Available online at www.sciencedirect.com
SCIENCE DIRECT®
European Journal of Pharmacology 464 (2003) 79–86
www.elsevier.com/locate/ejphar

Inhibitory effects of ursodeoxycholic acid on the induction of nitric oxide synthase in vascular smooth muscle cells

Ji Ma[a,b], Toshiaki Nakajima[a,*], Haruko Iida[a], Kuniaki Iwasawa[a], Kuniko Terasawa[a], Hitoshi Oonuma[a], Taisuke Jo[a], Toshihiro Morita[a], Hiroyuki Imuta[a], Jun-ichi Suzuki[a], Ken Hirose[a], Yukichi Okuda[b], Nobuhiko Yamada[b], Ryozo Nagai[a], Masao Omata[a]

[a] *Department of Cardiovascular, Respiratory Medicine and Gastroenterology, University of Tokyo, Graduate School of Medicine, 7-3-1 Hongo, Bunkyo, Tokyo, 113-8645, Japan*

[b] *Department of Internal Medicine, Institute of Clinical Medicine, University of Tsukuba, Tsukuba, Ibaraki-ken, 305-0005, Japan*

Received 30 September 2002; received in revised form 21 January 2003; accepted 24 January 2003

Abstract

The expression of inducible nitric oxide synthase (iNOS) and the resultant increased nitric oxide production are associated with endotoxemia and atherosclerotic lesions observed in transplant hearts or balloon-injured artery. Ursodeoxycholic acid has been shown to have cardiovascular protective effects, such as inhibition of the development of transplant arteriosclerosis, but its mechanism remains unclear. Here, we investigated the effects of ursodeoxycholic acid on nitric oxide production and the expression of iNOS in vascular smooth muscle cells isolated from adult rat aorta and rabbit coronary artery. Nitrite released from cells in the culture medium was measured with the Griess reaction. iNOS mRNA and protein were measured by Northern and Western blot analyses. Treatment with ursodeoxycholic acid (30–1000 μM) significantly inhibited lipopolysaccharide plus interferon-γ-induced nitric oxide production in a concentration-dependent manner, but ursodeoxycholic acid showed only small inhibitory effects on nitric oxide production that had already been induced by lipopolysaccharide plus interferon-γ. Ursodeoxycholic acid by itself did not affect basal nitric oxide production. Ursodeoxycholic acid also suppressed lipopolysaccharide plus interferon-γ-induced expression of iNOS mRNA and protein. Ursodeoxycholic acid had the most potent inhibitory effect among various kinds of bile acids examined, i.e. chenodeoxycholic acid, deoxycholic acid, cholic acid and conjugated bile acids such as tauroursodeoxycholic acid. These results suggest that ursodeoxycholic acid inhibits the induction of iNOS and then nitric oxide production in aortic and coronary artery smooth muscle cells, suggesting a possible mechanism for the cardiovascular protective effect of ursodeoxycholic acid under various pathophysiological conditions such as endotoxemia and atherosclerosis.

Keywords: Ursodeoxycholic acid; Smooth muscle cell, aortic; Smooth muscle cell, coronary artery; Nitric oxide (NO) synthase; Lipopolysaccaride; Interferon-γ

1. Introduction

Nitric oxide, derived from the vascular endothelium or other cells in the cardiovascular system, plays a vital role in regulating vascular tone (Moncada et al., 1991; Nathan, 1992). In addition, it is an important physiological and pathophysiological mediator in cardiovascular diseases. Nitric oxide is synthesized from L-arginine by three isoforms of nitric oxide synthase (NOS) (Forstermann and Kleinert, 1995). Two of the isoforms (neural NOS (nNOS) and endothelial NOS (eNOS)) are constitutive, and eNOS is found predominantly in endothelial cells. The third isoform, inducible NOS (iNOS), an inducible isoform, can be expressed in macrophages, vascular smooth muscle cells, and endothelial cells upon induction by cytokines and bacterial lipopolysaccharide. eNOS is a physiologically significant vasodilator and an inhibitor of vascular smooth muscle cell proliferation and platelet aggregation, and is generally considered to have cardiovascular protective and antiatherosclerotic roles. The induced expression of iNOS is believed to play a role in the pathogenesis of endotoxin shock, which contributes to suppress vessel contractility and subsequently induce severe hypotension (Thiemermann and Vane, 1990; Nava et al., 1991). In addition, a large amount of nitric oxide may induce apoptosis (Nathan and Xie, 1994; Iwashina et al., 1998), and peroxynitrite pro-

* Corresponding author. Tel.: +81-3-3815-5411; fax: +81-3-3814-0021.
E-mail address: masamasa@pb4.so-net.ne.jp (T. Nakajima).

0014-2999/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0014-2999(03)01373-6

duced from iNOS induces cell necrosis and death (Beckman and Koppenol, 1996) and may be associated with regression of advanced atheromatous plaques (White et al., 1994; Fukuo et al., 1995; Isner et al., 1995; Buttery et al., 1996). In fact, the expression of iNOS in endothelial cells and vascular smooth muscle cells and the resultant increased production of nitric oxide have been detected in atherosclerosis, injured vessels and related coronary atherosclerotic lesions (Yan and Hansson, 1998; Depre et al., 1999). It is also involved in the pathogenesis of severe chronic inflammatory actions, including transplant atherosclerosis and balloon-injured artery (Hansson et al., 1994; Akyurek et al., 1996; Lafond-Walker et al., 1997; Anderson et al., 2000).

Ursodeoxycholic acid, a hydrophilic tertiary bile acid, has been widely used to treat patients with chronic cholestatic liver diseases (Luketic and Sanyal, 1994). Recently, it has been demonstrated to be beneficial in the treatment of patients with liver diseases such as primary biliary cirrhosis and chronic viral hepatitis (Cirillo and Zwas, 1994; Makino and Tanaka, 1998). The basic mechanism has not yet been identified, but it may be due to a wide range of cellular actions of ursodeoxycholic acid, i.e. anti-inflammatory and immunomodulating effects (Makino and Tanaka, 1998). As a consequence, the potential of using ursodeoxycholic acid for extrahepatic diseases has been investigated. Ursodeoxycholic acid has been shown to have chemoprotective effects in an animal model of chemically induced colonic carcinogenesis (Earnest et al., 1994) and to reduce inflammation in experimental models of small bowel inflammatory disease.

