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## The role of laccase in prostaglandin production by *Cryptococcus neoformans*

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### Abstract

Recently, it has been demonstrated that the opportunistic fungal pathogen *Cryptococcus neoformans* can synthesize authentic immunomodulatory prostaglandins. The mechanism by which this takes place is unclear since there is no cyclooxygenase homolog in the cryptococcal genome. In this study, we show that cryptococcal production of both PGE<sub>2</sub> and PGF<sub>2α</sub> can be chemically inhibited by caffeic acid, resveratrol and nordihydroguaiaretic acid. These polyphenolic molecules are frequently used as inhibitors of lipoxygenase enzymes; however, BLAST searches of the cryptococcal genome were unable to identify any homologs of mammalian, plant or fungal lipoxygenases. Next we investigated cryptococcal laccase, an enzyme known to bind polyphenols, and found that either antibody depletion or genetic deletion of the primary cryptococcal laccase (*lac1Δ*) resulted in a loss of cryptococcal prostaglandin production. To determine how laccase is involved, we tested recombinant laccase activity on the prostaglandin precursors, arachidonic acid (AA), PGG<sub>2</sub> and PGH<sub>2</sub>. Using mass spectroscopy we determined that recombinant Lac1 does not modify AA or PGH<sub>2</sub>, but does have a marked activity toward PGG<sub>2</sub> converting it to PGE<sub>2</sub> and 15-keto-PGE<sub>2</sub>. This data demonstrates a critical role for laccase in cryptococcal prostaglandin production, and provides insight into a new and unique fungal prostaglandin pathway.

### Keywords

oxylipin; fungi; polyphenol; eicosanoid; laccase

### Introduction

The production of oxidized fatty acids (oxylipins) from single celled eukaryotes is an emerging area of research that has defined new mechanisms by which these organisms sense environment and community structure, signal morphological differentiation, and regulate virulence (Erb-Downward & Huffnagle, 2006, Noverr *et al.*, 2003b, Shea & Del Poeta, 2006, Tsitsigiannis & Keller, 2007). Among these discoveries is evidence that many fungi are capable of producing 20-carbon oxylipins known as prostaglandins (Noverr *et al.*, 2002, Tsitsigiannis *et al.*, 2005a). In particular, *Cryptococcus neoformans* and *Candida albicans*

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have been identified as capable of producing authentic prostaglandin-E<sub>2</sub> (PGE<sub>2</sub>) (Erb-Downward & Huffnagle, 2007, Erb-Downward & Noverr, 2007), a potent signaling molecule that can regulate inflammation in higher eukaryotes (Harris *et al.*, 2002).

Prostaglandins are 20-carbon fatty acid metabolites of arachidonic acid (AA). In higher eukaryotes, the synthesis of prostaglandins is dependent upon the action of cyclooxygenase enzymes which first generate the common precursor of prostaglandin synthesis, PGH<sub>2</sub> (Smith *et al.*, 2000). This precursor is then converted to the various prostaglandins by the action of prostaglandin synthases. The absolute dependence of prostaglandin synthesis in higher eukaryotes upon a cyclooxygenase has led to a biochemical paradox in that there is no cyclooxygenase homolog in either the *C. neoformans* or *C. albicans* genome (Erb-Downward & Huffnagle, 2007, Erb-Downward & Noverr, 2007, Tsitsigiannis *et al.*, 2005b) and classical inhibitors of prostaglandin synthesis, which are known to broadly inhibit cyclooxygenases, do not inhibit prostaglandin synthesis (Erb-Downward & Huffnagle, 2007, Erb-Downward & Noverr, 2007). 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 *Aspergillus nidulans* and *Aspergillus fumigatis* (Tsitsigiannis *et al.*, 2005b). Further analysis showed that the cultures produced prostaglandins (as detected by immunoassay) and ppo mutants produced less (Tsitsigiannis *et al.*, 2005a). But these enzymes do not exist in the *C. neoformans* genome (Tsitsigiannis *et al.*, 2005b). The current work details our investigation into the enzymes involved in cryptococcal prostaglandin synthesis, and the discovery that a cryptococcal laccase is required for prostaglandin synthesis in *C. neoformans*.

## Results

### Chemical inhibition of cryptococcal prostaglandin synthesis

To examine the effects of chemical inhibition on cryptococcal prostaglandin production we first established a lysate system by which multiple species of prostaglandins could be measured. Briefly, lysates were generated through mechanical disruption of stationary phase *C. neoformans* cells followed by a 2 hr. incubation with AA. Figure 1 demonstrates that multiple prostaglandin species can be efficiently generated using this method including PGF<sub>2α</sub> and PGE<sub>2</sub>. These also can be measured using a pan-specific prostaglandin immunoassay that detects multiple prostaglandin species (Fig. 1A). In this lysate system, proportionally more PGF<sub>2α</sub> is synthesized (Fig. 1C) than PGE<sub>2</sub> (Fig. 1B). Furthermore, production of prostaglandins is an enzymatic process (i.e. heat denaturable), because boiling of the lysates prior to incubation with AA results in a significant reduction in prostaglandin production.

Since we have previously demonstrated that cyclooxygenase enzymes are not present in *C. neoformans* (Erb-Downward & Huffnagle, 2007), we investigated whether other classes of oxygenases could be involved by using the inhibitors resveratrol, caffeic acid and nordihydroguaiaretic acid (NDGA) to inhibit prostaglandin synthesis in cell-free lysates. Caffeic acid (Fig. 2B) and NDGA (Fig. 2A) are well characterized for their ability to inhibit lipoxygenase enzymes (Koshihara *et al.*, 1984, Salari *et al.*, 1984, Tanaka *et al.*, 2003, Whitman *et al.*, 2002) whereas resveratrol (Fig. 2A), which shares a similar structure, inhibits both cyclooxygenase and lipoxygenase enzymes (Kimura *et al.*, 1985, Kimura *et al.*, 1995, Tanaka *et al.*, 2003, Pinto *et al.*, 1999). All of the polyphenols tested significantly decreased the total prostaglandin production (Fig. 2). For resveratrol and NDGA, the inhibition was equivalent to boiling the lysates prior to the addition of AA. These findings indicate that polyphenols are capable of inhibiting cryptococcal prostaglandin production.

