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# *Candida albicans* Induces Selectively Transcriptional Activation of Cyclooxygenase-2 in HeLa Cells: Pivotal Roles of Toll-Like Receptors, p38 Mitogen-Activated Protein Kinase, and NF-κB FREE

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# *Candida albicans* Induces Selectively Transcriptional Activation of Cyclooxygenase-2 in HeLa Cells: Pivotal Roles of Toll-Like Receptors, p38 Mitogen-Activated Protein Kinase, and NF-κB

**Rupal Deva,[1] Pattabhiraman Shankaranarayanan, Roberto Ciccoli, and Santosh Nigam[2]**

**Candidiasis, in its mucocutaneous form as well as in an invasive form, is frequently associated with high morbidity. $PGE_2$, which is generated by enzymatic activity of cyclooxygenases (COXs) 1 and 2, has been shown to trigger morphogenesis in *Candida albicans*. In the present study, we investigated whether *C. albicans* altered COX-2 expression in HeLa cells. RT-PCR and Western blot analyses revealed a time-dependent biphasic behavior of COX-2 mRNA expression and COX-2 protein level. COX-1 protein remained unaffected. Neutralization with Abs against Toll-like receptors (TLR) 2 and 4 inhibited the *Candida*-induced production of $PGE_2$, suggesting a vital role for TLRs in the recognition and signaling in mammalian cells upon infection with *C. albicans*. Transient transfections with COX-2 promoter-luciferase construct and various inhibitors of mitogen-activated protein kinases (MAPK), such as protein kinase C (PKC) inhibitor GF203190X, $p38^{MAPK}$ inhibitor SB203109, and extracellular-regulated kinases 1 and 2 inhibitor PD98509 showed that *C. albicans* up-regulates selectively COX-2, but not COX-1, through $p38^{MAPK}$ and PKC pathways. No involvement of other stress kinases, e.g., c-Jun $NH_2$-terminal kinase and extracellular-regulated kinases 1 and 2, was observed. Transient transfection of NF-κB promoter construct and dominant negative plasmid of IκBβ kinase showed that COX-2 transcription is mediated through $p38^{MAPK}$ and NF-κB pathways. That NF-κB up-regulates $p38^{MAPK}$ is novel and is in contradiction to earlier reports in which NF-κB was shown to inhibit $p38^{MAPK}$. In conclusion, multiple converging signaling pathways, involving TLRs followed by PKC, $p38^{MAPK}$, and/or NF-κB, are triggered by *C. albicans* in activation of COX-2 gene.** ***The Journal of Immunology,*** **2003, 171: 3047–3055.**

In recent years, invasive fungal infections increased, predominantly in cancer patients and other immunosuppressed individuals (1–5). Candidemia and invasive candidiasis are frequently associated with high morbidity and not seldom with high mortality rates. *Candida albicans* is dimorphic and can either grow as blastospores (budding) or switch to a filamentous form depending on the environmental status (3). It is represented in >50% of candidemia cases as the hallmark of bloodstream infections. It is also the causative agent in various mucocutaneous infections such as vulvovaginal candidiasis (VVC),[3] which is believed to affect ~75% of female patients during the child-bearing period (5). Although a number of antifungal drugs are applied, the treatment of VVC is often long lasting and unsatisfactory due to frequent recurrences and development of drug resistance. The latter is attributed to a great extent to formation of morphologically distinct phenotypes of *Candida albicans*, such as yeast-like blastospores, germ tubes, pseudohyphae, true hyphae, and chlamydospores.

The ability of *C. albicans* to establish a persistent infection is critically dependent on cellular signals that regulate release of factors from target cells responsible for replication of pathogen. This cell signaling is designed to serve the purpose of cell survival for both host and pathogen. Infection by *C. albicans* of host tissue and cells is mediated through surface receptors, such as mannose, β-glucan, integrins, etc. (6–8) and has been found to release proinflammatory cytokines and large amount of arachidonic acid (AA) from host cells. AA is subsequently converted by lipoxygenases and cyclooxygenases (COXs) to eicosanoids (9–12). In a previous report, we showed for the first time that AA stimulated not only growth of *C. albicans* and inhibited the antimycin A-resistant alternative oxidase but was also used exclusively as a sole carbon source (13). The most striking observation was, however, that two-thirds of it were transformed to carbohydrates via glyoxylate shunt of citrate cycle (13). Support for this observation came recently from Lorenz and Fink (14), who found that the phagocytosis of *C. albicans* by macrophages up-regulated genes encoding principal enzymes of glyoxylate cycle, isocitrate lyase, and malate synthase, which are required for the virulence of fungus.

Because *C. albicans* has been reported to produce $PGE_2$ in HeLa cells (15), and $PGE_2$ has been shown to induce germ-tube formation in *C. albicans* (16), a vicious cycle seems to be operative in mucocutaneous candidiasis, in which the infected host tissue releases AA and $PGE_2$. Earlier we have shown that AA is also converted by *C. albicans* to 3(*R*)-hydroxyoxylipins, e.g., 3-hydroxyeicosatetraenoic acid and 3,18-dihydroxyeicosatetraenoic acid (13, 17). In analogy to AA metabolism by *Dipodascopsis uninucleata*, both endogenous and host-derived 3-hydroxyoxylipins are subsequently used by *C. albicans* as positive bioregulators for its growth

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[3] Abbreviations used in this paper: VVC, vulvovaginal candidiasis; AA, arachidonic acid; BSO, L-buthionine sulfoximine; COX, cyclooxygenases; ERK, extracellular-regulated kinase; IKK, IκBβ kinase; IKK-DN, IκB kinase β dominant negative; JNK, c-Jun $NH_2$-terminal kinase; MOI, multiplicity of infection; MAPK, mitogen-activated protein kinase; $p38^{MAPK}$, p38 mitogen-activated protein kinase; NAC, *N*-acetylcysteine; RLU, relative light units; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; TLR, Toll-like receptors; PKC, protein kinase C.



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and virulence (13, 18). Inasmuch as $PGE_2$ exerts immunosuppressive and vasodilatory activities and enhances vascular permeability, a pathogenic invasion by *C. albicans* is even more facilitated.

Because cyclooxygenase is the rate-limiting enzyme that catalyzes the oxygenation of AA to PG endoperoxides, which in turn are transformed enzymatically to PGs, we addressed this study to investigate the regulation of COX-2 gene expression in *C. albicans*-treated HeLa cells. HeLa cells were chosen because in a previous study we found enhancement of $PGE_2$ synthesis in HeLa cells on infection with *C. albicans* (15). In addition, this model HeLa cell/*C. albicans* closely represents the VVC. Our data demonstrate that *C. albicans* induces selectively COX-2 gene expression and protein synthesis in HeLa cells. Moreover, these effects, which are enhanced by oxidant stress, are mediated in a redundant manner in which $p38^{MAPK}$ and/or NF-κB are part of mitogen-activated protein kinase (MAPK) and protein kinase C (PKC) signal transduction pathways. Thereby the critical role played by Toll-like receptors (TLR) 2 and 4 in the *C. albicans*-induced cell signaling pathways has also been emphasized.

