

Revisiting an Old Antimicrobial Drug: Amphotericin B Induces Interleukin-1- β -Converting Enzyme as the Main Factor for Inducible Nitric-Oxide Synthase Expression in Activated Endothelia

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ABSTRACT

We have investigated the impact of the widely used antifungal agent Amphotericin B (AmB) on cytokine activated aortic endothelial cells (AEC) and their inflammatory response as monitored by cytokine and inducible nitric-oxide synthase (iNOS) expression as well as high-output nitric oxide synthesis. Because both blood-borne infections and systemically administered drugs will first encounter vessel lining endothelial cells, this cell type represents an important participant in innate immune reactions against xenobiotics. Culturing cytokine-activated AEC in the presence of 1.25 $\mu\text{g/ml}$ AmB, a concentration equivalent to serum levels during patient treatment, we find increases in iNOS promoter activity up to 120%, in iNOS mRNA or protein expressions by factors of up to 3.5 ± 1.1 , and in iNOS activity of up to 180% compared with cells with cytokines

only. In parallel, a strong increase in endothelial interleukin (IL)-1 β -converting enzyme (ICE) and IL-1 β expression and activity was observed. Specific inhibition of ICE activity or IL-1 β functionality significantly reduces expression and activity of the iNOS to control values. Because ICE activity is essential for the endogenous synthesis of active IL-1 β , ICE overexpression represents the key signal in the AmB-induced and IL-1 β -mediated effects on iNOS activity. In summary, in endothelial cells, AmB strongly augments cytokine-induced iNOS expression and activity by increasing the expression and activity of the ICE. This adjuvant activity for augmented endogenous cytokine processing adds to the efficacy of the antimycotic activity of AmB. Furthermore, our data underline the relevance of the endothelial iNOS as a potent effector of the innate immune system.

Amphotericin B (AmB) is a highly efficient antifungal drug widely used for systemic infections (Gallis et al., 1990). Along with the therapeutic efficacy of AmB, serious toxicities are observed, among which nephrotoxicity as a result of AmB-induced renal arteriolar vasoconstriction (Reiner and Thompson, 1979; Sawaya et al., 1991) is the most important factor limiting its use (Carlson and Condon, 1994).

Despite its widespread use and clinical effectiveness, the mechanisms of AmB antibiotic actions are still under inves-

tigation. Previously, it has been observed that AmB potentiates the antimicrobial and tumoricidal activities of macrophages (Perfect et al., 1987), probably via induction of cytokine synthesis, such as tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β), as well as generation of a respiratory burst (Wolf and Massof, 1990). Antimicrobial defense of macrophages includes synthesis of reactive oxygen species and nitric oxide (NO) that contributes to the pathways by which cytokine- or endotoxin-activated inflammatory cells mediate cytotoxic defense against microbes (Nathan, 1992). Chemically generated NO or its derivatives (Alspaugh and Granger, 1991) as well as NO derived from macrophages

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ABBREVIATIONS: AmB, amphotericin B; TNF, tumor necrosis factor; IL, interleukin; iNOS, inducible nitric-oxide synthase; ICE, interleukin-1 β -converting enzyme; IFN, interferon; ELISA, enzyme-linked immunosorbent assay; LPS, lipopolysaccharide; eNOS, endothelial nitric-oxide synthase; FCS, fetal calf serum; RPMI, Roswell Park Memorial Institute; IEC, islet endothelial cells; AEC, aorta endothelial cells; DOC, desoxycholate; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EPR, electron paramagnetic resonance; kb, kilobase(s); RT, reverse transcription; NMA, *N*^G-monomethyl-L-arginine; YVAD, *N*-acetyl-Tyr-Val-Ala-Asp-aldehyde, an ICE inhibitor; ZVAD, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone pan-caspase inhibitor.

(Cameron et al., 1990), microglial cells (Blasi et al., 1995) or astrocytes (Lee et al., 1994) has been shown to be fungistatic or fungicidal. Recently, with murine macrophages, it has been shown that AmB-induced increase in proinflammatory cytokine secretion are responsible for augmenting their anticryptococcal activity through triggering the NO-dependent pathway (Tohyama et al., 1996).

The biochemical basis of NO-mediated effects involves inactivation of enzymes essential for energy metabolism and growth (James, 1995), inhibition of the DNA binding activity of several transcription factors (Kröncke et al., 1997), but also NO-mediated S-nitrosation of pathogenic viral, bacterial, fungal, and parasitic cysteine proteinases (Venturin et al., 2000), all of which may represent general mechanisms of antimicrobial and antiparasitic host defenses.

Endothelial cells display a capacity for cytokine synthesis similar to that of macrophages and represent an equivalent source for NO production by cytokine-induced expression of the inducible nitric-oxide synthase (iNOS) (Suschek et al., 1993). Because drugs administered via the circulation will encounter the blood vessel lining endothelial cells first before their transport into the tissue, we have focused on the impact of AmB on iNOS expression and activity of cytokine-activated endothelial cells. It has been shown that endothelial cell-derived, high-output NO synthesis exhibits antiapoptotic and cell-protective effects (Kim et al., 1999; Suschek et al., 1999). In addition, cytokine-activated and iNOS-expressing endothelial cells were shown to exert potent cytotoxic functions (Oswald et al., 1994; Fehsel et al., 1995; Steiner et al., 1997). Thus, although traditionally not considered part of the immune system, endothelial cells may play an important role in innate immunity and contribute to the resistance against pathogens.

We here have tested the effects of AmB on iNOS mRNA expression and enzyme activity as well as cytokine formation in primary cultures of endothelial cells. Our experiments give further evidence for the macrophage-like activity of endothelial cells in innate immune responses and demonstrate that in activated endothelial cells, AmB strongly augments nitric oxide production, exclusively because of augmented interleukin-1 β -converting enzyme (ICE) expression and activity, which, via increases in endogenous bioactive IL-1 β formation, further enhances iNOS expression and activity.

Materials and Methods

Reagents. Recombinant human IL-1 β and rat TNF- α was purchased from HBT (Leiden, Netherlands); recombinant human or murine γ -interferon (γ -IFN) and recombinant human TNF- α were purchased from Genzyme (Cambridge, MA). The ICE activity assay kit, polyclonal anti-human-IL-1 β and TNF- α antisera, monoclonal anti-rat-IL-1 β or TNF- α antibodies and the rat IL-1 β as well as rat TNF- α ELISA kits from were from R & D Systems (Wiesbaden, Germany). Endothelial cell growth supplement, LPS (from *Salmonella typhimurium*), Neutral Red (3% solution), type I collagen, collagenase (from *Clostridium histolyticum*), rabbit anti-human von Willebrand Factor antiserum, and anti- α -tubulin antibody were from Sigma-Aldrich (Deisenhofen, Germany). The rat endothelium specific monoclonal antibody Ox43 was from Serotec (Cameron, Wiesbaden, Germany). The monoclonal anti-iNOS and anti-eNOS antibodies were from Transduction Laboratories (Lexington, KY); peroxidase-conjugated porcine anti-rabbit IgG was from DAKO (Hamburg, Germany); peroxidase-conjugated goat anti-mouse IgG was from

Zymed Laboratories (South San Francisco, CA). Trypsin, EDTA, fetal calf serum (FCS, endotoxin free) and Luciferase Assay Kit were from Roche Applied Science (Mannheim, Germany); the caspase-inhibitor zVAD was from Enzyme Systems (Livermore, CA) and the ICE-inhibitor YVAD was from Alexis (Läufelfingen, Switzerland). RPMI 1640 (endotoxin free), oligo(dT16)-primer, Taq DNA polymerase, and amphotericin B (Fungizone) were from Invitrogen (Eggenstein, Germany); 3,3'-diaminobenzidine was from Serva GmbH (Heidelberg, Germany); and desoxycholate was from Squibb-Heyden (Munich, Germany).

Cell Cultures. Capillary islet endothelial cells (IEC) were isolated from hand-picked pancreatic islets by outgrowth on a collagen type I matrix as described previously (Suschek et al., 1994), whereas rat aorta endothelial cells (AEC) were isolated by outgrowth from rat aortic rings exactly as described previously (Suschek et al., 1994). Briefly, aortic segments or isolated whole islets were placed on top of a collagen gel (1.8 mg/ml collagen) in 24-well tissue culture plates and incubated in RPMI 1640 with 20% FCS and 100 μ g/ml endothelial cell growth supplement for 4 to 6 days depending on the degree of cellular outgrowth. Aortic explants or islets were then removed, cells were detached with 0.25% collagenase in Hanks' buffered saline solution and replated onto plastic culture dishes in RPMI 1640/20% FCS. Cells were subcultured for up to 10 passages, and removal from culture dishes for each passage was performed by treatment with 0.05% trypsin/0.02% EDTA in isotonic NaCl for 3 min. The human cell line A549/8 was purchased from the American Type Culture Collection (Manassas, VA).

