Biochemical Pharmacology, Vol. 59, pp. 719–726, 2000.

ELSEVIER

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(99)00358-5

Inhibitory Effects of Aclarubicin on Nitric Oxide Production in Aortic Smooth Muscle Cells and Macrophages

Ichiro Wakabayashi,† Bernd Mayer‡ and Klaus Groschner‡*

*Department of Hygiene and Preventive Medicine, School of Medicine, Yamagata University, Yamagata 990-9585, Japan; and ‡Karl-Franzens-Universität Graz, Department of Pharmacology and Toxicology, A-8010 Graz, Austria

ABSTRACT. The effects of aclarubicin (ACR), an anthracycline antibiotic, on inducible nitric oxide (NO) synthesis was investigated in rat aortic smooth muscle cells (RASMCs) and RAW macrophages. ACR at concentrations as low as 0.1 μM significantly inhibited NO production induced by interleukin-1β in RASMCs. About 5- to 10-fold higher concentrations of ACR were required for inhibition of interferon-γ and lipopolysaccharide-induced NO production in RAW cells. When ACR was subsequently administered to inducible NO synthase (iNOS) induction, the NO production was barely suppressed in RASMCs. Moreover, ACR (up to 10 μM) lacked direct inhibitory effects on iNOS activity in homogenates of these cells. ACR (0.1 μM) inhibited the expression of iNOS protein and mRNA in RASMCs without concomitant cytotoxic effects. ACR (>0.5 μM)-induced inhibition of NO production in RAW cells was associated with substantial cytotoxic effects as shown by measurement of lactate dehydrogenase release. These results suggest that ACR is a potent inhibitor of iNOS induction in vascular smooth muscle, but inhibits iNOS induction in macrophage only at high cytotoxic concentrations. BIOCHEM PHARMACOL **59**;6:719–726, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. aclarubicin; vascular smooth muscle cell; macrophage; inducible nitric oxide synthase; gene transcription; cytotoxicity

Anthracycline antibiotics are potent antineoplastic drugs that are widely used in the therapy of hematological malignancies such as leukemia and malignant lymphoma. Myelosuppression is a common side-effect which restricts the therapeutic dose of anthracyclines [1]. Moreover, as disorder of bone marrow function is a typical complication of hematological malignancies, severe infection due to leukopenia eventually occurs during chemotherapy. Consequently, sepsis is one of the most frequent causes of death during the therapy of blood malignancies [2]. Septic shock is a major cause of death during severe bacterial infection [3]. This circulatory failure is elicited by inflammatory cytokines such as IFN-γ§ and IL-1β, which induce iNOS expression in vascular smooth muscle cells as well as in macrophages. Excess production of NO from these cells causes a profound reduction of arterial tone, resulting in a severe drop in blood pressure and shock [4–6]. Nonetheless, NO and reactive nitrogen intermediates are known to exert cytocidal effects on microorganisms [7–9] and to play a pivotal role in the function of macrophages. NO production in macrophages is considered a central mechanism in resistance against bacterial infections [8, 10]. Thus, any drug-induced modulation of NO production appears of particular significance with respect to therapy of hematological malignancies.

Previous studies showed that anthracycline derivatives such as daunorubicin, doxorubicin, and ACR inhibit endothelium-dependent relaxation in rat aorta [11–13]. Moreover, a recent study has demonstrated that doxorubicin and ACR inhibit the activity of constitutive NO synthase isolated from rat cerebella [14]. It was therefore of interest to elucidate whether these drugs also interfere with iNOS-mediated NO production and to compare the effects of ACR on NO production by iNOS in vascular smooth muscle cells and in macrophages.

MATERIALS AND METHODS

Animals

Male Wistar rats (10–12 weeks old) were housed in clear plastic cages, fed normal rat chow *ad lib.*, and given free access to water. The care and use of the laboratory animals

† Corresponding author: Ichiro Wakabayashi, M.D., Department of Hygiene and Preventive Medicine, School of Medicine, Yamagata University, 2-2-2, Iida-Nishi, Yamagata 990-9585, Japan. Tel. 81-23-628-5252; FAX 81-23-642-7378; E-mail: wakabaya@med.id.yamagata-u.ac.jp

§ *Abbreviations:* ACR, aclarubicin; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible NO synthase; L-NMMA, N^G-monomethyl-L-arginine; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; NO, nitric oxide; NOS, nitric oxide synthase; RASMCs, rat aortic smooth muscle cells; PCR, polymerase chain reaction; RT–PCR, reverse transcription–polymerase chain reaction; and TBS, Tris-buffered saline.

Received 4 February 1999; accepted 8 September 1999.

were in accordance with guidelines established by the Yamagata University School of Medicine.

Preparation of RASMCs

The rats were anesthetized with sodium pentobarbital (50 mg/kg body weight) and then killed by exsanguination. RASMCs were isolated from the thoracic aorta of male Wistar rats (10–12 weeks old) by outgrowth of explants according to the method of Ross [15]. Briefly, segments of thoracic aorta were dissected and placed on 60-mm plastic dishes containing Ca^{2+}, Mg^{2+}-free PBS. Subsequently, fat and connective tissues were removed carefully. After longitudinal dissection, the intima was removed by a scraper. Then 1- to 2-mm^2 sections of the media were prepared and transferred to 35-mm wells in a 6-well plate containing Dulbecco's modified Eagle's medium with 5% FBS, 4 mM glutamate, 100 U/mL penicillin, and 100 μg/mL streptomycin. The dishes were incubated in a humidified atmosphere at 37° under 5% CO_2–95% air. After 2–3 weeks of incubation, cell layers showing the hill-and-valley pattern characteristic of smooth muscle cells grew to confluency. Cells from passages 15–30 were used for experiments.