## Materials and Methods

### *Materials*

DMEM, peptone, and glucose were purchased from Difco Laboratories (Augsburg, Germany). Indomethacin was supplied by Sigma (Taufkirchen, Germany). Inhibitors SB 202190, PD98059, PDTC, GF203190X, NS398, and dexamethasone were purchased from Calbiochem (Germany). Rabbit polyclonal Abs against human COX-2 and COX-1 were from Cayman (Ann Arbor, MI). Abs against $p38^{MAPK}$, extracellular regulated kinases (ERKs) 1 and 2, c-Jun $NH_2$-terminal kinase (JNK), and rabbit IgG conjugated to HRP were supplied by Santa Cruz Biotechnology (Heidelberg, Germany). Western blotting detection reagents (ECL) were purchased from Amersham Pharmacia Biotech., (Freiburg, Germany). Neutralization Abs (without sodium azide) against TLR-2 and TLR-4 were purchased from eBioscience, and those against IL-1β were purchased from Peprotech (London, U.K.). Plasmid pNF-κB-Luc was obtained from Clontech (Heidelberg, Germany). Plasmids were transfected using PolyFect transfection reagent (Qiagen, Hilden, Germany), and assayed using la uciferase reporter assay system obtained from Promega (Madison, WI). For extraction of RNA, the RNAeasy system from Qiagen was used.

### *Organism and cell culture*

HeLa cells and *C. albicans* 1386 (a clinical isolate from bronchomycosis) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). HeLa cells were grown in DMEM containing 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin. *C. albicans* was grown in Sabouraud glucose broth containing 1% peptone in a rotary shaker at 120 rpm for 36 h at 37°C. The cells were then separated from the medium by centrifugation at 3000 × *g* for 5 min, washed three times with PBS, and finally resuspended in 1 ml of fresh DMEM for each experiment. *C. albicans* with multiplicity of infection of 5 (MOI 5) was added to the HeLa cells in DMEM containing 1% FCS, and the mixture was incubated at 37°C in an atmosphere containing 5% $CO_2$ and 95% air.

### *SDS-PAGE and Western blot analysis*

Cell lysates were prepared by treating cells with buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 1% SDS, 10 mM sodium orthovanadate, 2 mM PMSF, 20 μg/ml leupeptin, 2 μM pepstatin A, and 20 μg/ml of aprotinin). Lysates were sonicated for 20 s on ice and centrifuged at 10,000 × *g* for 10 min. Protein concentration of the supernatant was determined with a Bio-Rad kit (Bio-Rad, Hercules, CA). SDS-PAGE performed under reducing conditions on 10% polyacrylamide gels The resolved protein were transferred onto a nitrocellulose membrane, incubated with desired primary Abs and then probed with appropriate HRP-conjugated secondary Ab. Blots were detected with a ECL Western blot detection system according to the manufacturer's instructions.

### *Effect of various antagonists on* C. albicans*-induced COX-2 or on $PGE_2$ formation*

HeLa cells ($10^6$), untransfected or transfected with COX-2 promoter construct, NF-κB-pLUC, IKKβ-DN (hereafter designated as IKK-DN) were treated either with diluent, $p38^{MAPK}$ inhibitor SB202190 (0.1–10 μM), ERK1/2 inhibitor PD98059 (10 μM), free radical scavenger pyrrolidinedithiocarbamate (PDTC; 20 μM), PKC inhibitor GF203190X (0.1–30 μM), COX-2 inhibitor NS398 (10 μM), or COX-1 inhibitor resveratrol (20 μM) and SC-560 (100 μM) for 1 h before infection with *C. albicans.* When necessary, concentrations of inhibitors were increased to achieve a more pronounced effect.

### *Neutralization experiments*

HeLa cells were preincubated for 1 h with 20 μg/ml Ab solution of TLR-2 and TLR-4 or IL-1β. *C. albicans* was then added at MOI 5:1 for 6 h. The cell culture supernatants were collected, and Abs were removed by incubation with protein A agarose. The stripped supernatants were analyzed for $PGE_2$ by ELISA.

### *Effect of pro- and antioxidant on* C. albicans*-induced COX-2*

HeLa cells, untransfected or transfected as described above, were pretreated either with 100 μM L-buthionine sulfoximine (BSO) for 24 h and then stimulated with *C. albicans* with MOI 1:5 for 6 h or were incubated for the same time with both 200 μM *N*-acetylcysteine (NAC) and *C. albicans* (MOI 1:5) for 6 h. Cells were harvested for Western analysis and ELISA as described above.

### *Measurement of COX activity*

HeLa cells were grown in 10% DMEM as a monolayer in a 12-well plate until 60% of confluency was attained. Cells were then treated with vehicle or specific inhibitors for 1 h before infection with *C. albicans* at MOI 5 for 6 h. Cells were then spun at 2000 × *g*, and supernatant was collected. $PGE_2$ synthesis was assayed by ELISA from Cayman, as described previously (15).

### *Measurement of COX-2 promoter activity*

COX-2 promoter activity was determined by transient expression of a COX-2 5′-flanking region in HeLa cells grown to 70% confluency. COX-2 promoter construct was made as described (19) with minor modifications. PCR amplification of the promoter region of COX-2 from human genomic DNA was performed with GGGGTACCACATTTAGCGTCCCTGCA (*Kpn*I) (forward), GGAAGCTTCGGACGTGCTCCT (*Hin*dIII) (reverse) primers. The fragment was digested and cloned into pGL3 basic vector (Promega). In each well,1.5 μg of plasmid DNA were introduced using PolyFect reagent (Qiagen). Control plasmid pRSVLACZ was used to normalize for transfection efficiency. The expressed luciferase activity was measured in a scintillation counter (Perkin-Elmer, Freidburg, Germany). In each experiment, pGL3 basic was used as an empty expression vector.

RT-PCR-cDNA was reverse transcribed from the total RNA (1 μg) extracted from HeLa cells using RNAeasy extraction kit according to the manufacturer's instructions. PCR amplification was performed for 30 cycles using the rat/ovine/human COX-2 gene-specific primers 5′-GTC ACA AGA TGG CAA AAT GCT G-3′ (sense) and 5′-TAA GAT AAC ACT GCA GTG GCT C-3′ (antisense). The cycling parameters were the following: 30 s at 94°C for denaturation; 30 s at 60°C for primer annealing; and 60 s at 72°C for polymerization. β-Actin RT-PCR with the same amount of cDNA was performed (amplification for 20 cycles) using specific GAPDH primers 5′-TCGGAGTCAACGGATTTGGTCGTA-3′ (sense) and 5′-ATGGACTGTGGTCATGAGTCCTTC-3′ (antisense) to normalize COX-2 expression. The PCR products were separated on 1% agarose gel containing ethidium bromide and visualized.

### *Transient transfections*

HeLa cells were transfected with respective plasmid, such as p-NF-κB-LUC (pNF-κB-LUC) (Clontech), IKK-DN plasmid (kindly provided by Dr. S. Ghosh, HHMI, Yale University, New Haven, CT), or COX-2 promoter construct according to manufacturer's instruction. pNF-κB-LUC consists of four tandem repeats of NF-κB consensus sequence coupled to the luciferase gene. IKK-DN construct conducted a point mutation in the kinase domain (*K44A*), thus inhibiting the phosphorylation and subsequent cleavage of the IκB proteins. Briefly, cells were grown in DMEM containing 10% FCS until 50% confluency. Cells were then washed and transfected with 1.5 μg of plasmid DNA using PolyFect reagent. Experiments were performed 18–24 h after transfection. The ratio of COX-2 promoter construct to the respective dominant negative plasmids for transfection experiments was 1:1.

