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Release of prostaglandin E-2 in bovine brain endothelial cells after exposure to three unique forms of the antifungal drug amphotericin-B: role of COX-2 in amphotericin-B induced fever

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Abstract

Common formulations of amphotericin-B include a deoxycholate colloidal suspension (d-Amph), an amphotericin-B lipid complex (Ablc), and a liposomal product (l-Amph). The clinical incidence of infusion related fever is highest with d-Amph, intermediate with Ablc, and lowest with l-Amph. In the present study, we measured the activation of cyclooxygenase-2 (COX-2) and subsequent release of prostaglandin E-2 (PgE-2) from brain microvessel endothelium treated with these three formulations of amphotericin-B. Primary cultured bovine brain microvessel endothelial cells (BBMEC) were exposed to d-Amph, Ablc and l-Amph at concentrations that can be achieved in the plasma of patients receiving the drug. Media samples from the cells were collected and analyzed for PgE-2. Release of PgE-2 from BBMEC monolayers treated with l-Amph was similar to cells receiving culture media alone. In contrast, Ablc and d-Amph caused significantly greater release of PgE-2 from BBMEC monolayers compared to controls receiving culture media alone. PgE-2 release after d-Amph treatment was similar in magnitude to that observed with bacterial lipopolysaccharide (LPS). Western blot analysis indicated significant induction of COX-2 expression in BBMEC following LPS, Ablc or d-Amph treatment. Furthermore, PgE-2 release following exposure of BBMEC monolayers to either LPS or the various amphotericin-B formulations was reduced by the addition of the selective COX-2 inhibitor, NS-398. These studies indicate that amphotericin-B induces COX-2 expression in brain microvessel endothelium resulting in release of fever producing PgE-2. The magnitude of PgE-2 release from BBMEC following exposure to various amphotericin-B

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formulations mirrors the clinical observations regarding amphotericin-B induced fever and serves as initial support for the clinical use of COX-2 inhibitors to reduce amphotericin-B fever.

Keywords: Amphotericin; Brain endothelial cell activation; COX-2

Introduction

Amphotericin-B is a polyene antifungal agent that is the drug of choice for most systemic fungal infections [5]. While an effective agent, its use has been associated with a severe toxicity profile. It is associated with both acute and chronic renal failure which may require hemodialysis. The administration of amphotericin-B is also associated with infusion related reactions characterized by fever, chills, and rigors which can be severe and complicate the care of patients.

In an attempt to reduce toxicity various lipid formulations of amphotericin-B have been developed. The two most commonly used lipid formulations are an amphotericin-B lipid complex (Ablc; Abelcet®) and a liposomal product (l-Amph; Ambisome®). These lipid formulations reduce the incidence and severity of renal dysfunction compared to the older product formulated with deoxycholate (d-Amph; Fungizone®). The degree to which the lipid products reduce the incidence of infusion related fever is not completely clear. A randomized controlled trial did report a significantly lower incidence of infusion reaction with l-Amph compared to Ablc [22]. However, despite l-Amph having less infusion related fever it remains an occasional limiting toxicity [4]. Ablc may have lower liability for fever than d-Amph but a controlled trial has not been performed.

Infusion related fevers associated with amphotericin-B exposure have generally been explained by a release of pyrogenic cytokines such as IL-6, TNF-alpha, and IL-1beta from leukocytes [1]. One of the prominent theories of fever pathogenesis is that systemic cytokines, released from leukocytes exposed to an exogenous pyrogen such as LPS or amphotericin-B, function as endogenous pyrogens and activate neurons that innervate the hypothalmic thermoregulatory centers [2].

One limitation of this hypothesis is that for the inflammatory cytokines to act as central mediators of fever, these macromolecules must first cross the blood brain barrier. The brain microvessel endothelial cells that form the blood brain barrier are well suited for keeping large macromolecules present in the blood from entering into the brain's extracellular environment and while investigators have shown that pyrogenic cytokines can transit into the CNS it is unclear their contribution to fever given the low concentrations achieved in the CSF [3]. The brain microvessel endothelial cells can act as a source of the endogenous pyrogens such as prostaglandins, IL-1beta, and TNF-alpha. Thus an alternative mechanism for fever involves the activation of brain microvessel endothelial cells through either peripheral or centrally released stimuli resulting in the release of prostaglandins that in turn activate hypothalamic thermoregulatory centers [2,6,14].