Ursodeoxycholic acid has been also reported to prevent ischemia and reperfusion injury in isolated hearts (Lee et al., 1999), and adjuvant treatment with ursodeoxycholic acid has been shown to reduce the incidence of acute cardiac allograft rejection (Friman et al., 1992; Bahrle et al., 1998). The underlying mechanisms are not clearly understood, but several reports show that ursodeoxycholic acid inhibits iNOS and the resultant nitric oxide production induced by bacterial lipopolysaccharide in a mouse macrophage cell line and by cytokines (interleukin and interferon) in human intestinal epithelial cells (Hattori et al., 1996; Invernizzi et al., 1997). Thus, it is very likely that ursodeoxycholic acid also has cardiovascular protective effects, by modulating iNOS activity in cardiovascular systems.

In this study, we examined the effects of ursodeoxycholic acid treatment on the induction of iNOS synthesis and iNOS gene expression induced by lipopolysaccharide plus interferon-γ in rat aortic and rabbit coronary artery smooth muscle cells. Here, we provide evidence that a therapeutic concentration of ursodeoxycholic acid inhibits the induction of iNOS in vascular smooth muscle cells, thereby providing a possible mechanism for the cardiovascular protective effect of ursodeoxycholic acid under various pathophysiological conditions such as endotoxemia and atherosclerosis.

2. Materials and methods

2.1. Materials

Ursodeoxycholic acid (Na salt), tauroursodeoxycholic acid (Na salt) and glycoursodeoxycholic acid (Na salt) were kindly provided by Mitsubishi Tokyo Pharmaceutical (Tokyo, Japan). Deoxycholic acid (Na salt), chenodeoxycholic acid (Na salt), cholic acid (Na salt), taurodeoxycholic acid (Na salt), taurocholic acid (Na salt) and taurochenodeoxycholic acid (Na salt) were purchased from Sigma (St. Louis, MO). Bacterial lipopolysaccharide and human interferon-γ were also obtained from Sigma.

2.2. Cell culture

Vascular smooth muscle cells were prepared by the explant method from thoracic aorta of male Wistar rats (6–8 weeks) or coronary artery of adult male Japanese White rabbits (2.5–3.0 kg). Briefly, aortas of male Wistar rats or rabbit coronary arteries (left anterior descending and left circumflex coronary artery) were stripped of adventitia, the endothelial cell layer of the intima was scraped off, and the aorta or coronary artery was cut into small pieces. These were placed in Dubecco's modified Eagle's Medium (DMEM, Sigma) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin, 100 μg/ml gentamicin and 0.25 μg/ml amphotericin B (GIBCO) in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. They exhibited a typical "hill and valley" growth pattern and also exhibited positive fluorescence with antibodies against β-actin. Medium was replaced twice a week. When the cells became confluent, they were subcultured in the medium using 0.5% trypsin in 0.02% EDTA. Confluent cells at passage numbers 3–6 were used for the experiments.

2.3. Nitrite assay

The amount of nitric oxide released from rat aortic or rabbit coronary artery smooth muscle cells was determined by measuring the concentration of nitrite (NO_2^-) in culture medium in a well using the Griess test, as shown previously (Nakajima et al., 2000; Chisaki et al., 2001). The accumulation of NO_2^-, a stable end product of nitric oxide formation, in the cell culture supernatant was used as a measure of nitric oxide production. One milliliter of cell-free supernatant was incubated with 1 ml Griess reagent for 10 min at room temperature, and the absorbance at 540 nm was measured in a Beckman DU-70 spectrophotometer (CA, USA). The concentration of NO_2^- (μM) was calculated by comparison with the absorbance at 540 nm of standard solutions of 0–150 μM $NaNO_2$ prepared with DMEM medium. Nitrite accumulation per well (μM/mg) was normalized to the total volume of the incubation medium and to the total protein content of the cells in a well.

2.4. Protein assay

After confluent smooth muscle cells in 35-mm plates were exposed to various conditions, the cells in a plate were washed three times in phosphate-buffered saline and then lysed in 0.5 M NaOH. The amount of protein in the cytolysate was measured with the Bradford protein assay.

2.5. Simultaneous isolation of total RNA and protein preparation

The simultaneous isolation of total RNA and the protein fraction for Northern blot analysis and Western blotting was performed using ISOGEN (Nippon Gene, Tokyo) according to the manufacturer's protocol, as briefly described below. Smooth muscle cells were lysed directly in the culture dish by the addition of the reagent. The lysate was mixed with chloroform and centrifuged, which yielded the top aqueous phase, interphase, and the bottom organic phase. Total RNA was precipitated from the aqueous phase by addition of isopropanol, washed and dissolved in water. In addition, mRNA was purified from total RNA using Gen elute mRNA mini prep kit (Sigma). DNA was removed from the phenol–ethanol phase of ISOGEN by the addition of isopropanol. The protein pellet was washed and dissolved in 5% sodium dodecyl sulfate.

2.6. Northern blot analysis

The mRNA was size-fractionated on 1.2% agarose gels containing 0.67 M formaldehyde, blotted onto a Hybond-N^+ (Amersham Bioscienses, Piscataway, NJ, USA) and fixed by UV transillumination. Reverse transcriptase/polymerase chain reactions (RT–PCR) for rat iNOS and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed. Specific primers for iNOS were forward, 5′-AGGCCACCTCGGATATCTCT-3′, and reverse, 5′-CCATGATGGTCACATTCTGC-3′; and for GAPDH forward, 5′-ACATTGTTGCCATCAACGAC-3′, and reverse, 5′-ATGTAGGCCATGAGGTCCAC-3′. The RT–PCR products were labeled using the AlKPhos direct labeling module kit (Amersham Bioscienses). Hybridization was done according to the AlKPhos direct protocol. The membrane was analyzed with LAS-1000 image analyzer (Fuji-Film, Tokyo, Japan).

2.7. Western blotting

For Western blotting, proteins were separated on 8% polyacrylamide gels for 90 min at 25 mA. Proteins were then transferred to Hybond-P (Amersham Biosciences) for 4 h at 60 V. Then, the membrane was blocked with 1% bovine serum albumin in Tris-buffered saline (137 mM NaCl and 25 mM Tris–HCl, pH 7.4) with 0.1% Tween-20 (TBS-T) for 1 h. The membrane was then probed with anti-iNOS monoclonal antibody diluted to 1:1000 (BD Transduction Laboratories, KY, USA) for 2 h at room temperature, and washed three times in TBS-T for 10 min each wash. The membrane was subsequently incubated with goat anti-rabbit immunoglobulin G linked to peroxidase (Vector Laboratories, Burlingame, CA) diluted to 1:5000 for 1 h at room temperature. After three additional washes, bound antibodies were detected with ECL-Plus (Amersham Pharmacia Biotech) and analyzed with a LAS-1000 image analyzer (Fuji-Film). The blot was stripped and reprobed with anti-β-actin monoclonal antibody (Sigma) to normalize lanes for the protein content.