BLAST searches of the cryptococcal genome for homologs of 5, 12, or 15-lipoxygenases from humans, mice, or rats did not reveal a lipoxygenase homolog (data not shown). Similarly, searches for homologs of plant lipoxygenases (1-LO from soy-beans, and 13-LO from *Arabidopsis thaliana*) did not yield any protein with significant homology. Even the 1-lipoxygenase found in the fungus *Neurospora crassa* does not have a homolog in *C. neoformans* (data not shown). Thus, we shifted our focus from lipoxygenases and cyclooxygenases to other enzymes known to interact with polyphenols (Li *et al.*, 2005).

### A role for laccase in cryptococcal prostaglandin production

Cryptococcal laccase (*lac1*) is a multi-copper oxido-reductase having a broad specificity that includes Fe (II), aminophenols, and polyphenols (Liu *et al.*, 1999, Williamson, 1994). Additionally, we have previously noted that growth conditions that promote prostaglandin expression also result in increased levels of prostaglandin production (Noverr *et al.*, 2001). To test whether this polyphenol-binding enzyme plays a role in cryptococcal prostaglandin synthesis, lysates were depleted of laccase prior to incubation with AA by the addition of a monoclonal antibody (G3P4D3) raised against the N-terminal portion of cryptococcal laccase. The laccase-depleted supernatants were then incubated with arachidonic acid, and prostaglandin levels were measured by EIA (Fig. 3A). Lysates treated with control antibody produced prostaglandins at the same levels as untreated lysates. However, immunoprecipitation of laccase from cryptococcal lysates significantly diminished the ability of the lysates to produce prostaglandins from arachidonic acid.

Recently, a second laccase gene (*LAC2*) was identified that shares 65% identity with *LAC1* at the nucleotide level (Zhu & Williamson, 2004). Sequence analysis suggests that the mAb directed against the protein product of *LAC1* might also recognize the product of *LAC2*. To assess the relative contribution of each of these enzymes to cryptococcal prostaglandin production, *LAC1* and *LAC2* deletion strains were incubated in the presence of exogenous AA, the prostaglandins purified from the supernatants using organic extraction followed by RP-HPLC fractionation, and the levels assayed by EIA (Fig. 3B). The *LAC2* deficient strain produced the same levels of prostaglandins as the wild-type strain upon the addition of AA. However, consistent with the lysate immunodepletion experiments, the *LAC1* deficient strain of *C. neoformans* produced significantly lower levels of prostaglandins. Thus, loss of *LAC1*, but not *LAC2*, decreases cryptococcal prostaglandin production

We next investigated how Lac1 is involved in prostaglandin synthesis. To address the question, recombinant Lac1 was tested for whether prostaglandins or intermediates in prostaglandin synthesis could be generated from AA, PGG<sub>2</sub>, or PGH<sub>2</sub>. If Lac1 functions as a cyclooxygenase, PGG<sub>2</sub> and PGH<sub>2</sub> should be generated from AA. Alternatively, if the activity of Lac1 is similar to a prostaglandin synthase, then Lac1 should convert PGH<sub>2</sub> to individual prostaglandin species such as PGE<sub>2</sub> or PGF<sub>2α</sub>. To this end, increasing concentrations of recombinant Lac1 (rLac) were incubated with 50 μM of substrate (AA, PGG<sub>2</sub> or PGH<sub>2</sub>) at 37°C for 3 hr. The products were then extracted into ethyl acetate, dried down and analyzed by tandem liquid chromatography-mass spectroscopy. Incubation of rLac with either AA or PGH<sub>2</sub> alone did not result in the generation of new prostaglandins (data not shown). However, increasing amounts of rLac incubated with 50 μM PGG<sub>2</sub> resulted in a dose-dependent decrease in PGG<sub>2</sub> and a concomitant increase in PGE<sub>2</sub> (Fig. 4). Thus, Lac1 does not carry out the cyclooxygenase reaction or convert PGH<sub>2</sub> to a prostaglandin like a prostaglandin synthase, but it does possess a unique activity catalyzing the conversion of PGG<sub>2</sub> to PGE<sub>2</sub>.

In addition to PGE<sub>2</sub>, several other peaks within the m/z range of 340–370 were notably different in the samples that contained PGG<sub>2</sub> + rLac when compared to those that contained PGG<sub>2</sub> alone. The most significant of these occurred with an elution time of 11.3 min. and

possessed a  $m/z = 349 [M-H]^-$ . Using R-package XCMS (Smith *et al.*, 2006) and the METLIN database (<http://metlin.scripps.edu/>) we were able to narrow down the possible related compounds to PGE<sub>3</sub>, 15-keto-PGE<sub>2</sub> and PGK<sub>2</sub>. Commercial standards of these compounds were obtained and assessed by LC-MS. Based on retention times, MS, and MS<sup>2</sup> this peak was identified as the PGE<sub>2</sub> metabolite 15-keto-PGE<sub>2</sub> (Fig. 5). Thus, while Lac1 did not form the endocyclic peroxide that a cyclooxygenase generates, Lac1 carries out the unique conversion of PGG<sub>2</sub> to PGE<sub>2</sub> and 15-keto-PGE<sub>2</sub> which clearly distinguishes it from a prostaglandin synthase.

