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Amphotericin B severely affects expression and activity of the endothelial constitutive nitric oxide synthase involving altered mRNA stability

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1 The therapeutic use of the antifungal drug amphotericin B (AmB) is limited due to severe side effects like glomerular vasoconstriction and risk of renal failure during AmB administration. As nitric oxide (NO) has substantial functions in renal autoregulation, we have determined the effects of AmB on endothelial constitutive NO synthase (ecNOS) expression and activity in human and rat endothelial cell cultures.

2 AmB used at concentrations of 0.6 to 1.25 μg ml^{-1} led to increases in ecNOS mRNA and protein expression as well as NO production. This was the result of an increased ecNOS mRNA half-life. In contrast, incubation of cells with higher albeit subtoxic concentrations of AmB (2.5–5.0 μg ml^{-1}) resulted in a decrease or respectively in completely abolished ecNOS mRNA and protein expression with a strongly reduced or inhibited ecNOS activity, due to a decrease of ecNOS mRNA half-life. None of the AmB concentrations affected promoter activity as found with a reporter gene construct stably transfected into ECV304 cells.

3 Thus, our experiments show a concentration-dependent biphasic effect of AmB on expression and activity of ecNOS, an effect best explained by AmB influencing ecNOS mRNA stability. In view of the known renal accumulation of this drug the results reported here could help to elucidate its renal toxicity.

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Abbreviations: AmB, amphotericin B; ecNOS, endothelial constitutive nitric oxide synthase; HUDEC human microvascular dermal endothelial cells; NO, nitric oxide; RAEC, rat aortic endothelial cells

Introduction

Amphotericin B (AmB) is the predominant antifungal drug used today (Gallis *et al*., 1990; Hay, 1991). Originally, AmB was administered primarily to patients with deep-seated mycoses whose premorbid state of health was relatively unremarkable (Seabury & Dascomb, 1958). Currently, an increasing number of patients are treated with conditions that predispose them to fungal infections, such as iatrogenic, viral or malignancy related immunosuppression, total parenteral nutrition, bacterial sepsis, and broad-spectrum antibiotic therapy (Clements & Peacock, 1990).

Along with the therapeutic efficacy of AmB, serious toxicities are observed, among which nephrotoxicity is the most limiting factor in its use (Carlson & Condon, 1994). Nephrotoxicity caused by AmB is manifested by reduction in glomerular filtration rate (Halpern & Lindeman, 1970), renal blood flow (Beard *et al*., 1960; Halpern & Lindeman, 1970; Sanford *et al*., 1962), renal plasma flow (Cheng *et al*., 1982), and tubular dysfunction (Coggins & Fang 1988), and is a result of AmB-induced renal arteriolar vasoconstriction (Reiner & Thompson, 1979; Sawaya *et al*., 1991). This vasoconstrictive effect appears to be one of the major mechanisms contributing to the development of renal failure during AmB administration. However, the drug may also act by direct toxicity on renal cells (Andreoli, 1973).

Currently, the mechanism of AmB toxicity and specifically its nephrotoxicity, is incompletely understood. We now have searched for a role of AmB in modulating NO production as this important vasoactive signal molecule may be important in the mechanism of AmB-induced nephrotoxicity (Raij & Baylis, 1995). In endothelial cells, different renal cells, neurones, and upon activation in macrophages and many other cells, NO and equal amounts of citrulline are synthetized from the guanidino nitrogen of L-arginine by nitric oxide synthases (NOS) (Moncada & Higgs, 1993). This enzyme family comprises of two different subfamilies: two constitutively expressed and calcium/calmodulin-dependent isoenzymes, the endothelial ecNOS and the neuronal ncNOS, and a cytokine-inducible and calcium-independent isoenzyme (iNOS). The constitutive, calcium-dependent enzyme generally produces, upon agonist-stimulation, low amounts of NO for short periods of time. In contrast, once expressed after cytokine activation iNOS synthesizes large amounts of NO for long periods of time (Förstermann *et al*., 1994).

In the kidney NO plays an important role in glomerular actions and renal autoregulation. Expression of ecNOS has

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been found in glomeruli and vasculature as well as in the macula densa, the collecting duct, and the inner medullary thin limb. Within the kidney, NO generated by endothelial ecNOS participates in the regulation of the glomerular microcirculation by modifying the tone of the afferent arteriole and mesangial cells. Nitric oxide generated by macula densa and the afferent arteriole controls glomerular haemodynamics *via* the tubuloglomerular feedback system and by modulating renin release. Therefore, NO is a key molecule in the regulation of glomerular capillary blood pressure, glomerular plasma flow, and the ultrafiltration coefficient (Raij & Baylis, 1995). Further, nitric oxide is an endogenous active molecule that participates in the control of various cell functions by acting on membrane ion channels. NO can directly act on these channels or indirectly *via* cyclic guanosine monophosphate (cyclic GMP) formation and cyclic GMP-dependent protein kinase activation (Fagni & Bockaert, 1996).

With the experiments presented here we show that AmB exhibits a concentration-dependent biphasic effect on ecNOS enzyme activity which appears to be due to AmB-induced modulation of the post-transcriptional regulation of ecNOS mRNA stability, whereas promoter activity is not affected.

