Inhibition of β -adrenergic binding by fungal metabolites

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Summary. Strains of *Aspergillus flavus*, *Fusarium* sp., *Rhizopus* sp. and *Candida albicans* all produced inhibitors of β -adrenergic receptor binding; strains of *Saccharomyces* sp. and *Schizosaccharomyces* sp. did not. In tests with glutamic acid as the sole nutrient source, a *Fusarium* sp. produced four-fold larger amounts of inhibitor than the other fungi. The inhibitor from the *Fusarium* sp. was further purified by lyophilisation and sequential solvent extraction in chloroform, ethyl acetate and butanol; 60% of the original activity was recovered. The inhibitor had an estimated molecular size of 650 Da, and did not absorb light in the visible or ultraviolet range. When compared with a similar inhibitor from *Escherichia coli*, the *Fusarium* sp. inhibitor appeared to be a more potent inhibitor of β -adrenergic and dopaminergic binding to mammalian cells.

Introduction

Micro-organisms, including yeast and fungi, are capable of interacting with various mammalian hormone systems. For example, *Aspergillus alliaceus* produces a structurally-related antagonist of cholecystokinin,¹ *Trichophyton mentagrophytes* has proteins that bind progesterone,² *Paracoccidioides brasiliensis* has proteins that bind-estradiol,³ and *Saccharomyces cerevisiae* may produce an oestrogen-like substance.⁴ The production of extracellular inhibitors of the β -adrenergic response by bacteria and fungi in mammalian cells is of particular interest.^{5–10} We have noted previously that certain bacteria produce a substance that can inhibit the binding and function of adrenergic receptor systems, and we made preliminary observations that certain fungi also produce an inhibitor of β -adrenergic binding.^{8–10} This report extends these preliminary observations to include representatives of the major groups, and the inhibitor from a *Fusarium* sp. was further purified and characterised.

Materials and methods

Cultures

A strain of *Fusarium* sp. was obtained from the Department of Laboratory Medicine, University of Connecticut Health Center, Farmington, CT. All other cultures were purchased from Presque Isle Company, Presque Isle, PA (table I). Cultures were maintained on Sabouraud's Dextrose Agar (Difco),

stored at 4°C, and passaged monthly. *Escherichia coli* strain 9637 was from a local collection.

Preparation of culture filtrates

Overnight standing cultures of bacteria or yeast were grown in 50 ml of trypticase soy broth at 37°C. After centrifugation at 9000 *g* for 10 min, cells were resuspended in nutrient solutions containing either glucose or glutamic acid, 1 mg/ml in phosphate-buffered saline (PBS) and agitated overnight at 37°C. Supernates were passed through 0.45- μ m filters and collected. Filamentous fungi were incubated in 50 ml of Sabouraud's maltose broth at room temperature until mycelial mats formed (generally 5–7 days). After washing three times with 50 ml of PBS, the culture media were replaced with 50 ml of nutrient solution, glucose or glutamic acid 1 mg/ml in PBS, followed by incubation for various periods (1–7 days) at room temperature. Replacement media were collected, filtered through cotton gauze, and subjected to further centrifugation and filtration.

Adrenergic binding assays

C6 rat glial or Y1 mouse adrenal tissue-cultured cells for assay were maintained in F10 medium.¹¹ The standard reaction mixture contained, in a final volume of 1 ml, 10^5 – 10^6 tissue-cultured cells, 0.1 μ Ci (*c.* 100 000 cpm) of 1×10^{-5} M 3 H-dihydroalprenolol (for β -adrenergic binding) or 0.1 μ Ci (*c.* 100 000 cpm) of 5×10^{-5} M 3 H-spiperone (for dopaminergic binding) in 50 μ l, with or without test solutions or unlabelled ligand.¹¹ After incubation at 37°C for 10 min, the cells

Fig. 1. Scheme for purification of β -adrenergic inhibitor from *Fusarium* sp.

were collected by filtration; the filters were washed three times with PBS before being dried and placed in scintillation fluid, and the number of filter-bound counts was determined in a β -scintillation counter. These reaction mixtures and incubation times ensured optimal binding (i.e. steady state) conditions.¹¹ The percentage of the total available counts that were specifically bound (Bsp) was used for probit analysis to calculate the volume required for 50% inhibition (I_{50}).¹² Bsp (i.e. specific binding) is the difference between total bound counts and non-specific counts, and is expressed as a percentage of the total available counts. Non-specific counts are the counts obtained in the presence of excess (1000-fold) unlabelled ligand, i.e., L-alprenolol (for β -adrenergic binding), or haloperidol (for dopaminergic binding). Experiments were repeated at least three times, with triplicate determinations of binding within each experiment. Student's *t*-test was used for statistical analysis.