## Discussion

*Cryptococcus neoformans* can synthesize authentic PGE<sub>2</sub> that is identical to mammalian PGE<sub>2</sub>; however, this process proceeds via unique enzymatic pathways which do not involve a cyclooxygenase enzyme (Erb-Downward & Huffnagle, 2007). We have identified the *C. neoformans* laccase Lac1 as a critical enzyme in this new pathway based on evidence that either the depletion or disruption of Lac1 all but eliminates specific cryptococcal prostaglandin production, and that the recombinant enzyme can convert PGG<sub>2</sub> to PGE<sub>2</sub> and 15-keto-PGE<sub>2</sub>. In addition to Lac1, these studies demonstrate that there are likely at least two other enzymes in the cryptococcal prostaglandin pathway: one upstream of Lac1 activities and the other downstream. The evidence for the existence of the upstream enzyme is that cryptococcal lysates can produce prostaglandins from exogenous AA, but Lac1 can only produce prostaglandins from PGG<sub>2</sub>, an oxidized product of AA. Similarly, the lysate data indicates that in addition to PGE<sub>2</sub>, cryptococcal lysates supplemented with exogenous AA synthesize PGF<sub>2 $\alpha$</sub> , but if Lac1 is removed from the system the enzymatic production of PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub>  ceases. However, Lac1 does not synthesize PGF<sub>2 $\alpha$</sub> , indicating that another enzyme must act on the products of Lac1 to synthesize PGF<sub>2 $\alpha$</sub> . Together, these data indicate that the cryptococcal prostaglandin pathway is unique not only in the enzymes involved, but also the structure of the pathway, which the evidence suggests is linear. This clearly differentiates it from the common precursor model that is seen in cyclooxygenase-dependent prostaglandin production (Fig. 6).

The demonstration that cryptococcal laccase is critical to the production of cryptococcal prostaglandins provides the first association between a laccase and prostaglandin synthesis. Classically, laccases are thought of as enzymes involved in the oxidation of polyphenols or the degradation of plant lignin (Leonowicz *et al.*, 2001). In *C. neoformans*, laccase catalyzes the formation of a polymer melanin from 2,4- or 3,5-dihydroxy polyphenolic precursors. Melanin, in turn, enhances the ability of the organism to survive the oxidative burst within a macrophage and ultraviolet radiation induced damage out in the environment (Gomez & Nosanchuk, 2003). However, recent lines of study have begun to explore the action of this class of enzymes on non-polyphenolic molecules (Casa *et al.*, 2003). In one report investigators studied the ability of a mushroom laccase to degrade olive oil mill waste effluents, a mixture of polyphenols, fatty acids and other compounds. They found that in addition to polyphenols, fatty acids were also oxidized by this enzyme (Karlsson *et al.*, 2001). Further study revealed that the oxidation of long-chain poly-unsaturated fatty acids by the mushroom laccase was specific and proceeded via a mechanism that was remarkably similar to that of a lipoxygenase (Zhang *et al.*, 2002). A recent study comparing enzymes from different organisms that, based on DNA sequence homology, are considered laccases, found that many members of the family do not possess the ability to degrade lignin or oxidize polyphenols (Valderrama *et al.*, 2003). This suggests that alternative activities for this enzyme might be more common than was previously believed. Our studies provide additional support for the activity of laccase enzymes beyond the oxidation of polyphenolic compounds and lignin degradation: the generation of oxylipins from polyunsaturated fatty acids.

In recent years much has been discovered about the critical importance of lipid mediators in single celled eukaryotic pathogens. These effects include the stimulation or inhibition of sexual development (depending on the mediator), quorum sensing, and the regulation of virulence (Erb-Downward & Noverr, 2007, Noverr & Huffnagle, 2004, Tsitsigiannis et al., 2005a, Tsitsigiannis & Keller, 2006, Tsitsigiannis & Keller, 2007, Angeli *et al.*, 2001). Some of the prostaglandin production in these organisms is attributable to the presence of a cyclooxygenase homolog (Tsitsigiannis et al., 2005a). However, there also exists a body of literature relating to prostaglandin production in non-vertebrate organisms that has attempted to fit, unsuccessfully, what is found in these organisms into model of cyclooxygenase dependent prostaglandin production (reviewed (Lamacka & Sajbidor, 1995, Noverr et al., 2003b)). Recent studies have examined *Trypanosoma brucei*, *Trypanosoma cruzii* as well as *Plasmodium falciparum*, in the absence of a host, and found that they produce prostaglandins that are not inhibited by aspirin or indomethacin (Kubata *et al.*, 2002, Kubata *et al.*, 2000, Kilunga Kubata *et al.*, 1998). This production also occurs in the absence of an identifiable cyclooxygenase enzyme. In these organisms, a  $\text{PGF}_{2\alpha}^-$  synthase has been identified which is homologous to the Old Yellow enzyme (OYE) found in *Saccharomyces cerevisiae* (Kubata et al., 2002, Kubata et al., 2000). It has also been reported that the parasite-derived prostaglandins of *Schistosoma mansoni* actively suppress the immune system to facilitate infection of the host (Herve *et al.*, 2003).  $\text{PGE}_2$  is well known for its ability to suppress inflammation (Harris et al., 2002), and 15-keto- $\text{PGE}_2$  has been recently identified as a ligand for  $\text{PPAR}\gamma$  (Chou *et al.*, 2007), a nuclear receptor involved in the regulation of macrophage activation. Thus, it is tempting to speculate that cryptococcal production of prostaglandins may help down-regulate local inflammatory responses. The recent finding that cryptococcus carries out its sexual cycle in association with plants (Xue *et al.*, 2007) is another area where these oxylipins may be involved since oxylipin regulation of sexual cycles of other fungi has been well established (Tsitsigiannis & Keller, 2006, Tsitsigiannis & Keller, 2007, Tsitsigiannis et al., 2005b). To date only two enzymes involved in cryptococcal prostaglandin synthesis have been identified: (phospholipase B (PLB) (Noverr *et al.*, 2003a, Cox *et al.*, 2001) and now laccase (Lac1). Both are known virulence factors and the currently proposed mechanisms of virulence do not account for their role in prostaglandin synthesis. It is our belief that as additional enzymes involved in cryptococcal prostaglandin synthesis are discovered, the contribution of prostaglandins to the pathogenesis of *C. neoformans* will likely become clear.

