NOTE

**Cryptococcus neoformans** Produces Authentic Prostaglandin E₂ without a Cyclooxygenase

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Many single-celled eukaryotes produce prostaglandin-like molecules, but these have not been absolutely verified by mass spectrometry. We have isolated, and identified by liquid chromatography-tandem mass spectrometry, authentic prostaglandin E₂ from Cryptococcus neoformans. Cyclooxygenase inhibitors did not inhibit prostaglandin synthesis, and the cryptococcal genome lacks a cyclooxygenase homolog. Thus, novel enzymes must exist.

Our laboratory previously discovered that the opportunistic fungal pathogen *Cryptococcus neoformans* produces an oxylipin from exogenously supplied arachidonic acid (AA) that possesses a bioactivity similar to that of prostaglandin E₂ (PGE₂) and is immunomodulatory in vitro (11, 12). There have been reports of the production of bioactive lipids from other fungi and single-celled eukaryotes that behave like prostaglandins (5–8, 11, 13). However, the sole use of prostaglandin immunono assays, inconsistencies in effective inhibitors, or the apparent lack of enzymes believed critical for prostaglandin production has sometimes cast doubt that these putative prostaglandins are truly authentic. It is often argued that isoprostanes, which lack of enzymes believed critical for prostaglandin production have sometimes cast doubt that these putative prostaglandins are stereoisomers of prostaglandins formed nonenzymatically by free radical oxidation (9), are actually the type of oxylipin being measured. Because of the difficulty in distinguishing such similar molecules, we have employed a highly selective liquid chromatography-tandem mass spectrometry (LC–MS-MS) analysis that can discriminate between prostaglandins and their isoprostane stereoisomers. Our objective was to conclusively identify the cryptococcal PGE₂-like oxylipin.

For LC–MS-MS analysis, stationary-phase *C. neoformans* cells were washed and resuspended at a concentration of 2 × 10⁷ cells/ml in phosphate-buffered saline. Cultures were incubated with and without 500 µM AA with shaking at 37°C overnight. Supernatants were then passed over PGE₂ affinity columns (Cayman Chemical), and the eluates were collected and dried down under N₂ gas. Samples were resuspended in 50% methanol in water, and the material was separated using a Symmetry 2.1- by 150-mm analytical column (Waters) and reverse-phase high-performance liquid chromatography (HPLC). Reverse-phase HPLC was carried out using a gradient elution starting at 75:25:0.1 (water:acetonitrile:acetic acid) for 10 min, followed by a linear shift to 0:100:0.1 over 75 min at 500 µl/min. In this system, PGE₂ standards (Cayman Chemical) elute between 14.5 and 15.5 min, and so this fraction was collected for the cryptococcal samples. This fraction was then extracted into ethyl acetate, and the organic layer was dried down under N₂ gas. LC–MS–MS was carried out using a ThermoFinnigan Surveyor HPLC (San Jose, CA) interfaced directly to a ThermoFinnigan LTQ linear ion-trap mass spectrometer (San Jose, CA). Samples were resuspended in a methanol: water solution (1:1 [vol/vol]) and injected onto a Phenomenex Luna 2.00- by 150-mm 3 μ phenyl-hexyl column (Phenomenex). Mobile-phase solvents were 10 mM ammonium acetate, pH 8.5, and methanol. Compounds were separated and eluted as reported by Yang et al. (17).

We first sought to determine whether we could identify authentic PGE₂ in the supernatants from *C. neoformans*. Our analysis focused upon the mass range of m/z 350.5 to 351.5 for the detection of the [M-H] ion of prostaglandin E₂. Focusing on a narrow m/z range rules out unrelated molecules, such as the products of β-oxidation and 3,18-dihETE, a recently discovered fungal oxylipin (2). We were unable to detect a product in the absence of exogenously supplied arachidonic acid (Fig. 1A). However, when arachidonic acid was added to the cells, a single strong peak was evident (Fig. 1B). In addition, there was a very minor peak, which corresponded to 8-iso-PGE₂. This was not unexpected, because overnight incubation of AA at 37°C will generate isoprostanes. However, this minor peak also served as an internal standard for our analysis and provided additional confirmation that the main cryptococcal product was not an isoprostane. This type of analysis readily distinguishes between these three prostaglandins, despite the remarkable structural similarity between 8-iso-PGE₂, PGD₂, and PGE₂ (structures are shown in Fig. 1C). Thus, based on the correct mass and chromatographic characteristics, *C. neoformans* produces PGE₂.

To confirm that the cryptococcal prostaglandin we identified was PGE₂, MS-MS analysis was performed and the fragmentation pattern of the cryptococcal prostaglandin was compared...
FIG. 1. Chromatographic analysis of the cryptococcal AA metabolite. Putative PGE$_2$ purified from the supernatants of \textit{C. neoformans} cultures was analyzed using LC–MS-MS. Peaks occur when an [M-H] ion within the mass range of $m/z$ 350.5 to 351.5 strikes the detector. (A) Sample without AA. (B) Sample with AA. (C) Elution times of commercial prostaglandin and isoprostane standards.
to that of a PGE$_2$ standard. As can be seen in Fig. 2, the
fragmentation patterns of the cryptococcal prostaglandin (Fig.
2A) and the PGE$_2$ standard (Fig. 2B) were identical. Alto-
gether, the data in Fig. 1 and 2 unequivocally demonstrate that
Cryptococcus neoformans can produce authentic PGE$_2$.