Cell Culture

RAW 264.7 murine macrophages and RASMCs were cultured in Dulbecco's modified Eagle's medium containing 5% FBS, 4 mM glutamate, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified atmosphere at 37° under 5% CO_2–95% air. The cells were spread in 24- or 6-well culture plates and cultured until confluency. Then, confluent cells were used for the assays. iNOS was induced in RASMCs by IL-1β (2 ng/mL) and in RAW cells by IFN-γ (10 U/mL) plus LPS (1 μg/mL).

Measurement of NO Production

Production of NO was assayed by measuring the accumulation of nitrite in the culture medium by the Griess reaction using sodium nitrite as a standard. Aliquots of culture medium were mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid), the mixture was incubated at room temperature for 10 min, and the absorbance at 540 nm measured using a spectrophotometer. Standard curves were determined using known concentrations of sodium nitrite. In preliminary experiments, it was confirmed that ACR, at least up to 1 μM, did not interfere with the Griess reaction. The amount of net NO production after stimulation with LPS and/or the cytokine was calculated by subtracting the basal NO production from the total production, and the quantity thus determined was expressed as the % nitrite release relative to the control (cells treated with vehicle) or as the absolute value of nitrite (nmol) per mg protein of the cell lysates. Basal levels of NO production (in the absence of any stimulation) were 3.62 ± 0.30 and 5.25 ± 0.39 nmol/mg protein in RASMCs and RAW cells, respectively. L-NMMA, a classical and potent inhibitor of NO synthase, was used to demonstrate that NO production stimulated by LPS and/or the cytokine was due to NO synthase activation (Fig. 1) and to compare the effect of complete inhibition of NO synthase by L-NMMA with the effects of ACR (Fig. 2).

Measurement of LDH Activity

LDH release into the cell culture medium was used to assess the cytotoxic effect of ACR. LDH was measured using a commercially available kit (Liquitech LDH, Boehringer Mannheim). Briefly, 10 μL of the sample was added to 350 μL of a solution containing 0.6 mM pyruvate and 50 mM phosphate buffer (pH 7.5), and incubated at 37° for 5 min. Next, 70 μL of 0.18 mM NADH solution was added and incubated for a further 5 min. Then, absorbance at 340 nm was measured by a spectrophotometer.

Measurement of iNOS Activity

iNOS activity was measured in cell homogenates by monitoring the formation of L-[2,3,4,5-^{3}H]citrulline from L-[2,3,4,5-^{3}H]arginine as described previously [16]. Cells were centrifuged and washed with PBS. The cell pellet was resuspended in a buffer containing 50 mM triethanolamine plus 10 mM mercaptoethanol at pH 7.4 (300 μL/sample) and sonicated for 30 sec (3 times 10 sec). Incubations were performed for 10 min at 37° in 0.1 mL of 50 mM triethanolamine/HCl buffer, pH 7.4, containing 20 μL of cell homogenate, 10 μM L-[2,3,4,5-^{3}H]arginine, 0.2 mM NADPH, 10 μM H_4 biopterin, 5 μM flavin adenine dinucleotide, 5 μM flavin mononucleotide and 0.2 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS).

Protein Separation and Western Blotting

RASMCs were cultured in a 6-well plate. Following incubation with the requisite treatments, the culture medium was removed, and the cells were washed twice in cold PBS, then solubilized in hot (70°) SDS–PAGE sample buffer (0.625 M Tris–HCl, pH 6.8, 5% SDS, 25% β-mercaptoethanol, 50% glycerol, 0.025% bromophenol blue). The samples were dispersed by repeated passage through a 25G needle and then transferred to Eppendorf tubes. The samples were boiled for 10 min and then stored at −80° until analysis. Aliquots (20 μg protein) were subjected to SDS–PAGE on 7.5% polyacrylamide slab gels and then blotted onto polyvinylidene difluoride membrane. Polyvinylidene difluoride blots were blocked for 1 hr in 150 mM NaCl, 20 mM Tris (TBS, pH 7.5), containing 5% non-fat milk, and then incubated overnight at 4° with anti-(mouse iNOS) immunoglobulin G (1:10,000 dilution) in TBS containing 5% non-fat milk. The membrane was then

washed in TBS and incubated with goat anti-mouse alkaline phosphatase-conjugated antibody (1:3000 dilution) for 1.5 hr. After further washing with TBS, blots were detected by the enhanced chemiluminescence method using an immunoblot assay kit (Bio-Rad).

RNA Isolation and RT–PCR Analysis

RASMCs were cultured in a 24-well plate. Following incubation with the requisite treatments, the culture medium was removed, and the cells were washed once in PBS, then scraped and collected by centrifugation. Total RNA was prepared from RASMCs using Isogen (Nippon Gene Co., Ltd.). The quality and yield of the RNA were assessed by the 260/280-nm optical density ratio. Total RNA (0.5 μg) from each sample was reverse-transcribed (RT) into complementary DNA (cDNA) using a commercial kit (Rever Tra Dash™ from Toyobo Co., Ltd.). The PCR amplification for iNOS and GAPDH was carried out in an automatic DNA thermal cycler (PCR Thermal Cycler PERSONAL TP240, Takara Shuzo Co., Ltd.) using Taq DNA polymerase and oligonucleotide primers. The sequence of the iNOS-specific primers was 5′-TCGAGCCCTGGAAGACCCACATCTG (sense) and 5′-GTTGTTCCTCTTCCAAGGTGTTTGCCTTAT (antisense). The sequence of the GAPDH-specific primers was 5′-ACCACAGTCCATGCCATCAC (sense) and 5′-TCCACCACCCTGTTGCTGTA (antisense). The PCR amplification of iNOS and GAPDH was carried out for each 30 cycles at 94° for 1 min, 70° for 2 min, and 72° for 3 min for iNOS and at 98° for 10 sec, 60° for 2 sec, and 74° for 30 sec for GAPDH. The PCR products were size-fractioned by agarose (2%) gel electrophoresis, stained with ethidium bromide, and visualized by using an ultraviolet transilluminator. The intensity of the iNOS- and GAPDH-specific bands was quantified by densitometry, and the data were expressed as the iNOS/GAPDH ratio.