### *Densitometric analysis*

The density of the bands of Western blotting or RT-PCR was quantified by scanning densitometry and expressed as normalized values to β-actin or GAPDH, respectively.

*Statistics*

Data are presented as mean ± SD. Statistical comparisons between groups were made using Student's *t* test for paired observations. Significance was achieved at $p < 0.05$.

## Results

C. albicans *induces synthesis of COX-2 protein in HeLa cells and enhances $PGE_2$ production*

On stimulation of HeLa cells ($10^6$) with *C. albicans* (MOI 1:5), RT-PCR and Western blot analyses showed a time-dependent induction of COX-2-mRNA (Fig. 1*A*) and COX-2 protein synthesis (Fig. 1*B*), respectively. The densitometric scanning measurement of the COX-2 immunoreactivity revealed an ~4-fold increase for protein and >1.5 fold for COX-2 mRNA within 6 h over the basal value (Fig. 1*C*). The mRNA increased further to almost 3-fold of the basal value in 24 h, whereas the COX-2 protein decreased slightly during this period. MOIs <5 for HeLa cells and *C. albicans* did not show any significant expression of COX-2 protein (not shown), suggesting a threshold for the infection status. Stimulation of HeLa cells with *C. albicans*, however, did not affect COX-1 immunoreactivity (Fig. 1*B*). To investigate whether the enhanced COX-2 protein synthesis also exhibited increased PG formation, $PGE_2$ was measured in supernatant of HeLa cell-*C. albicans* incubation mixture. A 4-fold increase in $PGE_2$ over the basal level was observed, and this increase was markedly inhibited by NS398, a selective COX-2 inhibitor, as well as by nonselective COX-1/COX-2 inhibitors indomethacin and diclofenac (Fig. 1*D*). These findings are consistent with our earlier report on $PGE_2$ synthesis in a vaginal *C. albicans* isolate from a patient with recurrent candidiasis (15). To investigate the differential pattern of induction for COX-2 protein and COX-2 mRNA, HeLa cells were treated with 100 nM $PGE_2$ for 6 h and compared with cells challenged with *C. albicans*. A significant increase in the levels of COX-2 mRNA in $PGE_2$-treated infected cells was observed (Fig. 2). Concomitant treatment of HeLa cells with cycloheximide (10 $\mu$M) and $PGE_2$ (100 nM) abolished the COX-2 up-regulation induced by $PGE_2$, and this effect did not differ from that of cycloheximide alone (not shown). These data indicate that the initially synthesized $PGE_2$ by *Candida*-infected HeLa cells functions as a trigger for further induction of COX-2 gene.

C. albicans *induces $PGE_2$ synthesis through TLRs*

TLRs have been shown earlier to be involved in the antifungal defense mechanism in *Drosophila* and bacterial defense in humans (20, 21). To determine the involvement of these receptors in the interaction of HeLa cells with *C. albicans*, neutralization experiments were performed. Surface TLR-2 and TLR-4 receptors were blocked by neutralizing concentrations of respective Abs before the cells were infected with *C. albicans* at MOI 5. In this system, the production of $PGE_2$ by HeLa cells induced by *Candida* infection was almost completely abrogated, underlining the importance of TLRs in the interaction of HeLa cells with *C. albicans* (Fig. 3). Abs against IL-1$\beta$ cytokine, which has been shown to be produced by *C. albicans* (22) and to trigger the signaling cascade in smooth muscle cells (23), however, failed to inhibit the induction of $PGE_2$ (Fig. 3).

C. albicans-*induced synthesis of COX-2 protein mimics oxidative stress-mediated signaling*

To find whether *C. albicans*-induced activation of COX-2 expression is dependent on antioxidant potential of HeLa cells, cells transfected with COX-2 promoter were depleted of glutathione either by pretreatment with 100 $\mu$M BSO, an inhibitor of glutathione, for 24 h or with 200 $\mu$M *N*-acetylcysteine, an antioxidant, for 18 h for enhancing cellular glutathione content. On incubation of cells with *C. albicans* at MOI 5, almost a 3-fold increase in COX-2 promoter activity was observed (Fig. 4). Taken into consideration that BSO-treated cells alone caused only 2-fold increase

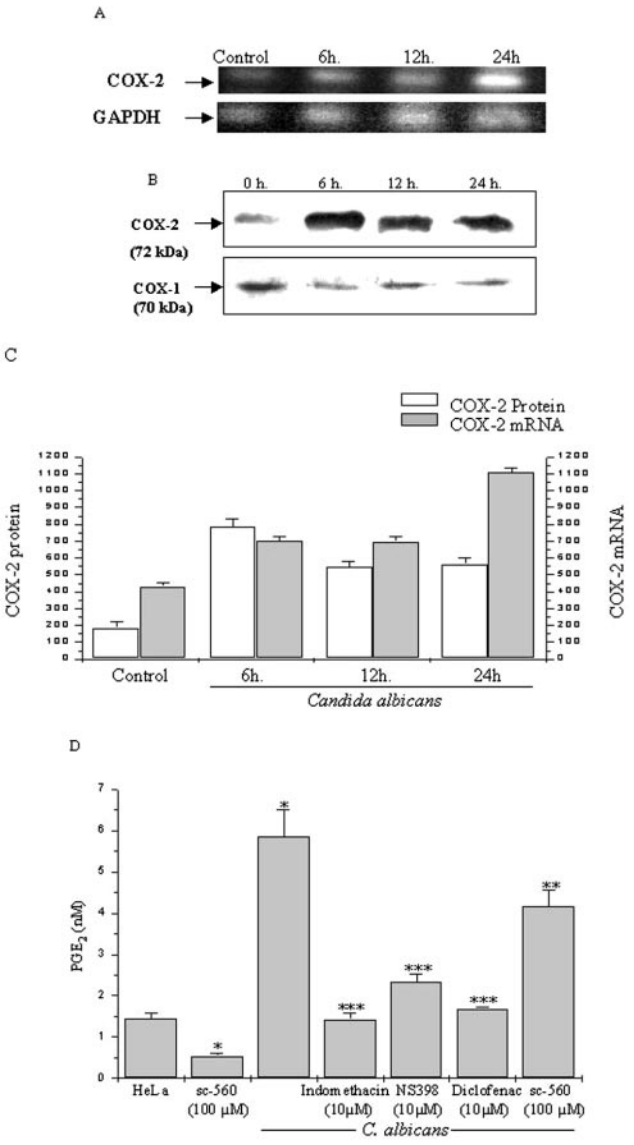


**FIGURE 1.** *C. albicans* induces the COX-2 mRNA expression and synthesis of COX-2 protein and $PGE_2$ but not COX-1 protein in HeLa cells. Cells were treated with *C. albicans* at MOI 5 for various periods of time. *A*, Total RNA (1 $\mu$g) was extracted, and RT-PCR analysis was performed. PCR was performed for COX-2 (*upper panel*), as well as for GAPDH (*lower panel*) as a control to ensure that RNA amounts were equal. Data are representative of four separate experiments. *B*, Lysate protein prepared from HeLa cells infected with *C. albicans* for 0, 6, 12, and 24 h was loaded onto a 10% SDS-PAGE and subsequently transferred onto nitrocellulose. Immunoblots were probed with Abs specific for COX-1 and COX-2. The immunoblot is representative of three separate experiments. *C*, Densitometric quantification of COX-2 protein normalized to $\beta$-actin and of COX-2 mRNA normalized to GAPDH. *D*, HeLa cells were treated with nonselective COX inhibitors indomethacin and diclofenac, selective COX-2 inhibitor NS398 and COX-1 inhibitor SC-560 for 1 h. After the medium was replaced, cells were infected with *C. albicans* (MOI 5) for 6 h. The supernatant was collected, and $PGE_2$ production was determined by ELISA. Data are given as mean ± SD ($n = 3$). Significant difference between untreated cells and infected cells is indicated by $p < 0.001$ (*), and that between infected cells plus inhibitors vs infected cells alone is indicated by $p < 0.05$ (**) or $p < 0.01$ (***).