2.8. Statistical analysis

Statistical comparison was carried out with three or more groups using one-way analysis of variance and Dunnett's test. The data represent means ± S.D., and values of $P < 0.05$ were statistically significant.

3. Results

3.1. Effects of ursodeoxycholic acid on nitric oxide production in vascular smooth muscle cells

Fig. 1 shows the time-dependent effects of lipopolysaccharide (10 μg/ml) plus interferon-γ (100 U/ml) on NO_2^- production in supernatants of rat aortic and rabbit coronary artery smooth muscle cells. Lipopolysaccharide plus interferon-γ increased NO_2^- production in a time-dependent manner (1–48 h) in both rat aortic and rabbit coronary artery smooth muscle cells. A significant elevation of NO_2^- concentration, adjusted to the total protein content of the

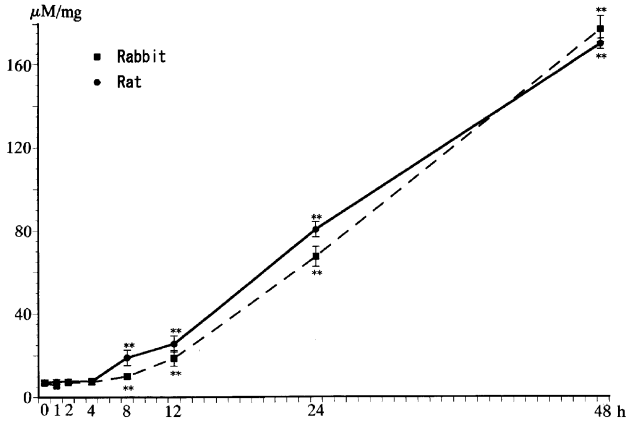

Fig. 1. Time-dependent effects of lipopolysaccharide (LPS) plus interferon-γ (IFN-γ) on NO_2^- production in rat aortic and rabbit coronary artery smooth muscle cells. The concentration of NO_2^- in cell culture medium and the total protein content of cells in a well were measured in control and 1–48 h after the application of LPS (10 μg/ml) plus INF-γ (100 U/ml). The data represent the concentration of NO_2^- in the culture medium, divided by the total protein content of the cells (μM/mg). *$P < 0.05$, **$P < 0.01$ vs. control (0 min). Each column represents mean ± S.D. value of six preparations.

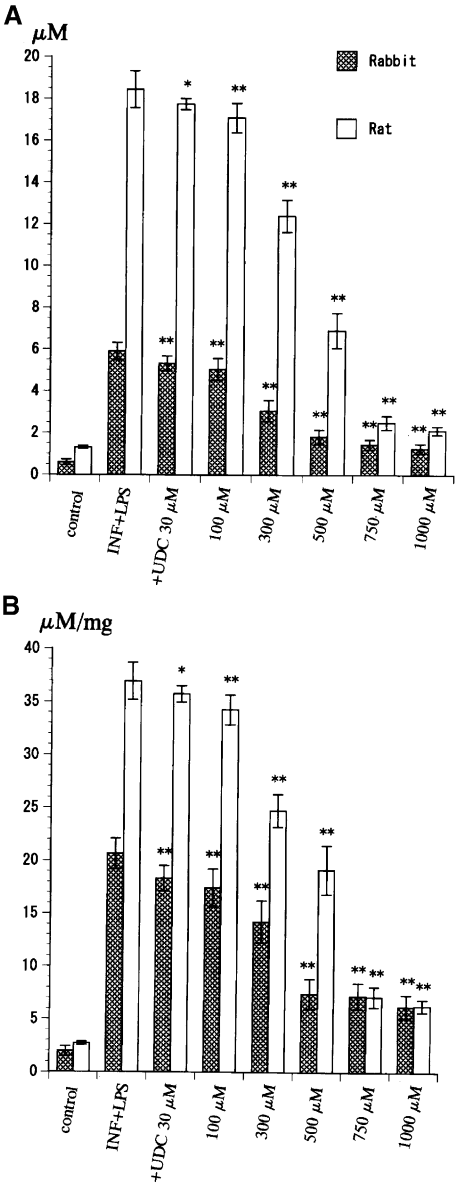

Fig. 2. Concentration-dependent inhibitory effects of ursodeoxycholic acid (UDC) on lipopolysaccharide (LPS) plus interferon (INF)-γ-enhanced NO_2^- production in rat aortic and rabbit coronary artery smooth muscle cells. NO_2^- production was measured 24 h after cells were stimulated with LPS (10 μg/ml) plus INF-γ (100 U/ml) in the absence and presence of various concentrations of UDC (30–1000 μM). The concentration of NO_2^- in the culture medium (A) and that adjusted by the total protein content of the cells in a well (B) were plotted for rat aortic (open bar) and rabbit coronary artery smooth muscle cells (shaded bar), respectively. *$P<0.05$, **$P<0.01$ vs. LPS plus INF-γ in the absence of UDC. Each column represents mean ± S.D. value of six preparations.

cells in a well (μM/mg), was observed at 8 h after the addition of lipopolysaccharide plus interferon-γ in both smooth muscle cells, followed by a progressive increase in NO_2^- production for 24–48 h. The basal NO_2^- production measured before the application of lipopolysaccharide plus interferon-γ was 7.27 ± 1.3 μM/mg ($n=6$) for rat aortic smooth muscle cells and 6.99 ± 0.96 μM/mg ($n=6$) for rabbit coronary artery smooth muscle cells. The NO_2^- production measured at 24 and 48 h after the addition of lipopolysaccharide plus interferon-γ was 80.4 ± 3.96 μM/mg ($n=6$) and 168.6 ± 2.5 μM/mg ($n=6$) for rat aortic smooth muscle cells as shown in Fig. 1. It was 67.3 ± 4.8 μM/mg ($n=6$) and 175.5 ± 6.17 μM/mg ($n=6$) for rabbit coronary artery smooth muscle cells, respectively.

