Biochimica et Biophysica Acta, 732 (1983) 251–255
Elsevier

BBA 71751

INFLUENCE OF AMPHOTERICIN B ON LEUCINE UPTAKE IN 3T3 CELLS

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(Received June 22nd, 1982)

Key words: Amphotericin B; Amino acid uptake; Leucine; (3T3 cells)

By studying the effect of leucine competitors we found that activation of the specific leucine-transport system underlies the enhancement of leucine uptake in mouse 3T3 fibroblast cells induced by sublethal doses of Amphotericin B (synergic effect). The relation of the antibiotic activity and the alteration of the membrane cholesterol interaction with lipids is discussed.

Introduction

The lipophilic polyene antibiotic, Amphotericin B, causes alterations in the permeability of artificial and biological sterol-containing membranes [1,2]. Fluorimetric and spectrophotometric studies have shown that Amphotericin B binds highly specifically to the cholesterol in the membrane [3,4], and it is thought that the resulting Amphotericin B-cholesterol complexes lead to the formation of 'pores' [5–7]. These pores form aqueous channels through the membrane that allow the passive diffusion of molecules to and from the external environment into the cells [4,5]. This uncontrolled flux of molecules soon unbalances the cellular metabolism and causes cell lysis.

It has been observed that sublethal concentrations of Amphotericin B increase the membrane permeability in fibroblast cell lines (synergic effect) [8].

We analyzed the effect of sublethal doses of Amphotericin B on leucine uptake in 3T3 cells to investigate if the antibiotic provokes a modification of the cell membrane which specifically alters the activity of transport systems.

Materials and Methods

Cells

Mouse 3T3 cells, mycoplasm-free, from our laboratory were propagated in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% calf serum and antibiotics. For incorporation measurements the cells were subcultured in 35-mm dishes at $5 \cdot 10^5$ cells/dish in 10% calf serum. After 18 h the cells were washed three times with warm phosphate-buffered saline to remove serum, and then incubated as described.

[^{3}H]Thymidine incorporation

[*methyl*-^{3}H]Thymidine (specific activity, 20 Ci/mmol) at 1 μCi/ml was added to the culture medium, and the cells were incubated for the desired period. The incorporation was terminated with three rapid washes in ice-cold phosphate-buffered saline, after which 5% trichloracetic acid was added to the dishes. Aliquots of the trichloroacetic acid extracts were used for scintillation counting.

[^{3}H]Leucine uptake

The leucine-uptake studies were performed basically according to the method of Nakamura and Weber [9]. Briefly, 3T3 cells were washed three times with warm phosphate-buffered saline. Subsequently, the cells were incubated for 1 h in phosphate-buffered saline supplemented with 0.1% D-glucose (medium 1), prior to measuring leucine uptake, to reduce intracellular levels of amino

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acids. These cells are referred to as 'depleted cells'. 3T3 cells were washed three times in Na^+-free medium 1, in which choline and K_2HPO_4 substituted NaCl and Na_2HPO_4, respectively, and then incubated in the same medium (0.5 ml) containing [^{3}H]leucine (5 μCi/ml) (specific activity, 60 Ci/mmol) and 1 μM leucine. The measurements were performed for 15 min at room temperature. After four rapid washes with ice-cold Dulbecco's modified Eagle's medium, the cells were lysed with 1 N NaOH (1 ml). After 8 h, 1 N HCl was added to the cells and aliquots were counted.

Amphotericin B (Fungizone Squibb) solutions were always prepared fresh. L-Leucine, L-phenylalanine, L-isoleucine, L-tryptophan and ouabain were all from Sigma. The radiochemicals were from New England Nuclear.

Results

The sublethal concentrations of Amphotericin B that increase the membrane permeability were determined by measuring the effect of the antibiotic on the amount of thymidine incorporation during a 2-h pulse. Logarithmically growing 3T3 cells in 10% calf serum were tested with increasing concentrations of Amphotericin B in the absence of serum to avoid any interference from sterols. As shown in Fig. 1, at concentrations around 1 μg/ml of the antibiotic, the incorporation of labelled thymidine started to fall. This value is in accordance with our previous observation [10] obtained by measuring labelled uridine uptake in the presence of serum in an 18-h pulse; 0.5 μg/ml of Amphotericin B seems to exert maximum activation of thymidine incorporation (30% over the controls).

