Effects of Aspirin and Other Nonsteroidal Anti-Inflammatory Drugs on Biofilms and Planktonic Cells of *Candida albicans*

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Prostaglandins are now known to be produced by *Candida albicans* and may play an important role in fungal colonization. Their synthesis in mammalian cells is decreased by inhibitors of the cyclooxygenase isoenzymes required for prostaglandin formation. In the present study, a catheter disk model system was used to investigate the effects of nonsteroidal anti-inflammatory drugs (all cyclooxygenase inhibitors) on biofilm formation by three strains of *C. albicans*. Seven of nine drugs tested at a concentration of 1 mM inhibited biofilm formation. Aspirin, etodolac, and diclofenac produced the greatest effects, with aspirin causing up to 95% inhibition. Celecoxib, nimesulide, ibuprofen, and meloxicam also inhibited biofilm formation, but to a lesser extent. Aspirin was active against growing and fully mature (48-h) biofilms; its effect was dose related, and it produced significant inhibition (20 to 80%) at pharmacological concentrations. Simultaneous addition of prostaglandin E_2 abolished the inhibitory effect of 25 or 50 μM aspirin. At 1 mM, aspirin reduced the viability of biofilm organisms to 1.9% of that of controls. Surviving cells had a wrinkled appearance, as judged by scanning electron microscopy, and consisted of both yeasts and hyphae. Treatment with other cyclooxygenase inhibitors, such as etodolac, resulted in biofilms that consisted almost entirely of yeast cells. In conventional assays for germ tube formation, these drugs produced significant inhibition, whereas aspirin had little effect. Our findings suggest that cyclooxygenase-dependent synthesis of fungal prostaglandin(s) is important for both biofilm development and morphogenesis in *C. albicans* and may act as a regulator in these physiological processes. Our results also demonstrate that aspirin possesses potent antibiofilm activity in vitro and could be useful in combined therapy with conventional antifungal agents in the management of some biofilm-associated *Candida* infections.

Pathogenic fungi in the genus *Candida* can cause both superficial and serious systemic diseases and are now widely recognized as important agents of hospital-acquired infection. Many *Candida* infections involve the formation of biofilms on implanted devices such as indwelling catheters or on tissue surfaces (10). Biofilms of *Candida albicans* normally consist of matrix-enclosed microcolonies of yeasts and hyphae and are resistant to a range of antifungal agents currently in clinical use, including amphotericin B and fluconazole (5, 6, 16, 27). Because of the drug resistance of biofilms, management of implant infections is difficult. Detachment of cells from an adherent population on a catheter can give rise to a septicemia that may respond to conventional drug therapy. However, biofilm cells are not killed by such treatment and remain as a reservoir of infection until the implant is removed. Non-device-related biofilm infections such as vaginitis and periodontitis are also frequently recalcitrant to treatment (10). Elevated prostaglandin levels have been observed in some of these chronic infections (33).

Prostaglandins are small lipid molecules that have diverse biological activities, including the modulation of host immune responses (14). They are now known to be produced by pathogenic fungi as well as by mammalian cells (22–24). Both *C. albicans* and *Cryptococcus neoforman*s secrete prostaglandins de novo or via conversion of exogenous arachidonic acid (23). In mammalian systems, arachidonic acid, formed by cleavage of phospholipids, is converted to prostaglandin H_2 (PGH_2) by the cyclooxygenase (COX) isoenzymes, COX-1 and COX-2. It is thought that COX-1 is expressed constitutively in most tissues of the body, whereas COX-2 is mainly an inducible enzyme involved in the regulation of inflammation (7). Prostaglandin synthases subsequently convert PGH_2 into a series of prostaglandins, including PGI_2, PGF_2, PGD_2, and PGE_2 (14). Treatment of *C. albicans* or *C. neoformans* infections with the COX inhibitor indomethacin significantly reduced the viabilities of the organisms and the production of prostaglandins, suggesting that an essential COX enzyme might be responsible for fungal prostaglandin synthesis (23). A PGE series lipid, possibly PGE_2, was purified from both fungal pathogens. It enhanced germ tube formation by *C. albicans* and was also biologically active on mammalian cells (23).

The role of prostaglandins in fungal biology is unknown. They may function as regulators of gene expression, as in animal cells. However, prostaglandin production could also represent an important virulence factor by promoting fungal colonization and chronic infection. Since colonization often involves a biofilm, we have determined the effects of a variety of COX inhibitors on biofilm formation by *C. albicans*, using both quantitative assays and scanning electron microscopy (SEM). All of the inhibitors tested are classified as nonsteroidal anti-inflammatory drugs (NSAIDs), which are among the most widely used therapeutics, primarily for the treatment of pain and inflammation, especially arthritis. These drugs spe-

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cifically block the biosynthesis of mammalian prostaglandins by inhibiting one or both of the COX isoenzymes, COX-1 and COX-2 (7). We demonstrate that the COX inhibitors etodolac, diclofenac, and, in particular, aspirin dramatically decrease biofilm production by *C. albicans*. We also show that some COX inhibitors completely block hyphal formation by biofilms. Overall, these results point to the existence of at least one COX-dependent pathway in *C. albicans* and suggest that the recently discovered fungal prostaglandin(s) may be involved in biofilm development.