Fig. 2 illustrates the effects of ursodeoxycholic acid on lipopolysaccharide plus interferon-γ-enhanced NO_2^- production in rat aortic and rabbit coronary artery smooth muscle cells. Treatment with lipopolysaccharide (10 μg/ml) plus interferon-γ (100 U/ml) for 24 h markedly increased NO_2^- production in both smooth muscle cells. Incubation with ursodeoxycholic acid (30–1000 μM) resulted in a significant reduction of NO_2^- production stimulated by lipopolysaccharide plus interferon-γ in both rat aortic and rabbit coronary artery smooth muscle cells. A significant inhibitory effect of ursodeoxycholic acid on lipopolysaccharide plus interferon-γ-enhanced NO production was observed at concentrations higher than 30 μM. The mean half-maximal inhibitory effect of ursodeoxycholic acid on lipopolysaccharide plus interferon-γ-induced nitric oxide production was 310 ± 50 μM ($n=3$) for rat aortic smooth muscle cells and 330 ± 40 μM ($n=3$) for rabbit coronary artery smooth muscle cells. Ursodeoxycholic acid (300 μM) by itself did not inhibit basal nitric oxide production by rat aortic smooth muscle cells, as shown in Fig. 3.

Fig. 4 compares the effects of various kinds of bile acids on lipopolysaccharide plus interferon-γ-enhanced NO_2^- production in rabbit coronary artery (Fig. 4A) and rat aortic

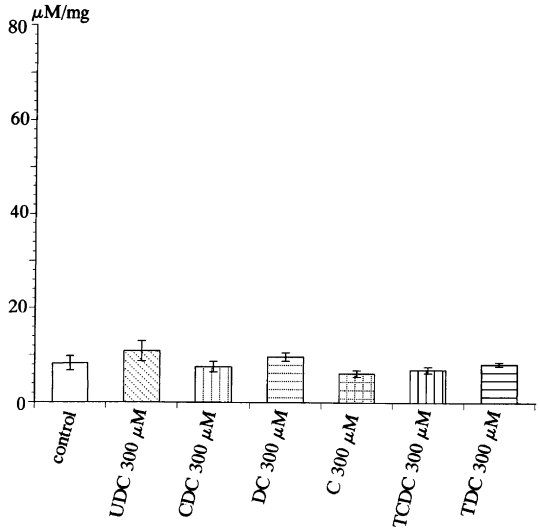

Fig. 3. Effects of various bile acids on NO_2^- production in rat aortic smooth muscle cells. The cells were treated with various bile acids (300 μM) for 24 h. NO_2^- production, adjusted to the total protein content of the cells (μM/mg), was plotted. Note that UDC by itself did not affect basal nitric oxide production. Ursodeoxycholic acid (UDC), chenodeoxycholic acid (CDC), deoxycholic acid (DC), cholic acid (C), taurochenodeoxycholic acid (TCDC) and taurodeoxycholic acid (TDC). Each column represents mean ± S.D. value of six preparations.

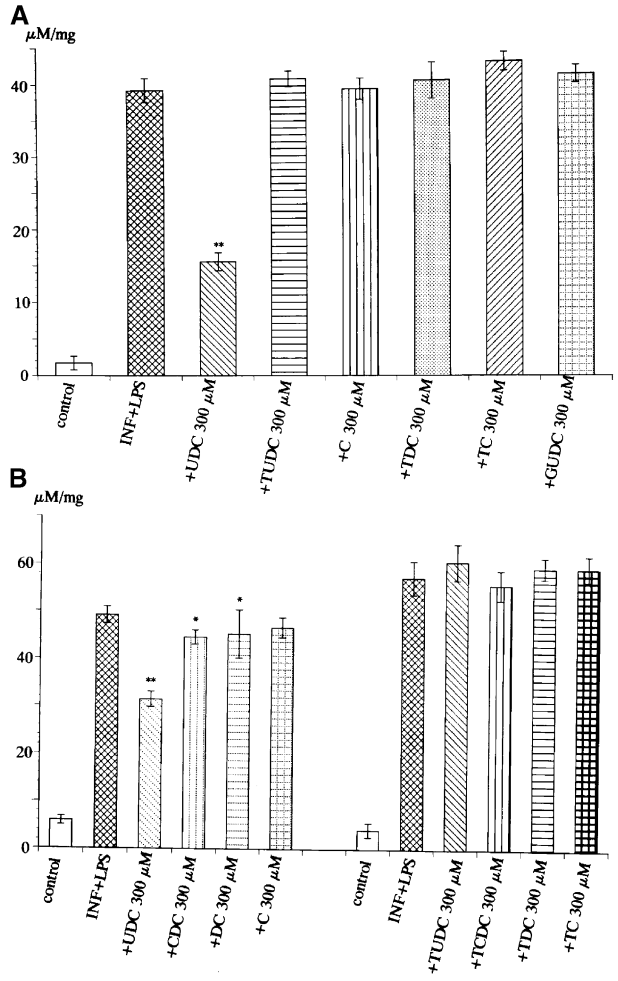

Fig. 4. Comparative effects of various bile acids on lipopolysaccharide (LPS) plus interferon (INF)-γ-enhanced NO_2^- production in rabbit coronary artery (A) and rat aortic smooth muscle cells (B). NO_2^- production was measured 24 h after cells were stimulated with LPS plus INF-γ in the absence and presence of various bile acids (300 μM). NO_2^- production, adjusted to the total protein content of the cells, was plotted. Ursodeoxycholic acid (UDC), chenodeoxycholic acid (CDC), deoxycholic acid (DC), cholic acid (C), tauroursodeoxycholic acid (TUDC), taurodeoxycholic acid (TDC), taurocholic acid (TC), and glycoursodeoxycholic acid (GUDC). $^*P<0.05$, $^{**}P<0.01$ vs. LPS plus INF-γ in the absence of UDC. Each column represents mean ± S.D. value of six preparations.

smooth muscle cells (Fig. 4B). Ursodeoxycholic acid (300 μM, $n=6$, $P<0.01$), chenodeoxycholic acid (300 μM, $n=6$, $P<0.05$) and deoxycholic acid (300 μM, $n=6$, $P<0.05$) significantly suppressed the lipopolysaccharide plus interferon-γ-enhanced nitric oxide production (Fig. 4B). However, the conjugated bile acids of ursodeoxycholic acid (tauroursodeoxycholic acid and glycoursodeoxycholic acid), and other bile acids such as taurodeoxycholic acid, taurochenodeoxycholic acid, and taurocholic acid and cholic acid at concentrations of 300 μM failed to inhibit the lipopolysaccharide plus interferon-γ-enhanced nitric oxide production in both smooth muscle cells. The bile acids by themselves did not affect basal NO_2^- production, as shown in Fig. 3.