Cellular Characterization of Cultured Cells. Rat endothelial cells (AEC, IEC) were characterized by using a cross reacting rabbit anti-human-von Willebrand Factor antiserum, the rat vascular endothelium-specific monoclonal antibody Ox43, the rat thymocyte- and brain endothelium-specific monoclonal antibody Ox2, and the respective secondary peroxidase-conjugated porcine anti-rabbit IgG or peroxidase-conjugated goat anti-mouse IgG antisera at conditions exactly as described previously (Suschek et al., 1994).

Rat IEC exhibited the phenotype vWF_{high} Ox2_{high} Ox43_{low}, whereas rat AEC showed the antigen phenotype vWF_{high} Ox2_{low} Ox43_{high}, exactly as published previously (Suschek et al., 1994). The labeling experiments also showed that the cell cultures consisted of pure cells, because the respective staining patterns with the endothelial cell cultures were found in virtually all cells (data not shown).

Experimental Design. All measurements were performed with cells from passages 2 to 8. Endothelial cells (1×10^5) were cultured in 12- or 24-well tissue culture plates in 600 μ l of RPMI 1640/20% FCS. Cytokine-challenge was performed by addition of IL-1 β , TNF- α , IFN- γ , or LPS at concentrations and combinations indicated, respectively. In addition, endothelial cell cultures were incubated for 24 to 48 h with AmB, the vehicle DOC, or the respective additives at concentrations indicated. LPS concentrations of RPMI 1640 and FCS were below 0.1 μ g/ml, and of cytokines, AmB, or DOC below 0.1 ng/ μ g, respectively.

Inhibition of Endogenous Endothelial IL-1 β or TNF- α Expression or Activity. Inhibition of endogenous bioactive IL-1 β formation was achieved using the pan-caspase inhibitor ZVAD (30 μ M) or the specific ICE inhibitor YVAD (30 μ M). Inhibition of endothelial IL-1 β activity or availability was achieved by addition of neutralizing anti-rat-IL-1 β antibodies [150 μ g/ml with a half-maximal neutralizing dose (ND₅₀) of 20 μ g/ml in the presence of \sim 1000 U/ml rat IL-1 β] or neutralizing anti-human-IL-1 β antibodies (100 μ g/ml with an ND₅₀ of 0.2 μ g/ml in the presence of \sim 500 U/ml recombinant human IL-1 β), respectively. Endogenous endothelial TNF- α production was inhibited by thalidomide (20 μ g/ml) (Wnendt et al., 1996; Sastry, 1999) or TNF- α activity was neutralized by addition of anti-rat-TNF- α antibodies (150 μ g/ml with an ND₅₀ of 25 μ g/ml in the presence of 500 U/ml rat TNF- α) or anti-human-TNF- α antibodies (25 μ g/ml with an ND₅₀ of 0.01 μ g/ml in the presence of 100 U/ml recombinant human TNF- α), respectively. Specificity, as well as the

neutralizing effectiveness of the anti-rat-TNF- α antibody, was characterized before its use (Table 1).

Nitrite Determination. In experiments examining the effects of AmB on endothelial high-output NO production by the iNOS, cells (1×10^5 in 600 μ l of medium) were preincubated with AmB and the respective cytokines or additives at concentrations indicated. Nitrite in culture supernatants accumulated during the last 24 h was determined using the diazotization reaction as described by Wood et al. (1990) and NaNO₂ as standard.

Growth Rates of Cell Cultures and Viability. Cell growth was determined at different times by Neutral Red staining. Cells were incubated for 90 min with Neutral Red (1:100 dilution of a 3% solution), washed twice with PBS, dried completely, and lysed with isopropanol containing 0.5% 1 N HCl. Extinctions of the supernatants, which show a linear correlation to the cell number, were then measured at 530 nm. Additionally, viability of endothelial cells was controlled routinely at the beginning and end of every experiment using the trypan blue exclusion assay.

Polymerase Chain Reaction (PCR). Total cellular RNA (1 μ g each) prepared from resting or cytokine-activated cells grown for 48 h in the presence or absence of AmB or DOC at concentrations indicated was used for cDNA synthesis using the dT16-oligonucleotide as primer. Reverse transcription was carried out at 42°C for 60 min. The cDNA (500 ng each) was used as template for PCR primed by using the oligonucleotides given in Table 2. PCR was carried out following standard protocols with the following cycle profiles: 25 cycles of 30 s at 94°C, 30 s at 60°C, 30 s at 72°C, and a final incubation step at 72°C for 10 min for GAPDH-cDNA amplification; 35 cycles of 30 s at 94°C, 30 s at 60°C, 45 s at 72°C, and a final incubation step at 72°C for 10 min for iNOS cDNA amplification; 30 cycles of 30 s at 94°C, 60 s at 60°C, 15 s at 72°C and a final incubation step at 72°C for 10 min for rat TNF- α cDNA amplification; 32 cycles

of 30 s at 94°C, 30 s at 58°C, 30 s at 72°C, and a final incubation step at 72°C for 10 min for IL-1 β cDNA amplification; 34 cycles with 30 s at 94°C, 30 s at 56°C, 30 s at 72°C, and a final incubation step at 72°C for 10 min for ICE cDNA amplification. Before PCR analysis, we routinely determine the relative amount of the respective specific amplification product at different PCR cycles and thus ensure that amplification conditions were always within the linear phase. In some experiments, aliquots of iNOS products were pooled with the GAPDH product and both were subjected to electrophoresis on 1.8% agarose gels. Bands were visualized by ethidium bromide staining. Densitometric analysis of the visualized amplification products was performed by using the Kodak software (Eastman Kodak, Stuttgart, Germany).

Sequence Analysis of the Amplified Rat mRNA Products. PCR products were purified via QIAGEN columns (QIAGEN, Hilden, Germany) and cycle sequenced with the ABI BigDye Terminator Kit (Applied Biosystems, Weiterstadt, Germany) using iNOS or IL-1 β forward and reverse amplification primers on an automated sequence analyzer (ABI 310 from Applied Biosystems).

Sequence analysis of the amplification products obtained from resting cells by priming with the iNOS-primer revealed a 100% homology with the published rat sequences (GenBank accession numbers AF085195, M98820, U14647, and L00981) of rat iNOS, rat IL-1 β , rat ICE, or rat TNF- α cDNA, respectively (data not shown).

Western Blot Analysis of the iNOS Protein. Resting or cytokine activated (200 U/ml IL-1 β) AEC (7×10^6) were incubated for 48 h in the absence or presence of 1.2, 2.5, or 5.0 μ g/ml AmB. Using the NuPAGE electrophoresis system (Invitrogen, Karlsruhe, Germany) endothelial iNOS protein expression was examined exactly as we described previously (Suschek et al., 2001). Briefly, cultures were washed, scraped from the dishes, lysed by the lauryl dodecyl sulfate sample buffer (4 \times), transferred to a microcentrifuge tube, and boiled for 5 min. Proteins (40 μ g per lane) were separated by electrophoresis in a 12%-Bis-Tris NuPAGE Novex precast polyacrylamide gel using the MOPS-SDS running buffer system under reducing conditions (500 mM dithiothreitol) and transferred to nitrocellulose membranes (Invitrogen) using the NuPAGE transfer buffer (25 mM Bis-Tris, 25 mM Bicine, 1 mM EDTA, and 20% methanol, pH 7.2) and following the manufacturer's instructions. Further incubations of the blots were: 2 h with blocking buffer (2% bovine serum albumin, 5% nonfat dry milk powder, 0.1% Tween 20 in PBS), 1 h at 37°C with a 1:2000 dilution of the monoclonal anti-iNOS antibody, and 1 h with a 1:2000 dilution of the secondary horseradish peroxidase-conjugated rabbit-anti-mouse-IgG antibody. Finally, blots were incubated for 5 min in enhanced chemiluminescence reagent (Pierce, Rockford, IL), and exposed to an autoradiographic film. To control equal loading of total protein in all lanes, blots were stained with a 1:2000 solution of the mouse anti- α -tubulin antibody. Otherwise, conditions were as described above. Densitometric analyses of the visualized iNOS protein or α -tubulin were performed by using the Kodak 1D software.

TABLE 1

Specificity-control and neutralizing capacity of the monoclonal anti-rat-TNF- α antibodies

Resting rat aorta endothelial cells (1×10^5) were incubated for 24 hours with IL-1 β (200 U/ml) and/or rat TNF- α (500 U/ml) or alternatively activated in the presence of 150 μ g/ml of the monoclonal anti-rat-TNF- α antibody. Then, nitrite concentrations were determined in culture supernatants using the Griess reaction. Values represent the mean \pm S.D. of three individual experiments.

	Nitrite	
	+ anti-TNF- α	
	μ M	
Resting	0.3 \pm 0.3	0.5 \pm 0.3
IL-1 β	2.2 \pm 0.6	2.8 \pm 1.0
TNF- α	0.5 \pm 0.3	0.7 \pm 0.2
IL-1 β + TNF- α	5.5 \pm 0.6 [#]	2.6 \pm 0.4*

*, $p < 0.001$ compared with respective cytokine activated cell cultures grown in the absence of the anti-rat-TNF- α antibody.