MATERIALS AND METHODS

Organisms. Three strains of *C. albicans* were used in this study. *C. albicans* GDH 2346 was originally isolated at Glasgow Dental Hospital from a patient with denture stomatitis. *C. albicans* CAI4 was obtained from Jacob Hornby, University of Nebraska, Lincoln, and *C. albicans* SC5314 was obtained from Neil Gow, University of Aberdeen, Aberdeen, Scotland. All strains were maintained on slopes of Sabouraud dextrose agar (Difco) and subcultured monthly. Every 2 months, cultures were replaced by new ones freshly grown from freeze-dried stocks.

Medium and culture conditions. Organisms were grown in yeast nitrogen base (YNB) medium (Difco) with 50 mM glucose. Medium for *C. albicans* CAI4 also contained uridine (40 mg liter^{-1}). Batches of medium (25 ml in 100-ml Erlenmeyer flasks) were inoculated from fresh culture slopes and incubated at 37°C in an orbital shaker at 60 rpm. All strains grew exclusively in the budding yeast phase under these conditions. Cells were harvested after 24 h and washed twice in 0.15 M phosphate-buffered saline (PBS; pH 7.2). Before use in biofilm experiments, washed cell suspensions were standardized to an optical density of 0.8 at 520 nm.

COX inhibitors and PGE₂. Stock solutions (100 mM) of diclofenac, ibuprofen, indomethacin, meloxicam, piroxicam, etodolac, celecoxib, and nimesulide (Sigma) were prepared in dimethyl sulfoxide. Stock solutions (100 mM) of aspirin (acetylsalicylic acid; Sigma) and salicylic acid (Sigma) were prepared in ethanol. In biofilm experiments, COX inhibitors were used at final concentrations ranging from 10 μM to 1 mM. PGE₂ (Cayman Chemicals, Ann Arbor, Mich.) was dissolved in ethanol and used at final concentrations of 10 nM, 100 nM, and 1 μM in biofilm experiments.

Biofilm formation. Biofilms were grown on small disks (surface area, 0.5 cm²) cut from polyvinyl chloride (PVC) Faucher tubes (French gauge 36; Vygon, Cirencester, United Kingdom), as described previously (4). Briefly, the disks were placed in the wells of 24-well Nunclon tissue culture plates, and a standardized cell suspension (80 μl) was applied to the surface of each one. Initially, incubation lasted for 1 h at 37°C (adhesion period). Nonadherent organisms were removed by washing, and the disks were then incubated for a further 48 h at 37°C while they were submerged in 1 ml of growth medium (biofilm formation). Unless stated otherwise, COX inhibitors and PGE₂ were added at the beginning of the 1-h adhesion period and again at time zero of the subsequent 48-h incubation. Controls included disks with no cells, disks with cells and solvent but no COX inhibitor or PGE₂, and disks with COX inhibitor or PGE₂ but no cells. At the end of the incubation period, the disks were gently washed twice with PBS and transferred to fresh 24-well plates for quantitative measurement of biofilm formation.

Quantitative measurement of biofilm formation. Biofilm growth was quantified colorimetrically by a 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) reduction assay, essentially as described earlier (11). XTT solution (250 μg ml^{-1}; Sigma) was prepared in PBS containing 1% (wt/vol) glucose, and 1 ml was added to each well of the fresh plates containing the biofilm disks. Menadione solution (4 μl; 1 mM in acetone; Sigma) was also added to the wells, and the plates were incubated for 5 h at 37°C. After incubation, the liquid was removed from each well and clarified by centrifugation, and XTT formazan production was measured by determining the absorbance at 492 nm. Control assays established that neither PGE₂ nor any of the COX inhibitors used in this study interfered with XTT reduction.

Germ tube formation. Cultures of *C. albicans* grown overnight in YNB-glucose medium were harvested, and the cells were washed twice in 50 mM potassium phosphate buffer (pH 6.5). The cells were then resuspended at 10⁷ cells ml^{-1} in the same buffer containing either 10 mM proline and 2.5 mM *N*-acetylglucosamine or 5% fetal bovine serum. Where indicated, at time zero, COX inhibitors were added to a final concentration of 100 μM and PGE₂ was added to a final concentration of 1 nM, 100 nM, or 1 μM. The cell suspensions were

incubated with gentle shaking at 37°C for 2 h. At 0 min and 2 h, the suspensions were examined for the percentage of germ tubes present by using a light microscope; 200 cells were counted each time.

SEM. The biofilms that formed on catheter disks were treated with glutaraldehyde and osmium tetroxide, as described previously (15). After dehydration in a series of ethanol solutions, samples were dried overnight in a desiccator, coated with gold with a Polaron coater, and viewed with a Philips 500 scanning electron microscope.