Fig. 5 shows the time-dependent inhibitory effects of ursodeoxycholic acid on NO_2^- production stimulated by lipopolysaccharide plus interferon-γ in rat aortic smooth muscle cells. When ursodeoxycholic acid (300–750 μM) and lipopolysaccharide (10 μg/ml) plus interferon-γ (100 U/ml) were applied to the cells at the same time, ursodeoxycholic acid concentration dependently inhibited the lipopolysaccharide plus interferon-γ-enhanced nitric oxide production at 24 and 48 h. Ursodeoxycholic acid (300 μM) inhibited lipopolysaccharide plus interferon-γ-induced nitric oxide production by 56 ± 12% at 24 h and 64 ± 7% at 48 h, which was calculated from mean values in Fig. 5A,B. Ursodeoxycholic acid (750 μM) inhibited it by 87 ± 4% at 24 h and 91 ± 2% at 48 h. When ursodeoxycholic acid was

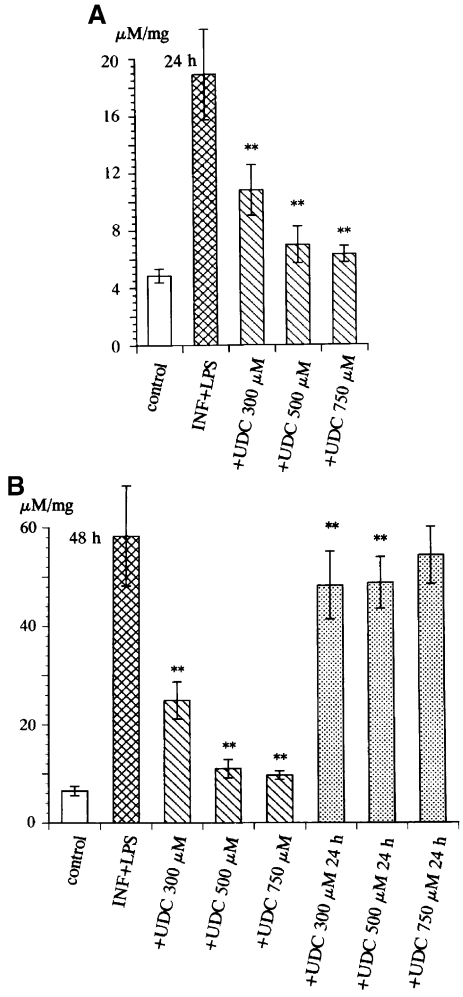

Fig. 5. Time-dependent effects of ursodeoxycholic acid (UDC) on lipopolysaccharide (LPS) plus interferon (INF)-γ-enhanced NO_2^- production. NO_2^- production, adjusted to the total protein content of the cells (μM/mg), was measured 24 (A) and 48 h (B) after they were stimulated with LPS (10 μg/ml) plus INF-γ (100 U/ml) and incubated for different periods with UDC (300–1000 μM). Note that the inhibitory effect of UDC was much less under the conditions where UDC was added 24 h after the cells were stimulated with LPS and INF-γ, as shown in B. $^*P<0.05$, $^{**}P<0.01$ vs. LPS plus INF-γ in the absence of UDC. Each column represents mean ± S.D. value of six preparations.

added to the cells 24 h after lipopolysaccharide plus interferon-γ stimulation, inhibition of nitric oxide production was only 19 ± 13% at 300 μM ursodeoxycholic acid and 7 ± 11% at 750 μM ursodeoxycholic acid.

3.2. Effects of ursodeoxycholic acid on lipopolysaccharide plus interferon-γ-induced iNOS mRNA and protein

The effects of ursodeoxycholic acid on lipopolysaccharide plus interferon-γ-induced iNOS mRNA and protein expression were investigated in rat aortic smooth muscle cells, as shown in Fig. 6. Northern blot analyses performed 24 h after lipopolysaccharide (10 μg/ml) plus interferon-γ (100 U/ml) stimulation of iNOS showed an intense band (Fig. 6A), which was not detected in cells without stimulation. Incubation with ursodeoxycholic acid significantly reduced the level of iNOS mRNA, adjusted to the internal control (GAPDH), as compared with the lipopolysaccharide plus interferon-γ-stimulated cells in the absence of ursodeoxycholic acid (0.56 ± 0.23 ($n = 3$, $P < 0.01$) at 100 μM and 0.34 ± 0.16 ($n = 3$, $P < 0.01$) at 300 μM, and 0.20 ± 0.15 ($n = 3$, $P < 0.01$) at 500 μM). Fig. 6B shows the effects of ursodeoxycholic acid on the expression of iNOS protein stimulated by lipopolysaccharide plus interferon-γ by using Western blot analyses with a monoclonal antibody raised against human iNOS. Incubation with ursodeoxycholic acid (100–500 μM) significantly reduced the expression of iNOS protein, adjusted to the internal control (β-actin), as compared with the lipopolysaccharide plus interferon-γ-stimulated cells in the absence of ursodeoxycholic acid (0.64 ± 0.23 ($n = 3$, $P < 0.01$) at 100 μM, 0.33 ± 0.14 ($n = 3$, $P < 0.01$) at 300 μM, and 0.21 ± 0.18 ($n = 3$, $P < 0.01$) at 500 μM).