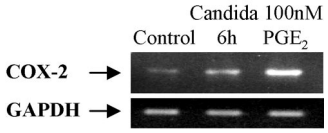


**FIGURE 2.** Induction of COX-2 gene expression by $PGE_2$. Cells were treated with 100 nM $PGE_2$ and with *C. albicans* at MOI 5 (control) for 6 h. Total RNA (1 $\mu$g) was extracted, and RT-PCR analysis was performed. PCR was performed for COX-2 as well as for GAPDH as a control to ensure that RNA amounts were equal. PCR data are representative of three separate experiments.

in COX-2 promoter activity (Fig. 4), BSO treatment of infected cells failed to increase the COX-2 promoter activity beyond the level obtained with *C. albicans* alone, suggesting that a maximum depletion of intracellular glutathione was achieved by *C. albicans* at MOI 5. In contrast, cells with increased glutathione levels abrogated the *C. albicans*-induced activation of COX-2 (Fig. 4), indicating a strong prooxidant behavior of *Candida* infection. Determination of glutathione content by ELISA in cells infected with *C. albicans* with and without pretreatment of BSO revealed glutathione levels of 28.3 $\pm$ 1.0 nmol/mg protein ($n = 3$) for untreated cells, 11.8 $\pm$ 1.1 nmol/mg protein ($n = 3$) for *C. albicans*-infected cells, and 8.0 $\pm$ 0.5 nmol/mg protein ($n = 3$) for BSO-pretreated cells infected with *C. albicans*. This supported our observations depicted in Fig. 4.

### C. albicans-*induced synthesis of COX-2 protein and* $PGE_2$ *formation is prevented by inhibitors of* $p38^{MAPK}$*, PKC, and reactive oxygen species (ROS)*

Oxidative stress has been suggested to activate cell signaling via MAPK family, which has been implicated in the regulation of cell growth and differentiation (24, 25). To investigate the pathways of *C. albicans*-induced activation of COX-2 in HeLa cells, various inhibitors of second messenger pathways were applied. Neither COX-2 expression nor $PGE_2$ formation was inhibited by ERK1/2 inhibitor PD98059 (Fig. 5, *A* and *B*). In contrast, inhibitors of p38MAPK (SB202190) and PKC (GF203190X) suppressed significantly *C. albicans*-induced COX-2 immunoreactivity (Fig. 5*A*). The suppression of COX-2 immunoreactivity was accompanied by a dose-dependent decrease in *C. albicans*-induced $PGE_2$ formation (Fig. 5*B*), whereby the inhibition by SB202190 was more pronounced than that by GF203190X. Whereas SB 202190 at a concentration of 0.1 $\mu$M inhibited almost 50% of $PGE_2$ synthesis and at 10 $\mu$M concentration abolished it, PKC inhibitor GF203190X suppressed the $PGE_2$ synthesis by only 50% at 30 $\mu$M (Fig. 5*B*). To investigate whether this deviation in suppression of COX-2 protein from the corresponding $PGE_2$ levels by PKC inhibitor GF203190X is caused by COX-1 contribution to $PGE_2$ synthesis, we pretreated the cells with increasing concentrations of COX-1 inhibitors resveratrol or SC-560. Whereas resveratrol suppressed $PGE_2$ synthesis completely (not shown), confirming its inefficacy as a specific COX-1 inhibitor (26), SC-560 reduced the $PGE_2$ level significantly in *Candida*-infected cells (Fig. 1*D*). Moreover, free radical scavenger PDTC was also capable of inhibiting *C. albicans*-induced COX-2 activation markedly, as shown by assaying COX-2 promoter activity (Fig. 5*C*) and $PGE_2$ formation (Fig. 5*D*), suggesting the involvement of ROS and NF-$\kappa$B.

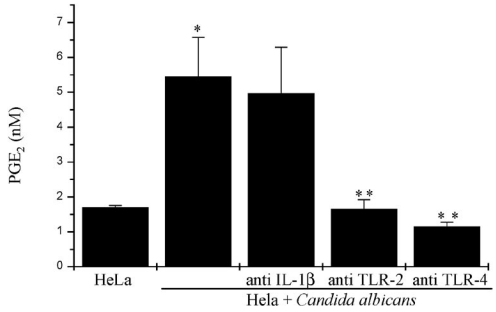


**FIGURE 3.** Neutralization with TLR-2 and 4 Abs inhibits the synthesis of $PGE_2$. HeLa cells were preincubated with 20 $\mu$g/ml anti-TLR-2, TLR-4, and IL-1$\beta$ Abs separately for 1 h. Cells were then challenged with *C. albicans* (MOI 5) for 6 h. The cell culture supernatants were stripped of the Abs by incubation with protein A-agarose and were subsequently used for $PGE_2$ ELISA. Data are given as mean $\pm$ SD ($n = 3$). Significant difference between untreated cells and infected cells is indicated by $p < 0.001$ (*), and that between infected cells treated with Abs vs infected cells alone is indicated by $p < 0.001$ (**). Cells treated with IL-1$\beta$ Ab do not show any significant difference in levels of $PGE_2$.

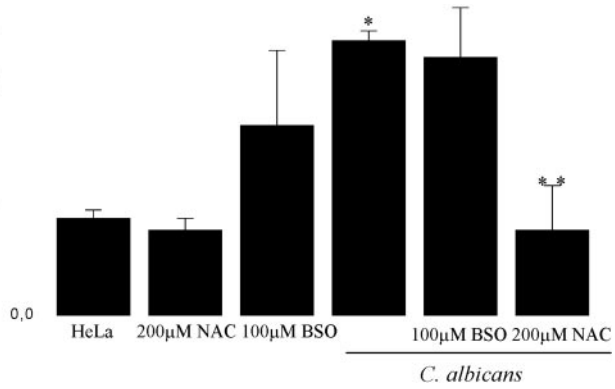


**FIGURE 4.** Prooxidant role of *C. albicans* in the induction of COX-2 expression. HeLa cells transfected with 1.5 $\mu$g of a human $-327/+59$ COX-2 promoter-*luciferase* reporter construct were either preincubated with 100 $\mu$M BSO for 24 h and then infected with *C. albicans* (MOI 5) for 6 h or coincubated with 200 $\mu$M NAC and *C. albicans* (MOI 5) for 6h. COX-2 promoter activity was measured and given as mean relative light units (RLU) $\pm$ SD ($n = 3$). Significant difference between untreated cells and infected cells is indicated by * $p < 0.01$ (*), and that between infected cells and NAC-treated cells is indicated by $p < 0.01$ (**).