Measurement of Cell Proliferation

Cells were spread to a 24-well dish at a density of 5×10^4/well. Twenty-four hours later, the medium was replaced with that containing 0.5% BSA instead of FBS. After 48 hr incubation, the medium was replaced with 1% FBS-containing medium, supplemented with ACR at various concentrations or vehicle (controls). [^{3}H]Thymidine (0.125 μCi/well) was added to each well. After further incubation for 24 hr, the cells were washed twice with 1 mL of PBS and then incubated with ice-cold 5% trichloroacetic acid for 10 min and dissolved in 1 N NaOH. This solution was finally neutralized by addition of 2N HCl, and the radioactivity was counted with a liquid scintillation analyzer.

Protein Determination

The cells were solubilized in 1 N NaOH. After neutralization with 2 N HCl, the protein concentration of the cell lysates was determined using Bradford reagent (Bio-Rad) with BSA as a standard.

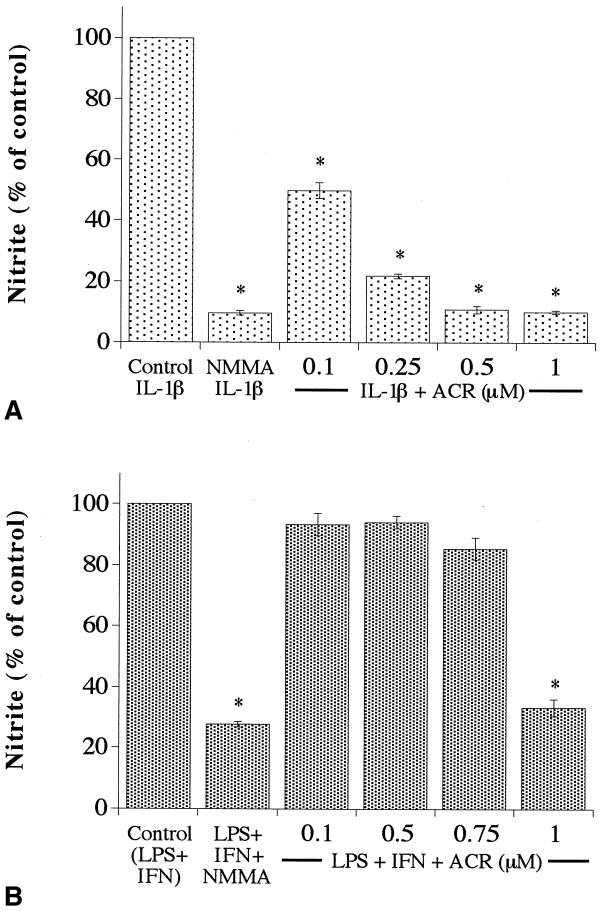

FIG. 1. Effects of ACR and L-NMMA on NO production in RASMCs (A) and RAW cells (B). NO production was assessed by measuring the nitrite accumulation in the culture medium at 24 hr after stimulating RASMCs with IL-1β (2 ng/mL) and RAW cells with LPS (1 μg/mL) plus IFN-γ (10 U/mL). Various concentrations of ACR or L-NMMA (2 mM) were added at the start of the stimulation with LPS and/or the cytokine. NO production is expressed as % of the control (incubation with vehicle). The mean actual values of the controls in A and B are 206.4 ± 10.5 and 320.2 ± 14.2 nmol/mg protein, respectively. Asterisks denote significant differences compared to the control ($P < 0.01$). The values are means ± SEM of 5 different experiments.

Chemicals

ACR hydrochloride (Wako) and L-NMMA and cycloheximide (both Sigma) were dissolved with PBS in solutions of 1, 100, and 1 mM, respectively, and stored at 4°. DRB (Sigma) was dissolved with dimethylsulfoxide in a solution of 100 mM and stored at −20°. Stock solutions of IL-1β (recombinant human IL-1β, Genzyme), LPS (lipopolysaccharide from *Escherichia coli* 055:B5, Sigma), IFN-γ (recombinant mouse interferon-γ, Biosource International), and anti-iNOS antibody (Transduction Laboratories) were stored at −20°. The concentration of each drug was expressed as the final concentration in the well.