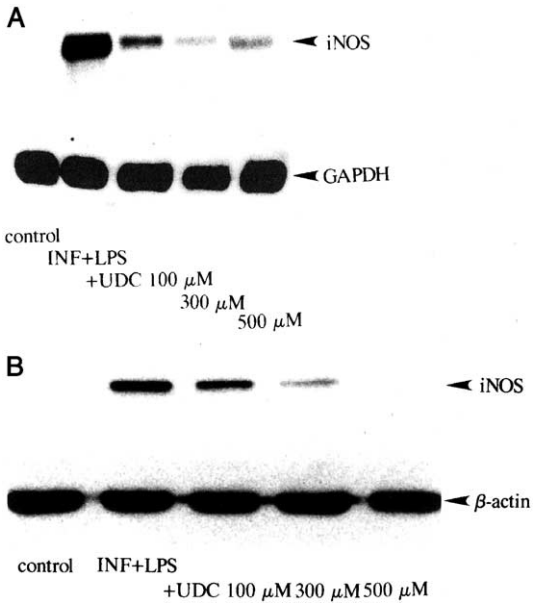

Fig. 6. Inhibitory effects of ursodeoxycholic acid (UDC) on lipopolysaccharide (LPS) plus interferon (INF)-γ-induced iNOS mRNA and protein expression in rat aortic smooth muscle cells. Typical Northern blot analysis for iNOS mRNA expression (A) and Western blotting analysis for iNOS protein (B) are shown in control cells (not stimulated with LPS plus INF-γ), cells stimulated with LPS (10 μg/ml) plus INF-γ (100 U/ml) for 24 h without or with different concentrations of UDC (100–500 μM). The expression of iNOS mRNA was compared with that of GAPDH mRNA. The expression of iNOS protein was compared with that of β-actin.

4. Discussion

The results of the present study indicate that ursodeoxycholic acid inhibits the induction of iNOS protein and the resultant synthesis of nitric oxide induced by lipopolysaccharide plus interferon-γ in vascular smooth muscle cells. The inhibitory effects of ursodeoxycholic acid on iNOS have previously been reported in other cell types (Hattori et al., 1996; Invernizzi et al., 1997), but this is the first study showing an inhibitory effect in vascular smooth muscle cells, both in aortic and rabbit coronary artery smooth muscle cells. Lipopolysaccharide plus interferon-γ induced the expression of iNOS protein, as shown by Western blot analysis, and the resultant production of nitric oxide in rat aortic smooth muscle cells, as reported previously (Hong et al., 2000). Similar findings were obtained for coronary artery smooth muscle cells isolated from the rabbit. Ursodeoxycholic acid at concentrations ranging from 30 to 1000 μM significantly decreased nitric oxide production induced by lipopolysaccharide plus interferon-γ. Ursodeoxycholic acid showed small inhibitory effects on nitric oxide production that had already been induced by lipopolysaccharide plus interferon-γ, which suggests that ursodeoxycholic acid interferes with the induction of iNOS rather than with its catalytic activity. Actually, ursodeoxycholic acid concentration dependently suppressed the expression of iNOS mRNA and protein. Thus, it is likely that ursodeoxycholic acid attenuated nitric oxide production by inhibiting the expression of the iNOS gene in vascular smooth muscle cells.

Several mechanisms may be proposed for the inhibitory effects of ursodeoxycholic acid. Bile acids are known to induce cell damage because of their detergent property. In general, the cytotoxic effects induced by bile acids increase with increasing hydrophobicity of the bile acid species (chenodeoxycholic acid, deoxycholic acid>ursodeoxycholic acid). However, in the present study, ursodeoxycholic acid had the most potent inhibitory effects on nitric oxide production in vascular smooth muscle cells, compared with the hydrophobic bile acids such as chenodeoxycholic acid and deoxycholic acid. Thus, it is unlikely that ursodeoxycholic acid inhibits iNOS via cytotoxic effects. Several bile acids, such as taurochenodeoxycholic acid, have been reported to inhibit the binding of cytokines, such as tumor necrosis factor-α, at high concentrations of 0.5–1 mM (Saitoh et al., 1998). However, the inhibitory effects of ursodeoxycholic acid were observed at much lower concentrations, and taurochenodeoxycholic acid and tauroursodeoxycholic acid failed to inhibit nitric oxide production

induced by lipopolysaccharide plus interferon-γ, suggesting that inhibitory effects of bile acids on cytokine binding to the cells are unlikely. We have reported that chenodeoxycholic acid and deoxycholic acid increased intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$), resulting in an increase in nitric oxide production in endothelial cells (Nakajima et al., 2000; Chisaki et al., 2001). However, ursodeoxycholic acid at concentrations of 100–300 μM did not significantly increase $[Ca^{2+}]_i$ (Nakajima et al., 2000; Chisaki et al., 2001). In addition, ursodeoxycholic acid was the most potent inhibitor of iNOS among the bile acids tested, suggesting that the cytosolic calcium ion does not play an important role in the effects of ursodeoxycholic acid on iNOS. Ursodeoxycholic acid and taurochenodeoxycholic acid have been reported to inhibit cyclic AMP production in hamster hepatocytes (Bouscarel et al., 1995). However, it is also unlikely that the inhibitory effect of ursodeoxycholic acid is mediated by cyclic AMP, since taurochenodeoxycholic acid did not inhibit nitric oxide production. Alternatively, the induction of iNOS appears to be dependent on the activation of nuclear factor κB, and steroid hormones such as cortisol reportedly inhibit the activation of iNOS via nuclear factor κB (Katsuyama et al., 1999). Recently, ursodeoxycholic acid has been demonstrated to activate the glucocorticoid receptor in a human hepatoma cell line (Tanaka and Makino, 1992; Tanaka et al., 1996). Thus, similar mechanisms may be involved in the effects of ursodeoxycholic acid on iNOS, but further studies are needed to clarify the basic mechanisms underlying the effects of ursodeoxycholic acid on iNOS in vascular smooth muscle cells.

The inhibitory effects of ursodeoxycholic acid were observed at concentrations of 30–100 μM in the present study. 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; Setchell et al., 1997; Kita et al., 1999) and to reach approximately 50 μM in patients with liver cirrhosis receiving ursodeoxycholic acid therapy (Chisaki et al., 2001), suggesting that therapeutic concentrations of ursodeoxycholic acid can affect iNOS in vascular smooth muscle cells. In addition, ursodeoxycholic acid inhibited the expression of iNOS induced by cytokines and bacterial lipopolysaccharide, but it had a small inhibitory effect on already activated iNOS, suggesting that ursodeoxycholic acid therapy may have a greater effect in preventing inflammation than in inhibiting pre-existing inflammatory conditions. However, further in vivo studies are needed to clarify this possibility.

