration Apparatus; Bio Rad, Hercules, CA, USA) as described previously (Cheley and Anderson, 1984). Total RNA (5 μg) was applied to each slot of the manifold and deposited onto nitrocellulose filters. The filter was hybridized with a random primed ^{32}P-labeled iNOS cDNA probe in rapid hybridization solution (QUIKHYB; Stratagene, La Jolla, CA, USA) at 68°C for 1 h. The hybridized filter was washed twice for 15 min at room temperature with 2 × SSC (sodium chloride/sodium citrate))/0.1% SDS (sodium dodecyl sulfate) and then twice for 30 min at 60°C with 0.1 × SSC/0.1% SDS. The filter was exposed to an imaging plate (Fuji Photo Film Co., Tokyo, Japan) at room temperature for 2 h and analyzed using a FUJIX bioimaging analyzer (BAS2000II, Fuji Photo Film Co.).

2.5. Materials

Ursodeoxycholic acid was a gift from Tokyo Tanabe Pharmaceutical Co. (Tokyo, Japan). Bacterial lipopolysaccharide (*E. coli*, serotype No. 0127:B8), and other chemicals were obtained from Sigma Chemical Co. (Saint Louis, MO, USA).

2.6. Statistical evaluation

Values are expressed as mean ± standard error of the mean of three observations. Student's unpaired *t*-tests were used to assess the statistical significance of differences. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

NO production by lipopolysaccharide-activated J774 macrophages is reflected in the accumulation of nitrite in the cell culture medium (Hattori et al., 1995). A lag phase of 5–6 h preceded the induction of nitrite synthesis, followed by a progressive increase in nitrite synthesis for at least 24 h. Nitrite accumulation in J774 macrophages at 24 h after stimulation with lipopolysaccharide was 18.3 ± 0.5 μM. Nitrite concentration in culture media of unstimulated cells was < 1 μM.

Studies with different concentrations of ursodeoxycholic acid (0.1–1000 μM) showed a concentration-dependent inhibition of nitrite production when ursodeoxycholic acid was added to J774 macrophages at the same time as lipopolysaccharide (Fig. 1A). At concentrations ≤ 125 μM, ursodeoxycholic acid did not inhibit cellular respiration (Fig. 1A). At higher concentrations (e.g. 250 or 1000 μM), however, ursodeoxycholic acid markedly inhibited both nitrite formation and cellular respiration (Fig. 1A).

To determine whether ursodeoxycholic acid affects the activity of NO synthase enzyme, fresh medium containing ursodeoxycholic acid (1–100 μM) with cycloheximide (4×10^{-6} M) was added to cells already treated with lipopolysaccharide (1 μg/ml for 24 h), and nitrite production was measured after a further 24 h. As shown in Fig. 1B, in J774 macrophages in which NO synthase had already been induced by lipopolysaccharide and any possible further induction was blocked by cycloheximide, ursodeoxycholic acid did not change nitrite production.

Unstimulated J774 macrophages had barely detectable inducible NO synthase mRNA. Levels of inducible NO synthase mRNA in J774 macrophages reached a maximum by approximately 8 h after treatment with lipopolysaccharide and began to fall by 24 h (Fig. 2A). Thus, the effect of ursodeoxycholic acid on the induction of NO synthase mRNA by lipopolysaccharide was examined after exposure of J774 macrophages to lipopolysaccharide for 8 h in the presence and absence of ursodeoxycholic acid (100 μM). By Northern blot analysis, a dominant transcript of ~ 4.6 kb was recognized by an inducible NO synthase-specific probe (data not shown). Then, RNA slot blot analysis using a bioimaging analyzer was employed to quantify inducible NO synthase mRNA abundance. As shown in Fig. 2B, ursodeoxycholic acid alone did not affect the expression of inducible NO synthase mRNA, but significantly inhibited the inducible NO synthase mRNA expression elicited by lipopolysaccharide. When we normalized the inducible NO synthase mRNA signal relative to the corresponding glyceraldehyde-3-phosphate dehydrogenase mRNA signal from the same sample, ursodeoxycholic acid at 100 μM, reduced the relative NO synthase mRNA levels by 57% in J774 macrophages.