Methods

Reagents

Recombinant human interleukin-1β (IL-1β), recombinant murine or human tumour necrosis factor-α (TNF-α), and recombinant murine or human gamma interferon (IFN-γ) were purchased from HBT (Leiden, Netherlands) or from Genzyme (Cambridge, MA, U.S.A.), endothelial cell growth supplement (ECGS), neutral red (3% solution), type I collagen, collagenase (from Cl. histolyticum), actinomycin D, phorbol-12-myristate-13-acetate (PMA), LPS (from *Salmonella typhimarium*), mouse anti α-tubulin antibody, and rabbit anti-human von Willebrand Factor (vWF) antiserum from Sigma (Deisenhofen, Germany), the monoclonal antibody Ox43 from Serotec (Camon, Wiesbaden, Germany), the monoclonal anti-ecNOS antibody from Transduction Laboratories (Lexington, KT, U.S.A.), peroxidase-conjugated porcine anti-rabbit IgG from DAKO (Hamburg, Germany), peroxidase-conjugated goat anti-mouse IgG from Zymed Laboratories, Inc. (San Francisco, CA, U.S.A.), trypsin, EDTA, RNase-free DNase I, RNase, T7 RNA polymerase, Luciferase assay system, DOTAP, foetal calf serum (FCS, endotoxin free), RPMI-1640 (endotoxin free), DNA molecular weight markers X and XIV, the oligo dT16-primer from Boehringer-Mannheim (Mannheim, Germany), Taq-polymerase, Geneticin G 418, and amphotericin B (Fungizone®) from Gibco/BRL (Eggenstein, Germany), 3,3′-diaminobenzidine (DAB) from Serva GmbH (Heidelberg, Germany), pGL_3-Basic from Promega (Madison, WI, U.S.A.), and oligonucleotides, restriction enzymes, were obtained from Pharmacia (Uppsala, Sweden). Desoxycholate was a kind gift from Squibb-Heyden (Munich, Germany).

Cell cultures

Rat aorta endothelial cells (RAEC) were isolated by outgrowth from aortic rings exactly as described (McGuire & Orkin, 1987). Briefly, aortic segments were placed on top of a collagen gel (1.8 mg collagen ml^{-1}) in 24-well tissue culture plates and incubated in RPMI 1640/20% FCS and 100 μg ECGS ml^{-1} in a humidified incubator at 37°C in a 95% air/5% CO_2 atmosphere for 4–5 days. Aortic explants were then removed, cells detached with 0.25% collagenase in HBSS and replated onto plastic culture dishes in RPMI 1640/20% FCS. Cells were subcultured for up to eight 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. Human dermal microvascular endothelial cells (HUDEC) were purchased from Promo Cell GmbH (Heidelberg, Germany).

Cellular characterization of cultured cells

Rat aorta endothelial cells (RAEC) were characterized by determining the antigenic phenotype using the anti-WF antiserum (1 : 50 dilution), the rat vascular endothelium specific monoclonal antibody Ox43 (1 : 50 dilution), and the anti-ecNOS-antibody (1 : 100 dilution). The secondary peroxidase-conjugated goat anti-mouse IgG or peroxidase-conjugated porcine anti-rabbit IgG were diluted 1 : 50 prior to use.

Pure cell cultures of rat aorta endothelial cells exhibited a positive antigen phenotype for vWF, Ox43, and ecNOS exactly as published (Suschek *et al*., 1993; 1994). These labelling experiments also showed that the cell cultures consisted of pure endothelial cells, since the respective staining patterns with the endothelial specific markers were found in all cells (data not shown).

Experimental design

All measurements were performed with cells from passages 2–8. Cells grown to near confluence prior to treatments were cultured in 12-, 24-well or 10 cm in diameter tissue culture plates in a humidified incubator at 37°C in a 95% air/5% CO_2 atmosphere in RPMI 1640/20% FCS in the presence or absence of amphotericin B or the tracer desoxycholate at concentrations indicated.

Nitrite determination

Nitrite was determined in culture supernatants using the diazotization reaction as modified by Wood *et al*. (1990) using $NaNO_2$ as standard.

Determination of growth rates and viability

Growth rates and viability of endothelial cells was controlled routinely at the beginning and the end of every experiment using neutral red staining or the trypan blue exclusion assay, respectively (Finter, 1969).

Polymerase chain reaction (PCR)

Total cellular RNA (1 μg each) prepared from resident endothelial cells grown in the presence or absence of amphotericin B or desoxycholate was used for cDNA synthesis using a 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 the oligonucleotides indicated in Table 1. PCR was carried out following standard protocols with the following cycle profiles: 25–27 cycles with 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 and 37–39 cycles with 30 s at 94°C, 45 s at 72°C and a final incubation step at 72°C for 10 min for ecNOS cDNA-amplification. PCR-products were subjected to electrophoresis on 1.8% agarose gels. With HUDEC aliquots of ecNOS and GAPDH products were

Table 1 Sequences and GenBank accession numbers of oligonucleotides used in the PCR

Species/product		*Sequence*	*Bases*	*GenBank accession number*	*Product size* (bases)
Rat ecNOS	sense:	5′-GATCAAAAGGAGTGGTTCCC-3′	415–434	AF085195	278
	antisense:	5′-CCGTGATGGCTGAACGAAGA-3′	673–692		
Human ecNOS	sense:	5′-TTAAGAGGAGCGGCTCCCAG-3′	433–452	M95296	278
	antisense:	5′-CACTGTGATGGCCGAGCGAA-3	691–710		
Rat GAPDH	sense:	5′-ACAGTCCATGCCATCACTGC-3′	569–588	M32599	266
	antisense:	5′-GCCTGCTTCACCACCTTCTT-3′	815–834		
Human GAPDH	sense:	5′-CAACTACATGGTTTACATGTTCC-3′	153–175	m17851	416
	antisense:	5′-GGACTGTGGTCATGAGTCCT-3′	549–568		

pooled and run in the same slot, as their product sizes allowed for this. Bands were visualized by ethidium bromide staining. Densitometric analysis of the visualized amplification products was performed by using the KODAK 1D software (KODAK, Stuttgart, Germany).