[#], $p < 0.001$ compared with IL-1 β only activated cell cultures.

TABLE 2

List of oligonucleotides used for rat iNOS-, rat IL-1 β -, rat TNF- α - or rat GAPDH-cDNA amplification

Species/Product		Sequence	Bases	GenBank Accession Number	Product Size (Bases)
Rat iNOS	Sense	5'-ATGCCCGATGGCACCATCAGA-3'	825-846	D14051	394
	Antisense	5'-TcTCCAGGCCCATCCTCCTGC-3'	1198-1218		
Rat IL-1 β	Sense	5'-ccaggatgaggacccaagca-3'	328-347	M98820	519
	Antisense	5'-tcccgaccattgtctgtttcc-3'	827-846		
Rat TNF- α	Sense	5'-TACTGAACTTCGGGGTGATTGGTCC-3'	4580-4604	L00981	295
	Antisense	5'-CAGCCTTGTCCTTGAAGAGAACC-3'	5761-5784		
Rat ICE	Sense	5'-GGAAGCAATTTATCAACTCAG-3'	29-49	U14647	807
	Antisense	5'-ATAACCTTGGGCTTGTCTTTC-3'	816-836		
GAPDH	Sense	5'-acagtccatgcatcactgc-3'	569-588	M32599	266
	Antisense	5'-gcctgcttcaccaccttctt-3'	815-834		
GAPDH	Sense	5'-caactacatggt ttacatgttcc-3'	153-175	M17851	416
	Antisense	5'-GGACTGTGGTCATGAGTCCT-3'	549-568		

Detection of Endothelial IL-1 β and TNF- α Production. Resting or cytokine-activated (200 U/ml IL-1 β) endothelial cells were cultured in the presence or absence of AmB or the TNF- α synthase inhibitor thalidomide at concentrations indicated. Thalidomide was present in culture supernatants 24 h before AmB challenge and for the 24 h after incubation with AmB or was present only during the 24 h of incubation with AmB. Then, in culture supernatants, the endothelial IL-1 β or TNF- α production was assayed using the specific ELISA kits exactly as recommended by manufacturer. The species-specific anti-rat-cytokine antibodies used in the test do not cross-react with human recombinant TNF- α that was used for cell activation in our experiments. Testing the specificity of the rat IL-1 β ELISA kit, we found 0.36% cross-reactivity with human IL-1 β . Regarding this problem, rat cell culture supernatants containing the added human IL-1 β were examined by ELISA in parallel to pure medium containing the same amount of the human cytokine, and values obtained from these controls were subtracted from the values obtained with the culture supernatants.

Detection of Endothelial ICE Activity. Endothelial ICE protein expression was determined as enzymatic activity of the caspase-1 protease using the caspase-1 colorimetric assay. The test is based on the principle that cells expressing ICE are first lysed to collect their intracellular contents. The cell lysate can then be tested for ICE activity by the addition of a ICE-specific peptide that is conjugated to the color reporter molecule *p*-nitroaniline. The cleavage of the peptide by the caspase releases the chromophore *p*-nitroaniline, which can be quantitated spectrophotometrically at a wavelength of 405 nm. The level of caspase enzymatic activity in the cell lysate is directly proportional to the color reaction.

Resting and cytokine-activated (single doses or combinations of 200 U/ml IL-1 β , 500 U/ml TNF- α , and 500 U/ml IFN- γ) endothelial cells (1×10^6) grown for 24 h in the presence or absence of 1.2 mg/ml AmB were harvested by trypsin treatment and collected by centrifugation in a conical tube at 250g for 10 min. The supernatant was gently removed and discarded, and the cell pellet was lysed by the addition of the lysis buffer. The protein content of the cell lysates was estimated using a protein determination assay (Pierce). The enzymatic reaction for caspase activity was carried out in a 96-well, flat-bottomed microplate that could be read with a microplate

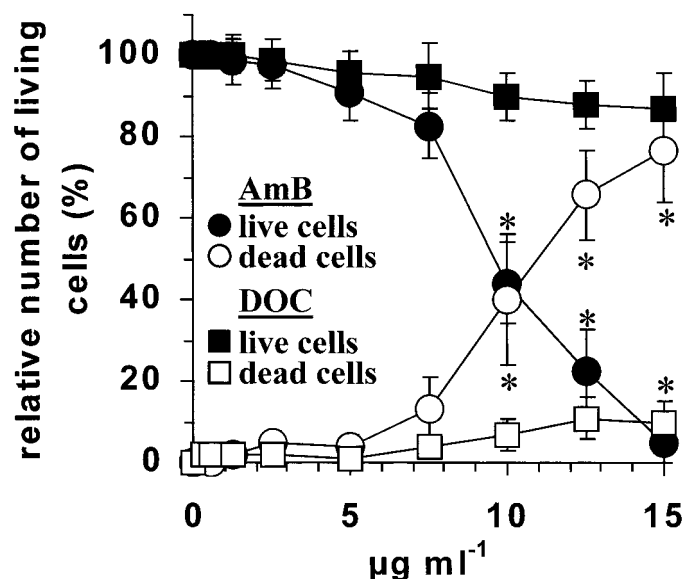


Fig. 1. Concentration-dependent cytotoxicity of amphotericin B. Cytokine-activated (200 U/ml IL-1 β) rat AEC (1×10^5) were incubated with AmB (●, ○) or the vehicle DOC (■, □) at concentrations indicated. After 48 h of incubation, the relative amount of live cells (● or ■, determined by Neutral Red) or dead cells (○ or □, determined by trypan blue) were determined, respectively. Values are the mean \pm S.D. of five individual experiments. *, $p < 0.001$ compared with the controls (0 μ g/ml AmB).

reader. If readings were not in the linear range of the instrument, assay was repeated with diluted cell lysates. Additional controls were probes with no cell lysate or no substrate, respectively. The results were expressed as -fold increase in caspase activity of AmB-treated cells over that grown in the absence of AmB.

EPR Spectrometry. For electron paramagnetic resonance (EPR) spectrometric analysis of AmB effects on iNOS activity, we used rat IEC because these cells, upon cytokine challenge, produce considerably more NO than other endothelial cell types (Suschek et al., 1994). IEC were cultured to near confluence in 10-cm dishes in RPMI 1640/20% FCS. Cells were then incubated with or without 500U/ml TNF- α for 48 h in the presence or absence of AmB (1.2 μ g/ml). Cells (1×10^7) were harvested by EDTA/trypsin treatment, washed twice with PBS, and transferred to quartz EPR tubes. After centrifugation (250g for 3 min), tubes were stored in liquid nitrogen. EPR spectra

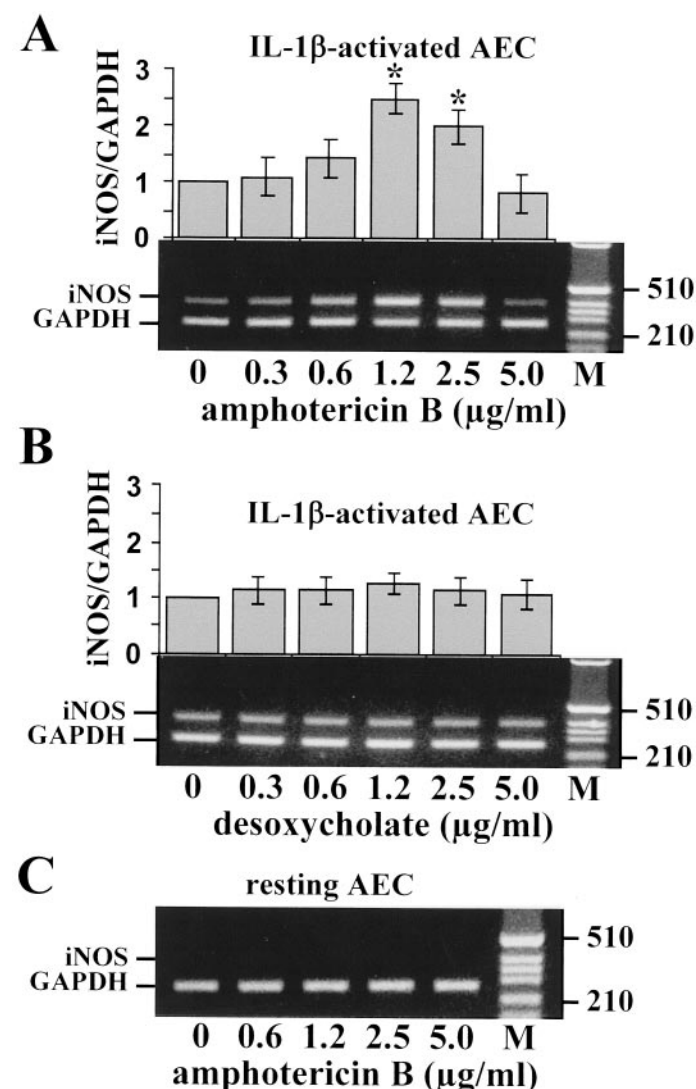


Fig. 2. Concentration-dependent effects of amphotericin B on iNOS-mRNA expression. Cytokine-activated (200 U/ml IL-1 β) rat AEC (1×10^5) were incubated with amphotericin B (A) or the vehicle desoxycholate (B) at concentrations indicated. After 48 h of incubation, iNOS- and GAPDH-mRNA expression was detected by RT-PCR. Bars represent the mean \pm S.D. of the iNOS/GAPDH ratio obtained by densitometric analysis of visualized amplification product-bands from four individual experiments. Values are normalized to the ratio obtained with cytokines only (0 μ g/ml AmB). C, Additionally, the effect of amphotericin B on iNOS-mRNA expression was examined in resting cells. *, $p < 0.001$ compared with the controls (iNOS-mRNA expression after 24-h culture in the presence of IL-1 β but the absence of AmB = 100%).

were taken using a computer controlled X-band spectrometer (ESP 300; Bruker, Karlsruhe, Germany). All samples were cooled to 90°K while recording the spectra. The machine parameters used were: 10 mW of microwave power, 100-kHz modulation frequency, 7.55-G

modulation amplitude, and a conversion time constant of 164 ms. The g-factor calibration was controlled using the 2,2'-diphenyl-1-picrylhydrazyl (DPH) radical signal at $g = 2.0036$.