## Experimental Procedures

### Strains and Culture Conditions

Throughout this study, *C. neoformans* strain 24067 was used except where *C. neoformans* deletion mutants were used. For these latter studies, the *LAC1* deletion mutant (*lac1*), the *LAC2* deletion mutant (*lac2Δ*), and the parent strain (H99) were utilized (Zhu & Williamson, 2004). All strains were grown shaking at  $\sim 37^\circ\text{C}$  in 30 ml Sabouraud dextrose broth (1% Neopeptone and 2% dextrose; Difco, Detroit, Mich.) until stationary phase was reached (72 hr or until the cells reached a concentration  $> 2 \times 10^8/\text{ml}$ ).

### C. neoformans Lysis Protocol

$1.2 \times 10^9$  cells were transferred to a 50 ml conical tube, spun down at 3000 rpm, and washed  $2 \times$  with 40 ml sterile saline. Next, 1.2 ml of cold phosphate buffer (pH 7.1) was added to the tube without disturbing the pellet. This was followed by the addition of  $0.5 \mu\text{m}$  glass beads until the beads just began to dry. The tube was then subjected to a vigorous regimen of vortexing  $5 \times$  for 1 min. followed by 1 min. on ice. The liquid was then transferred to another tube, and the beads washed  $2 \times$  with  $600 \mu\text{l}$  cold phosphate buffer, vortexing and transferring the liquid portion with each wash. This crude lysate was then spun down at 13,000 rpm for

45 min. to remove debris and unlysed cells. From these tubes the supernatant (lysate) was transferred to a fresh 15 ml conical and kept on ice.

### Prostaglandin Production and Measurement

Prostaglandins were generated from cryptococcal lysates by incubating 150  $\mu$ l of lysate in a 1.5 ml Eppendorf tube with 500  $\mu$ M AA (Cayman Chemical) at 37°C for 2 hr. The requirement for functional enzymes was tested by boiling a portion of *C. neoformans* lysate for 10 min. followed by spinning down the precipitated proteins prior to the addition of AA. The lipoxygenase inhibitors caffeic acid and nordihydroguaiaretic acid (NDGA) (Cayman Chemical) and resveratrol (Sigma Chemical) were dissolved in DMSO. Inhibition studies were performed by adding inhibitor or an equivalent amount of DMSO to lysate samples just prior to incubation with AA.

Prostaglandins were generated in whole cell supernatants by incubating  $1 \times 10^7$  cells in 1 ml phosphate buffer, pH 7.1, with 500  $\mu$ M AA or control at 37°C for 2 hr. Following incubation, the cells were spun down and the supernatant removed and assayed for prostaglandin content.

Prostaglandins were measured utilizing PGE<sub>2</sub> specific, PGF<sub>2 $\alpha$</sub>  specific or the Prostaglandin Screening enzyme immunoassay (EIA) (Cayman Chemical) according to manufacturer's protocols. EIA plates were read at 405 nm, and the concentrations of prostaglandin were calculated based on a bound vs. free (B/B<sub>0</sub>) measurement. 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.

### Organic Extraction and Reverse-Phase HPLC

To assay the organic compounds produced in cryptococcal supernatants, samples were transferred to a silyanized 12  $\times$  75 cm glass test tube, the organic components extracted twice into 500  $\mu$ l HPLC grade ethyl acetate (EtOAc) (Aldrich Chemical), and the organic phase transferred to a silyanized conical screw-top vial. The contents of the vial were dried down in a water bath under a stream of grade 5 nitrogen.

Reverse-phase high performance liquid chromatography (RP-HPLC) analysis was carried out using a Waters 600 HPLC system, with a 5  $\mu$ l sample loop and a Waters Symmetry 2.1 $\times$ 150 mm analytical column. The compounds were separated 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 (water:acetonitrile:acetic acid) over 75 min. The dry sample was brought up in 25  $\mu$ l HPLC grade EtOH, and 12.5  $\mu$ l was (5  $\mu$ l) injected. The remainder was dried down under nitrogen and stored at -20°C for not longer than 1 week. Fractions were collected, and the organic compounds were once again extracted twice into 500  $\mu$ l EtOAc, followed by drying down under nitrogen. These samples were then brought up in 150  $\mu$ l prostaglandin EIA buffer (Cayman Chemical) and assayed according to manufacturer's protocols as above.

The elution times of cyclooxygenase-derived prostaglandin standards (Cayman Chemical) were obtained using the same elution gradient.

### Purified Enzyme Studies

The recombinant Lac1 used in these studies was generated as previously described (Zhu *et al.*, 2001). Lyophilized recombinant Lac1 (rLac) was reconstituted in 50 mM phosphate buffer pH 6.5. Enzyme activity was determined by adding 1  $\mu$ l of rLac to 1 ml of 1 mM epinephrine bitartrate for 30 min. at 37°C and measuring the absorbance at 475 nm. One unit of enzyme activity was equal to change of 0.001 AU. To test rLac activity on prostaglandin

relevant substrates 100, 500 or 1000U of rLac was added to 50  $\mu$ M AA or PGG<sub>2</sub> in a 30  $\mu$ l reaction volume for 3 hr. at 37°C. Reactions were stopped by the addition of 150 $\mu$ l EtOAc, the organic phase was removed and dried down under N<sub>2</sub>.