Reporter gene assay

Human ECV 304 cells were stably transfected with pNOS III-Hu-3500-Luc-neo (human ecNOS promoter sequence – positions −3470 to +115 – in front of a luciferase reporter gene; construct contains a neomycin resistance gene) exactly as described in detail by Li *et al.* (1998). For analysis of the effects of amphotericin B on ecNOS promoter activity ECV 304 cells were incubated with AmB at concentrations indicated or additionally, as control with 10 nM phorbol-12-myristate-13-acetate (PMA) or 1000 u ml^{-1} tumour necrosis factor-alpha (TNF-α) plus 1000 u ml^{-1} interleukin-1-beta (IL-1β). Extracts (200 μl) were prepared 18 h later using the reporter lysis buffer. The luciferase activities of the extracts were determined using the Luciferase Assay System. The light units (LU) of the luciferase assay were normalized to the cell number of the respective probe (determined immediately prior to lysis by the neutral red method) after subtraction of extract background (LU_{Luc} minus background/absorption of neutral red_{530nm}) and were calculated as relative promoter activity given in per cent of controls (resident untreated cells = 100%).

Determination of ecNOS mRNA stability

HUDEC grown to near confluence on 10-cm culture plates were pre-treated with actinomycin D (Act D; 5 μg ml^{-1}) for 1 h before treatment with amphotericin B (AmB) at concentrations indicated. Additionally, control cells were incubated with TNF-α plus IL-1β (1000 u ml^{-1} each), cytokines which were shown to decrease endothelial ecNOS stability (Alonso *et al.*, 1997; Yoshizumi *et al.*, 1993). After 3, 6, 12, 24 or 48 h of incubation with AmB/Act D endothelial cells were trypsinized and washed twice with ice-cold phosphate-buffered saline. As under these transcription blocked conditions endothelial GAPDH mRNA expression failed to serve as an internal standard for mRNA extraction or amplification efficiency we used IL-2 mRNA expression of cytokine-activated EL-4-6.1 mouse thymocytes as an internal control for RNA isolation and the PCR procedure. Prior to cell lysis and RNA isolation we mixed 2×10^5 AmB/Act D treated HUDEC with 4×10^5 interleukin-1β (1000 u ml^{-1}) activated EL-4-6.1 mouse thymocytes which do not express the ecNOS. Cells mixtures were lysed and DNA free total RNA was isolated using the NucleoSpin RNA isolation kit (Macherey-Nagel GmbH, Düren, Germany) or the Qiagen RNA isolation kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions. ecNOS PCR was performed as described above. For mouse IL-2 cDNA amplification the oligonucleotides ATGTACAGCATG-CAGCTCGCATC (sense; mouse interleukin-2 mRNA, bases 48–70, GenBank accession no. X73040) and GGCTTGTTGAGATGATGCTTTGAC (antisense; mouse interleukin-2 mRNA, bases 526–549, GenBank accession no. X73040) were used. PCR was carried out following standard protocols with the following cycle profiles: 38–40 cycles with 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 ecNOS cDNA-amplification, and 34 cycles with 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 mouse IL-2 cDNA amplification. Aliquots of ecNOS and IL-2 products 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 1D software.

Sequence-analysis of the amplified ecNOS products

PCR products were purified *via* Qiagen columns (Qiagen, Hilden, Germany) and cycle sequenced with the ABI BigDye Terminator Kit (Perkin-Elmer, Weiterstadt, Germany) using ecNOS forward and reverse amplification primers on an automated sequence analyser (ABI 310 from Perkin-Elmer).

Sequence analysis of the amplification products obtained from resident cells by priming with the ecNOS-primer revealed a 100% homology with the published rat sequence (GenBank accession number: AF085195) or with the published sequences (GenBank accession number: M95296) of human ecNOS cDNA (data not shown).

Western-blot-analysis of the ecNOS protein

Endothelial cell cultures were washed, scraped from the dishes, transferred to a microcentrifuge tube, and boiled for 5 min. Proteins (40 μg per lane) were separated by electrophoresis in a 10%-SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Incubations of the blots were: 2 h with blocking buffer (2% BSA, 5% non fat milk powder, 0.1% Tween 20 in PBS-buffer), 1 h at 37°C with a 1 : 1500 dilution of the anti-ecNOS antibody, 1 h with a 1 : 1500 dilution of the secondary horseradish peroxidase conjugated rabbit-antimouse-IgG-antibody, incubated for 5 min in ECL reagent (Pierce, Rockford, IL, U.S.A.), and exposed to an enhanced 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. The secondary horseradish peroxidase conjugated goat anti-mouse IgG antibody was diluted 1 : 2000 prior to use. Densitometric analysis of the visualized ecNOS protein was performed by using the KODAK 1D software.

Statistical Analysis

Data are given as arithmetical means ± s.d. Values were calculated using analysis with Student's *t*-test (two-tailed for independent samples).

Results

Amphotericin B toxicity

Human microvascular dermal endothelial cells (HUDEC) or rat aortic endothelial cells (RAEC) were incubated with amphotericin B (AmB) or the tracer desoxycholate (DOC) at concentrations from 0.3 – 15 μg ml^{-1}. After 48 h of incubation the relative number of viable or dead cells was determined using neutral red staining or trypan blue exclusion, respectively. At concentrations up to 5.0 μg ml^{-1} as used in our experiments neither AmB nor DOC exerted any significant cytotoxic effects on HUDEC or RAEC (Figure 1).

Effects of amphotericin B on ecNOS mRNA expression of resident cells

Reverse transcription-polymerase chain reaction (RT – PCR) was performed with total RNA extracted from resident endothelial cells grown for 48 h in the presence or absence of AmB or DOC at concentrations indicated. As shown in Figure 2, in resident RAEC incubation with AmB between 0.3 and 1.2 μg ml^{-1} led to a reproducible increase in the amount of the ecNOS amplification product whereas, at 2.5 μg ml^{-1} AmB the signal decreased when compared to probes with 1.2 μg ml^{-1} AmB, and incubation with 5 μg ml^{-1} AmB completely abolished endothelial ecNOS mRNA expression (Figure 2A). The tracer DOC used at the same concentrations as AmB did not show any effect (Figure 2B). In none of our experiments AmB induced *de novo* iNOS mRNA expression (Figure 2C).