Analysis of the Human iNOS Promoter Activity in Stably iNOS-Transfected A549/8 cells. To generate A549/8 cells stably transfected with a construct containing a 16-kb fragment of the human iNOS promoter cloned in front of a luciferase reporter gene, cells were transfected by lipofection with *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate according to the manufacturers recommendations using 4.5 mg of pNOS2(16)Luc (de Vera et al., 1996) and 0.5 mg of pRc-CMV (Invitrogen) containing a neomycin resistance gene. The transfected cells were selected by G418-treatment (1 mg/ml). Different cell clones were analyzed for luciferase activity and checked for integration of the transfected DNA by PCR.

For analysis of the effects of amphotericin B on iNOS promoter activity, stably transfected A549/8 cells were incubated with a cytokine mix consisting of 1000 U/ml TNF- α plus 1000 U/ml IFN- γ , which was shown previously to lead to an increase in human iNOS promoter activity of ~2-fold. This represents about half-maximal induction of the iNOS-promoter-luc construct (de Vera et al., 1996). Cytokine-challenge was performed in the presence or absence of AmB at concentrations indicated. Extracts (200 μ l) were prepared 18 h later using the lysis buffer from the luciferase assay system and the same assay was used for determination of luciferase activities of the extracts (diluted 1:50 in lysis buffer), which were normalized to the number of cells in the respective probes, determined immediately before lysis by measuring neutral red uptake. Relative iNOS-promoter activity is given in counts per minute.

Statistical Analysis. Values were derived from several individual experiments ($n = 3-8$) from cultures independently derived from

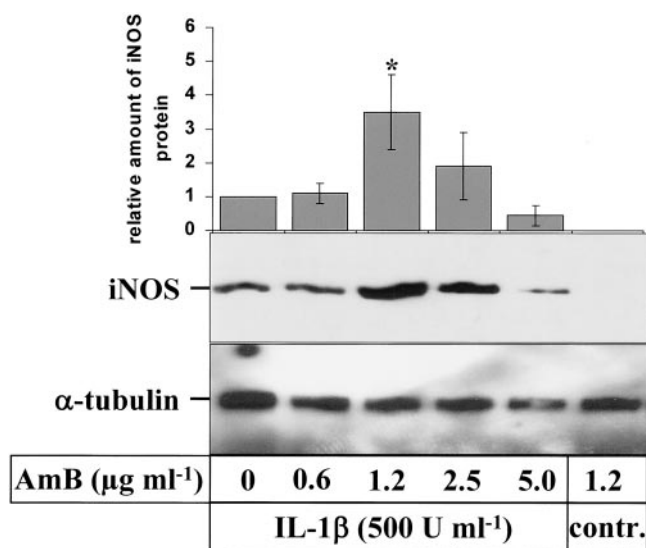


Fig. 3. Effect of amphotericin B on iNOS protein expression. Resting (contr.) or IL-1 β activated (200 U/ml) AEC were incubated with AmB at concentrations indicated. After 48 h of incubation, cultures were lysed and proteins (40 μ g per lane) were separated by electrophoresis, transferred to nitrocellulose membranes, and, using specific monoclonal antibodies and the enhanced chemiluminescence detection assay, iNOS- and α -tubulin-protein expression was determined. Bars represent the mean \pm S.D. of the iNOS/ α -tubulin-ratio obtained by densitometric analysis of visualized proteins from three individual experiments. Values were normalized against the ratio obtained with cytokine incubation only (0 μ g/ml AmB). *, $p < 0.01$ compared with control (0 μ g/ml AmB).

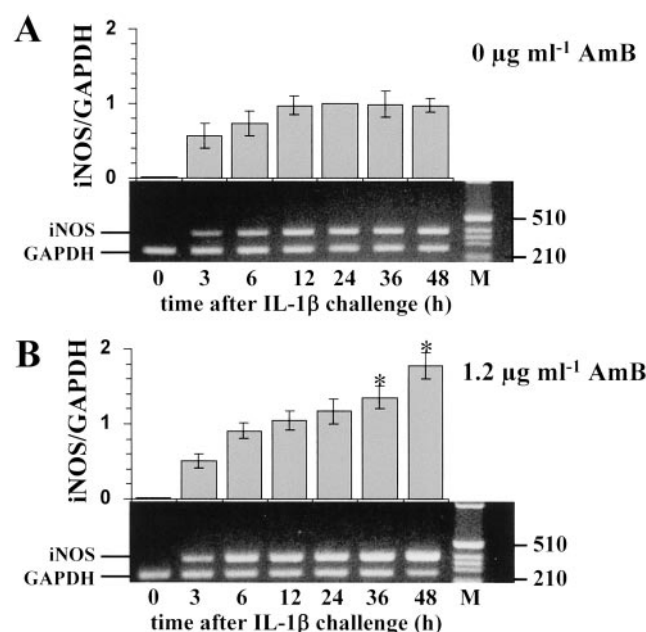


Fig. 4. Time course of AmB-mediated iNOS mRNA expression. AEC (1×10^5) were incubated simultaneously with IL-1 β (200 U/ml) plus AmB (A, 0 μ g/ml; B, 1.2 μ g/ml). At the indicated time points, iNOS- or GAPDH-mRNA expression was examined by RT-PCR. Bars represent the mean \pm S.D. of the iNOS/GAPDH-ratio obtained by densitometric analysis of visualized amplification product bands from three individual experiments. Values are normalized to the ratio obtained at 24 h with cytokines only. *, $p < 0.001$ compared with the controls (corresponding bars in A).

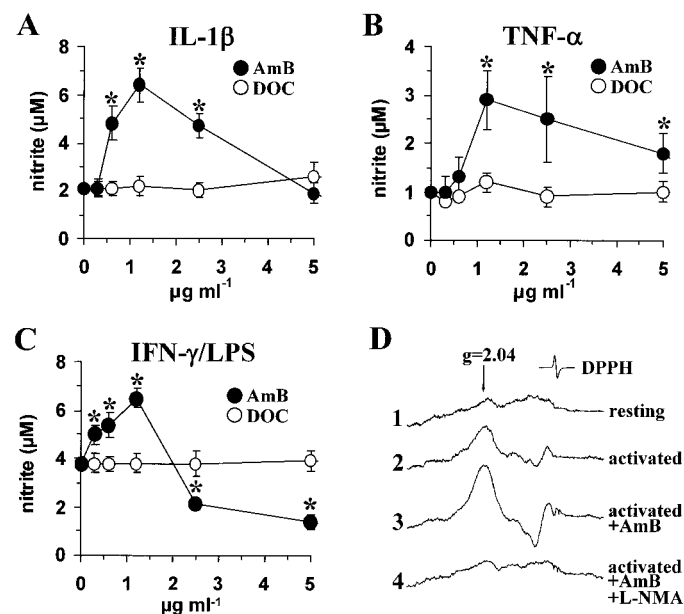


Fig. 5. Concentration-dependent effects of amphotericin B on iNOS enzyme activity in the presence of different activating stimuli. AEC (1×10^5) were activated with 200 U/ml IL-1 β (A), 500 U/ml TNF- α (B), or 100 U/ml IFN- γ + 100 ng/ml LPS (C) in the presence or absence of AmB (●) or vehicle DOC (○) at concentrations indicated. After 48 h of incubation, nitrite accumulation in culture supernatants during the last 24 h, as an indirect parameter for iNOS activity, was determined. Values are the mean \pm S.D. of three to six individual experiments. D, in addition, cytokine-activated (2) rat endothelial cell cultures were grown for 48 h in the presence of 1.2 μ g/ml AmB (3) or AmB plus the NOS inhibitor L-NMA (1 mM) (4) and then were subjected to EPR spectroscopy to detect intracellularly trapped NO (1, resting cells). The characteristic axial feature appears at $g = 2.04$. *, $p < 0.001$ compared with the respective culture grown in the presence of DOC.

different animals. Values were reported as mean \pm S.D. For statistical analysis, we used analysis of variance followed by an appropriate post hoc multiple comparison test (Tukey method); $p < 0.05$ was considered significant.