RESULTS

Effect of PGE₂ on biofilm formation by *C. albicans*. Earlier work showed that purified fungal prostaglandin from *C. albicans* enhanced germ tube formation by this organism when it was added to incubation mixtures (23). Commercially available PGE₂ also enhanced germ tube formation, although to a lesser extent (18, 23). This finding was confirmed in the present study. Addition of PGE₂ (at 10 nM, 100 nM, or 1 μM) to *C. albicans* GDH 2346 incubated in 5% serum increased germ tube formation by approximately 100% over 2 h (data not shown). Moreover, when PGE₂ was added to developing biofilms of *C. albicans* GDH 2346 at time zero, biofilm formation increased slightly. For example, in the presence of 100 nM PGE₂, biofilm formation was 114.8% ± 2.4% (mean ± standard error of the mean; $P < 0.05$) of that of control biofilms incubated in the absence of PGE₂.

Effects of COX inhibitors on biofilm formation. A variety of COX inhibitors were tested in biofilm assays with three strains of *C. albicans*. The compounds were all used at final concentrations of 1 mM and were present during the adhesion period of the assay and throughout the subsequent 48-h incubation. Aspirin, etodolac (a COX-2 inhibitor), and diclofenac produced the greatest effects, with aspirin inhibiting biofilm formation by up to 95% (Table 1). Celecoxib, nimesulide, ibuprofen, and meloxicam all inhibited biofilm formation to a lesser, but still significant, extent. Indomethacin and piroxicam failed to produce significant inhibition with any of the strains tested (Table 1). Salicylic acid, which, like aspirin, has been a known

TABLE 1. Effects of COX inhibitors on biofilm formation by three strains of *C. albicans*

Inhibitor	XTT reduction (%)[a] by biofilms after 48 h		
	Strain GDH 2346	Strain SC5314	Strain CAI4
Aspirin	20.6 ± 2.8[b]	12.3 ± 2.2[b]	4.6 ± 0.8[b]
Diclofenac	57.6 ± 3.6[b]	31.2 ± 1.1[b]	59.7 ± 2.6[b]
Ibuprofen	82.7 ± 3.9[c]	81.7 ± 6.3[c]	88.4 ± 6.3[d]
Indomethacin	92.7 ± 2.8	95.8 ± 4.2	85.7 ± 13.8
Piroxicam	93.7 ± 2.9	84.4 ± 6.0	80.6 ± 7.4
Celecoxib[e]	77.3 ± 2.7[b]	79.5 ± 7.4[c]	94.0 ± 3.9
Etodolac[e]	62.9 ± 1.5[b]	49.4 ± 3.8[b]	73.4 ± 3.6[b]
Meloxicam[e]	88.4 ± 7.0[d]	84.5 ± 3.0[b]	81.3 ± 2.4[b]
Nimesulide[e]	84.8 ± 3.5[c]	86.7 ± 7.8[d]	94.4 ± 6.3

[a] Inhibitors were present during the adhesion period and throughout the 48-h growth period. XTT reduction is expressed as a percentage of that of control biofilms of the same strain incubated in the absence of inhibitor. The results are means ± standard errors of the means of at least two independent experiments carried out in triplicate. Mean ± standard error of the mean values (A_{492}) for the controls were 2.413 ± 0.069, 2.676 ± 0.066, and 2.833 ± 0.072 for strains GDH 2346, SC5314, and CAI4, respectively.
[b] Value significantly different at $P < 0.001$ from that for the control.
[c] Value significantly different at $P < 0.01$ from that for the control.
[d] Value significantly different at $P < 0.05$ from that for the control.
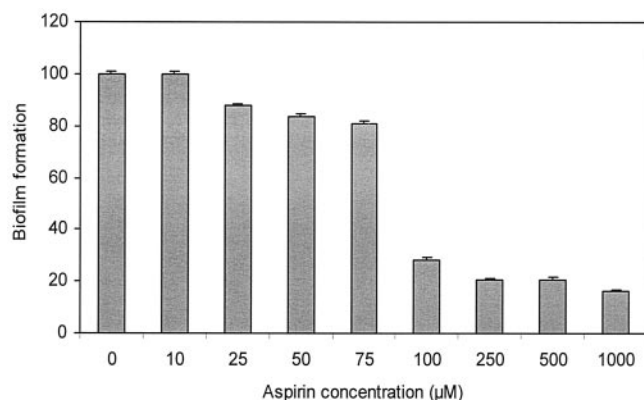[e] COX-2 inhibitor.

FIG. 1. Effects of different concentrations of aspirin on biofilm formation by *C. albicans* GDH 2346. Biofilm formation (as measured by XTT reduction) is expressed as a percentage of that of control biofilms incubated in the absence of aspirin. Most results are means ± standard errors of the means of at least two independent experiments with a total of seven or more replicates. Results for 250 and 500 μM aspirin are means ± standard errors of the means of triplicate determinations. The mean ± standard error of the mean control value (A_{492}) was 2.212 ± 0.084.

NSAID for more than a century, also inhibited biofilm formation by more than 90% (data not shown). Salicylate has virtually no activity against purified COX-1 or COX-2, and until recently, its mechanism of action was not understood. It is now thought to inhibit prostaglandin synthesis in intact cells by suppressing COX-2 gene transcription (35).