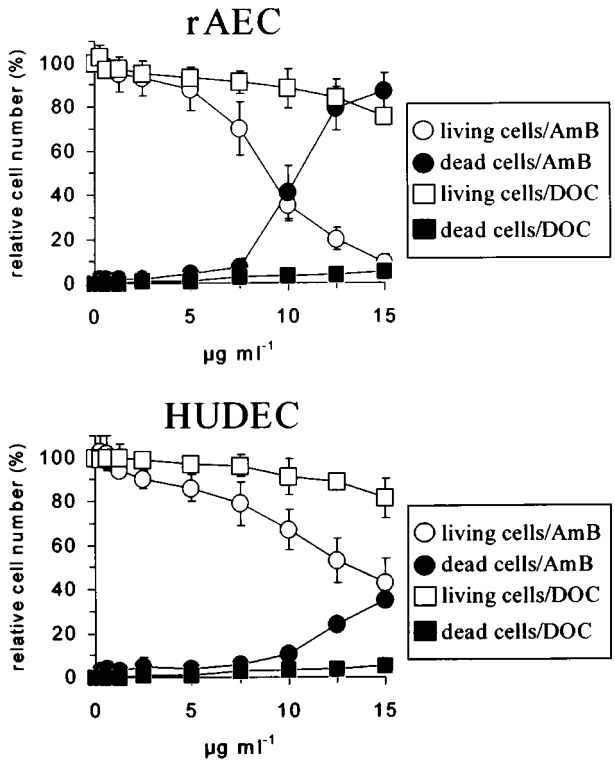

Figure 1 Cytotoxicity of amphotericin B and desoxycholate. Rat aortic endothelial cells (A, RAEC) or human dermal endothelial cells (B, HUDEC) were incubated with amphotericin B (AmB) or the tracer desoxycholate (DOC) at concentrations from 0.3 – 15 μg ml^{-1}. After 48 h of incubation viable cells or dead cells were visualized using neutral red staining or trypan blue exclusion. Then the relative number of cells, as compared to untreated controls, was calculated. At concentrations up to 5.0 μg ml^{-1} as used in our experiments neither AmB nor DOC exerted any significant cytotoxic effects in the cell cultures examined. Values are the mean ± s.d. of five individual experiments.

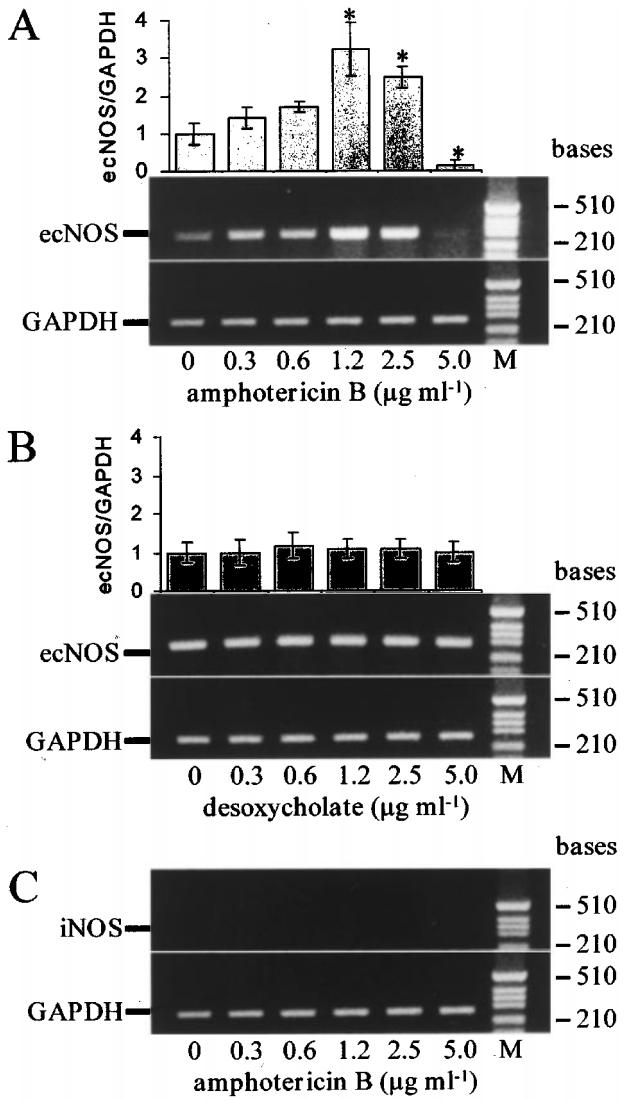

Figure 2 Concentration-dependent effect of amphotericin B on endothelial ecNOS mRNA expression. Reverse transcription and polymerase chain reaction (RT – PCR) was performed with total RNA extracted from resident rat aortic endothelial cells (RAEC) cells grown for 48 h in the presence or absence of amphotericin B (AmB) or the tracer desoxycholate (DOC) at concentrations indicated. PCR was primed by oligonucleotides for ecNOS (A and B) or iNOS (C). Gels shown represent one out of three experiments with identical results. Bars represent the mean ± s.d. of relative ecNOS/GAPDH-ratios obtained by densitometric analysis of amplification products from three individual experiments. (A) In RAEC incubation with AmB between 0.3 and 2.5 μg ml^{-1} led to a reproducible concentration-dependent increase in the amount of the ecNOS amplification product with a peak at 1.25 μg ml^{-1} and a subsequent decrease with a complete loss of ecNOS mRNA expression at 5 μg ml^{-1} AmB. (B) The tracer DOC used at the same concentrations did not show any effects. (C) In none of the experiments AmB led to *de novo* iNOS expression. Lanes M, DNA-size marker showing the 210, 298, 344, 396 and 510 bp DNA-fragments. *$P<0.001$ as compared to controls.