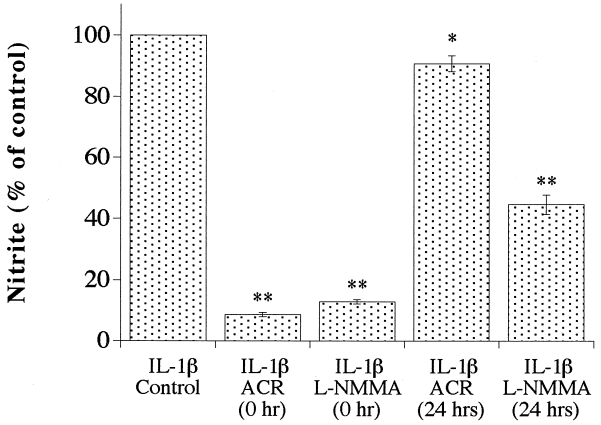

FIG. 2. Inhibition of IL-1β-induced NO production in RASMCs by ACR depends on the timing of incubation. RASMCs were stimulated with IL-1β (2 ng/mL). ACR (0.5 μM) or L-NMMA (2 mM) was added at 0 or 24 hr after the stimulation, and the nitrite levels of the supernatant were measured at 48 hr after the stimulation. Nitrite levels are expressed as % of the control (incubation with IL-1β plus vehicle). The mean absolute value of the control was 567.2 ± 29.5 nmol/mg protein. Asterisks denote significant differences compared to the control (*, $P < 0.05$; **, $P < 0.01$). The values are means ± SEM of 5 different experiments.

Statistics

Data are expressed as means ± SEM. Analysis of variance followed by Dunnett's *post hoc* test was used for statistical analysis. *P* values less than 0.05 were taken to be significant.

RESULTS

Inhibitory Effect of ACR on NO Production in RASMCs and RAW Cells

ACR concentration dependently (0.1–1 μM) inhibited the L-NMMA-sensitive NO production induced by IL-1β in RASMCs (Fig. 1A). Inhibition of IL-1β-induced NO production in RASMCs was statistically significant at concentrations as low as 0.1 μM. In contrast, ACR failed to inhibit the L-NMMA-sensitive, LPS plus IFN-γ-stimulated NO production in RAW cells up to 0.75 μM (Fig. 1B). Under these experimental conditions, significant inhibition of NO production in RAW cells required 1 μM ACR. When RAW cells were stimulated with LPS (1 μg/mL) alone, ACR at concentrations of 0.5 μM or more was required to significantly inhibit NO production (143.8 ± 10.1 [control] vs 121.9 ± 7.4 [0.5 μM ACR-treated] nmol/mg protein, $P < 0.05$).

Effects of ACR on iNOS Activity

Inhibition of NO production in RASMCs by ACR required that the antibiotic be administered before or during challenge of the cells with IL-1β. When ACR (0.5 μM) was added at a later time point (24 hr after stimulation with IL-1β), the antibiotic barely suppressed NO production in RASMCs. In contrast, the NOS inhibitor L-NMMA (2 μM) remarkably inhibited NO production when administered 24 hr after induction of iNOS expression (Fig. 2). Figure 3, A and B illustrates that ACR lacks the inhibitory effects of iNOS activity measured in cell homogenates of activated RASMCs and RAW cells, up to concentrations of 10 and 100 μM, respectively.

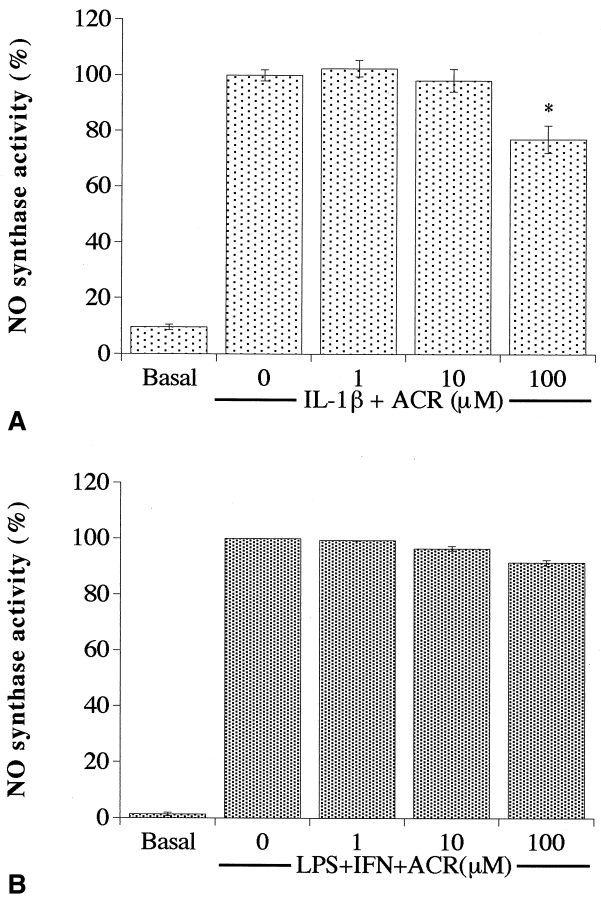

FIG. 3. Effects of ACR on iNOS activity in homogenates of activated RASMCs (A) and RAW cells (B). The mean absolute levels of NO synthase activity in the controls were 49.4 ± 20.2 pmol/min/mg protein in RASMCs and 66.6 ± 3.2 pmol/min/mg protein in RAW cells. An asterisk denotes significant difference compared to the control ($P < 0.05$). The values are means ± SEM of 4 different experiments.