Infusion related reactions associated with amphotericin-B while not as severe as those seen with LPS administration are similar in character [15,22]. Previous studies have reported an induction of COX-2 in the blood brain barrier following LPS administration [6]. The effects of amphotericin on brain endothelium have not been studied. In the present study, the relative pyrogenic potential of three

clinically used forms of amphotericin were evaluated using an in vitro model for pyrogenicity that measures the release of prostaglandin E-2 (PgE-2) from primary cultured bovine brain microvessel endothelial cells (BBMEC). The ability of amphotericin to increase the expression of COX-2 in BBMEC monolayers and the effects of a selective COX-2 inhibitor on PgE-2 release from BBMEC following exposure to the various amphotericin formulations were also examined. The results of the current study suggest that BBMEC can be used to qualitatively determine the pyrogenic potential of the different formulations of amphotericin and provide preliminary evidence in support of the use of COX-2 inhibitors to reduce amphotericin-induced fever.

Materials and methods

Materials

Minimal essential medium and Ham's F-12 medium were purchased from Fisher (St. Louis, MO). The COX-2 inhibitor NS-398 was purchased from Research Biochemicals International (Natick, MA). Bovine fibronectin, bovine serum albumin, and *E. coli* lipopolysaccharide was purchased from Sigma (St. Louis, MO). Bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Indianapolis, IN). Amphotericin was obtained from the manufacturers with d-Amph (Fungizone®;MSD) diluted with 5% dextrose then with culture media to deliver 5 mcg/ml into each well, Ablc (Abelcet®; Elan, Inc) was supplied as a 5 mg/ml suspension and diluted and delivered in an identical manner as d-Amph, and l-Amph (Ambisome®; Fujisawa, Inc) was supplied as a liposomal powder and diluted with 5% dextrose and further diluted in culture media to deliver 50 mcg/ml final concentration in each well.

Isolation and Culturing of BBMECs

Primary BBMECs were collected from the gray matter of fresh bovine cerebral cortices using enzymatic digestion and centrifugal separation methods as previously described [16]. The BBMECs were seeded (50,000 cells/cm^2) on collagen coated, fibronectin-treated 75 cm^2 tissue culture flasks or collagen and fibronectin treated 6 well plates. The culture media consisted of: 45% minimal essential medium, 45% Ham's F-12 nutrient mix, 10 mM HEPES, 13mM sodium bicarbonate, 50 mcg/ml gentamicin, 10% equine serum, and 100 mcg/ml heparin. The BBMECs were cultured in a humidified 37 °C incubator with 5% CO_2, with media replacement occurring every other day until the monolayers reached confluency (approximately 11–14 days).

Amphotericin, LPS, and NS-398 treatment of BBMECs

LPS (10 ng/ml), d-Amph (5 mcg/ml), Ablc (5 mcg/ml), and l-Amph (50 mcg/ml) were added in triplicate wells and 30 microliters of media collected at times 0, 1 hour, 2 hours, 4 hours, 8 hours, and 12 hours after addition. Responses to LPS and the various amphotericin products were compared to control monolayers receiving only culture media. To determine the effect of a COX-2 inhibitor on the production of PgE-2, experiments identical to those described above except for the addition of NS-398 (0.1 microM) at the same time as LPS or amphotericin, were performed.

Enzyme Immunoassay for Determination of PgE-2

The effects of LPS and amphotericin on the release of PgE-2 were determined by measuring the amounts of PgE-2 secreted into the culture medium of BBMEC monolayers following exposure to LPS, d-Amph, Ablc, and l-Amph. Baseline or time zero samples were collected immediately prior to the addition of LPS or amphotericin formulation. The concentrations of PgE-2 were determined using a specific PgE-2 enzyme immunoassay kit (Assay Designs, Ann Arbor, MI). The lower limit of sensitivity was 36 pg/ml and intra-assay and inter-assay relative standard deviation was less than 10%. All PgE-2 concentrations fell within the dynamic range of the assay and were corrected for the amount of cellular protein in each sample as determined using the BCA protein assay.