Next, we examined the time course of AmB effects on rat endothelial ecNOS mRNA expression. During the 48 h incubation period ecNOS mRNA expression in controls was constant (Figure 3A). When grown in the presence of 1.2 μg ml^{-1} AmB (Figure 3B) a steady increase in the ecNOS mRNA amplification product over time was observed, whereas in cultures incubated with 5.0 μg ml^{-1} AmB expression steadily decreased and was undetectable after 48 h of incubation (Figure 3C).

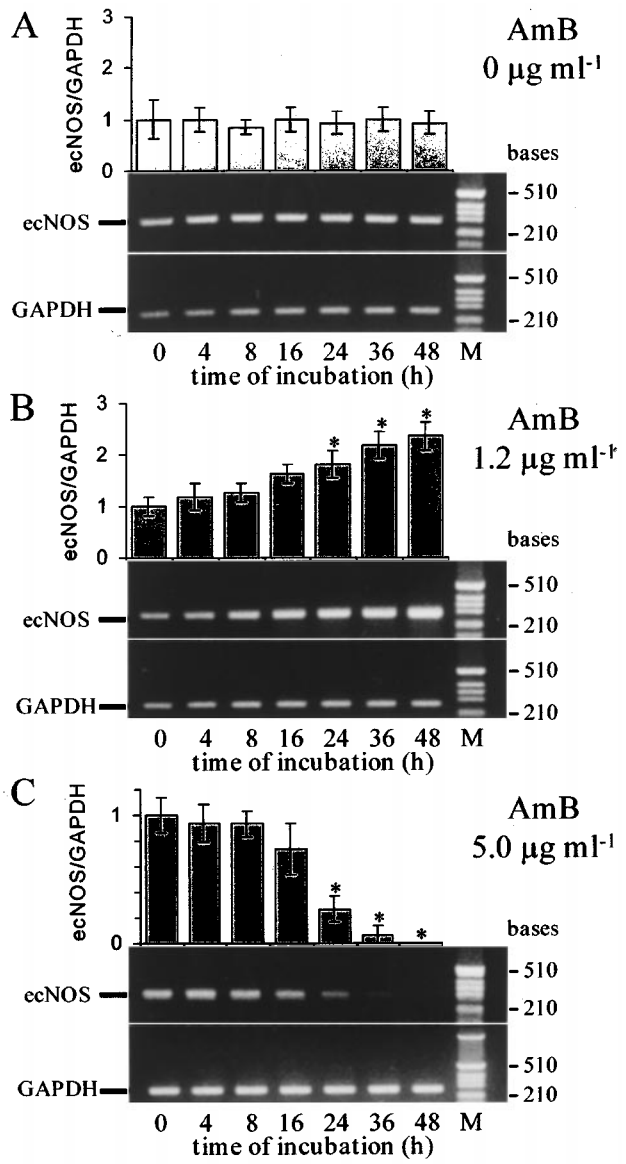

Figure 3 Time course of endothelial ecNOS mRNA expression after incubation with different concentrations of amphotericin B. Reverse transcription and polymerase chain reaction (RT–PCR) specific for ecNOS mRNA was performed with total RNA extracted from rat aortic endothelial cells (RAEC) at different times of AmB incubation. Each gel shown represents one of three experiments with similar results. Bars represent the mean ± s.d. of the relative values of the ecNOS/GAPDH-ratios from three individual experiments. (A) During the 48 h period examined the ecNOS mRNA expression in control cells was constant. (B) In RAEC cultures grown in the presence of 1.2 μg ml^{-1} AmB a continuous increase in ecNOS mRNA was found. (C) In RAEC cultures incubated with 5.0 μg ml^{-1} AmB a strong decrease in ecNOS mRNA expression was found with complete loss of ecNOS mRNA after 48 h. Lanes M, DNA-size marker showing the 210, 298, 344, 396 and 510 bp DNA-fragments. *$P<0.001$ as compared to the controls.

Effects of amphotericin B on endothelial ecNOS protein expression and enzyme activity

RAEC (7×10^6) were incubated for 48 h in the absence or presence of AmB at concentrations indicated (Figure 4). Cytosolic proteins were subjected to SDS–PAGE, and transferred to a nitrocellulose membrane. Staining with an ecNOS antibody revealed that incubation of resident RAEC with 1.2 μg ml^{-1} AmB led to a 4 fold increase in ecNOS protein expression as compared to control cells. With 2.5 μg ml^{-1} AmB a 2 fold increase was found and incubation with 5 μg ml^{-1} AmB led to a strongly significant reduction or complete inhibition (20 ± 17% of the control) of ecNOS protein expression, respectively (Figure 4). The same cells were used to monitor enzyme activity by determination of nitrite in culture supernatants. As shown in Figure 5A, in resident RAEC the presence of AmB at concentrations between 0.1 and 1.2 μg ml^{-1} led to a concentration-dependent increase in the nitrite concentrations which significantly peaked at 1.2 μg ml^{-1}. Above concentrations of 1.2 μg ml^{-1} AmB, the endothelial NO production decreased again reaching a minimum at 5.0 μg ml^{-1} AmB. Additional incubation of resident endothelial cell cultures with the NOS-inhibitor N^G-monomethyl-L-arginine (L-NMA: 1 mM) significantly suppressed the AmB-induced increases in nitrite formation (Figure 5B).