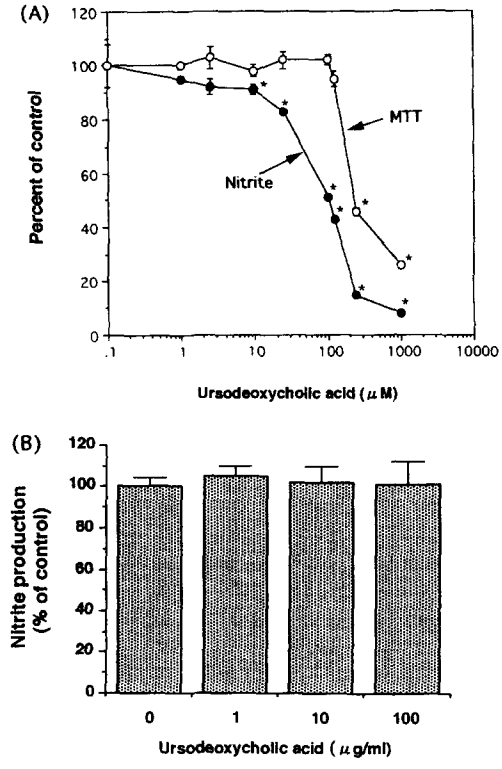

Fig. 1. (A) Effect of ursodeoxycholic acid on accumulation of nitrite and cellular respiration in J774 macrophages. Nitrite production (closed circle) and MTT value (open circle) were measured 24 h after cells were stimulated with lipopolysaccharide (1 μg/ml). (B) Effect of ursodeoxycholic acid on the activity of NO synthase in J774 macrophages. Fresh medium containing ursodeoxycholic acid (1–100 μg/ml) and cycloheximide (4×10^{-6} M) was added to J774 macrophages already treated with lipopolysaccharide (1 μg/ml for 24 h) and nitrite production was measured after a further 24 h. Data are mean ± S.E. of triplicate observations and expressed as percent values of control (100%: no ursodeoxycholic acid). $^{*}P < 0.01$ represents significant difference as compared with control.

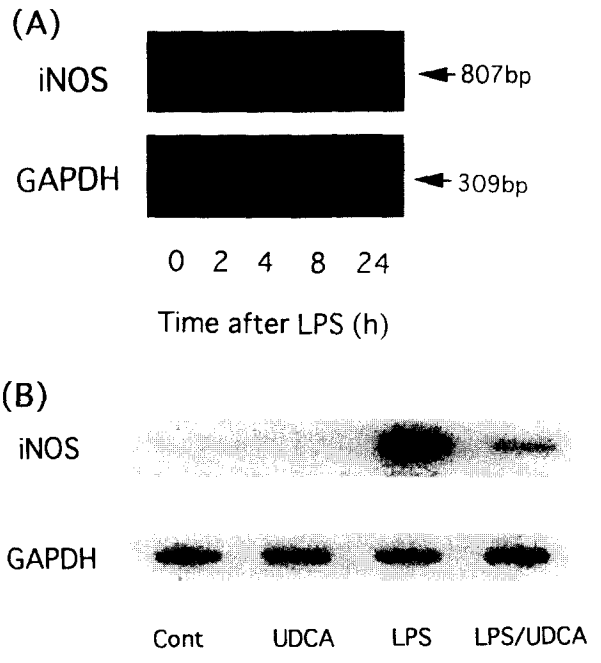

Fig. 2. (A) Time course of changes in mRNA levels for inducible NO synthase (iNOS). J774 macrophages were harvested at the indicated times after stimulation with lipoplysaccharide (1 μg/ml) and RNA was prepared and assayed by RT-PCR. Results using primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Terada et al., 1992) are shown for comparison. (B) Cells, untreated or stimulated with lipopolysaccharide (1 μg/ml), were incubated for 8 h in the absence and presence of ursodeoxycholic acid (UDCA: 100 μM). Total RNA was prepared and assayed by slot blot analysis using an iNOS probe and subsequently a GAPDH probe.

4. Discussion

The present findings demonstrated that ursodeoxycholic acid reduced the synthesis of NO in lipopolysaccharide-activated J774 macrophages. Bile acids are endogenous molecules with detergent properties because of their peculiar amphiphilic structure and show the relative cytotoxicity attributed to an interaction with cell membrane. Indeed, ursodeoxycholic acid at higher concentrations (250 or 1000 μM) showed the marked inhibition in cell respiration. However, ursodeoxycholic acid at concentrations ranging from 10–125 μM significantly decreased NO synthesis without evidence of cytotoxicity. This indicates that the reduction of NO production was not due to a cytotoxic effect of ursodeoxycholic acid. The addition of ursodeoxycholic acid after the induction of NO synthase did not significantly inhibit nitrite production, indicating that ursodeoxycholic acid interferes with NO synthase induction rather than with its catalytic activity. How, then, does ursodeoxycholic acid interfere with the induction of NO

synthase? It may well be through an inhibition of the cytokine formation which mediates the induction of NO synthase by lipopolysaccharide (Szabo et al., 1993; Thiemermann et al., 1993), for ursodeoxycholic acid inhibits production of various cytokines in monocytes (Yoshikawa et al., 1992). In the present study, ursodeoxycholic acid significantly inhibited the levels of NO synthase mRNA induced by lipopolysaccharide. We therefore conclude that ursodeoxycholic acid attenuated NO production by inhibiting the expression of the inducible NO synthase gene in J774 macrophages.

Ursodeoxycholic acid has a very low rate of clinical toxicity and shows promising results in immune-mediated diseases (Poupon et al., 1994). Recent reports suggest that inducible NO synthase contribute to tissue damage in some inflammatory and immunological diseases (Kolb and Kolb-Bachofen, 1992; Weinberg et al., 1994; Farrell et al., 1992; Sakurai et al., 1995). Plasma levels of ursodeoxycholic acid peak at about half an hour the concentrations of which may inhibit NO formation in vitro (the mean post-dose 0.5-h plasma drug concentrations observed were > 10 μM in the single-dose [300 mg] study; data provided by Tokyo Tanabe Pharmaceutical Co.), but steady-state levels appear to be much lower. However, ursodeoxycholic acid which circulates in the enterohepatic circulation could be at higher levels in the bile during treatment with ursodeoxycholic acid (Fedorowski et al., 1977). Thus, ursodeoxycholic acid may exert its beneficial effect on patients with immune-mediated liver diseases, in part, by inhibiting the induction of NO synthesis.

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