### C. albicans *induces activation of* $p38^{MAPK}$ *via PKC in HeLa cells*

Western blot analysis using specific Abs against $p38^{MAPK}$ and phosphorylated $p38^{MAPK}$ revealed that *C. albicans* rapidly increased both $p38^{MAPK}$ and phosphorylated $p38^{MAPK}$ levels, with a maximal effect occurring at MOI 5 (Fig. 6*A*). This increase of both proteins was, however, abrogated when HeLa cells were pretreated with 10 $\mu$M $p38^{MAPK}$ inhibitor SB202109 (Fig. 6*B*). To determine the upstream kinases, we pretreated HeLa cells with PD98059 (10 $\mu$M), PDTC (20 $\mu$M), and GF203190X (10 $\mu$M) for 1 h before incubating with *C. albicans* at MOI 5. Western analysis of the lysate showed significant inhibition of phosphorylated and non-phosphorylated $p38^{MAPK}$ proteins by GF203190X and PDTC (Fig. 6*B*), which was also confirmed by densitometric scanning analysis (not shown), pinpointing PKC and NF-$\kappa$B as upstream regulators of p38MAPK. No such inhibition was, however, observed with PD98059, an inhibitor of ERK-1/2 (Fig. 6*A*). Also, Western blot analysis failed to show any activation of ERK1/2 and JNK by *C. albicans* (Fig. 6*C*). NF-$\kappa$B is commonly located in cytoplasma in an inactive state as a heterodimer bound to its inhibitory protein I$\kappa$B. After stimulation with agonists, NF-$\kappa$B is detached from I$\kappa$B and translocates to the nucleus to activate the target genes (27). To investigate the role of NF-$\kappa$B in the *C. albicans*-induced activation

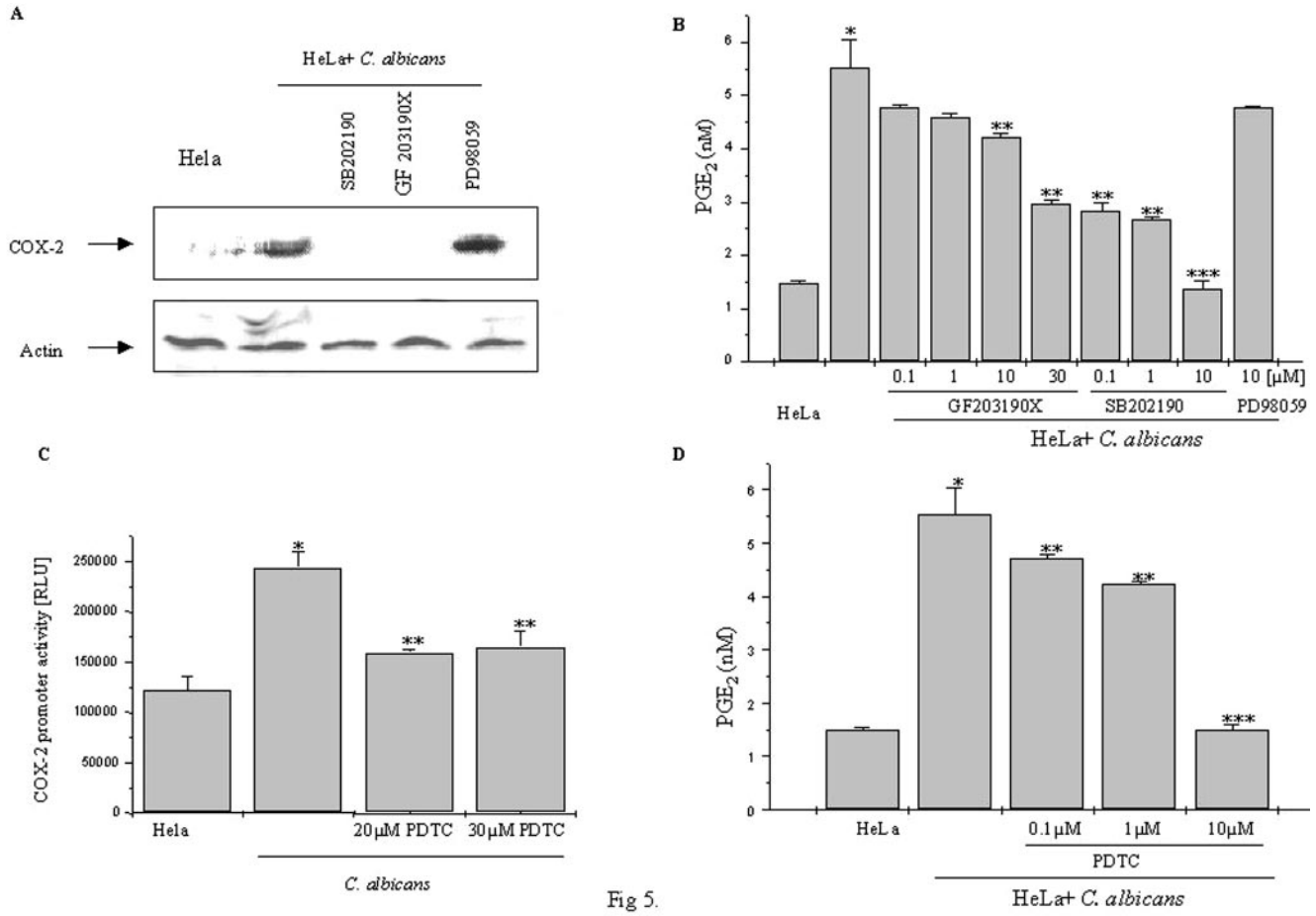


Fig 5.