Induction of iNOS has been suggested to contribute to the pathophysiological mechanism of a variety of cardiovascular diseases, such as endotoxin shock (Thiemermann and Vane, 1990; Nava et al., 1991) and atherosclerosis, including transplant atherosclerosis and balloon-injured artery (Hansson et al., 1994; Akyurek et al., 1996; Buttery et al., 1996; Lafond-Walker et al., 1997; Yan and Hansson, 1998; Depre et al., 1999; Anderson et al., 2000). Endotoxemia in obstructive jaundice induces iNOS. The subsequent overproduction of nitric oxide may lead to impairment of autoregulation of the renal vascular bed, which may contribute to renal failure in obstructive jaundice (Inan et al., 1997). Also, when blood vessels are injured, as in atherosclerosis, balloon catheter injury, and cardiac allograft (Hansson et al., 1994; Akyurek et al., 1996; Buttery et al., 1996; Lafond-Walker et al., 1997; Yan and Hansson, 1998; Depre et al., 1999; Anderson et al., 2000), cytokines and other factors induce iNOS, which produces sustained high levels of nitric oxide that induce apoptosis (Nathan and Xie, 1994; Iwashina et al., 1998) and peroxynitrite is produced from iNOS, leading to cell necrosis and death of cells such as endothelial cells (Beckman and Koppenol, 1996). In humans, atherosclerotic lesions and coronary plaque strongly express iNOS (Hansson et al., 1994; Akyurek et al., 1996; Buttery et al., 1996; Lafond-Walker et al., 1997; Yan and Hansson, 1998; Depre et al., 1999; Anderson et al., 2000), causing damage to the endothelium. Therefore, inhibition of excessive nitric oxide production may provide some therapeutic benefits under these pathophysiological conditions. In fact, recent papers showed that adjuvant ursodeoxycholic acid treatment reduced the incidence of acute cardiac allograft rejection in humans (Bahrle et al., 1998). Furthermore, in an experimental transplantation model with cardiac allograft rats, prolonged graft survival under ursodeoxycholic acid treatment was demonstrated (Friman et al., 1992). Thus, the unique properties of ursodeoxycholic acid may make it a candidate for preventing the stimulation of iNOS activity in pathological conditions associated with excessive nitric oxide production, such as various cardiovascular diseases.

In conclusion, the present data show that a therapeutic concentration of ursodeoxycholic acid inhibits iNOS expression and the resultant nitric oxide production in rat aortic and rabbit coronary artery smooth muscle cells, thereby providing a possible mechanism for the cardiovascular protective effect of ursodeoxycholic acid under various pathophysiological conditions such as endotoxemia and atherosclerosis.

Acknowledgements

This work is partly supported by grants from the Ministry of Education, Science and Culture of Japan to T. Nakajima, and from the Ministry of Education, Science, Sports, and Culture of Japan (13670055, COE) to Y. Okuda.

References

Akyurek, L.M., Fellstrom, B.C., Yan, Z.Q., Hansson, G.K., Funa, K., Larsson, E., 1996. Inducible and endothelial nitric oxide synthase expression during development of transplant arteriosclerosis in rat aortic grafts. Am. J. Pathol. 149, 1981–1990.

Anderson, P.G., Boerth, N.J., Liu, M., McNamara, D.B., Cornwell, T.L., Lincoln, T.M., 2000. Cyclic GMP-dependent protein kinase expression in coronary arterial smooth muscle in response to balloon catheter injury. Arterioscler. Thromb. Vasc. Biol. 20, 2192–2197.

Bahrle, S., Szabo, G., Stiehl, A., Theilmann, L., Dengler, T.J., Zimmermann, R., Kubler, W., 1998. Adjuvant treatment with ursodeoxycholic acid may reduce the incidence of acute cardiac allograft rejection. J. Heart Lung Transplant. 17, 592–598.

Beckman, J.S., Koppenol, W.H., 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am. J. Physiol. 271, C1424–C1437.

Bouscarel, B., Gettys, T.W., Fromm, H., Dubner, H., 1995. Ursodeoxycholic acid inhibits glucagon-induced cAMP formation in hamster hepatocytes: a role for PKC. Am. J. Physiol. 268, G300–G310.

Buttery, L.D., Springall, D.R., Chester, A.H., Evans, T.J., Standfield, E.N., Parums, D.V., Yacoub, M.H., Polak, J.M., 1996. Inducible nitric oxide synthase is present within human atherosclerotic lesions and promotes the formation and activity of peroxynitrite. Lab. Invest. 75, 77–85.

Chisaki, K., Nakajima, T., Iwasawa, K., Iida, H., Matsumoto, A., Tada, M., Komatsu, Y., Hirose, K., Miyamoto, K., Okuda, Y., Shiratori, Y., Goto, A., Hirata, Y., Nagai, R., Omata, M., 2001. Enhancement of endothelial nitric oxide production by chenodeoxycholic acids in patients with hepatobiliary diseases. Jpn. Heart J. 42, 339–353.

Cirillo, N.W., Zwas, F.R., 1994. Ursodeoxycholic acid in the treatment of chronic liver disease. Am. J. Gastroenterol. 89, 1447–1452.

Depre, C., Havaux, X., Renkin, J., Vanoverschelde, J.L., Wijns, W., 1999. Expression of inducible nitric oxide synthase in human coronary atherosclerotic plaque. Cardiovasc. Res. 41, 465–472.

Earnest, D.L., Holubec, H., Wali, R.K., Jolley, C.S., Bissonette, M., Bhattacharyya, A.K., Roy, H., Khare, S., Brasitus, T.A., 1994. Chemoprevention of azoxymethane-induced colonic carcinogenesis by supplemental dietary ursodeoxycholic acid. Cancer Res. 54, 5071–5074.

Forstermann, U., Kleinert, H., 1995. Nitric oxide synthase: expression and expressional control of the three isoforms. Naunyn-Schmiedeberg's Arch. Pharmacol. 352, 351–364.

Friman, S., Mjornstedt, L., Persson, H., Karlberg, I., Olausson, M., 1992. Ursodeoxycholic acid reduces acute rejection in heart allografted rats. Transplant. Proc. 24, 344–345.

Fukuo, K., Inoue, T., Morimoto, S., Nakahashi, T., Yasuda, O., Kitano, S., Sasada, R., Ogihara, T., 1995. Nitric oxide mediates cytotoxicity and basic fibroblast growth factor release in cultured vascular smooth muscle cells. A possible mechanism of neovascularization in atherosclerotic plaques. J. Clin. Invest. 95, 669–676.

Hansson, G.K., Geng, Y.J., Holm, J., Hardhammar, P., Wennmalm, A., Jennische, E., 1994. Arterial smooth muscle cells express nitric oxide synthase in response to endothelial injury. J. Exp. Med. 180, 733–738.