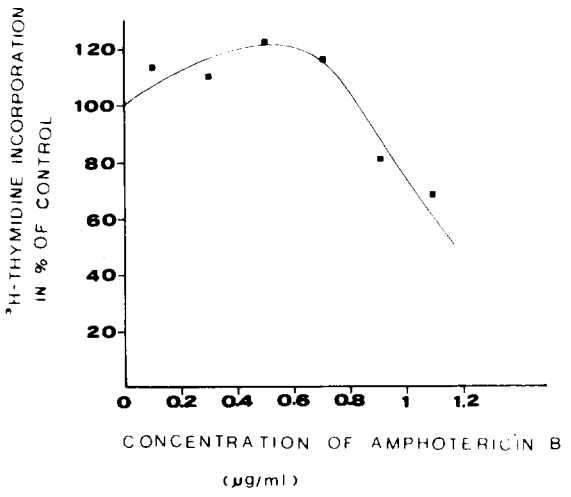

Fig. 1. Effects of Amphotericin B on thymidine incorporation. 3T3 cells were grown as described in Materials and Methods and washed three times with Dulbecco's modified Eagle's medium, and then incubated for 2 h in Dulbecco's modified Eagle's medium containing [^{3}H]thymidine (1 μCi/ml) plus different amounts of Amphotericin B.

We investigated the effect of sublethal doses of Amphotericin B on the control of the leucine-transport system. In 3T3 cells, leucine is incorporated into the cell mainly by the Na^+-independent L-system; however, it can use other Na^+-dependent transport systems, although with a lower efficiency [11–14]. We measured leucine uptake mediated by the L system in 3T3 cells growing exponentially. The cells were preincubated in medium 1 at 37°C for 1 h to increase the endogenous level of amino acids. Under these conditions the initial rates of L-leucine uptake rise (probably by a trans-stimulation characteristic of the L system) because the increased endogenous amino acids increase the potential for the exchange [15,16].

In Fig. 2 we show that Amphotericin B at 0.5 μg/ml stimulates leucine uptake more than 2-fold, while at higher concentrations it progressively depresses leucine uptake until cell lysis occurs.

Even though leucine is mainly transported by the Na^+-independent system (L system), we considered the possibility that under our experimental conditions leucine could be also incorporated by other systems. It is possible, in fact, that a minor fraction of leucine is incorporated by the Na^+-dependent system (A system) even under Na^+-free conditions since the Na^+ concentration in the assay medium may be decreased but not completely eliminated.

If cells are grown in the presence of ouabain, the Na^+-dependent component of leucine incorporation is inhibited [17]. Leucine uptake is altered only very slightly by ouabain since at concentrations of 10^{-5} M it gives an incorporation rate 94% of that of controls, and at 10^{-4} and 10^{-3} M ouabain gives 94 and 89% incorporation, respectively. Therefore, under our conditions the Na^+-dependent transport system seems to be barely, if at all, active.

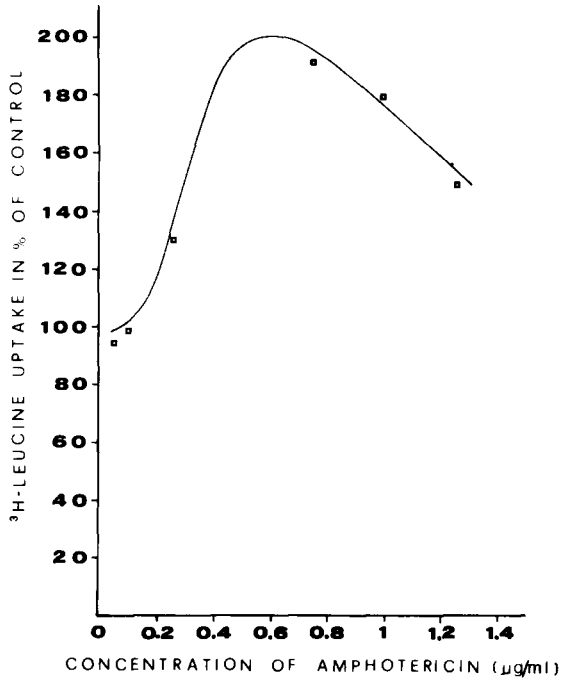

Fig. 2. Effect of Amphotericin B on leucine uptake. 3T3 cells were preincubated for 60 min as described in Materials and Methods, and incubated in Na^+-free medium 1 containing [^{3}H]leucine (5 μCi/ml), 1 μM leucine and Amphotericin B at the indicated concentrations.

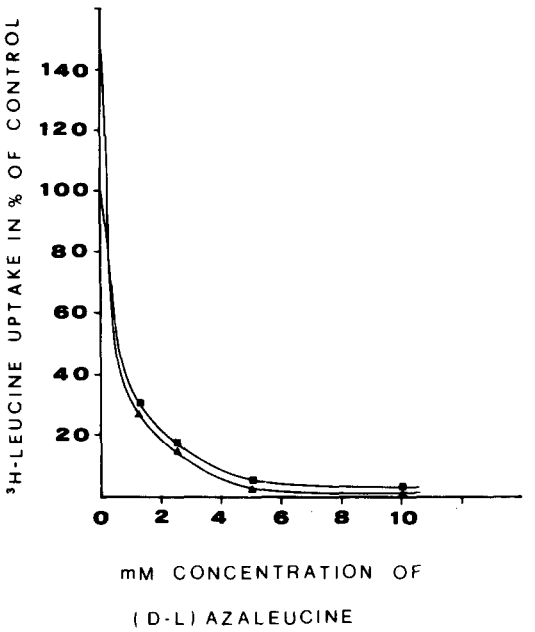

Fig. 3. Effect of DL-azaleucine on the leucine uptake. 3T3 cells treated as described previously were incubated in the presence of: ■, DL-azaleucine; ▲, DL-azaleucine plus Amphotericin B at 0.5 μg/ml. The assay medium contained [^{3}H]leucine (5 μCi/ml) and 1 μM leucine.