**FIGURE 5.** *C. albicans*-induced COX-2 and $PGE_2$ synthesis is suppressed by inhibitors of p38MAPK, PKC, and free radical scavenger. HeLa cells untransfected or transfected with 1.5 $\mu$g of a human $-327/+59$ COX-2 promoter-*luciferase* reporter were treated with various inhibitors, e.g., SB202190, GF203190X, PD98059 or PDTC for 1h before infection with *C. albicans* at MOI 5 for 6 h. *A*, Cell lysate protein was prepared from untransfected cells pretreated with 10 $\mu$M SB202190 or 10 $\mu$M PD98059 for 1 h and then challenged with *C. albicans* for 6 h. Lysate protein was prepared, loaded onto a 10% SDS-PAGE, and subsequently transferred onto nitrocellulose. Immunoblots were probed with Ab specific against COX-2. Actin served as a control for loading protein. The exposure is representative of a single experiment performed three times. *B*, Untransfected cells were treated with 0.1 $\mu$M, 1.0 $\mu$M, and 10 $\mu$M SB203190; with 0.1 $\mu$M, 1.0 $\mu$M, 10 $\mu$M, and 30 $\mu$M GF203190X; or with 10 $\mu$M PD98059 for 1 h before infection with *C. albicans* (MOI 5). After 6 h, supernatant was collected by centrifugation of incubation mixture and used for $PGE_2$ assay by ELISA. Values are mean $\pm$ SD for three separate experiments. Significant difference between untreated cells and infected cells is indicated by $p < 0.01$ (*), between infected cells plus 10 $\mu$M or 30 $\mu$M GF203190X vs infected cells alone by $p < 0.05$ (**), and between infected cells plus 10 $\mu$M SB203190 vs infected cells alone by $p < 0.01$ (***). *C*, HeLa cells transfected with 1.5 $\mu$g of a human $-327/+59$ COX-2 promoter-*luciferase* reporter construct were preincubated with vehicle, 20 $\mu$M PDTC, or 30 $\mu$M PDTC before infection with *C. albicans* at MOI 5 for 6 h. Luciferase activity was measured, and values are reported as mean RLU $\pm$ SD ($n = 3$). DNA content in each reaction was kept constant at 2 $\mu$g by using empty vector. Significant difference between untreated cells and infected cells is indicated by $p < 0.01$ (*), and between infected cells plus PDTC vs infected cells alone by $p < 0.01$ (**). *D*, Untransfected HeLa cells were preincubated with vehicle, 0.1 $\mu$M PDTC, 1 $\mu$M PDTC, or 10 $\mu$M PDTC before infection with *C. albicans* at MOI 5 for 6 h. The supernatant obtained after centrifugation of incubation mixture was used for $PGE_2$ assay by ELISA. Values are mean $\pm$ SD for three separate experiments. Significant difference between untreated cells and infected cells is indicated by $p < 0.001$ (*), and that between infected cells plus PDTC vs infected cells alone is indicated by $p < 0.01$ (**) and by $p < 0.001$ (***).

of $p38^{MAPK}$ and COX-2, HeLa cells were transfected with IKK dominant negative plasmid before challenging with *C. albicans*. Significant inhibition of $p38^{MAPK}$ was observed in Western blot analysis (Fig. 6*B*), suggesting the regulation of $p38^{MAPK}$ via the NF-$\kappa$B-dependent pathway.

### *Role of NF-$\kappa$B in* C. albicans*-induced activation of COX-2 in HeLa cells*

In COX-2 gene promoter, elements for NF-$\kappa$B, NF, IL-6, and cAMP-responsive element have been found to be crucial for the regulation of transcription (28, 29). Inasmuch as free radical scavenger PDTC partially inhibited COX-2 activation and $PGE_2$ synthesis, we performed experiments without exposing cells to TNF or cytokines, thus simulating in vivo conditions during the infection. To investigate whether *C. albicans* stimulated NF-$\kappa$B, HeLa cells were transfected with pNF-$\kappa$B-Luc plasmid or COX-2 promoter construct and subsequently infected with *C. albicans* for 6 h at MOI 5. Luciferase activity was measured using luciferase reporter assay. *C. albicans* up-regulated NF-$\kappa$B and COX-2 activities 4-fold of the basal value (Fig. 7*A*). Surprisingly, pretreatment of cells with SB202190 and GF203190X, inhibitors of $p38^{MAPK}$ and PKC, respectively, did not abrogate NF-$\kappa$B and COX-2 promoter activities, indicating alternative pathways for the COX-2 activation. To clarify the role of NF-$\kappa$B in COX-2 activation,

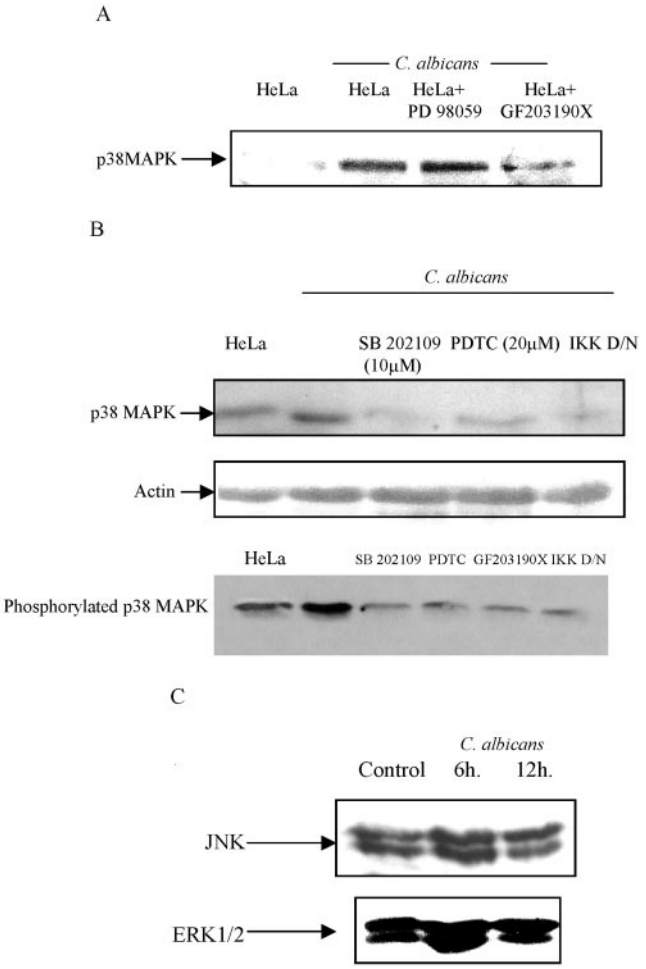


**FIGURE 6.** *C. albicans*-induced COX-2 is $p38^{MAPK}$-dependent but independent of JNK and ERK1/2. HeLa cells with or without pretreatment with inhibitors PD98059 (10 $\mu$M), GF203190X (20 $\mu$M), SB202109 (10 $\mu$M), or PDTC (20 $\mu$M) were treated with *C. albicans* at MOI 5 for 6 h. Lysate protein was prepared, and Western analysis was performed. Immunoblots shown are representative of three separate experiments. *A*, Lysate protein from cells (25 $\mu$g/lane) with or without pretreatment with inhibitors PD98059 (10 $\mu$M) or GF203190X (20 $\mu$M) was prepared after infection with *C. albicans* (MOI 5), electrophoresed, transferred onto nitrocellulose, and probed with Ab specific for $p38^{MAPK}$. *B*, Lysate protein from cells with or without pretreatment with inhibitors SB202109 (10 $\mu$M) or PDTC (20 $\mu$M) or GF203190X (20 $\mu$M) was prepared after infection with *C. albicans*, electrophoresed, and probed with Abs specific for p38MAPK (*upper panel*) or phosphorylated $p38^{MAPK}$ (*lower panel*). In some experiments, HeLa cells were transfected with 1 $\mu$g of expression vector for dominant negative I$\kappa$B kinase (IKK-DN) before challenging with *C. albicans*. Here also, lysate protein was electrophoresed, transferred onto nitrocellulose, and probed with Ab specific for $p38^{MAPK}$ (*far right lane*). Actin served as a control for loading protein. *C*, HeLa cells were treated with vehicle and *C. albicans* (MOI 5) for 6 and 12 h. Lysate protein was prepared, electrophoresed, and probed with Abs specific for JNK and ERK1/2.