Western Blot Analysis of COX-2 in BBMECs

Confluent BBMEC monolayers in 6 well plates were treated with d-Amph (5 mcg/ml), Ablc (5mcg/ml), and l-Amph (50 mcg/ml) for 12 hours. The cells were solubilized with 1% SDS/1% protease inhibitor, and the protein was quantified using the BCA method. COX-2 (5 ng; 72 kDa) protein standards (Cayman Chemicals, Ann Arbor, MI) were used as positive controls. The samples (25–30 mcg) of the BBMEC lysates were loaded onto a 7.5% polyacrylamide gel and electrophoresed at 45–65 V. The proteins were transferred from the polyacrylamide gel to a polyvinylidene fluoride membrane at 4 °C for one hour with 240 mA. Following the transfer, the membrane was blocked with Tween-20/1% BSA and then incubated overnight at 4 °C with the COX-2 primary antibody (1:1000 dilution). After incubating with the primary antibody, the membrane was washed a series of times with blocking buffer consisting of 1% BSA with Tween-20 (0.3–3%). The membrane was incubated with anti-mouse secondary antibody (1:1500) for 30 minutes at 4 °C and then washed a final series of times with blocking buffer [1% BSA with Tween-20 (0–3%)]. The protein bands were developed using the enzyme chemiluminescence method (Amersham Pharmacia Biotech, Cleveland, OH).

Statistical analysis

A repeated measures model was used in the data analysis. The model includes an effect for treatment, time, and a treatment-time interaction. Time was modeled as a continuous variable. Pairwise differences between the treatment groups were examined at each of the time-points and the p-values adjusted using Tukey's method.

Results

Effects of Amphotericin on PgE-2 production/release

Table 1 gives the average PgE-2 concentrations 4 and 12 hours after treatment with either LPS, d-Amph, Ablc, or l-Amph. Exposure of BBMEC monolayers to LPS, d-Amph, or Ablc caused significantly higher concentrations of PgE-2 to be released from the cells compared to media only controls. Of the amphotericin formulations l-Amph was the one that did not significantly increase PgE-2 release from BBMEC over media alone. Of the two formulations that did increase PgE-2

Table 1
Average PgE-2 concentrations (pg/mcg protein) in BBMEC

Treatment	4 hours		12 hours	
	PgE-2 (S.D.)	p-value[a]	PgE-2 (S.D.)	p-value[a]
Media Control	47.3 (11.7)		50.7 (0.9)	
LPS Control[b]	72.9 (3.0)	0.0002	101.6 (12.9)	< 0.0001
d-Amph[c]	70.2 (7.6)	0.0001	110.1 (12.0)	< 0.0001
Ablc[b,c]	51.6 (11.7)	0.0011	81.3 (14.8)	0.015
l-Amph	41.1 (5.0)	0.99	62.8 (14.6)	0.98

[a] Compared to media only controls using a repeated measures model.
[b] LPS > Ablc; p = 0.0047; Repeated measures model with Tukey's correction.
[c] d-Amph > Ablc; p = 0.0002; Repeated measures model with Tukey's correction.

release there was a significantly greater amount of PgE-2 at 12 hours associated with d-Amph compared to Ablc.

Fig. 1 illustrates time versus PgE-2 concentrations. In control wells, concentrations of PgE-2 were consistently around 50 pg/mcg protein. LPS, which serves as a positive control produced a robust PgE-2 release from BBMEC monolayers with maximal release of PgE-2 of approximately 100 pg/mcg protein after 12 hours of exposure. LPS, d-Amph, and Ablc treatment led to a significantly higher PgE-2 response compared to either media controls or l-Amph treatment as early as 1 or 2 hours after addition. d-Amph produced an effect similar to LPS with PgE-2 concentrations slightly exceeding those produced by LPS at 12 hours. Release of PgE-2 following Ablc treatment was intermediate in magnitude.