Inhibition of biofilm formation by different concentrations of aspirin. Although aspirin has been reported to have antifungal activity (9), its dramatic inhibition of biofilm formation was unexpected, and it was therefore investigated further at concentrations lower than 1 mM. The inhibitory effect on biofilms appeared to be dose related. Over 70% inhibition was observed at aspirin concentrations between 100 μM and 1 mM (Fig. 1). Lower concentrations (50 to 75 μM) produced only about 20% inhibition, while 10 μM aspirin had no effect on biofilm formation. Aspirin concentrations of 50 to 200 μM can be achieved in humans by the use of therapeutic doses of the drug (34, 35), suggesting that the antibiofilm effect observed in vitro might also be relevant in vivo.

Aspirin addition during different stages of biofilm growth. The results presented in Table 1 and Fig. 1 could conceivably be explained by the antifungal activity of aspirin on planktonic (free-floating) cells used as the inoculum for biofilm formation. To discount this possibility, the effect of adding aspirin at different times during biofilm growth was investigated. After the 1-h adhesion period, all remaining planktonic cells were removed from the disks by washing. Disks with firmly attached cells were then submerged in fresh growth medium, and aspirin was added at 0, 2, 4, 24, or 48 h of the incubation period at a final concentration of 1 mM. Biofilm formation, as determined by the metabolic activity of biofilm cells in the XTT reduction assay, was measured after 48 h (Table 2). In one experiment (experiment B), aspirin was also present during the 1-h adhesion period. Biofilm growth was severely inhibited by aspirin addition at any time up to 24 h. For example, addition of aspirin to relatively mature, 24-h biofilms reduced their meta-

bolic activity at 48 h by over 80% (Table 2). Drug addition at 48 h produced little or no effect because at this stage in the experiment the biofilms were immediately harvested, washed, and tested in the XTT assay. However, the result at 48 h in experiment B does indicate that the presence of aspirin during the adhesion period had only a minimal effect on biofilm formation. Overall, these findings demonstrate that the antibiofilm effect of aspirin is not simply due to an antifungal effect on the biofilm inoculum.

Aspirin addition to mature biofilms. In a further series of experiments, mature, 48-h biofilms grown in the absence of aspirin were transferred to fresh growth medium containing different concentrations of the drug and incubated for further periods of 5 to 48 h. All of the aspirin concentrations tested (75 μM to 1 mM) significantly inhibited biofilm activity after 16 h (Table 3). After 48 h of additional incubation, biofilm activity was reduced by 20 to 80%. Moreover, physiological concentrations of the drug (75 to 200 μM) reduced biofilm activity by 20 to 80%, suggesting that aspirin could have a significant inhibitory effect on mature biofilms in vivo.

TABLE 2. Effect of aspirin addition at different times during biofilm formation by *C. albicans* SC5314

Time of aspirin (1 mM) addition (h)	XTT reduction (%)[a] by biofilms at 48 h	
	Expt A	Expt B
0	10.8 ± 0.8[b]	8.0 ± 1.8[b]
2	13.4 ± 1.2[b]	7.6 ± 0.6[b]
4	18.7 ± 2.4[b]	8.3 ± 0.7[b]
24	18.8 ± 4.7[b]	12.1 ± 1.5[b]
48	103.6 ± 4.0	94.3 ± 3.4

[a] XTT reduction is expressed as a percentage of that of control biofilms incubated for 48 h in the absence of aspirin. Results are means ± standard errors of the means of four replicate determinations. In experiment B, aspirin was initially present at a concentration of 1 mM during the 1-h adhesion period of all assays, after which it was removed along with unattached yeasts during washing. The drug was then added at the indicated times during the subsequent incubation period. In experiment A, no aspirin was present during the adhesion period. Mean ± standard error of the mean values (A_{492}) for the controls were 2.532 ± 0.081 (experiment A) and 2.979 ± 0.122 (experiment B).
[b] Value significantly different at $P < 0.001$ from that for the control.

TABLE 3. Effect of aspirin on XTT reduction by mature (48-h) biofilms of *C. albicans* GDH 2346

Period of further incubation (h)	XTT reduction (%)[a] after further incubation in medium containing aspirin at a concn of:			
	75 μM	100 μM	200 μM	1 mM
5	100.8 ± 2.7	99.8 ± 2.7	79.3 ± 3.3[b]	66.0 ± 2.6[b]
16	89.7 ± 4.1[d]	87.8 ± 3.2[c]	80.1 ± 4.0[b]	58.2 ± 3.0[b]
24	94.4 ± 5.2	85.6 ± 4.1[c]	78.3 ± 2.6[b]	41.8 ± 2.0[b]
48	80.8 ± 6.5[c]	44.4 ± 2.7[b]	20.2 ± 1.6[b]	10.3 ± 1.1[b]

[a] Different concentrations of aspirin were added to mature (48-h) biofilms grown in the absence of the drug, and incubation was continued for a further 5 to 48 h. XTT reduction is expressed as a percentage of that of control 48-h biofilms incubated for the same time period without aspirin. The results are means ± standard errors of the means of two independent experiments carried out in quadruplicate. Mean ± standard error of the mean values (A_{492}) for the controls for different incubation periods ranged from 2.556 ± 0.033 to 2.807 ± 0.080.
[b] Value significantly different at $P < 0.001$ from that for the control.
[c] Value significantly different at $P < 0.01$ from that for the control.
[d] Value significantly different at $P < 0.05$ from that for the control.