LC-MS/MS was carried out using a ThermoFinnigan Surveyor HPLC (San Jose, CA) interfaced directly to the electrospray ionization source of a ThermoFinnigan LTQ linear ion-trap mass spectrometer (San Jose, CA). Samples were resuspended in a methanol:water solution (1:1, v/v) and 20  $\mu$ L aliquots were injected onto a Phenomenex Luna 2.00  $\times$  150mm 3 $\mu$ m C-18(2) column (Torrance, CA). Mobile phase solvents were water + 0.1% acetic acid (A) and acetonitrile + 0.1% acetic acid (B). Compounds were separated and eluted from the analytical column with a linear gradient of 25% to 100% B over 40 min. at a flow rate of 0.3 mL/min. The sample tray was cooled to 4 °C throughout the analysis. Concentrations of compounds were calculated by taking the ratio of area of peak to the area of the internal standard peak (15-deoxy- $\Delta^{12,14}$ -PGJ<sub>2</sub>) and multiplying by the response factor. The response factor was calculated as a ratio of the peak areas of known concentrations of commercial standards of PGG<sub>2</sub>, 15- keto-PGE<sub>2</sub>, and PGE<sub>2</sub> to the peak area of a 1 $\mu$ g/ml 15-deoxy $\Delta^{12,14}$ -PGJ<sub>2</sub>.

### Immunoprecipitation

Immunoprecipitation of laccase from cryptococcal lysates was carried out by incubating 600  $\mu$ l lysates on ice with 15  $\mu$ g/ml anti-laccase mouse monoclonal antibody (clone G3P4D3) raised against full length recombinant laccase enzyme or control mouse IgG, for 2 hr. on ice prior to the addition of 50  $\mu$ l of protein-A beads (Sigma). Tubes were set to rock overnight at 4°C and the following day, beads were spun down at 13,000 rpm for 1 min. and the supernatant transferred to a fresh tube. This depleted lysate was then treated as above to generate prostaglandins.

### Statistical Analysis

Statistical analysis of results was performed using Prism 4.0 (GraphPad Software, Inc.). Statistical significance was determined using either a One-Way ANOVA with Bonferroni's Multiple Comparison post test. For the purposes of comparing the degree of inhibition of the various inhibitors, the data was represented as a % of control (Lysate + AA).

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### Abbreviations used

<b>PG</b>	prostaglandin
<b>AA</b>	arachidonic acid
<b>RP-HPLC</b>	reverse-phase high performance liquid chromatography
<b>EtOAc</b>	ethyl acetate
<b>rLac</b>	recombinant laccase (Lac1)
<b>PGE<sub>2</sub></b>	prostaglandin E <sub>2</sub>

<b>PGF<sub>2α</sub></b>	prostaglandin F <sub>2α</sub>
<b>C.neo</b>	<i>C. neoformans</i>
<b>NDGA</b>	nordihydroguaiaretic acid
<b>PLB</b>	phospholipase B
<b>OYE</b>	Old Yellow Enzyme