Inhibitory Effect of ACR on iNOS Expression in RASMCs

Western blot experiments demonstrated that ACR (0.1–1 μM) suppressed IL-1β-stimulated iNOS synthesis (Fig. 4). Under basal conditions, iNOS expression was not detectable in RASMCs, as shown in lane a of Fig. 4. Activation of the cells with IL-1β (2 ng/mL) for 24 hr resulted in substantial expression of iNOS protein, as illustrated in lane b of Fig. 4. iNOS expression was concentration dependently suppressed by pretreatment of the cells with ACR (Fig. 4, lanes c–e). For comparison, an experiment with cycloheximide (1 μM), which

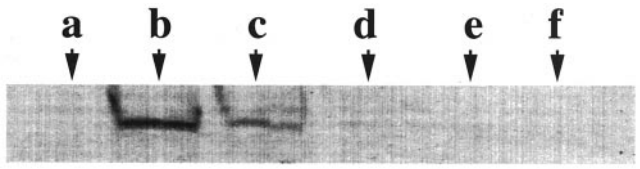

FIG. 4. ACR inhibits iNOS expression in RASMCs. Western blot analysis of iNOS expression in RASMCs incubated under different conditions: a, basal; b, IL-1β (2 ng/mL) + vehicle; c, IL-1β (2 ng/mL) + ACR (0.1 μM); d, IL-1β (2 ng/mL) + ACR (0.5 μM); e, IL-1β (2 ng/mL) + ACR (1 μM); f, IL-1β (2 ng/mL) + cycloheximide (1 μM).

completely inhibited iNOS expression, is shown in Fig. 4, lane f.

Inhibitory Effect of ACR on the mRNA Level of iNOS

The effect of ACR on the mRNA level of iNOS was determined by using semi-quantitative RT–PCR. ACR (0.1–1 μM) significantly and concentration dependently reduced the mRNA level of iNOS in RASMCs (Fig. 5, A and B).

Cytotoxicity of ACR

ACR failed to induce LDH release from RASMCs up to 0.5 μM, i.e. 5 times the minimum concentration required for significant inhibition of iNOS expression in these cells (Fig. 6A). ACR induced LDH release from RAW cells already at 0.5 μM (Fig. 6B), whereas 1 μM ACR was necessary to show cytotoxic effect in RASMCs (Fig. 6A).

Inhibitory Effect of ACR on Proliferation of RASMCs and RAW Cells

To assess the effect of ACR on cell proliferation, incorporation of [^{3}H]thymidine was measured. ACR concentration dependently suppressed proliferation of both RASMCs and RAW cells. [^{3}H]Thymidine incorporation into RASMCs was significantly suppressed by ACR at concentrations as

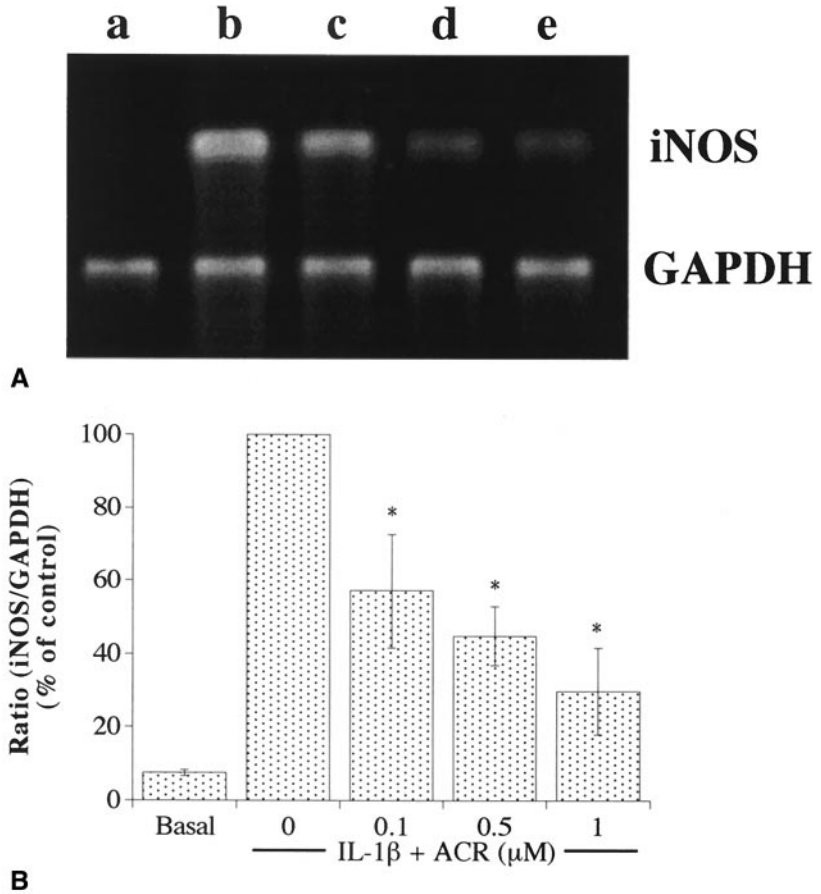

FIG. 5. ACR inhibits iNOS mRNA levels in RASMCs. (A) Representative gel photograph of PCR-amplified cDNA derived from iNOS and GAPDH mRNA in RASMCs cultured for 24 hr without IL-1β (a, basal) or with IL-1β (2 ng/mL) + vehicle (b, control), IL-1β (2 ng/mL) + ACR (0.1 μM) (c), IL-1β (2 ng/mL) + ACR (0.5 μM) (d), and IL-1β (2 ng/mL) + ACR (1 μM) (e). GAPDH was not increased by IL-1β with or without ACR. (B) Densitometric analysis of the gel photograph. The mRNA levels of iNOS and GAPDH on the gel photograph were quantified by densitometry. The results were expressed as a percentage of the IL-1β-stimulated control without ACR treatment for the ratio of iNOS to GAPDH. The values were means ± SEM of four different experiments. Asterisks denote significant differences compared to the control incubated with vehicle ($P < 0.01$).