TABLE 4. Effect of simultaneous addition of aspirin and PGE$_2$ on biofilm formation by *C. albicans* GDH 2346

Addition	XTT reduction (%)[a] by biofilms at 48 h
Aspirin (25 μM)	87.7 ± 9.5[c]
Aspirin (25 μM) + PGE$_2$ (1 nM)	106.1 ± 6.0
Aspirin (25 μM) + PGE$_2$ (100 nM)	101.3 ± 8.9
Aspirin (50 μM)	79.0 ± 7.6[b]
Aspirin (50 μM) + PGE$_2$ (1 nM)	101.2 ± 11.3
Aspirin (50 μM) + PGE$_2$ (100 nM)	102.9 ± 7.8

[a] XTT reduction is expressed as a percentage of that of control 48-h biofilms incubated in the absence of aspirin and PGE$_2$. The results are means ± standard errors of the means of two independent experiments carried out with a total of seven replicates. The mean ± standard error of the mean value (A_{492}) for the controls was 2.500 ± 0.112.

[b] Value significantly different at $P < 0.001$ from that for the control.

[c] Value significantly different at $P < 0.01$ from that for the control.

Effect of simultaneous addition of aspirin and PGE$_2$ on biofilm formation. To investigate whether the antibiofilm effect of aspirin might be related to an inhibition of fungal prostaglandin production, PGE$_2$ was added simultaneously with aspirin at the beginning of the 1-h adhesion period and again at time zero of the subsequent 48-h incubation. Aspirin alone, at a concentration of either 25 or 50 μM, significantly inhibited biofilm formation (Table 4). However, 1 or 100 nM PGE$_2$ completely abolished the inhibition due to aspirin at these relatively low concentrations, indicating a possible role for prostaglandin(s) in the regulation of biofilm formation.

Viabilities of biofilms after treatment with COX inhibitors. Viable counts of both biofilm and planktonic cells of *C. albicans* GDH 2346 were determined after growth for 24 h in the presence of 1 mM aspirin, etodolac, indomethacin, or piroxicam. Aspirin, whose antifungal properties have been reported previously (9), drastically reduced the viability of planktonic cells to 1.0% of that of untreated control cells (Table 5). Surprisingly, however, it also reduced the viability of normally recalcitrant biofilm cells to 1.9% of that of untreated controls. A longer incubation period with aspirin (48 h) decreased viability still further to 0.1% of that of controls for both cell types (data not shown). With the other COX inhibitors, viability in most cases was reduced to about 40% after 24 h, although,

TABLE 5. Viability of planktonic and biofilm cells of *C. albicans* GDH 2346 after growth for 24 h in the presence of different COX inhibitors[a]

Inhibitor (1 mM)	Viability (%)	
	Planktonic cells	Biofilm cells
Etodolac	39.2 ± 1.7	38.7 ± 2.3
Indomethacin	41.6 ± 0.7	98.7 ± 1.0
Piroxicam	40.6 ± 3.1	37.9 ± 2.6
Aspirin	1.0 ± 0.1	1.9 ± 0.1

[a] Viability is expressed as a percentage of that of control cells incubated under identical conditions in the absence of inhibitor. The results are means ± standard errors of the means of at least four replicate determinations. Control counts gave mean values of $14.7 \times 10^6 \pm 1.5 \times 10^6$ cells/ml for planktonic cells and $1.9 \times 10^6 \pm 0.1 \times 10^6$ cells/disk for biofilms. Viable counts were determined by the standard procedure of serial dilution followed by plating. For biofilm counts, cells from triplicate disks were resuspended in 3 ml of PBS and were then diluted and plated.

interestingly, biofilm cells were completely resistant to indomethacin.

SEM of biofilms exposed to COX inhibitors. Morphogenesis appears to be an important feature of the development of *C. albicans* biofilms under many environmental conditions (10). Biofilms grown on catheter disks normally consist of two distinct layers: a thin, basal region of densely packed yeast cells and an overlying, mainly hyphal layer (5). In this study, the morphological appearance of biofilms of *C. albicans* GDH 2346 was examined by SEM after growth in the presence of various COX inhibitors. Aspirin reduced biofilm formation substantially, as determined by quantitative measurements (Table 1), but in areas of the catheter disks where biofilms could be observed, large numbers of yeasts and hyphae were present, just as in untreated controls (Fig. 2A and B). However, examination of the cells at higher magnification revealed that aspirin-treated fungi had very wrinkled surfaces (Fig. 2C and D). Piroxicam affected the appearance of biofilms very little (Fig. 3B), whereas treatment with either indomethacin or etodolac resulted in biofilms that consisted almost entirely of yeast cells (Fig. 3C and D). The change in morphology due to indomethacin is particularly noteworthy, since this drug affected neither cell viability (Table 5) nor the extent of biofilm formation, as determined by XTT assays (Table 1).