Purification of inhibitor

Filtrates of supernates of *Fusarium* sp. incubated for 5 days with glutamic acid 1 mg/ml were harvested and concentrated by lyophilisation. Identical volumes of uninoculated medium were prepared in the same way as controls for all procedures. No inhibitory activity was lost after lyophilisation. After reconstituting the lyophilised material in deionised distilled water to an 100-fold increase in concentration, sequential solvent extractions were performed with chloroform, ethyl acetate and butanol.¹³ Solvent extracts of culture

filtrates and control solutions were evaporated to dryness in a heating block. When equal samples of extracts and residues of controls and culture filtrates were examined for β -adrenergic binding inhibition, no activity was found in either chloroform or ethyl acetate extracts. All of the inhibitor activity was found in the butanol extracts and unextracted residue. Butanol-extracted inhibitor was then subjected to dialysis with Type C 0.5 tubing (Spectrum Medical Industries Inc., Los Angeles, CA, USA), which has a mol. wt exclusion of 500. Extract reconstituted in 5 ml of deionised distilled water was dialysed at 5°C against 2 L of deionised distilled water, changed daily on three successive days. After dialysis, 60% of the original activity was recovered. This scheme of purification is shown in fig. 1. A further attempt at purification of the *Fusarium* sp. inhibitor was made with Sephadex G-50 gel filtration. This eliminated much, but not all of the highly pigmented material. Butanol-extracted inhibitor was also dried, reconstituted with methanol to the original volume, and subjected to separation by thin-layer chromatography. A solvent system of methanol: water (9:1, v:v) was used on K2 cellulose plates (250- μ m thickness, without binder, Whatman Paper Ltd). Sections (1 cm²) were scraped from the plate covering zones outside the point of sample application as well as areas covering regions from the point of application to the solvent front. Cellulose scrapings were then resuspended in the solvent, mixed and subjected to centrifugation (3000 rpm for 3 min) in an Eppendorf micro-centrifuge. Supernates were dried in a heat block, and the residues were assayed for β -adrenergic binding inhibition activity.

Results

Production of inhibitors by filamentous fungi and yeasts

A. flavus and the *Fusarium* sp. produced the greatest amount of inhibitors/volume of replacement nutrient

Table 1. Inhibition of β -adrenergic receptor binding to C6 cells by fungal filtrates

Species	I_{50} (μ l)	I_{50} /ml (units)
Filamentous fungi		
<i>Aspergillus flavus</i>	50	20
<i>Fusarium</i> sp.	70	14
<i>Rhizopus nigricans</i>	400	2.5
Yeasts		
<i>Candida albicans</i>	640	1.5
<i>Saccharomyces cerevisiae</i>	...	...
<i>Saccharomyces octosporus</i>	...	...
<i>Schizosaccharomyces ellipsoideus</i>	...	...

* I_{50} , μ l required for 50% inhibition of specific binding; I_{50} /ml is expressed as the number of inhibitory units/ml of culture filtrate, where 1.0 ml = 1.0 unit.

..., No inhibition observed with 800 μ l of filtrate.

Fig. 2. Production of inhibitors by *A. flavus* (●) and *Fusarium sp.* (○) in glucose 1 mg/ml in PBS. Samples were taken at the times indicated and 100- μ l volumes were tested for inhibition of β -adrenergic binding to C6 cells. Control, uninoculated medium was also tested and gave no inhibition of binding.

(table I). The *Saccharomyces sp.* and *Schizosaccharomyces ellipsoideus* produced no detectable inhibitors. When examined for the relationship between days in culture and production of inhibitor from glucose, production of inhibitor was first noted after incubation for 3 days; inhibition continued to be present over a 7-day period with *Fusarium sp.* and actually increased in the case of *A. flavus* (fig. 2).

Nutrient requirements for production of inhibitor

Various biochemical intermediates and products were tested as sources of replacement nutrients for the formation of β -adrenergic inhibitor by *A. flavus* and

Fusarium sp. Both *Fusarium sp.* and *A. flavus* produced inhibitor from sodium citrate and sodium succinate, but *Fusarium sp.* produced four times as much as *A. flavus* from glutamic acid. As similar studies with *E. coli* showed that glutamic acid was a favourable substrate for the formation of inhibitor by bacteria,⁹ and as this substrate was more suitable for future attempts to purify inhibitors, most investigations were conducted with glutamic acid as the substrate.

Characterisation of inhibitor

When subjected to thin-layer chromatography, inhibitor was located in a homogeneous area with a peak R_f value of 0.6. Material recovered from this region was found not to absorb light in either the visible or ultraviolet ranges.

An attempt to define the molecular size of the inhibitor by gel filtration with Sephadex G-10 is shown in fig. 3. According to this method, the estimated molecular size of the fungal inhibitor is 650 Da similar to that noted for the bacterial inhibitors.⁸

Comparison of fungal and bacterial inhibitors

Inhibitors from *Fusarium sp.* and *E. coli* produced with glutamic acid as substrate were compared for inhibition of β -adrenergic and dopaminergic binding in Y1 and C6 cells (table II). These results indicate that the fungal inhibitor activity is greater than that of *E. coli*, and that both β -adrenergic and dopaminergic binding appear to be affected to similar degrees by each inhibitor.

Fig. 3. Gel permeation (Sephadex G-10) chromatography of partially purified β -adrenergic binding inhibitor from *Fusarium sp.* The indicated mol. wt standards were subjected to chromatographic separations in parallel experiments. V_o = void volume.