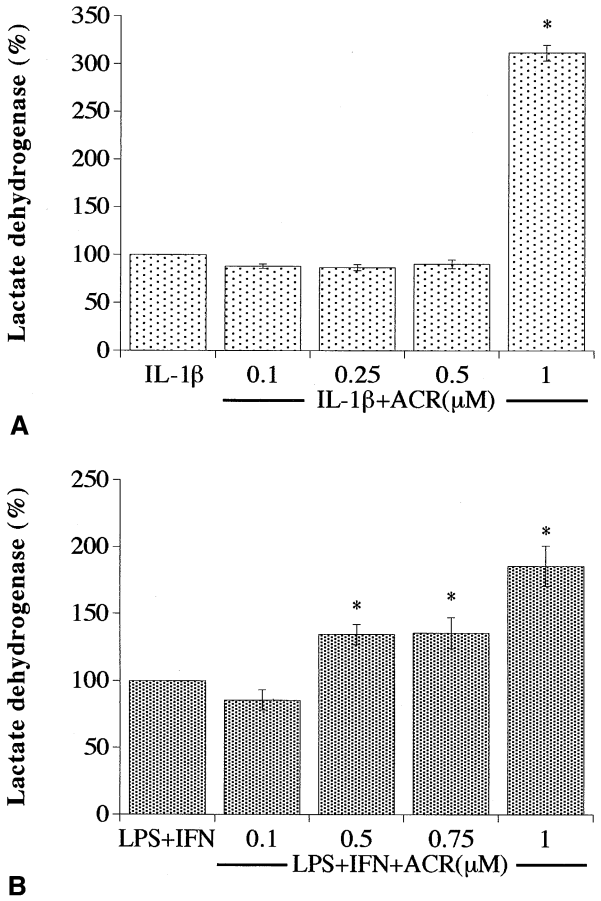

FIG. 6. Effects of ACR on LDH release from RASMCs (A) and RAW cells (B). LDH levels in the culture medium were measured at 24 hr after stimulating RASMCs with IL-1β (2 ng/mL) and RAW cells with LPS (1 μg/mL) plus IFN-γ (10 U/mL). LDH levels are expressed as % of the control (incubation with vehicle). The mean absolute LDH levels of the controls were 56.7 ± 4.8 IU/mg protein in RASMCs and 791 ± 69 IU/mg protein in RAW cells. Asterisks denote significant differences compared to the control incubated with vehicle ($P < 0.01$). The values are means ± SEM of 5 different experiments.

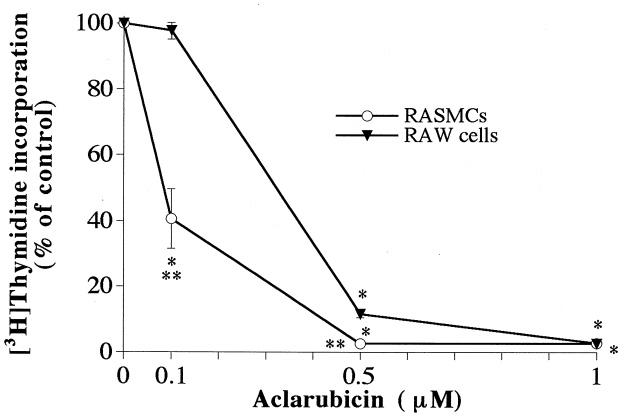

FIG. 7. Effects of ACR on [^{3}H]thymidine incorporation in RASMCs and RAW cells. Data are presented as % control values (incubation with vehicle). The mean absolute values of the controls in RASMCs and RAW cells were (837 ± 92) × 10^2 and (1463 ± 69) × 10^2 dpm/well, respectively. *, significantly different compared to the controls ($P < 0.01$); **, significantly different compared to the values in RAW cells ($P < 0.01$). The values are means ± SEM of 5 different experiments.

low as 0.1 μM, whereas 5-fold higher concentrations of ACR were required for inhibition of [^{3}H]thymidine incorporation into RAW cells (Fig. 7).

Inhibitory Effect of DRB on NO Production and iNOS Expression

DRB at 25–100 and 50–100 μM significantly inhibited NO production in RASMCs and RAW cells, respectively (Fig. 8A). The inhibitory effect of DRB at 25 and 50 μM on NO production was stronger in RASMCs than in RAW cells. iNOS expression was concentration dependently suppressed by pretreatment of RASMCs with DRB (25–100 μM) (Fig. 8B).

DISCUSSION

The present study demonstrates that ACR is a potent inhibitor of iNOS-mediated NO production in vascular smooth muscle, whereas NO production in macrophages is suppressed by this anthracycline antibiotic only at high concentrations. We provide evidence that the profound effect of ACR on vascular smooth muscle NO production is based on inhibition of protein expression. Our results show that ACR inhibits NO production in RASMCs at concentrations as low as 0.1 μM, i.e. at concentrations which clearly lack cytotoxic effects. In contrast, in macrophages, suppression of NO production by ACR required relatively high concentrations (>0.5 μM), which exerted significant cytotoxicity as evident from induction of LDH release from the cells. Thus, non-specific cytotoxicity may be involved in the inhibitory action of ACR on NO production in macrophages, but not in vascular smooth muscle.