Effect of COX inhibitors on germ tube formation. Germ tube formation by planktonic *C. albicans* GDH 2346 in the presence of COX inhibitors (100 μM) was determined in proline–*N*-acetylglucosamine buffer over 2 h. Indomethacin had the greatest effect, inhibiting germ tube formation by almost 80% (Fig. 4). This is consistent with the absence of hyphae in developing biofilms exposed to the drug (Fig. 3C). Treatment with celecoxib or ibuprofen also resulted in significant inhibition (>70%) (Fig. 4). The compound that inhibited germ tube formation the least was aspirin, a finding that correlated with the presence of both yeasts and hyphae in biofilms exposed to this drug (Fig. 2B).

DISCUSSION

Microbial biofilms are notoriously resistant to antimicrobial agents of various types, including biocides, antibiotics, and antiseptics (13, 20). For example, clinically important antifungal agents such as amphotericin B, fluconazole, and itraconazole are all much less active against *C. albicans* biofilms than against planktonic cells (16). Drug resistance has been demonstrated for *Candida* biofilms growing on surfaces such as cellulose (3, 5), polystyrene (27, 28), and denture acrylic (6), as well as polyvinyl chloride (16). Recently, however, it has been reported that the newly introduced antifungal agent caspofungin is active against *C. albicans* biofilms in vitro (2, 19, 30). The results presented here show that aspirin, one of the oldest and most widely used anti-inflammatory drugs, also dramatically decreases biofilm formation by *C. albicans*. Moreover, some aspirin concentrations (50 to 200 μM) producing significant levels of antibiofilm activity in vitro fall within the range of those frequently achieved by therapeutic doses of aspirin in humans (34, 35). Other nonsteroidal anti-inflammatory drugs, particularly etodolac and diclofenac, also inhibited biofilm formation to a significant but lesser extent.

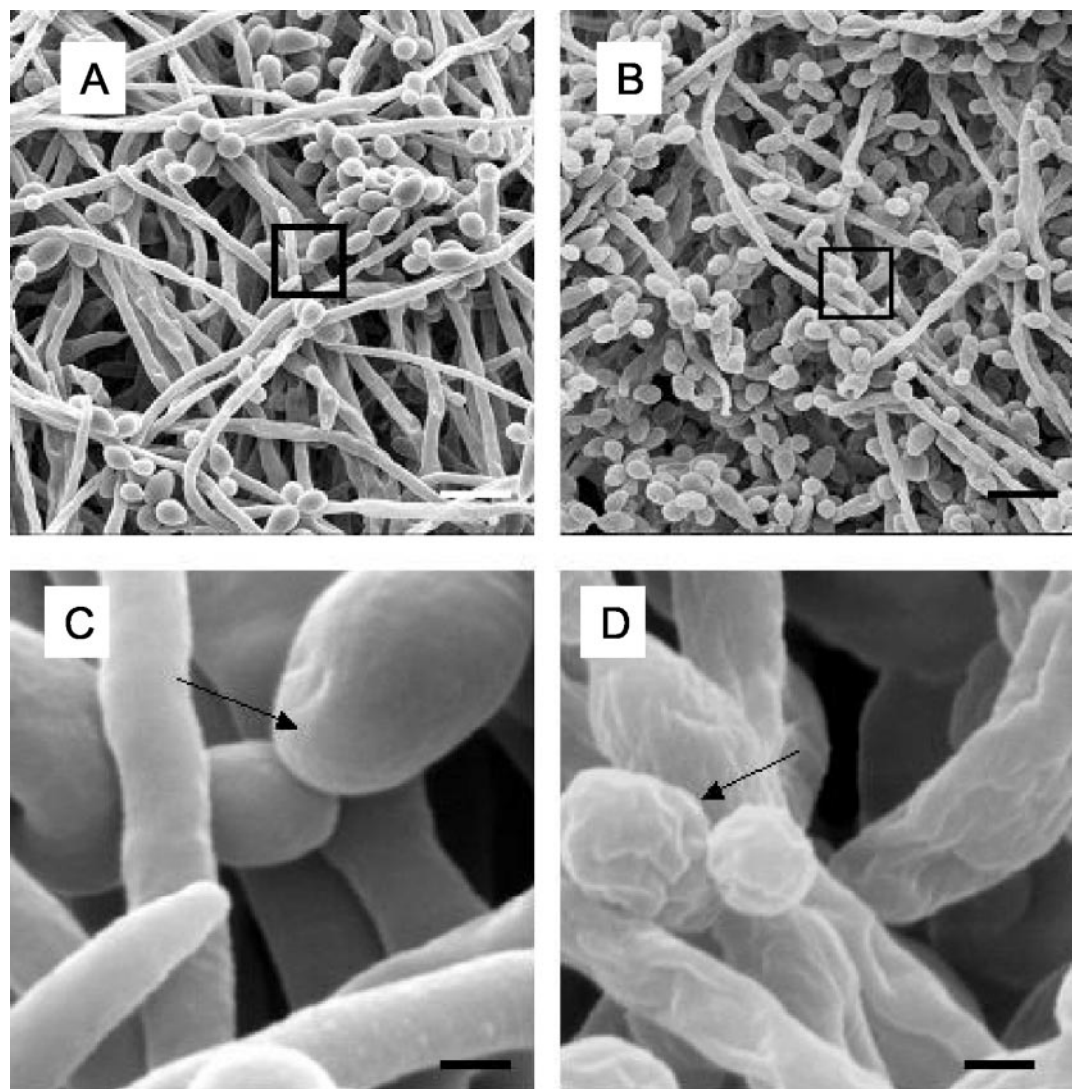Aspirin (acetylsalicylic acid) has a short half-life in circulat-