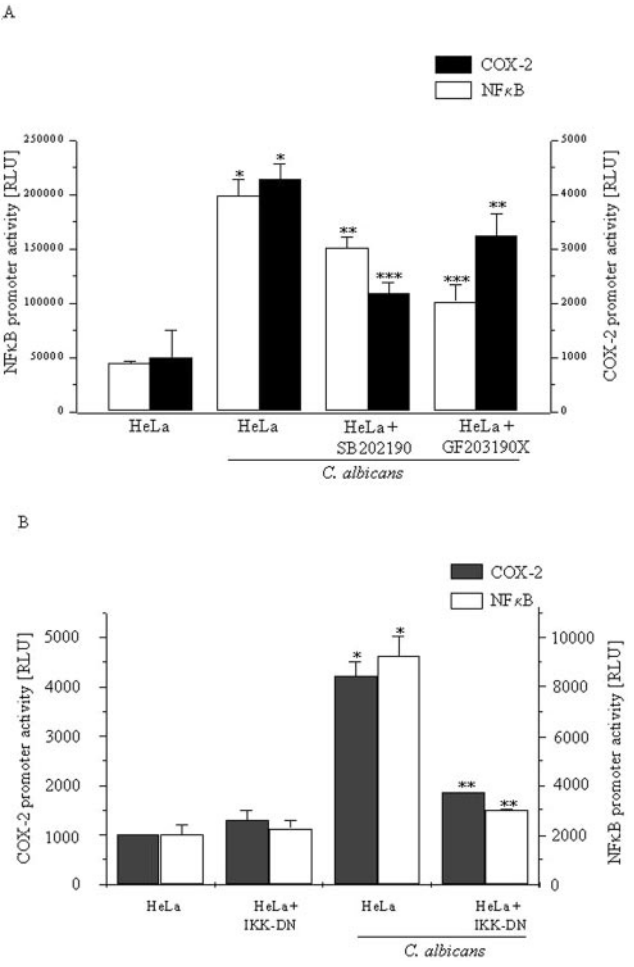


**FIGURE 7.** *C. albicans*-induced NF-$\kappa$B activity is partially dependent on PKC and $p38^{MAPK}$ and mediates the induction of COX-2. *A*, In one set of experiments HeLa cells were transfected with 1.5 $\mu$g of a human COX-2 promoter construct ligated to luciferase and 0.2 $\mu$g of empty vector. In another set of experiments, HeLa cells were transfected with 1.5 $\mu$g of pNF-$\kappa$B-LUC plasmid ligated to luciferase and 0.2 $\mu$g of empty vector. Cells were then pretreated with vehicle or 20 $\mu$M SB202190 or 10 $\mu$M GF203190X for 1 h, followed by infection with *C. albicans* (MOI 5) for 6 h. Luciferase activity was measured for COX-2 and NF-$\kappa$B promoter activities, and values are reported as mean RLU $\pm$ SD ($n = 3$). For NF-$\kappa$B activity significant difference between control and infected cells is indicated by $p < 0.001$ (*), between infected cells and infected cells plus SB202190 by $p < 0.05$ (**), and between infected cells and infected cells plus GF203190X by $p < 0.01$ (***). For COX-2 promoter activity, significant difference between control and infected cells is indicated by $p < 0.001$ (*), between infected cells and infected cells plus SB202190 by $p < 0.01$ (***) and between infected cells and infected cells plus GF203190X by $p < 0.05$ (**). *B*, In one set of experiments, HeLa cells were transfected with 1.5 $\mu$g of a human COX-2 promoter construct ligated to luciferase and 0.2 $\mu$g of empty vector. In another set of experiments, HeLa cells were transfected with 1.5 $\mu$g of pNF-$\kappa$B-LUC plasmid ligated to luciferase and 0.2 $\mu$g of empty vector. Columns representing IKK-DN received 1.5 $\mu$g of dominant negative plasmid for IKK. In both sets of experiments, cells were infected with vehicle or *C. albicans* for 6 h. Luciferase activity was measured for COX-2 und NF-$\kappa$B promoter activities, and values were reported as mean RLU $\pm$ SD ($n = 3$). Significant difference between control and infected cells is indicated by $p < 0.001$ (*) and between infected cells and infected cells plus IKK-DN by $p < 0.01$ (**).

HeLa cells were cotransfected with COX-2 promoter construct and IKK-dominant negative plasmid or with pNF-$\kappa$B-Luc plasmid and IKK-dominant negative plasmid. After challenging transfected cells with *C. albicans* (MOI 5) for 6 h, inhibition of >60% of COX-2 and >70% of NF-$\kappa$B promoter activities were observed (Fig. 7*B*). These observations show that signaling pathways other than via $p38^{MAPK}$ and NF-$\kappa$B may be operative in *C. albicans*-induced activation of COX-2 gene.

## Discussion

$PGE_2$ plays a crucial role in the morphogenesis of *C. albicans* and has been shown to stimulate germ tube formation (16). Conversely, we have shown recently that *C. albicans* isolated from

vaginal tract of a patient with VVC when cocultured with HeLa cells caused significant release of $PGE_2$ (15). These observations led us to investigate the activation of COXs in HeLa cells by *C. albicans* in a coculturing model of *C. albicans*-HeLa cells maintaining MOI 5:1.

In the present study, we demonstrate for the first time that COX-2 mRNA, COX-2 protein, and COX-2 activity are selectively induced by *C. albicans* in HeLa cells in a time- and dose-dependent manner, whereas the constitutively expressed COX-1 gene was barely affected (Fig. 1, *A* and *B*). The differential pattern in time course of COX-2 mRNA expression (peak at 24 h) and COX-2 protein level (maximum at 6 h) is, however, intriguing. The fact that exogenously added $PGE_2$ (100 nM) augmented the expression of COX-2 mRNA in 6 h (Fig. 2) and cycloheximide caused significant reduction of COX-2 expression (not shown) pinpoints a de novo protein synthesis triggered by initially formed $PGE_2$. Although EP receptors have rarely been suggested as a link for signaling via $p38^{MAPK}$ or PKC (30), our observations provide evidence that $PGE_2$ induces both $p38^{MAPK}$ and PKC possibly via $PGE_2$ receptors (EP). The $PGE_2$-induced up-regulation of COX-2 leads to drastic increase of $PGE_2$ formation (23), which plays a major role in morphogenesis of *C. albicans* (16) and candidiasis (22). Absence of increase in COX-2 protein expression after 6 h may also be attributed to posttranscriptional regulation of the COX-2 gene, in which RNA-binding proteins bind to the 3′-untranslated region of the COX-2 mRNA and inhibit its translation (31). Also, we do not rule out the occurrence of $PGE_2$-mediated positive feedback regulation at the transcriptional level in a comparable time course between COX-2 mRNA and COX-2 protein, as shown by Schrör et al. (32), although differences in experimental setups between both laboratories prohibit any direct comparison. The selective activation of COX-2 gene was in agreement with the inflammatory status of the *Candida*-infected HeLa cells, because at MOI $\leq$1, which describes the common microbial status of *C. albicans* in organs, COX-2 protein as well as $PGE_2$ synthesis were hardly detectable (not shown).