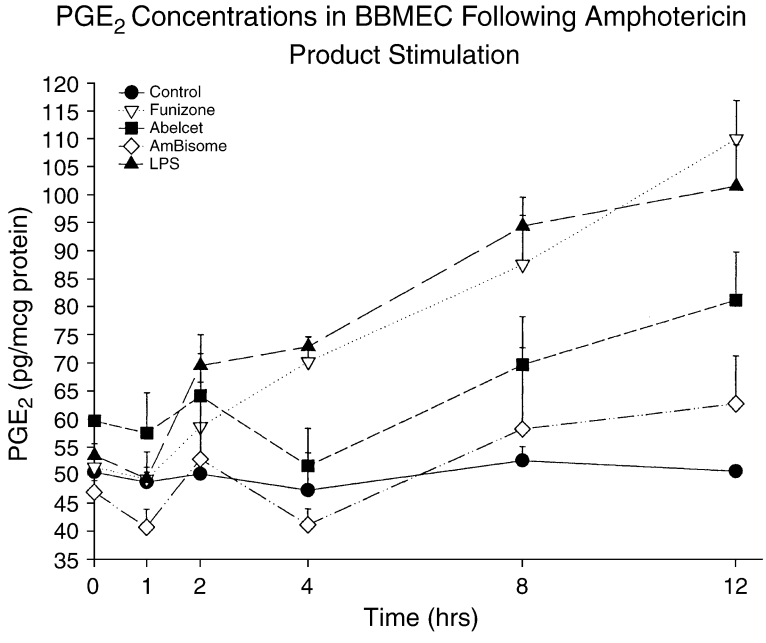

Fig. 1. PgE-2 generation in BBMEC treated with LPS (10 ng/ml), d-Amph (5 mcg/ml), Ablc (5 mcg/ml), and l-Amph (50 mcg/ml). Each time point represents the Mean of triplicate samples with respective SEM.

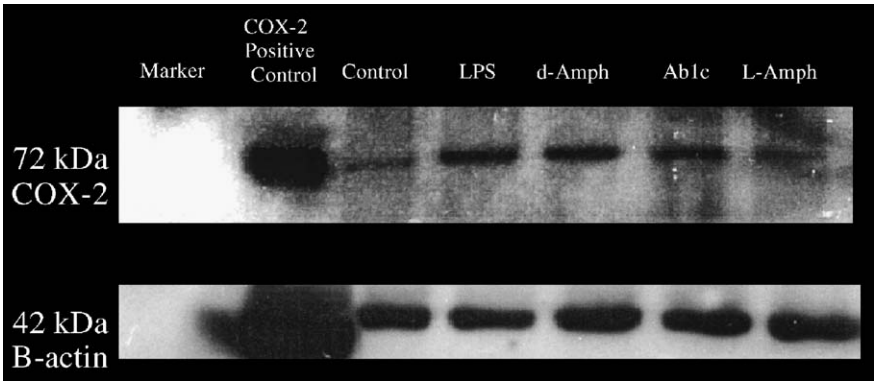

Fig. 2. Western blot analysis of COX-2 protein expression in BBMEC. Confluent BBMEC monolayers were treated with culture media alone (negative control), LPS 10 ng/ml (positive control), d-Amph (5 mcg/ml), Ablc (5 mcg/ml), and l-Amph (50 mcg/ml) for 12 hours. The amount of BBMEC lysate loaded was 25–30 mcg.

Effects of Amphotericin on COX-2 expression

Western blot analysis shows expression of the COX-2 enzyme in BBMEC monolayers following treatment with LPS and each amphotericin formulation compared with control cells receiving culture medium alone. BBMEC monolayers treated with LPS, d-Amph, and Ablc showed a substantial increase

