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 alliacetus produces a structurally-related antagonist of cholecystokinin, Trichophyton mentagrophytes has proteins that bind progesterone, Paracoccidioides brasiliensis has proteins that bind-estriadiol, and Saccharomyces cerevisiae may produce an oestrogen-like substance. The production of extracellular inhibitors of the β-adrenergic response by bacteria and fungi in mammalian cells is of particular interest. 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. 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-μm 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. The standard reaction mixture contained, in a final volume of 1 ml, 10E-10E tissue-cultured cells, 0.1 μCi (c. 100000 cpm) of 1 × 10E-3 M 3H-dihydroalprenolol (for β-adrenergic binding) or 0.1 μCi (c. 100000 cpm) of 5 × 10E-4 M 3H-spiperone (for dopaminergic binding) in 50 μl, with or without test solutions or unlabelled ligand. After incubation at 37°C for 10 min, the cells
counts. Non-specific counts are the counts obtained in
Purification and is expressed as a percentage of the total available
evaluations of binding within each experiment. Student’s
test was used for statistical analysis.

Reconstituted at 100-fold increased concentration
Extract with chloroform 60% v/v
Chloroform phase Aqueous phase

Extract with ethyl acetate 60% v/v
Ethyl acetate phase Aqueous phase

Extract with 40% (v/v) n-butanol
n-butanol phase Aqueous phase

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.a 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}).b The
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specifically bound (Bsp) was used for probit analysis to
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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.c 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-
μm 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>
<thead>
<tr>
<th>Species</th>
<th>I_{50} (μl)</th>
<th>I_{50}/ml (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filamentous fungi</td>
<td></td>
<td></td>
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<tr>
<td>Aspergillus flavus</td>
<td>50</td>
<td>20</td>
</tr>
<tr>
<td>Fusarium sp.</td>
<td>70</td>
<td>14</td>
</tr>
<tr>
<td>Rhizopus nigricans</td>
<td>400</td>
<td>2-5</td>
</tr>
<tr>
<td>Yeasts</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Candida albicans</td>
<td>640</td>
<td>1:5</td>
</tr>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Saccharomyces octosporus</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Schizosaccharomyces ellipsoides</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

*a I_{50}, μl required for 50% inhibition of specific binding; I_{50}/ml is
expressed as the number of inhibitory units/ml of culture filtrate.

††† No inhibition observed with 800 μl 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-μl volumes were tested for inhibition of β-adrenergic binding to C6 cells. Control, uninoculated medium was also tested and gave no inhibition of binding.

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, 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 Rf 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.

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.
Discussion

Several fungi produce inhibitors of $\beta$-adrenergic and dopaminergic binding. The inhibition is similar to that previously observed with bacteria, 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. 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. 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; the inhibitor appeared to elute with a molecular size of c. 650 Da.

$\beta$-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 $\beta$-adrenergic hormone binding occurs in C6 cells, but not in Y1 cells. 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., choleratoxin) to C6 and Y1 cells, which utilise different receptors, and which activate adenylate cyclase by different mechanisms, is not inhibited by the bacterial inhibitor. As both $\beta$-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. Although not yet evaluated for the fungal inhibitors, the bacterial inhibitors also block $\beta$-agonist (e.g., isoproterenol)-induced functions (i.e., activation of adenylate cyclase), presumably secondary to their effects on receptor binding.

Characterisation of the inhibitors should aid investigations to understand the role of microorganisms in both pathogenic and non-pathogenic infections. Fusarium sp., as well as A. flavus and other filamentous fungi and yeasts, can infect the immune-compromised patient. If $\beta$-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 $\beta$-receptors. Patients with respiratory tract infections may experience bronchospasm that can be relieved with the use of $\beta$-adrenergic agonists. Should the inhibitors described here be involved in interfering with $\beta$-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.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$\beta$-adrenergic binding</th>
<th>Dopaminergic binding</th>
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<tbody>
<tr>
<td></td>
<td>Y1 Cells (Bsp SD)</td>
<td>C6 Cells (Bsp SD)</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fungal inhibitor</td>
<td>2.04 (0.02)*</td>
<td>3.30 (0.34)</td>
</tr>
<tr>
<td>(Fusarium sp.)</td>
<td>0.16 (0.04)*</td>
<td>0.37 (0.40)*</td>
</tr>
<tr>
<td>Bacterial inhibitor</td>
<td>0.63 (0.46)*</td>
<td>1.31 (0.74)</td>
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Bsp, percentage specific binding.

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