Effects of amphotericin B on ecNOS mRNA expression in cytokine activated cells

The reverse transcription-polymerase chain reaction (RT–PCR) was performed with total RNA extracted from

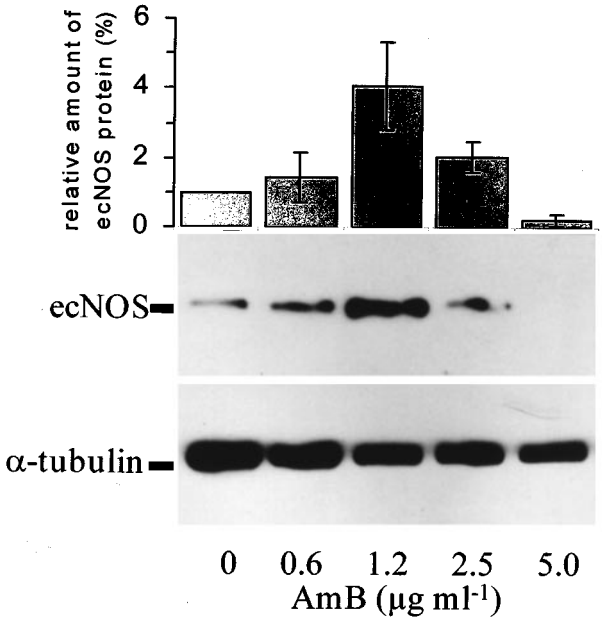

Figure 4 Concentration-dependent effects of amphotericin B on ecNOS protein expression. Total protein (40 μg) obtained from rat aortic endothelial cells (RAEC) grown for 48 h in the absence or presence of amphotericin B (AmB) was separated on a SDS-polyacrylamide gel, blotted, incubated with an ecNOS antibody, and visualized using ECL detection. Bars represent the relative amount of ecNOS protein as compared to controls (0 μg ml^{-1} AmB) obtained by densitometric analysis of the blots. With RAEC 1.2 μg ml^{-1} AmB increases the ecNOS protein expression by the factor of approximately 4 and at 2.5 μg ml^{-1} AmB by the factor of approximately 2 as compared to the controls (0 μg ml^{-1}). In contrast, incubation of RAEC with 5 μg ml^{-1} AmB led to a loss of ecNOS protein.

resident or cytokine activated (200 u ml^{-1} IL-1β plus 500 u ml^{-1} TNF-α plus 100 u ml^{-1} human IFN-γ) HUDEC grown for 48 h in the presence or absence of AmB at concentrations indicated. RT–PCR was primed by ecNOS-specific oligonucleotides. As shown in Figure 6, after cytokine challenge ecNOS mRNA expression of HUDEC decreased by a factor of approximately 0.5 as compared to resident cells. With cytokine activated HUDEC the decrease in ecNOS mRNA expression could be compensated by incubation with 0.6 μg ml^{-1} AmB and incubation with 1.2 μg ml^{-1} AmB led to a reproducible and highly significant enlargement of the ecNOS amplification product. In contrast, incubation with 5 μg ml^{-1} AmB again completely eliminated endothelial ecNOS mRNA expression.

Effect of amphotericin B on ecNOS gene transcription

To analyse the effect of amphotericin B (AmB) on the activity of the endogenous human ecNOS promoter, human ECV304 cells stably transfected with pNOS III-Hu-3500-Luc-neo that contained a 3470 bp fragment of the 5′-flanking sequence of the human ecNOS gene cloned before a luciferase reporter gene, were incubated with amphotericin B at concentrations indicated. As a positive control, incubation with 10 nM phorbol-12-myristate-13-acetate (PMA) and as a negative control incubation with TNF-α plus IL-1β (1000 u ml^{-1} each) was included. Increasing concentrations of AmB (0.3–5.0 μg ml^{-1}) or cytokine-challenge had no effects on ecNOS promoter activity (Figure 7) whereas PMA treatment resulted in a 2.7 fold increase exactly as shown previously (Li *et al.*, 1998).

Effect of amphotericin B on ecNOS mRNA stability

Human dermal microvascular endothelial cells (HUDEC) were transcriptionally blocked by addition of actinomycin D (Act D; 5 μg ml^{-1}) 1 h prior to treatment with amphotericin B (AmB) at concentrations indicated. Additionally, as positive control, cells were incubated with TNF-α plus IL-1β (1000 u ml^{-1} each). After 3, 6, 12, 24 or 48 h ecNOS mRNA expression was determined by RT–PCR exactly as described in Methods. As shown in Figure

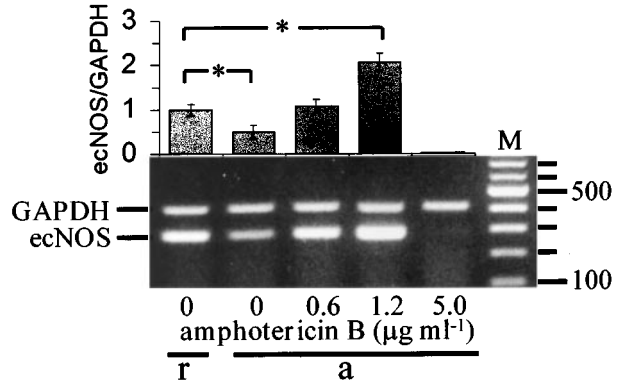

Figure 6 Effects of amphotericin B on ecNOS mRNA expression in cytokine activated cells. Reverse transcription-polymerase chain reaction (RT–PCR) was performed with total RNA extracted from resident (r) or cytokine activated (a, 200 u ml^{-1} IL-1β plus 500 u ml^{-1} TNF-α plus 100 u ml^{-1} human IFN-γ, respectively) human microvascular dermal endothelial cells. After cytokine challenge (a) ecNOS mRNA expression in HUDEC was diminished. This decrease could be compensated by incubation with 0.6 μg ml^{-1} AmB and 1.2 μg ml^{-1} AmB led to a highly significant augmentation in ecNOS mRNA expression as compared to the resident untreated controls (r). Again, incubation of cytokine activated (a) HUDEC with 5 μg ml^{-1} AmB completely eliminated endothelial ecNOS mRNA expression. Bars represent the mean$\pm$s.d. of relative values of the ecNOS/GAPDH-ratios obtained by densitometric analysis of amplification products from four individual experiments. Lane M, DNA-size marker showing the 100, 200, 300, 400, 500, 600 and 700 bp DNA-fragments. *$P<0.001$.