FIG. 2. Scanning electron micrographs of *C. albicans* GDH 2346 biofilms grown on PVC catheter disks for 48 h in the presence of 1 mM aspirin. (A) Control biofilm; (B) aspirin-treated biofilm; (C) enlargement of marked square in panel A; (D) enlargement of marked square in panel B. Arrows indicate the smooth surface of a control cell (C) and the wrinkled surface of an aspirin-treated cell (D). Bars, 8 μm (A and B) and 1 μm (C and D).

ing blood (about 20 min) and is rapidly deacetylated to form salicylic acid in vivo (34). Sodium salicylate and related compounds such as aspirin are known to have a variety of effects on microorganisms. Growth of certain bacteria in the presence of salicylate can induce multiple resistance to antibiotics. Paradoxically, it can also reduce resistance to some antibiotics (26). *Escherichia coli*, for example, exhibits increased resistance to chloramphenicol, ampicillin, nalidixic acid, and tetracycline after such treatment (31). On the other hand, *E. coli* cells grown in the presence of salicylate are more sensitive to aminoglycosides (1). The activities of antifungal agents can also be affected by salicylate. A combination of fluconazole with either sodium salicylate or ibuprofen results in synergistic activity against *C. albicans* (25, 32). Clearly, it would be of interest to investigate such combinations of antifungal agents and COX inhibitors in *Candida* biofilm assays, with a view to their pos-

sible use in combined therapy for the management of some biofilm-associated infections.

Sodium salicylate inhibits biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* on contact lenses and medical polymers such as polyethylene and polystyrene. Bacterial adhesion also decreases in a dose-dependent manner (12). Some strains of *S. epidermidis* secrete mucoid extracellular polymers (polysaccharides, proteins, and teichoic acid) that promote biofilm formation and become important components of the biofilm matrix. Salicylate can inhibit the production of some of these components by as much as 95% (11, 21). It has been suggested that coating of catheters with salicylate or incorporation of salicylate into contact lens solutions might decrease the incidence of some device-related infections (11, 12).

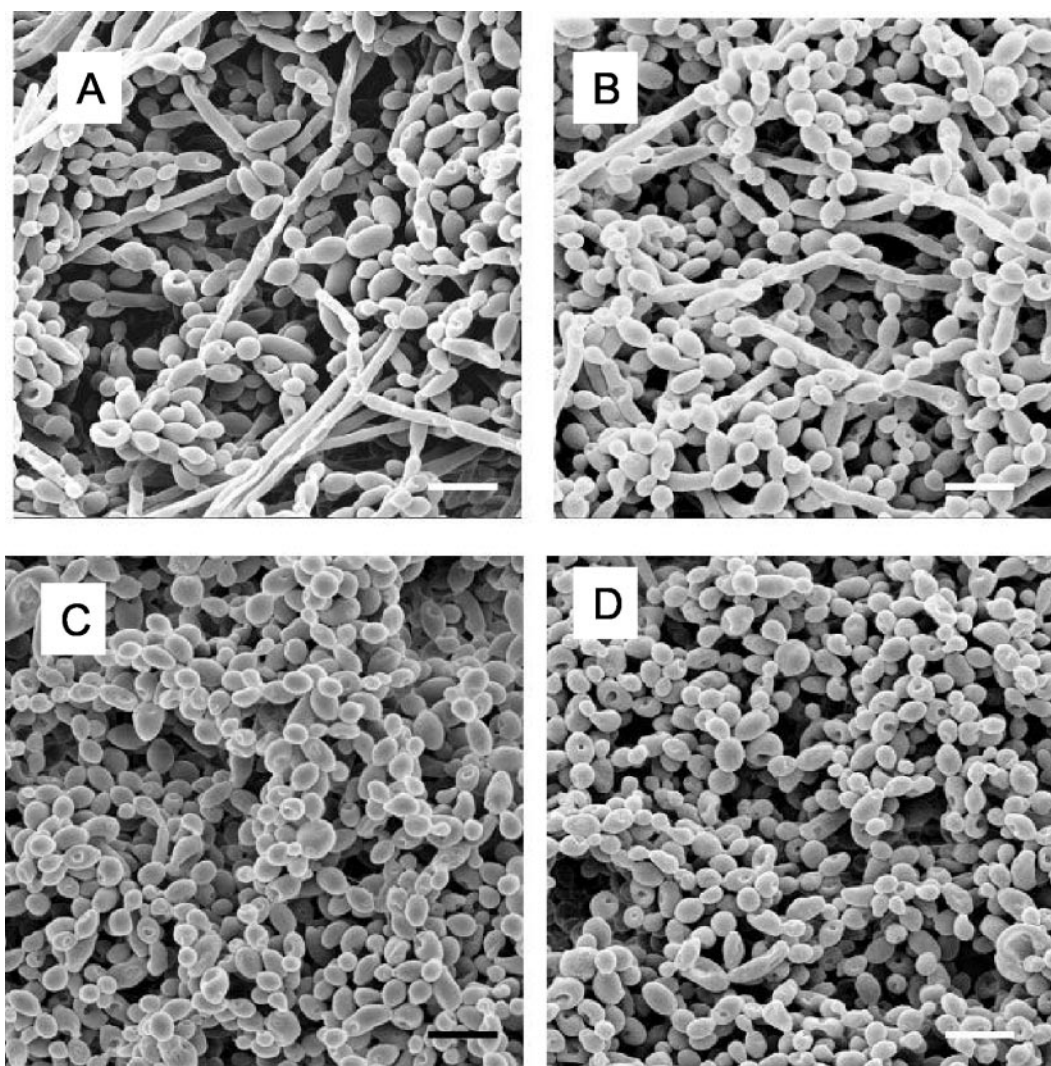In mammalian systems, all of the classic NSAIDs such as