TLRs have been identified in humans as an important component of innate immunity against microbial pathogens. Spaetzle/Toll and other similar proteins were first discovered in *Drosophila melanogaster*, and their interactions were shown to induce antifungal defense by the production of drosomycin, an antifungal compound (21). TLRs recognize various components of bacterial pathogen such as peptidoglycans and lipoproteins (TLR-2) (33, 34), lipopolysaccharides (TLR-4) (34), and bacterial DNA (TLR-9) (35). Experiments with TLR-4-deficient mice revealed an important role for TLRs in combating candidiasis (36). The signaling pathways induced by TLRs vary with respect to interactions involving MyD88, TIRAP, Tollip, and p85 proteins (37), as well as NF-$\kappa$B (38). Neutralization experiments with Abs blocking TLR-2 and TLR-4 demonstrate that these receptors play a critical role in the signal transduction pathway induced by *C. albicans* (Fig. 3). *Candida* infection also induces the production of cytokines such as IL-1$\beta$ (22), which may subsequently trigger signal transduction cascade in HeLa cells. However, neutralization experiments performed with specific IL-1$\beta$ Abs ruled out this pathway (Fig. 3).

Increased ROS formation is a common consequence of many pathologies, including infection and inflmmation, and provides a link between signaling pathways and transcriptional events that regulate the large number of genes. The consequences of enhanced oxidative stress are expressed in release of proinflammatory cytokines, growth factors, and a large number of transcription factors (39). Assay of intracellular glutathione content in HeLa cells revealed that the *Candida* infection alone caused drastic depletion of glutathione, thus enhancing the oxidative stress and consequently COX-2 promoter activity. Conversely, NAC, an antioxidant, and PDTC, a free radical scavenger, strongly decreased the COX-2 activity (Figs. 4 and 5). These findings are consistent with the earlier reports in which oxidative stress has been assigned a key role in the activation of COX-2 (40), and depletion of glutathione was shown to be associated with increased susceptibility to infection (41).

Oxidative stress has also been linked with intracellular MAP kinase signaling pathways (24, 25), which lead to up-regulation of COX-2 gene expression (42, 43). $p38^{MAPK}$s are members of the MAPK superfamily and can be activated in response to cellular stress (44), ischemia/reperfusion (45), endotoxins, and inflammatory cytokines (46). Although $p38^{MAPK}$ activation in other cell systems has been reported to be mediated by G protein-coupled receptors (47, 48), the upstream signaling mechanisms for its activation is far from clear. Thus, *C. albicans*-induced activation of COX-2 showed the stimulation of $p38^{MAPK}$ pathway without any involvement of ERK1/2 and stress-activated protein kinase/JNK pathways (Fig. 6, *A–C*). Moreover, the $p38^{MAPK}$ pathway was not only suppressed by $p38^{MAPK}$ inhibitor SB202190, but also by GF203190X, a PKC inhibitor (Fig. 6, *A* and *B*), which suggests that the PKC activation occurs upstream of $p38^{MAPK}$ and is an obligatory event in *C. albicans*-mediated COX-2 induction in HeLa cells. Furthermore, total suppression of COX-2 protein expression by SB202190 and GF203190X (Fig. 5*A*), but their differential inhibitory activities with respect to concomitant $PGE_2$ synthesis is striking. Whereas SB202190 completely inhibited the $PGE_2$ synthesis, GF203190X suppressed $PGE_2$ only partially (Figs. 5, *A* and *B*). If it holds true that the residual $PGE_2$ in GF203190X-pretreated cells is contributed by COX-1 as shown by the use of selective COX-1 inhibitor SC-560 (Fig. 1*D*), it can be concluded that SB202190-mediated inhibition of $PGE_2$ is caused by both COX-1 and COX-2. This conclusion was also supported by the presence of residual COX-2 promoter activity found in COX-2- and NF-$\kappa$B-transfected cells pretreated with SB202190 and GF203190X (Fig. 7*A*) as well as in cells cotransfected with IKK-DN plasmid and COX-2 promoter construct or pNF-$\kappa$B-Luc plasmid (Fig. 7*B*).

One of the redox-sensitive transcription factors was recognized as NF-$\kappa$B (49). NF-$\kappa$B commonly exists as a heterodimer of p50 and p65 peptides, bound to its inhibitory protein I$\kappa$B, which masks its nuclear localization. After stimulation with agonists, I$\kappa$B is cleaved and phosphorylated by IKK complex and then degraded by the 26S proteasome (50, 51). Subsequently, NF-$\kappa$B translocates to the nucleus, where it docks to DNA at binding sites within the promoter region of target genes (24, 27). Cleavage of I$\kappa$B requires an oxidizing milieu and appears to be one of the mechanisms by which ROS activate NF-$\kappa$B (52). Assay of NF-$\kappa$B activity in presence of the free radical scavenger PDTC and in cells transiently transfected with NF-$\kappa$B plasmid and dominant negative vector of IKK, showed the important signaling role of NF-$\kappa$B in *C. albicans*-induced COX-2 activation (Figs. 5 and 7). This is not surprising, because several consensus sequences for NF binding, e.g., NF-$\kappa$B, NF-IL6 and cAMP-responsive element binding sites, found in the 5′-flanking region of the COX-2 gene, have been reported as regulatory sequences in COX-2 induction by various agonists in different cell types (29). However, as discussed above, careful analysis of NF-$\kappa$B and COX-2 promoter activities using various inhibitors and dominant negative vectors showed a residual COX-2 promoter activity, which pinpoints the involvement of pathways other than via NF-$\kappa$B and $p38^{MAPK}$ for COX-2 activation. This is not fully in agreement with previous reports, because

pharmacological inhibition of NF-κB activation has been shown to abolish the COX-2 gene expression (53).

The NF-κB-mediated activation of $p38^{MAPK}$ protein synthesis (Fig. 6*B*) is novel and has been described for the first time in this study. However, this pathway is totally in contradiction to earlier reports, in which sustained activation of $p38^{MAPK}$ has been shown to inhibit the NF-κB activity (54). The mechanism involved in this cross-talk between NF-κB and $p38^{MAPK}$ is not yet clear and needs further investigation. However, it is tempting to assume that NF-κB after translocating and binding to DNA promoter may turn on synthesis of proteins which might be directed to activate $p38^{MAPK}$. This assumption is supported by a recent report, in which IKK has been shown to negatively modulate TNF-α-induced JNK activity most probably through the induction of NF-κB target genes encoding proteins such as X-chromosome-linked inhibitor of apoptosis (55).

In conclusion, the overall mechanism for COX-2 activation in candidiasis is likely to involve multiple converging signal transduction pathways and oxidative stress. This holds especially true for NF-κB-mediated cellular actions as well as for MAP kinases, where different combinations of stress kinases, such as ERK, $p38^{MAPK}$, etc., together with protein kinase C and tyrosine kinases lead to different actions in the cell. The same may apply for *Candida* infection of HeLa cells, in which specific inhibition of PKC, $p38^{MAPK}$, NF-κB as well as their activator proteins revealed that these signaling pathways for the activation of COX-2 by *C. albicans* are required, albeit these are dissociated from morphogenetic alterations in *C. albicans* itself. For the first time, we have shown the vital role of TLR-2 and 4 in the recognition and subsequent signaling in HeLa cells, suggesting its importance in VVC. Thus, our study offers a deeper insight into the host-pathogen interaction, which may lead to development of effective therapeutic strategies.

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