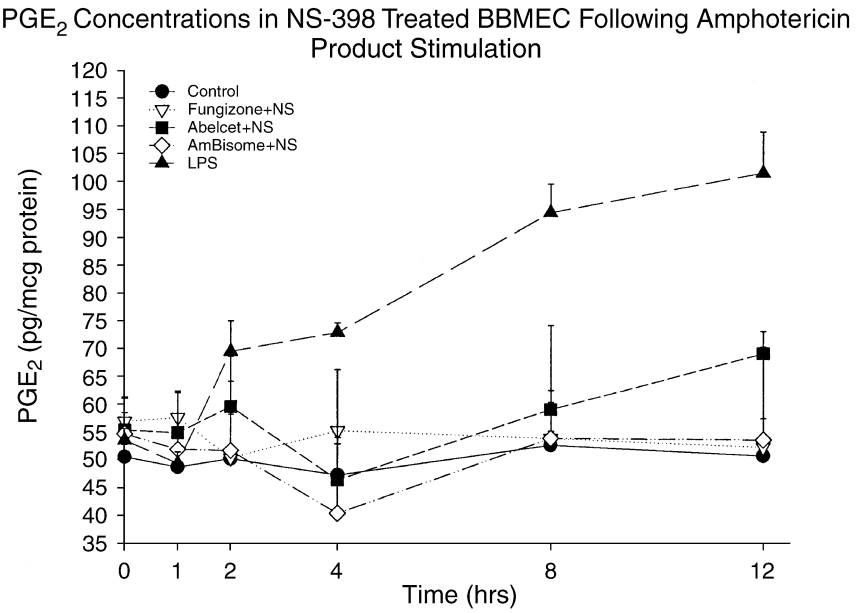

Fig. 3. PgE-2 generation in BBMEC treated with LPS (10 ng/ml), d-Amph (5 mcg/ml), Ablc (5 mcg/ml), and l-Amph (50 mcg/ml) with co-administration of the COX-2 inhibitor NS-398. LPS (10 ng/ml) positive control without NS-398 co-treatment included. Each time point represents the Mean of triplicate samples with respective SEM.

in the expression of COX-2 protein when compared with control cells. Expression of COX-2 was similar in l-Amph treated cells and media controls (Fig. 2).

Effects of the COX-2 inhibitor NS-398 on PgE-2 production/release

NS-398 reduced PgE-2 release under all conditions. Treatment with NS-398 resulted in no statistically significant difference in PgE-2 concentrations between media control, LPS, d-Amph, Ablc, and l-Amph treated cells (Fig. 3). LPS without NS-398 produced the usual increase in PgE-2 indicating the model was intact.

Discussion

Inflammatory response to infectious and other noxious stimuli leads to activation of an acute phase reactant system which includes the release of inflammatory cytokines, hepatic proteins, and the activation of various cellular enzyme systems. Clinical manifestations of this activation can include vasodilation and subsequent hypotension, increase in hepatic enzymes, and fever. Fever can also occur from the administration of various drugs including interferons, anti-thymocyte globulin, and GM-CSF. The mechanisms involved in fever produced by these protein drugs include the release of inflammatory cytokines from peripheral blood leukocytes [20].

The mechanism by which non-protein drugs produce fever has always been less clear. Idiosyncratic fevers associated with drug classes like antibiotics and anti-arrhythmics are poorly understood. In addition, the sporadic occurrence of fever with non-protein drugs makes it difficult to characterize and to determine causality. Amphotericin, is an example of a pyrogenic non-protein drug. It produces fever in the majority of patients who receive it for treatment or prevention of systemic fungal infections [12]. Despite many studies evaluating amphotericin fever the mediators responsible have not been completely identified. Investigators have reported that amphotericin exposure will increase mRNA and protein for the inflammatory cytokines IL-1beta and TNF-alpha in mononuclear cells [11,17]. Despite these in vitro experiments there is little data on the elevation of inflammatory cytokines in the systemic circulation after the adminstration of amphotericin. In six patients administered different formulations of amphotericin there was an inconsistent elevation in systemic TNF-alpha and IL-6. While all patients had fever none of the formulations produced a consistent elevation in systemic cytokines [1].

Clearly, local release of mediators of fever are important. Since cytokines and PgE-2 will not easily transit into the CNS, amphotericin must activate cells outside the CNS that then can release endogenous pyrogens into the brain. Brain endothelium is the likely candidate as it represents the cellular interface between the blood and the central nervous system and activation of these cells can result in the release of PgE-2 into the brain [6,14].