Results

Amphotericin B Toxicity in the Presence of Proinflammatory Cytokines. Incubation of AEC with IL-1 β alone is a stimulus sufficient for de novo endothelial iNOS expression and activity (Suschek et al., 1993, 1994). Activated (200 U/ml IL-1 β) AEC were incubated with AmB or the vehicle DOC at concentrations indicated. After 48 h of culture, the relative number of viable or dead cells was determined using Neutral Red or trypan blue staining, respectively (Fig. 1). Incubation of IL-1 β -activated AEC with AmB at concentrations higher than 5 μ g/ml led to cell death in a dose-dependent manner. After 48 h of incubation with 15 μ g/ml AmB, only $5 \pm 4\%$ of the cells had survived. Half-maximal cytotoxicity was found at 10.0 μ g/ml AmB. We therefore restricted our experiments using nontoxic concentrations of AmB up to 5.0 μ g/ml. Controls using the solvent DOC gave no effects. Toxicity of AmB in the absence of cytokines was similar (Suschek et al., 2000) (data not shown).

iNOS mRNA and Protein Expression. IL-1 β (200 U/ml) activated AEC were cultured in the presence or absence of AmB at concentrations of 0.3 to 5.0 μ g/ml. Reverse transcrip-

tion and polymerase chain reaction (RT-PCR) was performed with total RNA after 48 h of incubation.

Culture in the presence of IL-1 β alone led to endothelial de novo iNOS mRNA expression at submaximal levels. Simultaneous presence of AmB significantly augmented iNOS expression in a dose-dependent mode with a maximal increase by the factor of 2.5 ± 0.25 at 1.2 μ g/ml AmB and a decrease at higher AmB concentrations (Fig. 2A), whereas the solvent alone was without effects (Fig. 2B). AmB alone in the absence of the cytokine did not result in de novo iNOS mRNA expression (Fig. 2C).

Next, we analyzed the amount of iNOS protein by Western blotting. Incubation with IL-1 β + 1.2 μ g/ml AmB led to a 3.8-fold increase in iNOS protein levels compared with the IL-1 β only treatment. Resting cultures incubated with 1.2 μ g/ml AmB only gave no positive signal (Fig. 3).

When examining the time course of iNOS mRNA induction in endothelial cells during IL-1 β challenge only, we observed an increase during the first 12 h to a level remaining constant for up to 48 h (Fig. 4A). In contrast, cells coincubated with 1.2 μ g/ml AmB showed a constant increase in iNOS mRNA levels (Fig. 4B) throughout the observation period, resulting in significantly higher levels at 36 and 48 h.

iNOS Enzyme Activity. Next we examined whether AmB-mediated increases in iNOS activity are found in com-

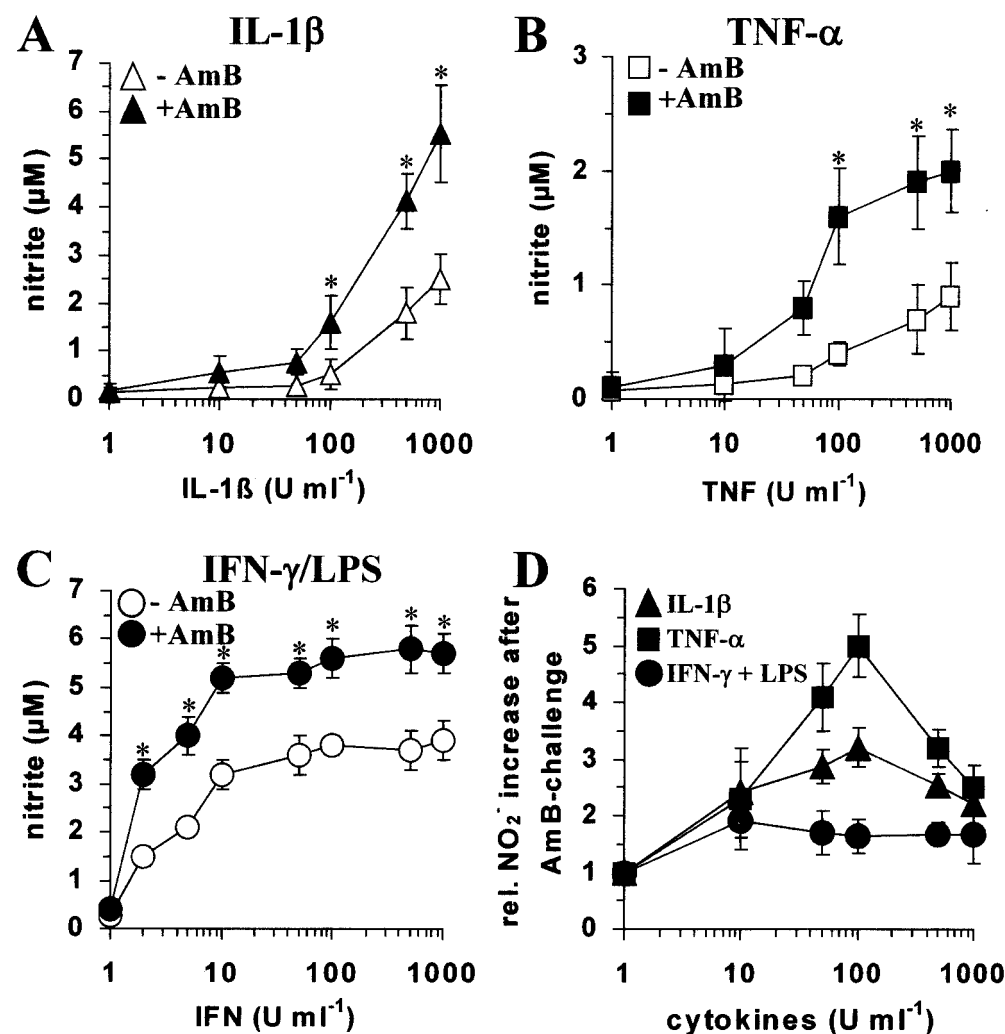


Fig. 6. Amphotericin B-mediated increases in iNOS activity depending on cytokine concentrations. Rat aorta endothelial cells (AEC, 1×10^5) were activated with IL-1 β (A, \blacktriangle , \triangle), TNF- α (B, \blacksquare , \square), or IFN- γ + 100 ng/ml LPS (C, \bullet , \circ) at the concentrations indicated in the presence (\blacktriangle , \blacksquare , \bullet) or absence (\triangle , \square , \circ) of 1.2 μ g/ml AmB. After 48 h of incubation, nitrite accumulation in cultured supernatants during the last 24 h, as an indirect parameter for iNOS activity, was determined. The relative increases in iNOS activities mediated by AmB compared with activated cultures grown in the absence of AmB are shown in D (\blacksquare , 100 U/ml TNF- α ; \blacktriangle , 100 U/ml IL-1 β ; \bullet , 500 U/ml γ -IFN + 100 ng/ml LPS). Values are the mean \pm S.D. of three to six individual experiments. *, $p < 0.001$ compared with the respective cultures grown in the absence of AmB.

bination with IL-1 β only or whether combinations with any proinflammatory stimulus will increase iNOS-activity.

Activation of AEC with 200 U/ml IL-1 β (Fig. 5A), 500 U/ml TNF- α (Fig. 5B), or 500 U/ml IFN- γ + 100 ng/ml LPS (Fig. 5C) in the presence of 0.3–1.2 μ g/ml AmB led to a concentration-dependent increase in nitrite concentrations in culture supernatants, which peaked at 1.2 μ g/ml AmB and was always significantly different from the cytokine and/or DOC-only controls.