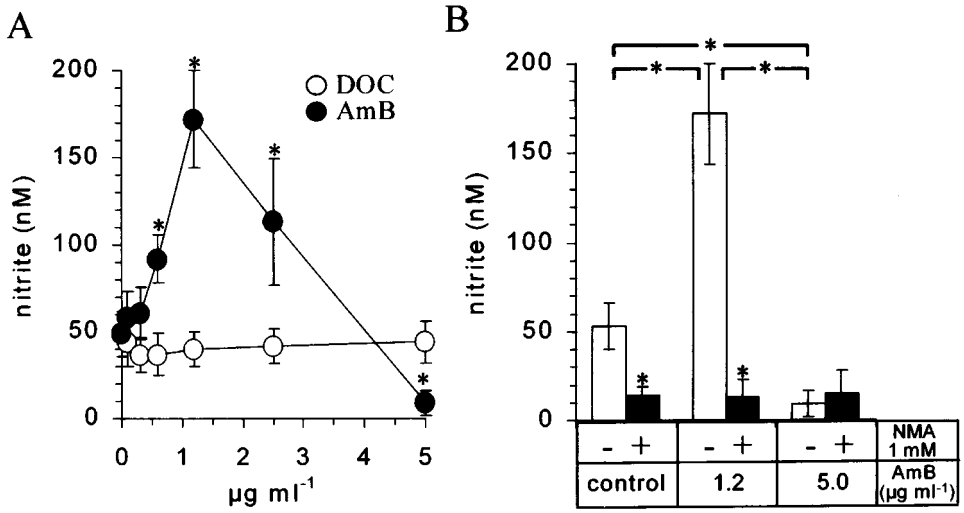

Figure 5 Effects of amphotericin B on endothelial ecNOS activity. Rat aortic endothelial cells (RAEC) were incubated for 24 h with amphotericin B (AmB) or the tracer desoxycholate (DOC) at concentrations indicated. Cells were washed and after an additional incubation for 24 h in the presence of the same additives, nitrite was determined in culture supernatants using the diazotization reaction as described in Methods. (A) In resident RAEC the presence of AmB at concentrations between 0.1 and 1.2 μg ml^{-1} led to a concentration-dependent increase in the ecNOS activity. At concentrations above 1.2 μg ml^{-1} of AmB a fall in endothelial constitutive NO production can be seen with a highly significant decrease of constitutive NO formation at 5.0 μg ml^{-1} AmB as compared to controls. The tracer desoxycholate used in the same concentration range as AmB showed no significant effects. (B) Additional incubation of endothelial cell cultures with the NOS inhibitor N^{G}-monomethyl-L-arginine (1 mM) led to a highly significant suppression of AmB-induced effects on increases in nitrite formation as evidence for the NOS-specificity. Values are the mean$\pm$s.d. of 3–6 individual experiments. *$P<0.001$.

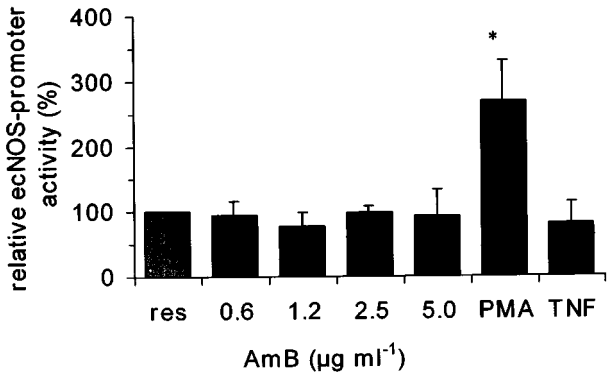

Figure 7 Amphotericin B does not affect ecNOS gene transcription. To analyse the effect of amphotericin B (AmB) on ecNOS promoter activity, human cell line ECV 304 (stably transfected with a 3470-b fragment of the 5′-flanking sequence of the human ecNOS gene cloned before a luciferase reporter gene) were incubated with AmB at concentrations indicated and luciferase activity was measured after 18 h of incubation. As controls, cells were incubated with phorbol-12-myristate-13-acetate (PMA) or with TNF-α plus IL-1β (1000 u ml^{-1} each). Bars represent the relative mean$\pm$s.d. of the relative ecNOS promoter activity of 4–6 individual experiments. Incubation of the pNOS III-Hu-Luc-neo stably transfected ECV 304 cells with AmB or TNF-α plus IL-1β had no influence on ecNOS promoter activity. In contrast, control cultures consisting of ECV 304 cells incubated with PMA exhibited a 2.7 fold increase in promoter activity. *$P<0.01$.

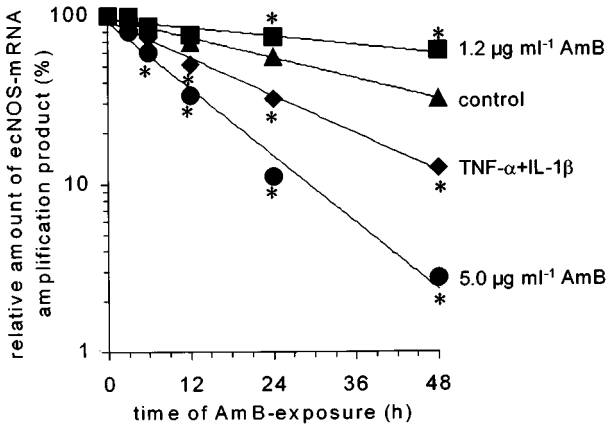

Figure 8 Amphotericin B concentration-dependent affects ecNOS mRNA stability. In human dermal endothelial cells (HUDEC) the effect of amphotericin B (AmB, 1.2 and 5.0 μg ml^{-1}) or TNF-α plus IL-1β (1000 u ml^{-1} each) on the ecNOS mRNA half-life was examined at 3, 6, 12, 24 or 48 h of incubation under transcriptional block (5 μg ml^{-1} actinomycin D). The plot data represent the relative mean$\pm$s.d. of relative ecNOS mRNA amplification product obtained from three individual experiments exactly as described in Methods. In untreated HUDEC (0 μg ml^{-1} AmB) the approximate half-life of the ecNOS mRNA was 29 h, whereas in cells treated with 1.2 μg ml^{-1} AmB the half-life was strongly increased. In contrast, incubation of HUDEC with 5.0 μg ml^{-1} AmB or with cytokines strongly decreased the ecNOS mRNA half-life to approximately 8 or 12 h. *$P<0.05$.