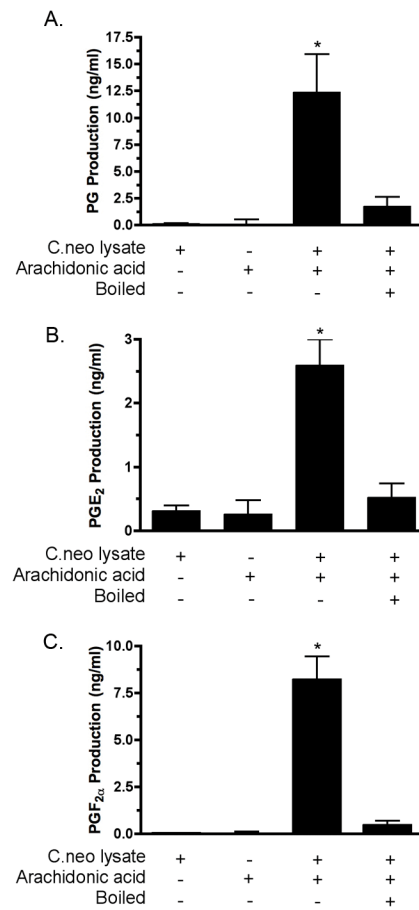
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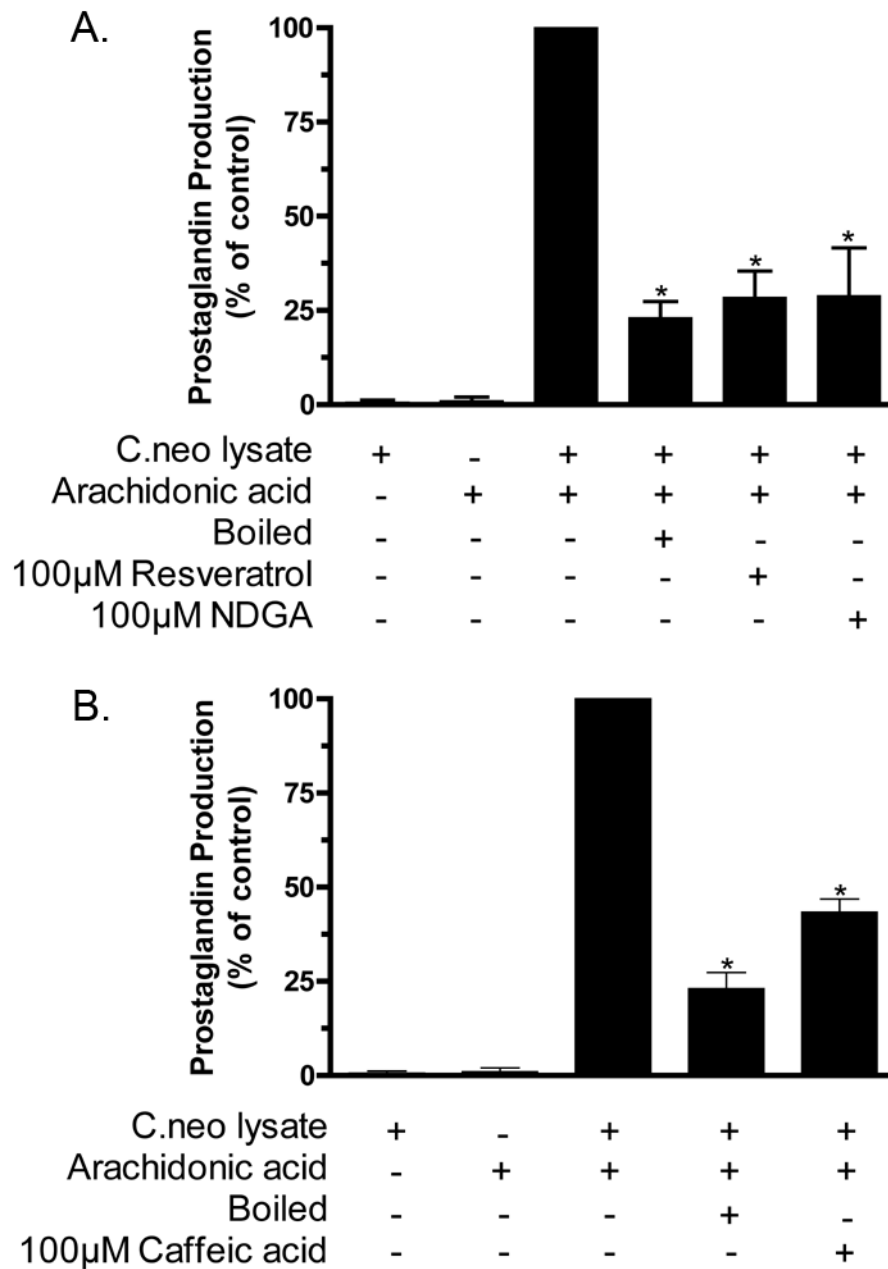
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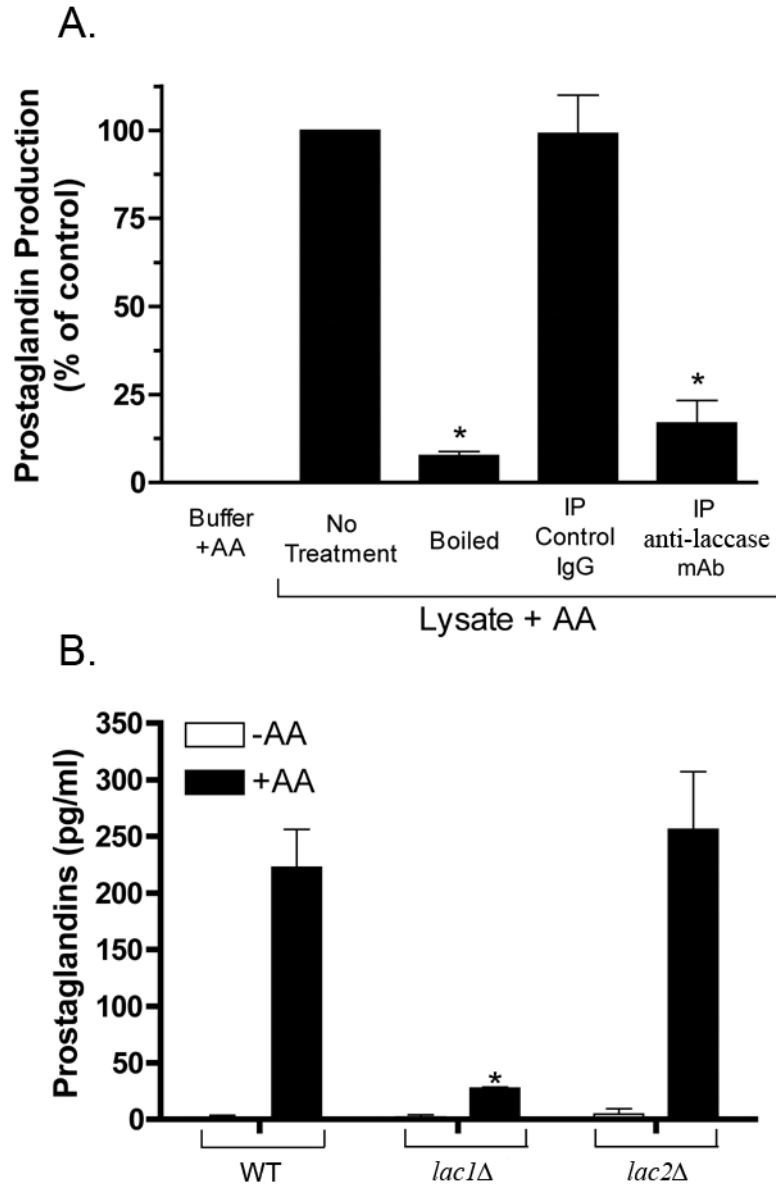