Since prostaglandins produced in mammalian cells are cyc-
loxygenase dependent, we next tested for the presence of a
cyclooxygenase-like enzyme in cell-free lysates from C. neoform-
ans. Lysates were generated by mechanically disrupting the
cells in a pH 6.5 phosphate buffer using 0.5-μm glass beads and
spinning down the debris at 15,000 × g for 45 min at 4°C. Lysates were incubated in the presence of 500 μM AA for 2 h
at 37°C. Prostaglandin production was then assayed using a
prostaglandin-screening enzyme immunoassay (Cayman Chemi-
cal). Background values in the absence of lysate were subtracted
from each corresponding sample condition to obtain a specific
measurement of prostaglandin production due to the conversion
of AA. Lysates of C. neoformans that were incubated with AA
produced readily detectable amounts of prostaglandins (Fig. 3A).

The requirement for functional enzymes in the lysate was
tested by boiling a portion of the lysate for 10 min (followed by
spinning down the precipitated proteins) prior to the addition
of AA. The boiling of lysates resulted in a significant reduction
in PGE$_2$ production (Fig. 3B). While some residual production
remains, this amount was not statistically different from that
seen for the buffer control. The denaturability of this process
demonstrated that the production of PGE$_2$ by C. neoformans is
dependent on a heat-denaturable enzymatic process.

We next sought to determine the effect of cyclooxygenase
inhibitors. Aspirin and indomethacin (Sigma) were added to
the lysates just prior to the addition of AA. As can be seen in
Fig. 3B, cryptococcal prostaglandin production was not signif-
icantly inhibited by aspirin or indomethacin. This would seem
to stand in contrast to previous work where cryptococcal cells
treated with indomethacin decreased prostaglandin produc-
tion; however, indomethacin treatment also decreased the
number of viable cells in the culture (11). This suggests that the
decrease seen previously was not due to a specific inhibition of
the enzyme but rather to an effect on cell viability.

Recently, a family of fatty acid dioxygenase enzymes (PpoA,
PpoB, and PpoC) which possess homology to the catalytic
domains of mammalian cyclooxygenases were identified in As-

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FIG. 2. MS-MS of the cryptococcal AA metabolite. MS-MS was performed on the 4.1-min peak found for the C. neoformans-plus-AA sample
and a PGE$_2$ standard. (A) Fragmentation pattern of the m/z 351.2 peak from C. neoformans-plus-AA samples. (B) Fragmentation pattern of the
m/z 351.2 peak from the PGE$_2$ standard (Std).
Chemical approaches or by homology searches. Thus, despite the fact that we identi-
fied the production of PGE<sub>2</sub> by C. neoformans, we could not demonstrate the presence of a cyclooxygenase either by bio-
chemical approaches or by homology searches.

Here we show that despite the lack of a cyclooxygenase, C. neoformans synthesizes PGE<sub>2</sub> that is identical to mammalian PGE<sub>2</sub>. The absence of a cyclooxygenase goes against the cur-
rent paradigm, which holds that without the cyclooxygenase the production of prostaglandins should not be possible. Addi-
tionally, cryptococcus is not known to possess 20-carbon fatty acids, so they must be obtained from the environment. This fac-
t is highlighted in the current study, where almost no pro-
taglandin production occurs in the absence of exogenous AA. Previous work (11) has shown greater amounts of prostaglan-
din produced in the absence of AA, but these studies were carried out in a rich growth medium in which free fatty acids were available. In contrast, the current work was performed in a simple phosphate buffer devoid of free fatty acids unless they were deliberately added. Within a mammalian host, cryptococ-
cus-derived prostaglandins could be generated utilizing host AA liberated by cryptococcal phospholipase B (PLB) (1, 3, 4,
10, 14). Should cryptococcal prostaglandin synthesis prove to be important in pathogenesis, the existence of different pro-
taglandin-synthetic enzymes could provide novel targets for future antifungal therapies.

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**FIG. 3.** The effect of broad-spectrum cyclooxygenase inhibitors on cryptococcal prostaglandin production. Lysates from C. neoformans (C.neo) cells were incubated with or without AA with or without inhibitor for 2 h at 37°C. Enzymatic involvement was demonstrated using boiled lysate. Background, i.e., nonenzymatic, conversion of AA was measured for a sample with AA alone and subtracted from all values. (A) Total prostaglandin production was measured using a pro-
taglandin-screening enzyme immunoassay (*, P value of <0.05 by unpaired t test). (B) Inhibitory effect of cyclooxygenase inhibitors ex-
pressed as percent inhibition (where the lysate-plus-AA sample value was set at 100% *) (*, P value of <0.001 by one-way analysis of variance with Bonferroni’s posttest). Statistically, results for the boiled lysates are not different from those for buffer alone.
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