Hattori, Y., Murakami, Y., Hattori, S., Kuroda, H., Kasai, K., Shimoda, S., 1996. Ursodeoxycholic acid inhibits the induction of nitric oxide synthase. Eur. J. Pharmacol. 300, 147–150.

Hong, Y., Suzuki, S., Yatoh, S., Mizutani, M., Nakajima, T., Bannai, S., Sato, H., Soma, M., Okuda, Y., Yamada, N., 2000. Effect of hypoxia on nitric oxide production and its synthase gene expression in rat smooth muscle cells. Biochem. Biophys. Res. Commun. 268, 329–332.

Inan, M., Sayek, I., Tel, B.C., Sahin-Erdemli, I., 1997. Role of endotoxin and nitric oxide in the pathogenesis of renal failure in obstructive jaundice. Br. J. Surg. 84, 943–947.

Invernizzi, P., Salzman, A.L., Szabo, C., Ueta, I., O'Connor, M., Setchell, K.D., 1997. Ursodeoxychololate inhibits induction of NOS in human intestinal epithelial cells and in vivo. Am. J. Physiol. 273, G131–G138.

Isner, J.M., Kearney, M., Bortman, S., Passeri, J., 1995. Apoptosis in human atherosclerosis and restenosis. Circulation 91, 2703–2711.

Iwashina, M., Shichiri, M., Marumo, F., Hirata, Y., 1998. Transfection of inducible nitric oxide synthase gene causes apoptosis in vascular smooth muscle cells. Circulation 98, 1212–1218.

Katsuyama, K., Shichiri, M., Kato, H., Imai, T., Marumo, F., Hirata, Y., 1999. Differential inhibitory actions by glucocorticoid and aspirin on cytokine-induced nitric oxide production in vascular smooth muscle cells. Endocrinology 140, 2183–2190.

Kita, Y., Sakakura, H., Hirata, M., Harihara, Y., Tanaka, H., Ito, M., Yoshino, H., Takayama, T., Kubota, K., Hashizume, K., Makuuchi, M., 1999. Ursodeoxycholic acid in serum and liver tissue in patients with end-stage cholestatic liver cirrhosis. Transplant. Proc. 31, 2897–2898.

Lafond-Walker, A., Chen, C.L., Augustine, S., Wu, T.C., Hruban, R.H., Lowenstein, C.J., 1997. Inducible nitric oxide synthase expression in coronary arteries of transplanted human hearts with accelerated graft arteriosclerosis. Am. J. Pathol. 151, 919–925.

Lee, W.Y., Han, S.H., Cho, T.S., Yoo, Y.H., Lee, S.M., 1999. Effect of ursodeoxycholic acid on ischemia/reperfusion injury in isolated rat heart. Arch. Pharm. Res. 22, 479–484.

Luketic, V.A., Sanyal, A.J., 1994. The current status of ursodeoxycholate in the treatment of chronic cholestatic liver disease. Gastroenterologist 2, 74–79.

Makino, I., Tanaka, H., 1998. From a choleretic to an immunomodulator: historical review of ursodeoxycholic acid as a medicament. J. Gastroenterol. Hepatol. 13, 659–664.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.

Nakajima, T., Okuda, Y., Chisaki, K., Shin, W.S., Iwasawa, K., Morita, T., Matsumoto, A., Suzuki, J., Suzuki, S., Yamada, N., Toyo-oka, T., Nagai, R., Omata, M., 2000. Bile acids increase intracellular Ca^{2+} concentration and nitric oxide production in vascular endothelial cells. Br. J. Pharmacol. 130, 1457–1467.

Nathan, C., 1992. Nitric oxide as a secretory product of mammalian cells. FASEB J. 6, 3051–3064.

Nathan, C., Xie, Q.W., 1994. Nitric oxide synthases: roles, tolls, and controls. Cell 78, 915–918.

Nava, E., Palmer, R.M., Moncada, S., 1991. Inhibition of nitric oxide synthesis in septic shock: how much is beneficial? Lancet 338, 1555–1557.

Saitoh, O., Nakagawa, K., Sugi, K., Matsuse, R., Uchida, K., Kojima, K., Tanaka, S., Teranishi, T., Hirata, I., Katsu, K., 1998. Bile acids inhibit tumor necrosis factor α-induced interleukin-8 production in human colon epithelial cells. J. Gastroenterol. Hepatol. 13, 1212–1217.

Setchell, K.D., Rodrigues, C.M., Clerici, C., Solinas, A., Morelli, A., Gartung, C., Boyer, J., 1997. Bile acid concentrations in human and rat liver tissue and in hepatocyte nuclei. Gastroenterology 112, 226–235.

Stiehl, A., Rudolph, G., Raedsch, R., Moller, B., Hopf, U., Lotterer, E., Bircher, J., Folsch, U., Klaus, J., Endele, R., 1990. Ursodeoxycholic acid-induced changes of plasma and urinary bile acids in primary biliary cirrhosis. Hepatology 12, 492–497.

Tanaka, H., Makino, I., 1992. Ursodeoxycholic acid-dependent activation of the glucocorticoid receptor. Biochem. Biophys. Res. Commun. 188, 942–948.

Tanaka, H., Makino, Y., Miura, T., Hirano, F., Okamoto, K., Komuro, K., Sato, Y., Makino, I., 1996. Ligand-independent activation of the glucocorticoid receptor by ursodeoxycholic acid. Repression of INF-γ-induced MHC class II gene expression via a glucocorticoid receptor-dependent pathway. J. Immunol. 156, 1601–1608.

Thiemermann, C., Vane, J., 1990. Inhibition of nitric oxide synthesis reduces the hypotension induced by bacterial lipopolysaccharides in the rat in vivo. Eur. J. Pharmacol. 182, 591–595.

White, C.R., Brock, T.A., Chang, L.Y., Crapo, J., Briscoe, P., Ku, D., Bradley, W.A., Gianturco, S.H., Gore, J., Freeman, B.A., Tarpey, M., 1994. Superoxide and peroxynitrite in atherosclerosis. Proc. Natl. Acad. Sci. U. S. A. 91, 1044–1048.

Yan, Z., Hansson, G.K., 1998. Overexpression by inducible nitric oxide synthase by neointimal smooth muscle cells. Circ. Res. 82, 21–29.