**Figure 1. Prostaglandin production in cryptococcal lysates**

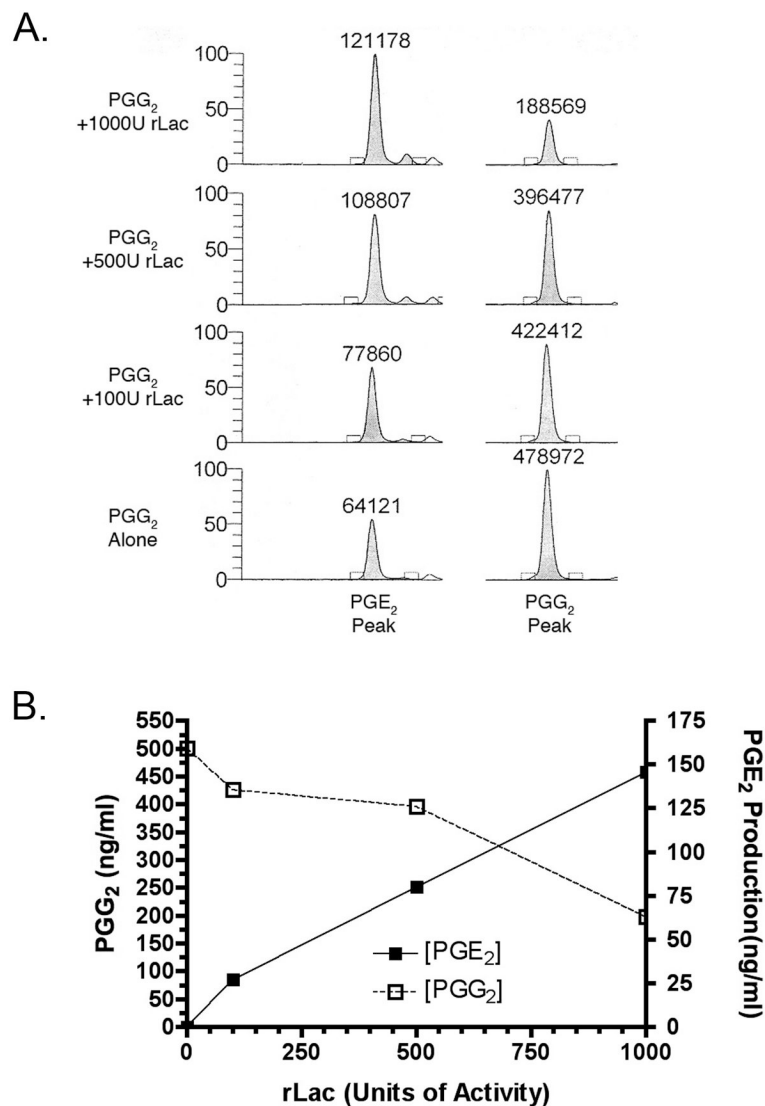
*C. neoformans* cells were lysed and the lysates incubated  $\pm$  AA for 2 hr. at 37°C. *C. neoformans* lysates were incubated with AA and total prostaglandins were measured using a prostaglandin screening EIA (Panel A (n = 11; \* = p < 0.001)); PGE<sub>2</sub> were levels assayed using a PGE<sub>2</sub> monoclonal EIA (Panel B (n = 7; \* = p < 0.001)); and PGF<sub>2α</sub> levels were assayed using a PGF<sub>2α</sub> monoclonal EIA (Panel C (n = 5; \* = p < 0.001)).



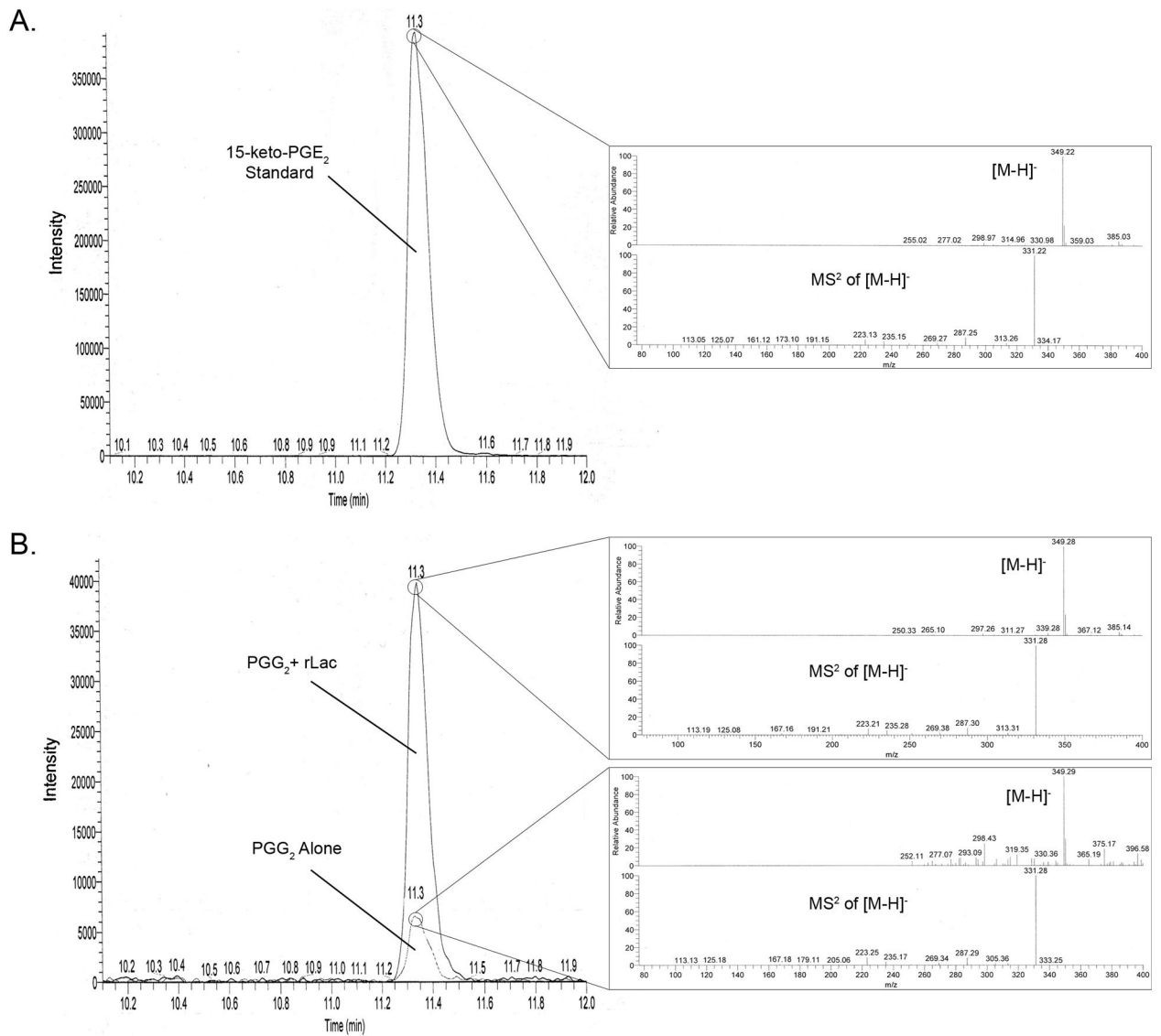
**Figure 2. The effect of inhibitors on total prostaglandin production from *C. neoformans* lysates**  
*C. neoformans* cells were lysed, the lysates incubated  $\pm$  AA for 2 hr. at 37°C and total prostaglandin production was measured using a prostaglandin screening EIA. Panels A and B document the effect of various polyphenols on cryptococcal prostaglandin production. The results are normalized as a percent inhibition (where the lysate + AA was set to 100%). (Panel A n = 5, and Panel B n = 2 ; \* = p < 0.001).