LPS and amphotericin are both potent pyrogens. In addition to producing fever, both have been reported to cause hypotension and hypoxia [15,21]. They also have similar physicochemical properties with both being amphiphatic molecules that bind to lipid components of blood and are able to form self-aggregates [7,15]. These similarities suggest that the mechanism of amphotericin infusion related reactions may be similar to LPS induced fever.

The data presented here supports similar magnitude and kinetics of PgE-2 release after treatment of BBMECs with LPS and d-Amph. Both produced an increased expression of the COX-2 protein and a

corresponding increase in PgE-2 concentrations. PgE-2 release was effectively inhibited in amphotericin treated cells by a selective COX-2 inhibitor (NS-398).

LPS is known to have a cellular receptor identified on various white blood cells and endothelium. CD14 is the initial LPS binding protein on the cell membrane which then leads to the activation of Toll-Like receptor 4, activation of NFκB and increased transcription of various inflammatory cytokines [9]. The binding of LPS to CD14 ultimately results in an activated cell that produces and releases inflammatory mediators including IL-1beta, TNF-alpha, IL-6, and PgE-2. Because of the similarities in the physical properties of LPS and amphotericin and the resultant fever, it is possible that amphotericin fever may result from the activation of a Toll-Like receptor 2 or 4 on brain endothelium [10].

While the release of endogenous pyrogens from various white blood cells may be important, in the following study we have shown for the first time that amphotericin can directly increase release of PgE-2 from brain endothelium and thus is directly able to activate central fever mediators. The relative importance of the release of inflammatory cytokines from white blood cells versus direct effects on brain endothelium is not known. Both mechanisms of amphotericin fever are probably important but the use of agents to prevent amphotericin fever that target systemic TNF-alpha and IL-1beta are unlikely to be completely effective at preventing infusion related reactions.

The use of COX inhibitors to prevent infusion reactions is much more likely to be successful in that these drugs can effect both the leukocyte mediated production of PgE-2 and brain endothelial cell release of PgE-2 [8]. TNF-alpha or IL-1beta released from peripheral blood white cells will upregulate COX-2 in brain endothelium and will increase PgE-2 production [23]. Similar effects have been observed in cultured BBMEC monolayers following TNF-alpha exposure [14]. If amphotericin fever has a final pathway of brain endothelial cell production of PgE-2 then the COX-2 inhibitors may be ideal as preventive agents for infusion related reactions.

The populations which receive amphotericin such as patients being treated for cancer often are thrombocytopenic and inhibition of platelet function which is an effect of COX-1 inhibition is not desirable. The COX-2 specific inhibitors have limited platelet inhibiting activity which may allow their safe use in thrombocytopenic cancer patients [19].

Amphotericin commonly produces renal dysfunction which can be severe. The mechanism by which renal damage occurs is not entirely clear but involves renal vasoconstriction and ischemic damage to the kidneys [18]. COX enzymes are responsible for producing renal prostaglandins which are important in maintaining renal blood flow. The use of non-steroidal anti-inflammatory drugs which block COX-1 and COX-2 may potentiate nephrotoxicity induced by renal vasoconstriction [13]. COX-2 inhibitors have been thought to produce less nephrotoxicity but the role of COX-2 in producing renal prostaglandins suggests that their use in patients on amphotericin should proceed with care. The average duration of amphotericin empiric therapy in cancer patients is approximately one week and the associated risk of nephrotoxicity from a week of COX-2 inhibitor therapy is likely small [18,22]. Because of the possibility of nephrotoxicity and potential subjectivity of measuring infusion related reactions the use of COX-2 inhibitors to prevent amphotericin infusion reactions should be studied in a blinded randomized controlled trial.

In conclusion, amphotericin directly stimulates the production of PgE-2 from brain endothelial cells by activating COX-2. This direct effect of amphotericin is likely to be a major mechanism for infusion related fever. The use of a COX-2 specific inhibitor led to a complete inhibition of amphotericin mediated PgE-2 release and if tolerated in patients may be an important addition to prophylaxis regimens for amphotericin infusion reactions.

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