8, in untreated HUDEC the approximate half-life of the ecNOS mRNA was 29 h. In cells treated with 1.25 μg ml^{-1} AmB ecNOS mRNA stability was strongly augmented and in our experiments half-life was calculated as 54 h. In contrast, both, incubation of HUDEC with 5.0 μg ml^{-1} AmB or with cytokines strongly destabilized the ecNOS mRNA and a half-life of 8 or 12 h was calculated, respectively.

Discussion

Identifying the molecular events involved in renal toxicity as a result of AmB treatment is clinically important, as nephrotoxicity is the major limiting factor in the use of this otherwise highly effective antifungal agent. Recent results using both, *in vivo* administration and cultured endothelial cells, indicate that the vasoconstrictor endothelin is not involved therein (Heymann *et al*., 1992). Otherwise, several recent studies have shown that blockade of nitric oxide production induces kidney damage associated with renal vasoconstriction, decreased renal blood and plasma flow, and fall in glomerular filtration rates (Elsner *et al*., 1992; Granger *et al*., 1992; Tolins *et al*., 1990) followed by substantial microvascular and glomerular damage (Baylis *et al*., 1992; Ribeiro *et al*., 1992; Salazar *et al*., 1992).

Due to the similarity in effects, these findings point to a possible involvement of NO synthesis in the mechanism of AmB-induced nephrotoxicity. Both, the known AmB accumulation in the kidney (Lawrence *et al*., 1980), and the data presented here demonstrate a reduction of ecNOS mRNA stability with higher AmB concentrations followed by inhibited ecNOS protein expression and endothelial NO production. Considering the vasodilating and antithrombogenic properties of nitric oxide, impaired constitutive NO formation will favour vasoconstriction, platelet and leukocyte adhesion to the endothelium, and thrombus formation with the possible outcome of ischaemia, hypoperfusion, and hypoxic tubular destruction (Raij *et al*., 1996), results which parallel those seen as a consequence of chronic AmB-treatment. Indeed, one early study had already attempted to attribute the fall in renal blood flow and renal vascular resistance after AmB application in rats to diminished EDRF release by simultaneous infusion of the NO-donor nitroprusside but found small effects only (Heidemann *et al*., 1983). However, it is important to note that authors examined effects of short term infusion of AmB in concentrations far exceeding those used here. Further, the effects of nitroprusside are difficult to predict, as the major site of nitroprusside metabolism is in the liver (Bates *et al*., 1991; Rao *et al*., 1991). In contrast, calcium channel antagonists, known dilators of vascular smooth muscle cells, in hypertension due to impaired ecNOS activity inhibit AmB-induced vasoconstriction, improve renal haemodynamics and thus protect from chronic AmB nephrotoxicity (Tolins & Raij, 1988; 1991).

Interestingly, AmB at the physiological dose of 1.2 μg ml^{-1} which is in the clinically relevant range of peak serum levels (Fields *et al*., 1970), leads to a significant increase in endothelial NO production. These increases are not due to expression of inducible NO synthase. Furthermore, the drug-induced increase in endothelial NO production strongly correlates with increases in ecNOS mRNA and protein expression and could be the result of AmB-mediated increases in ecNOS mRNA half-life.

Since AmB therapy is usually given during inflammatory conditions of patients, we also examined the effects on ecNOS mRNA expression during pro-inflammatory cytokine challenge. The known downregulation of ecNOS mRNA expression by pro-inflammatory cytokines was completely abolished in the presence of lower AmB concentrations where even potent increases in constitutive NO production are maintained in addition to iNOS generated NO due to the presence of pro-inflammatory cytokines.

Semi-quantitative RT–PCR as used here does not allow for exact quantitation of mRNA contents. However, protein expression levels measured by immunoblots as well as nitrite determination in culture supernatants exactly paralleled the

mRNA levels measured by RT–PCR. The ecNOS mRNA half-life determined by RT–PCR in resident or cytokine treated human endothelial cells was calculated as approximately 29 or 12 h respectively and is thus comparable to those determined previously (Lamas *et al*., 1992; Laufs *et al*., 1997; Ziesche *et al*., 1996). We thus conclude that the effects of AmB on ecNOS activity may be due to modulating effects of the drug on ecNOS mRNA stability. This is further strengthened by our findings that AmB did not affect ecNOS reporter gene transcription efficiency, whereas PMA-stimulation showed the expected increase as described previously (Li *et al*., 1998).

Stabilization or destabilization of a mRNA is the result of a combined actions of different factors. The list of putative mRNA regulatory factors and mRNA-binding proteins involved is large and growing rapidly. Moreover, chaperones, cytokines, growth mediators as well as intracellular Ca^{2+} or Mg^{2+} concentrations also affect mRNA decay rates (Ross, 1996). Thus, modulation of heat shock protein, cytokine, chemokine or growth factor expression by AmB as well as AmB-modulated binding of mRNA-binding proteins *via* unspecific effects on intracellular Ca^{2+} or Mg^{2+} homeostasis may represent likely mechanisms by which AmB influences endothelial ecNOS mRNA stability.

In conclusion, with the experiments presented here we show a concentration-dependent and biphasic effect of amphotericin B on expression and activity of endothelial constitutive NO synthase: With AmB at concentrations comparable to patient serum levels a highly significant increase in ecNOS activity was found, whereas higher concentrations of AmB, as are present in kidney due to drug accumulation during therapy, led to a strong decrease of ecNOS mRNA expression, ecNOS protein, and ecNOS activity, effects attributed to AmB's influence on the ecNOS mRNA stability.

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