We then investigated the effects of specific inhibitors of leucine uptake in 3T3 cells treated with 0.5 μg/ml Amphotericin B to obtain direct evidence that the activation of leucine uptake causes a stimulation of the specific transport systems without affecting the control mechanisms.

A very efficient inhibitor of leucine, DL-azaleucine, was used [18]. In Fig. 3 we show that leucine uptake decreased in a dose-dependent manner when azaleucine was added to 3T3 cells treated with 0.5 μg/ml of Amphotericin B. The inhibitory effect of DL-azaleucine was the same whether or not the cells were treated with the antibiotic. Moreover, addition of the competitor completely abolished the Amphotericin B activation.

This result was confirmed with other inhibitors of leucine uptake. L-Isoleucine, L-phenylalanine and L-tryptophan are substrates for the L system for 3T3 cells [14]. This indicates a more than 95% inhibition of leucine uptake. These inhibitors, all at 5 mM, were tested in the absence or presence of Amphotericin B. As shown in Table I, no differences in the levels of the inhibition of leucine

TABLE I

EFFECT OF INHIBITORS OF LEUCINE UPTAKE IN THE ABSENCE AND PRESENCE OF 0.5 μg/ml AMPHOTERICIN B

Inhibitors (5 mM)	No Amphotericin B		+ Amphotericin B		
	cpm	%	cpm	%	% of control
None	93333	100	147000	100	
L-Phenylalanine	2171	2.3	2339	1.6	2.3
L-Isoleucine	1944	2.0	1710	1.2	1.8
L-Tryptophan	2476	2.6	2271	1.5	2.4

uptake were observed compared with the untreated control; therefore, yet again the enhancement produced by the antibiotic addition is promptly eliminated, showing that Amphotericin B acts specifically on the transport system.

Discussion

Amphotericin B is a polyene antibiotic that complexes the sterols of the cell membrane and forms aqueous channels through the membrane. Its lethal action is believed to be mainly due to these pores, which permit a passive flux of molecules across the membrane and soon cause cell lysis. Furthermore, the synergic effect has also been correlated with the formation of these membrane structures [8,19]. In this communication we report evidence that sublethal doses of Amphotericin B act on the cell's membrane by increasing the activity of a transport system. This interpretation was prompted by the finding that low concentrations of Amphotericin B increase the permeability of the cell membrane of several fibroblast cell lines [20].

The control of leucine uptake was studied in order to investigate the effects of Amphotericin B on the membrane permeability of 3T3 cells. The observed stimulation of leucine incorporation is dependent on the concentrations used.

When a specific inhibitor of leucine uptake such as DL-azaleucine was added to 3T3 cells, its inhibitory effect was the same whether Amphotericin B was present or not. This demonstrates that the amino acid always enters the cells via their own transport system, even when its uptake is activated by Amphotericin B. If the increased uptake was caused by simple diffusion there would be a remaining incorporation not suppressible by the inhibitor. Also, other inhibitors, such as isoleucine, phenylalanine and tryptophan, which compete with leucine for the same transport system had the same effect.

Treatment of cells with ouabain demonstrated that the Na^+-dependent transport system is barely active in our conditions. This is in accordance with the observation that the competitors we used are specific for the L system and act very efficiently under our conditions.

Our results indicate that the increase in the uptake observed with Amphotericin B treatment caused an activation of the specific transport system, probably following alterations in the fluidity of the membrane induced by the antibiotic. Amphotericin B, in fact, prevents the cholesterol of the membranes from interacting with the lipids. This effect causes an alteration in the fluid state of the membrane [21] and perhaps also alterations in the membrane protein activity [22,23].

It is interesting to recall that cholesterol added to the membrane of erythrocytes inhibits leucine transport [24]; thus, the Amphotericin B stimulation of leucine uptake observed by us seems to indicate that Amphotericin B-cholesterol association is physiologically similar to cell membrane cholesterol depletion.

Acknowledgements

We are indebted to Dr. A. Pannuti for valuable suggestions in the preparation of the manuscript and to Jean Gilder for editing the text. The technical assistance of Miss R. Terrecciano is gratefully acknowledged. This work was supported by grants 79.00366.84 and 80.00576.84 from the CNR Progetto Finalizzato Virus.

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