Interestingly, in the presence of TNF- α alone, which will not induce iNOS expression or increased nitrite accumulation, as described previously (Suschek et al., 1993; Bonmann et al., 1997), addition of AmB will result in significant in-

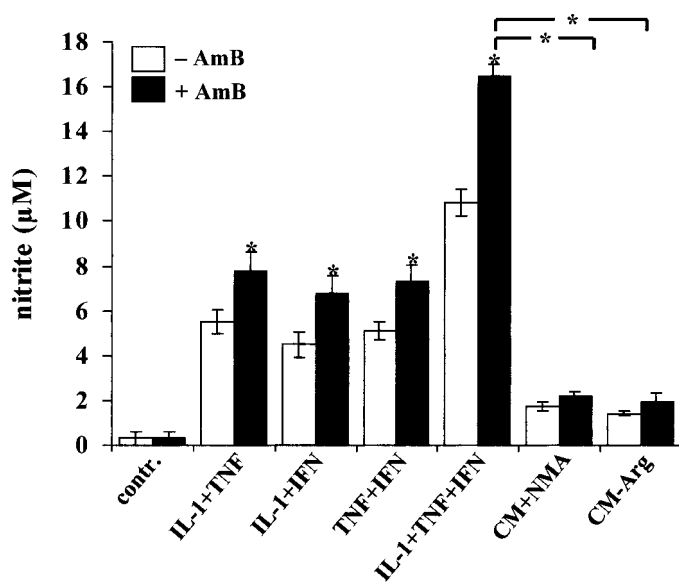


Fig. 7. Amphotericin B-mediated increases of nitrite formation during activation with various cytokine combinations. AEC (1×10^5) were activated for 48 h with indicated combinations of 200 U/ml IL-1 β or 500 U/ml TNF- α or 100 U/ml IFN- γ in the presence (■) or absence (□) of 1.25 μ g/ml AmB. After 48 h of incubation nitrite accumulation in culture supernatants during the last 24 h, as an indirect parameter for iNOS activity, was determined. CM+NMA, cultures incubated with the cytokine-mix, consisting of all three cytokines, in the presence of the NOS-inhibitor N^G -monomethyl-L-arginine (1 mM). CM-Arg, cultures incubated with the cytokine-mix in L-arginine-free medium. Desoxycholate used at the same concentration as AmB did not affect iNOS activity (data not shown). Values are the mean \pm S.D. of three to six individual experiments. *, $p < 0.01$ compared with the respective cultures grown in the absence of AmB

TABLE 3

Effects of amphotericin B on endothelial expression of IL-1 β and TNF- α

Resting or cytokine activated (200 U/ml IL-1 β) endothelial cells (1×10^5) were cultured in the presence or absence of AmB or the TNF- α synthase inhibitor thalidomide (20 μ g/ml) at concentrations indicated. Thalidomide was present in culture supernatants 24 hours before AmB challenge and for 24 hours after incubation with AmB or only during the 24 hours of incubation with AmB. Values represent the mean \pm S.D. of three individual experiments.

	IL-1 β	TNF- α	Thalidomide (20 μ g/ml) + TNF- α	
			During AmB	Before and After AmB
			pg/ml	
Resting	6 \pm 2	57 \pm 4	64 \pm 9	21 \pm 11 [#]
+0.6 μ g/ml AmB	21 \pm 9	64 \pm 16	57 \pm 11	12 \pm 9 [#]
+1.2 μ g/ml AmB	26 \pm 8	70 \pm 10	67 \pm 12	17 \pm 7 [#]
+5.0 μ g/ml AmB	26 \pm 12	67 \pm 22	63 \pm 42	14 \pm 8 [#]
Activated	84 \pm 15*	140 \pm 31*	51 \pm 37	17 \pm 6 [#]
+0.6 μ g/ml AmB	147 \pm 31**	229 \pm 35**	57 \pm 30 [#]	18 \pm 18 [#]
+1.2 μ g/ml AmB	222 \pm 38**	317 \pm 72**	65 \pm 40 [#]	13 \pm 10 [#]
+5.0 μ g/ml AmB	213 \pm 36**	279 \pm 12**	48 \pm 33 [#]	15 \pm 15 [#]

*, $p < 0.01$ compared with resting cells.

**, $p < 0.001$ compared with activated cells grown in the absence of AmB.

[#], $p < 0.001$ compared with TNF- α values obtained with the respective cultures grown in the absence of thalidomide.

crease in nitrite production. Additionally, and as a direct means to demonstrate intracellular NO formation, we also performed EPR spectroscopy. As shown in Fig. 5D, cytokine activated endothelial cells gave the characteristic NO-specific axial feature at $g = 2.04$, which was strongly increased

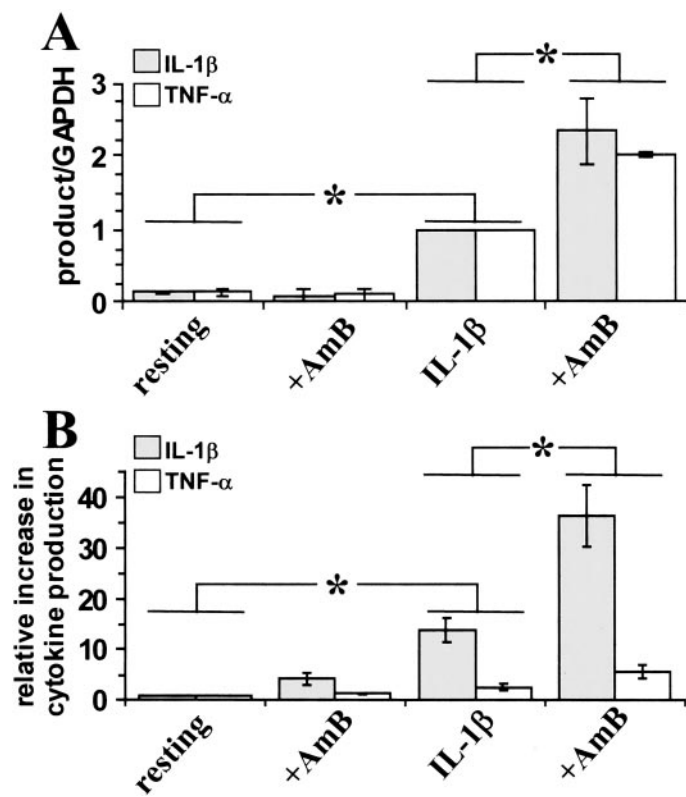


Fig. 8. Input of AmB on endothelial IL-1 β and TNF- α expression. Resting or cytokine activated (IL-1 β , 200 U/ml) aortic endothelial cell cultures were grown in the absence or the presence of 1.2 mg/ml amphotericin B (+AmB). After 48 h of incubation IL-1 β - (■) and TNF- α -mRNA (□) expression was determined by RT-PCR (A). Bars represent the mean \pm S.D. of the IL-1 β or TNF- α /GAPDH-ratio obtained by densitometric analysis of visualized amplification product-bands from three individual experiments. Values are normalized to the ratio obtained with cytokine activated cultures (IL-1 β) only. B, in cultured supernatants of sham-treated cells, IL-1 β and TNF- α protein production was examined using the cytokine-specific ELISAs. Values are the mean \pm S.D. of four individual experiments. Values are normalized to cytokine amounts determined in resting cultures. *, $p < 0.0001$