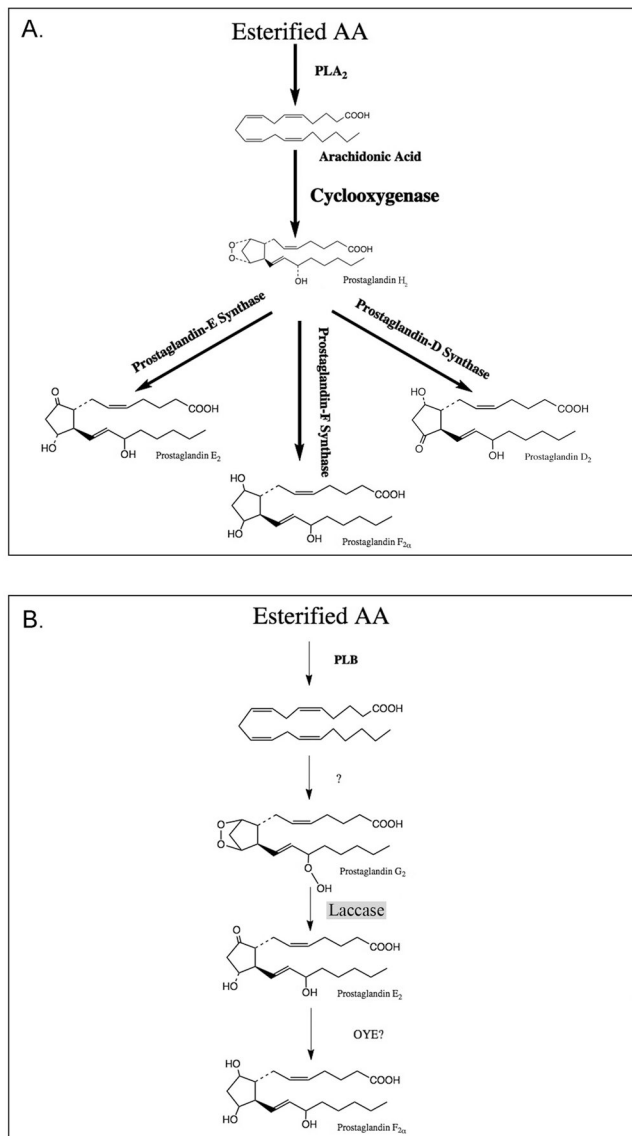
**Figure 3. The effect of the removal of laccase on cryptococcal prostaglandin production**  
 Panel A details the effect of the immunoprecipitation (IP) of laccase from cryptococcal lysates prior to the addition of AA. Inhibitory effect is displayed as a percent of control (the non-immunodepleted lysate + AA), which was set to 100 % (n = 4 ; \* = p <0.001). Panel B demonstrates the effect of deleting the cryptococcal laccase genes on prostaglandin production into culture (when incubated with AA). Culture supernatants were taken from wild-type *C. neoformans*, *lac1Δ*, or *lac2Δ* strains that had been incubated for 2 hr. at 37°C in the presence of AA. Fungal prostaglandins were extracted from the culture supernatant into ethyl acetate and purified using RP-HPLC. Total prostaglandins were measured by EIA (n = 2; \* = p <0.05).



**Figure 4. The effect of recombinant Lac1 on PGG<sub>2</sub> to PGE<sub>2</sub> conversion**  
 Increasing concentrations of rLac1 were incubated with PGG<sub>2</sub>, and the products were separated and analyzed using LC-MS as described in Materials and Methods. Panel A is the extracted ion chromatograms of PGE<sub>2</sub> (m/z range = 351–352 [M–H]<sup>–</sup> ion) and PGG<sub>2</sub> (m/z range = 367–368 [M–H]<sup>–</sup> ion) with increasing concentrations of rLac1. The numbers above each peak denote the area under the peak. Panel B demonstrates the increase in PGE<sub>2</sub> production over background levels and the decrease in PGG<sub>2</sub>.



**Figure 5. The effect of recombinant Lac1 on 15-keto-PGE<sub>2</sub> levels**  
 Chromatograms of samples containing 15-keto-PGE<sub>2</sub> standard (Panel A) or PGG<sub>2</sub> ± 1000 U recombinant Lac1 (Panel B) focusing on the m/z range = 349.1–349.5. Panel insets indicate the parent [M-H]<sup>-</sup> ion mass and MS<sup>2</sup> of this ion.



**Figure 6. The common precursor vs. a linear model of cryptococcal prostaglandin synthesis**  
 Panel A depicts the common precursor model of prostaglandin synthesis where cyclooxygenase enzymes act on free arachidonic acid to produce PGH<sub>2</sub>, which then serves as the common substrate for the individual prostaglandin synthases. Panel B depicts the proposed linear model of cryptococcal prostaglandin synthesis, where each step is dependent upon the product of the step before. Dashed arrows indicate other possible synthetic routes.