A recent study by Luo and Vincent demonstrated that ACR inhibits neuronal NOS in a non-competitive manner with a K_i of 50 μM [14]. Previous experiments using rat aortic tissues showed that ACR decreased endothelium-dependent relaxation and concomitant increases in cyclic GMP levels [11]. Thus, this effect has been suggested to be due to inhibition of constitutive NOS in endothelial cells. However, to our knowledge, there is no direct evidence for inhibition of constitutive NOS in isolated/cultured endothelial cells by ACR. In the present study, direct inhibition of iNOS activity as a mechanism of inhibition of iNOS-mediated NO production in RASMCs appeared unlikely, since ACR was barely effective when administered 24 hr after induction of iNOS expression. Moreover, ACR failed to inhibit iNOS activity in homogenates of RASMCs up to a concentration of 10 μM, and no significant inhibition of iNOS activity in RAW cells was detected up to 100 μM. This prompted us to investigate the effects of ACR on expression of iNOS. Western blotting experiments demonstrated that expression of iNOS in activated RASMCs was indeed substantially reduced by ACR.

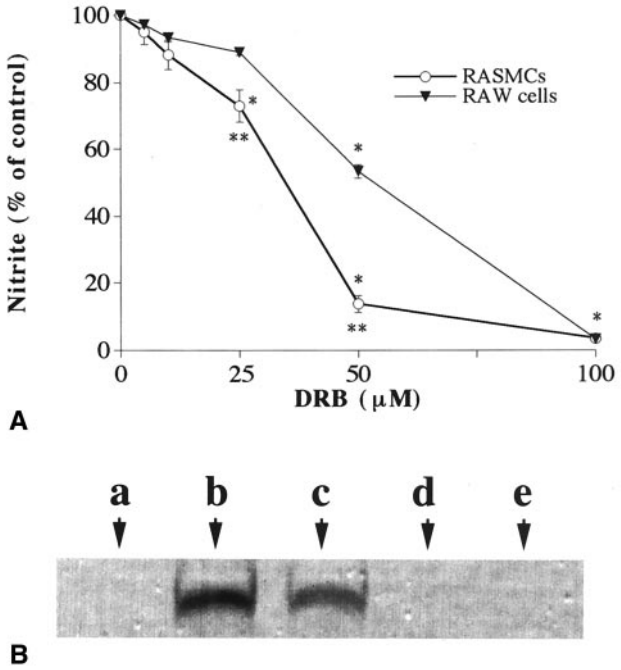

FIG. 8. A. Effects of DRB on NO production in RASMCs and RAW cells. NO production was assessed by measuring the nitrite accumulation in the culture medium at 24 hr after stimulating RASMCs with IL-1β (2 ng/mL) and RAW cells with LPS (1 μg/mL) plus IFN-γ (10 U/mL). DRB or vehicle (0.1% dimethylsulfoxide) was added to the medium simultaneously with the stimulation. NO production is expressed as % of the control (incubation with vehicle). The mean actual values of the controls in RASMCs and RAW cells were 210.4 ± 9.9 and 230.4 ± 19.0 nmol/mg protein, respectively. *, significantly different compared to the controls ($P < 0.01$); **, significantly different compared to the values in RAW cells ($P < 0.01$). The values are means ± SEM of 5 different experiments. B. DRB inhibits iNOS expression in RASMCs. Western blot analysis of iNOS expression in RASMCs incubated under different conditions: a, basal; b, IL-1β (2 ng/mL) + vehicle; c, IL-1β (2 ng/mL) + DRB (25 μM); d, IL-1β (2 ng/mL) + DRB (50 μM); e, IL-1β (2 ng/mL) + DRB (100 μM).

A recent study showed that doxorubicin (10 μM) inhibits the relaxation elicited by exogenous L-arginine in rat aortae pretreated with LPS, and that LPS-induced NO synthase activity of rat lung homogenate was reduced when the rats had been pretreated *in vivo* with doxorubicin [13]. Our present findings suggest that these effects of doxorubicin are due to inhibition of iNOS induction. Interestingly, high concentrations (>10 μM) of doxorubicin have been reported to stimulate NO production in EMT-6 cells, a murine breast cancer cell line [17], and elevated NOS activity in brain was observed after *in vivo* treatment of rats with daunorubicin [18]. Thus, anthracycline antibiotics may stimulate NOS activity at high concentrations via an as yet unknown mechanism. Nonetheless, the present study demonstrates unequivocally that iNOS expression in vascular smooth muscle is reduced significantly at submicromolar concentrations of ACR. Thus, this action of ACR may be considered of particular therapeutical relevance. The molecular mechanism of this action of ACR remains to be elucidated in detail. It is well established that ACR suppresses the growth of neoplastic cells by generating free radicals and inhibiting the catalytic activity of both type I and type II DNA topoisomerases, which regulate DNA topology during DNA replication, transcription, and repair [19–22]. In the present study, ACR significantly reduced the mRNA level of iNOS in RASMCs. Inhibition of NO production in both RASMCs and RAW cells was associated with reduced proliferation. RASMCs exhibited a higher sensitivity than RAW cells with respect to both ACR-induced suppression of proliferation and inhibition of iNOS expression. Our results, therefore, strongly suggest that ACR exerts the observed effects on NO signaling predominantly via interference with gene transcription. Moreover, DRB, a classical inhibitor of transcription, significantly inhibited NO production and iNOS expression. DRB mimicked the effects of ACR in that the inhibition was stronger in RASMCs than in RAW cells. Thus, these results are again in line with our suggestion that ACR may inhibit NO production at the transcription level.

In septic patients, excessive production of NO is involved in the pathogenesis of circulatory shock [4]. Since ACR inhibits iNOS induction preferentially in the vasculature, it might consequently stabilize circulation in these patients. On the other hand, NO production of macrophages at the infectious focus may be impaired at higher ACR concentrations, resulting in decreased resistance against infection [7]. Further studies including *in vivo* experiments are required to evaluate the clinical significance of ACR's effect on inducible NO synthesis during sepsis.