FIG. 3. Scanning electron micrographs of *C. albicans* GDH 2346 biofilms grown on PVC catheter disks for 48 h in the presence of no inhibitor (A), 1 mM piroxicam (B), 1 mM indomethacin (C), or 1 mM etodolac (D). Bars, 8 μm.

aspirin, diclofenac, and indomethacin inhibit both the COX-1 and COX-2 isoenzymes (7). The COX active site is created by a long hydrophobic channel that is also the site of drug binding. Aspirin is the only known NSAID that covalently binds to a serine residue and that inhibits COX-1 more than COX-2 (7). In this study, seven of the nine COX inhibitors tested decreased biofilm formation by *C. albicans*, with aspirin, diclofenac, and etodolac, a preferential COX-2 inhibitor, producing the greatest effects. Aspirin and etodolac also significantly reduced the viability of biofilm cells. Indeed, aspirin appears to show an even greater effect on viability than on biofilm formation; presumably, aspirin-treated biofilm cells are largely incapable of cell division but still retain some metabolic activity, including that measured in the XTT assay. Nothing is known of COX enzymes in *C. albicans*, but the complex effects of COX inhibitors on viability, biofilm formation, and morphogenesis demonstrated here and those on prostaglandin production described earlier (23) suggest the existence of one or more COX-dependent pathways in this organism. At relatively high con-
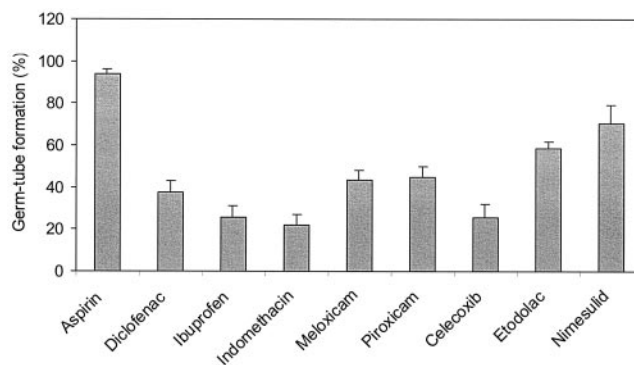

FIG. 4. Effects of COX inhibitors (final concentration, 100 μM) on germ tube formation by *C. albicans* GDH 2346. Germ tube formation is expressed as a percentage of that for control cells incubated in the absence of inhibitors. Results are means ± standard errors of the means of triplicate determinations. The mean ± standard error of the mean value for the controls was 72.6 ± 12 germ tube-forming cells/200 counted cells.

centrations (1 mM), however, the toxicity of aspirin for *C. albicans* is such that the drug probably has multiple, nonspecific effects on fungal physiology.

Prostaglandin secretion by *C. albicans* is likely to be an important factor in the pathogenesis of many infections. In experiments with mammalian cells, purified fungal prostaglandin down-modulated chemokine production, tumor necrosis factor alpha production, and splenocyte proliferation, while it up-regulated interleukin 10 production (23). Its role in fungal biology is less clear. Synthetic PGE_2 enhanced biofilm formation and germ tube formation by *C. albicans*, whereas COX inhibitors adversely affected both processes. Moreover, exposure to some COX inhibitors (indomethacin and etodolac) produced biofilms that consisted almost entirely of yeast cells. Recently, farnesol has been identified as an extracellular quorum-sensing molecule in *C. albicans* that prevents mycelial development (17) and inhibits biofilm formation (29). Fungal prostaglandins may represent signaling molecules of a similar type. (3*R*)-Hydroxyoxylipins, which, like prostaglandins, are derived from arachidonic acid, have also been identified in *C. albicans* (8). The synthesis of these compounds appears to take place in hyphae but not yeast cells and is suppressed by aspirin (8, 9). It is not yet clear whether (3*R*)-hydroxyoxylipins are excreted from the cell or how they relate to fungal prostaglandins. However, it seems likely that a range of biologically active lipid molecules are involved in the regulation of biofilm formation, morphogenesis, and other major physiological processes in *C. albicans*.

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