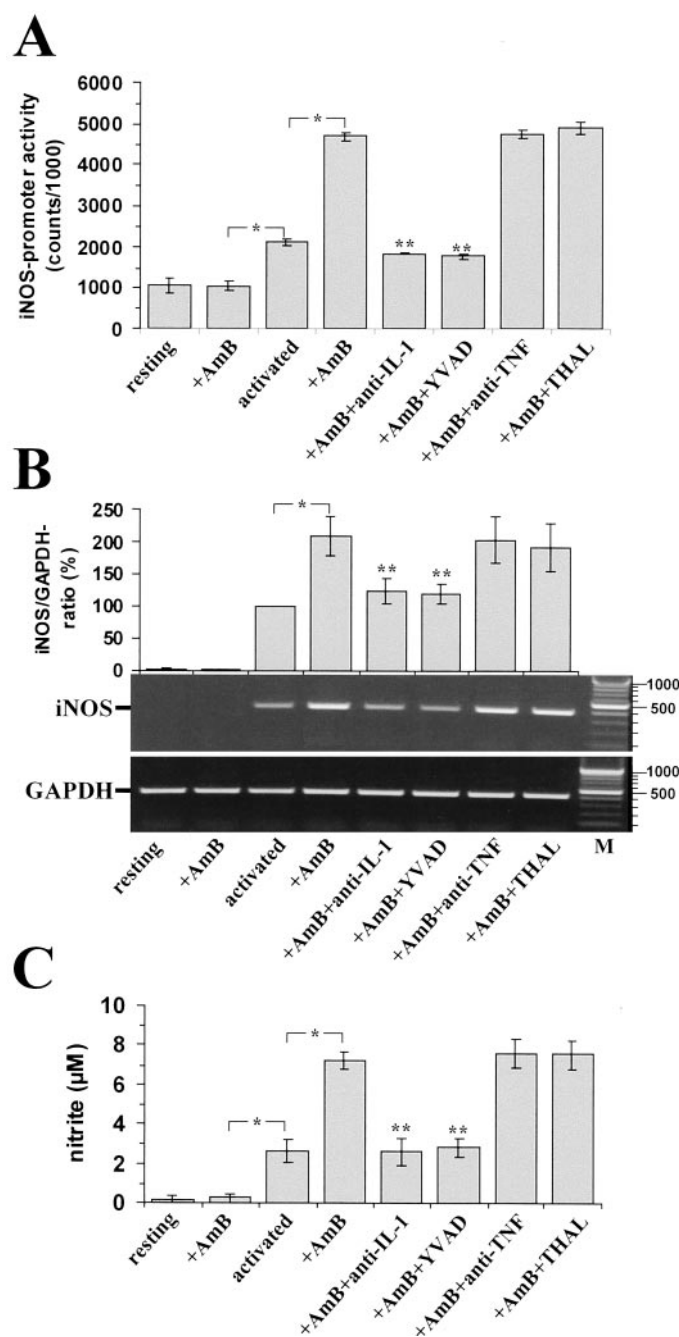


Fig. 9. Impact of endothelial cytokine production on iNOS promoter activity, iNOS-mRNA expression, and iNOS activity. A, in a reporter gene assay, human A549/8 cells (1×10^5) stably transfected with a construct containing a 16-kb fragment of the human iNOS promoter cloned in front of a luciferase reporter gene (A549/8-iNOS-Luc) were used. Resting or cytokine-activated cells (after challenge with 1000 U/ml TNF- α plus 1000 U/ml IFN- γ) were grown in the absence or presence of 1.2 μ g/ml amphotericin B (+AmB). Furthermore, activated cells grown in the presence of AmB were incubated with neutralizing anti-human-IL-1 β antibodies (+AmB+anti-IL-1), the ICE inhibitor YVAD (30 μ M, +AmB+YVAD), neutralizing anti-human-TNF- α antibodies (+AmB+anti-TNF- α), or the TNF- α formation-inhibitor thalidomide (20 μ g/ml, +AmB+THAL), respectively. After 18 h of incubation, luciferase activities of the extracts were determined. Bars represent the mean \pm S.D. of the iNOS-promoter activity given in counts per minute of four individual experiments. B, after the same protocol, iNOS-mRNA expression or (C) iNOS activity was determined in rat AEC cultures. Bars represent the mean \pm S.D. of four individual experiments. *, $p < 0.001$; **, $p < 0.001$ compared with cytokine plus AmB-challenged cells.

in the presence of 1.2 μ g/ml AmB. Identical incubations but in the presence of the NOS inhibitor *N*^G-monomethyl-L-arginine (NMA, 1 mM) abolished the NO-specific signal.

Although AmB, via modulation of the constitutive eNOS, can augment NO synthesis in resting cells (Suschek et al., 2000), very low eNOS-mediated NO amounts that are found in the range between 50 and 200 nM cannot be detected by either the EPR method or the Griess-test as used here. Thus, in the present study, eNOS activity representing less than 1% of the iNOS activities measured will not lead to a significant difference.

We also tested the effects of various concentrations of cytokines or cytokine combinations at a fixed AmB concentration of 1.2 μ g/ml. At any concentration of the various different cytokine combinations used, AmB led to a significantly enhanced iNOS activity. AmB treatment in the presence of TNF- α (100 U/ml) resulted in a 5-fold increase in iNOS activity, a 3-fold increase with IL-1 β (100 U/ml), and a 2-fold increase with γ -IFN + LPS (Fig. 6D).

Unexpectedly, we find that AmB significantly increases iNOS activity AmB even during maximal activation by combining all three cytokines (Fig. 7). Addition of NMA (1 mM) or absence of arginine inhibited the cytokine- as well as AmB-induced increases in nitrite formation as evidences for the specificity (Fig. 7). Again, the vehicle control showed no effects (data not shown).

Endothelial Cytokine and ICE Expression. To elucidate the enhancing effects of AmB on endothelial iNOS expression by using specific PCR or ELISA, we then examined whether the AmB-induced increases are caused by endoge-

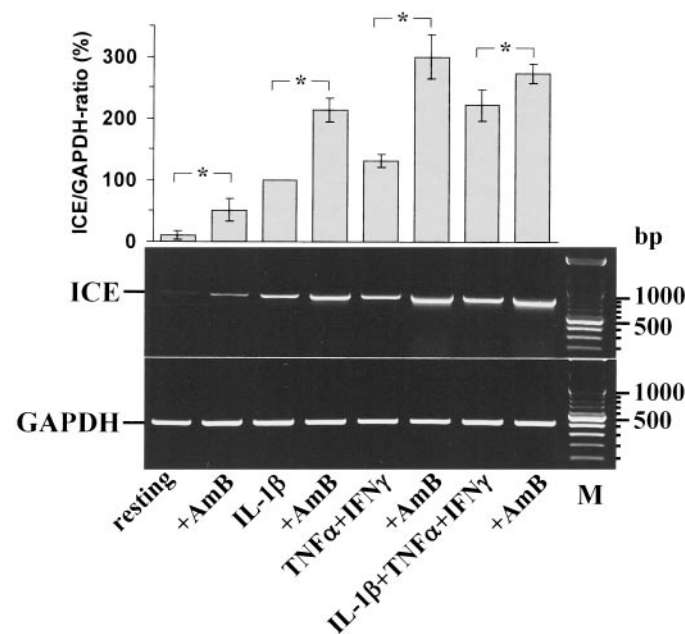


Fig. 10. The dominant role of amphotericin B on endothelial ICE expression. Resting or cytokine-activated (single doses or indicated combinations of 200 U/ml IL-1 β , 500 U/ml TNF- α , and 500 U/ml IFN- γ) rat aorta endothelial cells were incubated in the absence or presence of 1.25 μ g/ml AmB. After 24 h of incubation, ICE- and GAPDH-mRNA expression was evaluated by RT-PCR. Bars represent the mean \pm S.D. of the ICE/GAPDH-ratio obtained by densitometric analysis of visualized amplification product-bands from three individual experiments. Values are normalized to the ratio obtained with IL-1 β only. *, $p < 0.001$ compared with IL-1 β -challenged cultures grown in the absence of AmB (=100%).

nous induction of the proinflammatory cytokines IL-1 β and/or TNF- α .

Although untreated AEC with or without AmB do not express significant levels of IL-1 β or TNF- α mRNA, cytokine challenge (200 U/ml IL-1 β) leads to both IL-1 β - (2.4 \pm 0.4-fold) and TNF- α -specific mRNA (2.0 \pm 0.1-fold) expression and both are significantly increased in the additional presence of AmB (Fig. 8). Cytokine-specific ELISA measurement of culture supernatants (Table 3 and Fig. 8B) from resting cells show that AmB does not influence TNF- α production but slightly increases endothelial IL-1 β production (4.2 \pm 1.2-fold). Cytokine-challenge (200 U/ml IL-1 β) of AEC leads to high increases in IL-1 β (13.8 \pm 2.5-fold) and to some TNF- α (2.5 \pm 0.6-fold) production. In the additional presence of AmB (1.2 μ g/ml), formation of both cytokines was further augmented (IL-1 β , 36 \pm 6.1-fold and TNF- α , 5.6 \pm 1.3-fold over production in resting cells), and TNF- α secretion was effectively blocked by thalidomide (20 μ g/ml).

Impact of Endothelial IL-1 β and TNF- α Production on iNOS Promoter Activity, iNOS-mRNA Expression, and iNOS Activity. The increases in iNOS mRNA expression (Figs. 2 and 4) after coincubation of AEC with IL-1 β + 1.2 μ g/ml AmB strongly correlate with increases in endothelial IL-1 β or TNF- α expression (Fig. 8), indicating that endogenous cytokine expression may be involved in AmB-mediated enhancement of iNOS expression. We therefore analyzed the effect of AmB as well as the impact of endothelial cytokine production on iNOS promoter activity using the A549/8 cell line stably transfected with a 16-kb fragment of the human iNOS promoter cloned in front of a luciferase reporter gene (A549/8-iNOS-Luc) as well as iNOS-mRNA expression and iNOS enzyme activity in AEC.

As expected from the work from de Vera et al. (1996), cytokine challenge of human A549/8 cells with 1000 U/ml TNF- α plus 1000 U/ml IFN- γ led to a half-maximal 2.1 \pm 0.3-fold increase in iNOS promoter activity (Fig. 9A), whereas the additional presence of AmB (1.2 μ g/ml) led to a 4.6 \pm 0.8-fold increase, which represents the maximally possible activation level achievable with this construct. These AmB-mediated increases in human iNOS promoter activity were reduced to control levels (cytokines only) in the presence of a neutralizing anti-human-IL-1 β antibody. Interest-

ingly, the blocking of ICE activity by addition of the ICE inhibitor YVAD was as effective as blocking IL-1 β itself (Fig. 9A). In contrast, neither the inhibition of endogenous TNF- α formation by thalidomide nor neutralization with anti-human-TNF- α antibodies had any influence on the AmB-induced increases in iNOS-promoter activity (Fig. 9A).

Next, we compared the data from human A549/8 cells described above with results from studying the impact on endothelial cells and found that in rat AEC cultures, iNOS-mRNA expression (Fig. 9B) or endothelial iNOS activity (Fig. 9C) paralleled the results obtained by studying the human iNOS promoter activity under the respective treatments. These results indicate that IL-1 β but not TNF- α is the pivotal mediator of AmB-induced effects on endothelial iNOS expression and activity.