In conclusion, our study demonstrates that ACR is a potent inhibitor of iNOS induction in vascular smooth muscle. This action of ACR may be beneficial in therapy of septic patients since it moderates NO-mediated vasodepression.

This work was supported by the Austrian Research Foundation (SFB Biomembranes, F715 and P-11478 Med).

References

1. Hortobagyi GN, Anthracyclines in the treatment of cancer. An overview. *Drugs* **54**(Suppl 4): 1–7, 1997.
2. Jacobs P, Myelodysplasia and the leukemias. *Dis Mon* **43:** 505–597, 1997.
3. Parrillo JE, Parker MM, Natanson C, Suffredini AF, Danner RL, Cunnion RE and Ognibene FP, Septic shock in humans. Advances in the understanding of pathogenesis, cardiovascular dysfunction, and therapy. *Ann Intern Med* **113:** 227–242, 1990.
4. Moncada S, Palmer RM and Higgs EA, Nitric oxide: Physiology, pathophysiology, and pharmacology. *Pharmacol Rev* **43:** 109–142, 1991.
5. Thiemermann C, The role of the L-arginine: Nitric oxide pathway in circulatory shock. *Adv Pharmacol* **28:** 45–79, 1994.
6. MacMicking JD, Nathan C, Hom G, Chartrain N, Fletcher DS, Trumbauer M, Stevens K, Xie QW, Sokol K, Hutchinson

N, Chen H and Mudgett JS, Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* **81:** 641–650, 1995.
7. Nathan CF and Hibbs JB Jr, Role of nitric oxide synthesis in macrophage antimicrobial activity. *Curr Opin Immunol* **3:** 65–70, 1991.
8. Anthony LS, Morrissey PJ and Nano FE, Growth inhibition of *Francisella tularensis* live vaccine strain by IFN-γ-activated macrophages is mediated by reactive nitrogen intermediates derived from L-arginine metabolism. *J Immunol* **148:** 1829–1834, 1992.
9. Pacelli R, Wink DA, Cook JA, Krishna MC, DeGraff W, Friedman N, Tsokos M, Samuni A and Mitchell JB, Nitric oxide potentiates hydrogen peroxide-induced killing of *Escherichia coli*. *J Exp Med* **182:** 1469–1479, 1995.
10. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA and Bloom BR, An essential role for interferon γ in resistance to *Mycobacterium tuberculosis* infection. *J Exp Med* **178:** 2249–2254, 1993.
11. Wakabayashi I, Sakamoto K and Hatake K, Inhibitory effect of aclarubicin on endothelium-dependent relaxation of rat aorta. *Pharmacol Toxicol* **68:** 187–191, 1991.
12. Wakabayashi I, Hatake K, Yoshimoto S and Sakamoto K, Inhibitory effects of daunorubicin on endothelium-dependent vasorelaxing response to acetylcholine of rat aorta. *J Pharm Pharmacol* **46:** 296–299, 1994.
13. Sakai T, Muramatsu I, Hayashi N and Ishii Y, Inhibition of NO synthase induction by an anticancer drug 4′-epi-doxorubicin in rats. *Gen Pharmacol* **27:** 1367–1372, 1996.
14. Luo D and Vincent SR, Inhibition of nitric oxide synthase by antineoplastic anthracyclines. *Biochem Pharmacol* **47:** 2111–2112, 1994.
15. Ross R, The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J Cell Biol* **50:** 172–186, 1971.
16. Mayer B, Klatt P, Werner ER and Schmidt K, Molecular mechanisms of inhibition of porcine brain nitric oxide synthase by the antinociceptive drug 7-nitro-indazole. *Neuropharmacology* **33:** 1253–1259, 1994.
17. Lind DS, Kontaridis MI, Edwards PD, Josephs MD, Moldawer LL and Copeland EM III, Nitric oxide contributes to adriamycin's antitumor effect. *J Surg Res* **69:** 283–287, 1997.
18. Joshi P, Vig PJ, Veerisetty V, Cameron JA, Sekhon BS and Desaiah D, Increase in brain nitric oxide synthase activity in daunorubicin-treated rats. *Pharmacol Toxicol* **78:** 99–103, 1996.
19. Komiyama T, Kikuchi T and Sugiura Y, Interactions of anticancer quinone drugs, aclacinomycin A, adriamycin, carbazilquinone, and mitomycin C, with NADPH–cytochrome P-450 reductase, xanthine oxidase and oxygen. *J Pharmacobiodyn* **9:** 651–664, 1986.
20. Jensen PB, Sorensen BS, Demant EJ, Sehested M, Jensen PS, Vindelov L and Hansen HH, Antagonistic effect of aclarubicin on the cytotoxicity of etoposide and 4′-(9-acridinylamino) methanesulfon-*m*-anisidide in human small cell lung cancer cell lines and on topoisomerase II-mediated DNA cleavage. *Cancer Res* **50:** 3311–3316, 1990.
21. Chabner BA, Allegra CJ, Curt GA and Calabresi P, Antineoplastic agents. In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th ed. (Eds. Hardman JG, Limberd LE, Molinoff PB, Ruddon RW and Gilman AG), pp. 1233–1287. McGraw–Hill, Health Professions Division, New York, 1996.
22. Pommier Y, Pourquier P, Fan Y and Strumberg D, Mechanism of action of eukaryotic DNA topoisomerase I and drugs targeted to the enzyme. *Biochim Biophys Acta* **1400:** 83–106, 1998.