Table II. Comparison of fungal and bacterial inhibitors of adrenergic binding in mammalian cells

Inhibitor	β -adrenergic binding				Dopaminergic binding			
	Y1 Cells		C6 Cells		Y1 Cells		C6 Cells	
	Bsp (SD)	Inhibition (%)	Bsp (SD)	Inhibition (%)	Bsp (SD)	Inhibition (%)	Bsp	Inhibition (%)
None	2.04 (0.02)	...	3.30 (0.34)	...	2.02 (0.04)	...	3.39 (0.40)	...
Fungal inhibitor (<i>Fusarium</i> sp.)	0.16 (0.04)*	92.2	0.37 (0.40)*	89.1	0.11 (0.09)*	94.6	0.32 (0.45)*	90.6
Bacterial inhibitor (<i>E. coli</i>)	0.63 (0.46)*	69.2	1.31 (0.74)	61.4	0.61 (0.46)*	69.9	1.20 (0.71)*	64.7

Bsp, percentage specific binding.

* Volumes tested over four-fold dilution range; $p = < 0.05$.

Discussion

Several fungi produce inhibitors of β -adrenergic and dopaminergic binding. The inhibition is similar to that previously observed with bacteria,^{8,9} although the fungal inhibitor may be more potent than that produced by bacteria. However, further purification and standardisation of the inhibitors will be needed to validate these preliminary conclusions. Fungi produce the inhibitor when incubated with glucose alone, and maximal levels appear after 3–5 days. Inhibitor is also synthesised when succinic acid or the amino acids isoleucine or glutamic acid are used as substrate. Among the fungi and yeasts tested, *Fusarium* sp. and *A. flavus* produced the highest levels of inhibitor. The mechanisms of induction and control of inhibitor production remain to be defined. Loss of *Fusarium* sp. inhibitor activity was observed when extracts were treated with some ion-exchange resins, silica gel, and cellulose-containing binders (unpublished observations). Inhibitor activity was recoverable after thin-layer chromatography on cellulose plates. The fungal inhibitor was found in butanol extracts by a procedure applied to the isolation of toxic substances produced by *Fusarium* sp.¹³ The loss of some inhibitor activity by dialysis may have been due to the loss of a co-factor or of inhibitor molecules. *Fusarium* sp. produce substances that chelate divalent cations and are particularly difficult to isolate by routine procedures.¹⁴ The inhibitor contains no amino acids, nucleic acids, or other substances capable of absorption of light in the ultraviolet and visible ranges tested. The inability to use many routine procedures for isolation and purification, coupled with the presence of the inhibitor in small amounts, have hampered further purification.

Since there is no information about charges or diffusion of the inhibitor, there is limited accuracy in mol. wt estimation by gel exclusion chromatography with Sephadex G10;¹⁵ the inhibitor appeared to elute with a molecular size of *c.* 650 Da.

β -Adrenergic and dopaminergic receptor binding were both inhibited by products of *Fusarium* sp. and *E. coli* when tested in C6 and Y1 cells. Activation of adenylate cyclase secondary to β -adrenergic hormone

binding occurs in C6 cells, but not in Y1 cells.¹¹ If binding had been inhibited in only one cell type, it could be possible that bacteria or fungal products, or both, affect the internal cell mechanisms first, and that such an alteration would then modify the external binding site as a secondary effect. Since this is not the case, it is likely that the inhibitor blocks binding directly. Binding of other ligands (e.g., cholera toxin) to C6 and Y1 cells, which utilise different receptors, and which activate adenylate cyclase by different mechanisms, is not inhibited by the bacterial inhibitor.^{8,9} As both β -adrenergic and dopaminergic binding were affected, and because the receptors are different for these two somewhat related hormones, our results would predict that either the two receptors are located near each other, and that the inhibitor affects the micro-environment of the two receptors, or, less likely, that the inhibitor had some structural similarity to both ligands. By Scatchard analysis, the bacterial inhibitor appears to affect binding affinity and not receptor numbers.^{8,9} Although not yet evaluated for the fungal inhibitors, the bacterial inhibitors also block β -agonist (e.g., isoproterenol)-induced functions (i.e., activation of adenylate cyclase), presumably secondary to their effects on receptor binding.^{8,9}

Characterisation of the inhibitors should aid investigations to understand the role of micro-organisms in both pathogenic and non-pathogenic infections. *Fusarium* sp., as well as *A. flavus* and other filamentous fungi and yeasts, can infect the immunocompromised patient.^{16,17} If β -adrenergic and dopaminergic responses can be significantly altered during the course of infection and disease, host responses may themselves be compromised. In the lung, for example, several different bacteria (e.g., *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Bordetella pertussis*) have been noted to affect the number of β -receptors.⁷ Patients with respiratory tract infections may experience bronchospasm that can be relieved with the use of β -adrenergic agonists. Should the inhibitors described here be involved in interfering with β -adrenergic responses, further understanding of the nature of the inhibitors and their mechanism of action may lead to improved means to prevent or reverse the inhibition.

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