The Dominant Role of Endothelial ICE Expression and Activity. ICE activity represents a prerequisite for endogenous generation of bioactive IL-1 β (Watkins et al., 1999). Therefore, we further analyzed the role of endothelial ICE expression and activity on AmB-induced iNOS overexpression. We find that under all conditions, in resting and cytokine activated cells, AmB (1.2 μ g/ml) strongly augments ICE mRNA expression (Fig. 10) as well as ICE-enzyme activity (Table 4). A careful investigation of the effects of ICE inhibition under the various culture conditions was performed (Table 4). We find that specific inhibition of ICE activity completely reverses the AmB-induced iNOS overexpression as well as the increases in IL-1 β formation. Moreover, these data also reveal that ICE inhibition will completely shut down iNOS expression in any condition that does not contain exogenous IL-1 β and will inhibit any additional AmB effects under all conditions in which IL-1 β was added. Thus these data demonstrate that the main impact of AmB lies in the overexpression of ICE and any of the other effects (i.e., increases in iNOS, IL-1 β , or TNF- α) are an indirect result of increased caspase-1 activity.

Discussion

The resistance to fungal infections is associated with up-regulation of innate and acquired antifungal Th1-like responses, such as production of proinflammatory cytokines and NO. It has been shown that a pharmacological inhibition

TABLE 4
Impact of AmB on ICE activity, IL-1 β formation, and iNOS activity

Resting or cytokine-activated endothelial cells were cultured in the presence or absence of AmB (1.25 μ g/ml) or of the ICE-inhibiting peptide YVAD (40 μ M) added to culture supernatants 3 hours before and during cytokine and/or AmB addition. After 24 hours of incubation, endogenous ICE activity, formation of bioactive IL-1 β , and nitrite accumulation in culture supernatants were determined. Values represent the mean \pm S.D. of three to five individual experiments.

AEC	ICE Activity		IL-1 β		Nitrite	
		+YVAD		+YVAD		+YVAD
	<i>fold increases</i>		<i>pg/ml</i>		<i>μM</i>	
Resting	1 [#]	0.7 \pm 0.6	15 \pm 4 ^{##}	11 \pm 3	1.0 \pm 0.3 ^{##}	0.9 \pm 0.5
+AmB	1.4 \pm 0.5 ^{##}	0.6 \pm 0.6	14 \pm 3 ^{##}	15 \pm 5	0.9 \pm 0.6 ^{##}	1.0 \pm 0.3
IL-1 β	2.8 \pm 0.6	1.1 \pm 0.5 [#]	59 \pm 13	12 \pm 6 [#]	3.3 \pm 0.7	3.1 \pm 0.6
+AmB	6.2 \pm 0.8 [*]	1.0 \pm 0.8 [#]	161 \pm 33 [*]	11 \pm 9 [#]	6.4 \pm 0.8 [*]	3.3 \pm 0.9 [#]
TNF- α + IFN- γ	3.5 \pm 0.4	0.9 \pm 0.4 [#]	90 \pm 24	12 \pm 4 [#]	9.7 \pm 1.5	0.8 \pm 0.5 ^{*,**}
+AmB	7.5 \pm 1.2 [*]	0.9 \pm 0.5 [#]	190 \pm 42 [*]	15 \pm 5 [#]	16.6 \pm 2.2 [*]	0.8 \pm 0.5 ^{*,**}
IL-1 β + TNF- α + IFN- γ	3.9 \pm 0.6	1.2 \pm 0.7 [#]	119 \pm 18	12 \pm 7 [#]	14.3 \pm 2.5	4.5 \pm 0.9 [#]
+AmB	8.5 \pm 1.1 [*]	0.9 \pm 0.6 [#]	259 \pm 38 [*]	17 \pm 10 [#]	19.7 \pm 1.9 [*]	4.6 \pm 0.9 [#]

^{*}, p < 0.001 compared with cells grown in the absence of AmB.

[#], p < 0.001 compared with respective cultures grown in the absence of YVAD.

^{**}, p < 0.001 compared with YVAD-treated cultures containing exogenous IL-1 β .

^{##}, p < 0.001 compared with cytokine-activated cell cultures.

of NO production greatly reduces the resistance to fungal infections (Del Sero et al., 1999).

AmB is a classic (and one of the most effective) antifungal drug for the treatment of systemic fungal infections (Gallis et al., 1990; Carlson and Condon, 1994). AmB may act directly on fungi by a mechanism involving pore formation in fungal membranes (Clements and Peacock, 1990; Gallis et al., 1990) but has also been shown to increase the NO-dependent anti-cryptococcal activity of macrophages, which correlated with the endogenous production of proinflammatory cytokines (Tohyama et al., 1996).

After systemic application of AmB, in patients with cryptococcal infections, serum concentrations of 1.0 to 3.0 $\mu\text{g/ml}$ have to be reached for drug efficacy (Louira, 1958; Bindschadler and Bennett, 1969; Fields et al., 1970). AmB, systemically administered, will act directly on the blood vessel lining endothelial cells; thus, this cell population and its response to AmB are of prime interest. Recently, we could show a concentration-dependent and biphasic effect of amphotericin B on expression and activity of the constitutive eNOS in resting aortic endothelial cells (Suschek et al., 2000). With AmB at clinically relevant concentrations, a highly significant increase in eNOS activity was found, which may support the antithrombotic state of the endothelium and inhibit the *trans*-vascular migration events of leukocytes or pathogens. In contrast, at higher AmB concentrations, as are postulated to occur in the kidney because of drug accumulation during therapy, a strong decrease in expression and activity of the eNOS can be observed, an effect that will favor thrombus formation and vasoconstriction, both potentially contributing to the serious side effects observed during AmB therapy (Sawaya et al., 1991).

Migration of fungal pathogens across the endothelial cell layer is considered a prerequisite for the organ invasion in systemic fungal infections (Zink et al., 1996). Activated endothelial cells are capable of tumor cell killing as well as parasite, bacteria, virus, or tissue destruction via the iNOS/NO pathway (Steiner et al., 1997) and were shown to augment the antifungal activity of polymorphonuclear cells (Roseff and Levitz, 1993). Endothelial cells have been shown to synthesize NO in similar high-output concentrations as is known for macrophages (Suschek et al., 1993) and they express iNOS *in vivo* as a consequence of cryptococcal infection (Goldman et al., 1996). Thus, endothelial cells will be involved in antifungal reactions.

Furthermore, as shown here with iNOS-expressing endothelial cells after cytokine challenge, AmB exhibits a powerful enhancing activity on iNOS-promoter as well as mRNA and protein expression and enzyme activity, even in fully activated cells. These increases are paralleled by a strongly enhanced endothelial production of the proinflammatory cytokines IL-1 β and TNF- α , which are known inducers/enhancers of endothelial iNOS expression (Suschek et al., 1993). Blocking the formation or availability of IL-1 β by specific antibodies completely reverses AmB-induced effects on the iNOS, whereas inhibition of TNF- α formation or activity had no effect. Consequently, data presented give evidence that with cytokine-activated endothelial cells, AmB-induced increases in iNOS activity are dependent on either exogenously added or produced IL-1 β . Examining the events upstream of IL-1 β formation, we find that AmB-induced increases of bioactive IL-1 β and iNOS can be completely blocked by the

specific ICE inhibitor YVAD. Thus, for the first time, we show herein that the core mechanism of AmB affecting cytokine and iNOS expression is predominantly based on AmB-induced increases in ICE activity.

The data presented here differ in important aspects from those of an earlier study (Tohyama et al., 1996), in which AmB at comparable concentrations strongly augmented iNOS activities in IFN- γ activated mouse macrophages accompanied by augmented formation of IL-1 β and TNF- α . In this earlier study, an impact of both cytokines on the AmB-induced iNOS increases activity had been postulated, due to inhibition obtained by neutralizing antibodies. Interestingly, inhibition studies using neutralizing anti-TNF- α antibodies revealed a weak reduction of the AmB-induced effects on iNOS activity, and IL-1 β depletion led to an even more striking reduction of the AmB-induced iNOS activity (Tohyama et al., 1996). These results indicate that in addition to induction of proinflammatory cytokine synthesis, AmB may use additional or supplementary mechanisms to influence iNOS expression and activity.

In contrast, we here now further support and underline previous findings on the pivotal role of IL-1 β in endothelial iNOS expression (Suschek et al., 1993; Bonmann et al., 1997) and, in addition, demonstrate for the first time the rate-limiting role for ICE activity in iNOS expression. Thus, AmB-induced ICE increases will aid innate responses, because IL-1 β is involved not only in iNOS expression but also induces endothelial adhesion molecules such as ICAM-1, VCAM-1, and E-selectin, essential for leukocyte recruitment to sites of inflammation, and induces or augments expression of other cytokines, including TNF- α , IL-6, IL-8, MCP-1, and gro/MSA, thereby adding to proinflammatory and antifungal responses (Sahnoun et al., 1998).

In conclusion, the experiments presented here show significant AmB-induced increases in endothelial iNOS expression and activity in cytokine-activated endothelial cells. These AmB-induced effects are predominantly mediated by endogenous IL-1 β formation because of the action of AmB on expression and activity of ICE as a rate-limiting step in IL-1 β synthesis. Furthermore, these data underscore the relevance and independent role of the vascular endothelium and endothelial iNOS as potent machinery of the innate immune defense against microorganisms.

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