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Host-pathogen interaction in vulvovaginal candidiasis: Regulation of COX-2 and iPLA$_2$ genes in host cell survival and apoptosis in *Candida albicans*-infected HeLa cells.

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SUMMARY

Candida albicans is generally found as a commensal colonizer of the mucocutaneous surfaces of the oral cavity, gastrointestinal tract and lower female reproductive tracts of humans. Normally, not pathogenic and causing no damage to host in its commensal state, *Candida* can become pathogenic usually in association with host immunosuppression, such as organ transplantation and chemotherapy and is responsible for recurrent vulvovaginal candidiasis. Upon infection with *C. albicans,* several signal transduction pathways are triggered in host cells. In HeLa cells, maximum expression of COX-2 mRNA was observed after 6 hours of infection with *C. albicans*. In addition, initial increase in PGE_2 production, which decreased as infection progressed, was observed. Since infection describes an oxidative stress phenomenon NAC, a potent free radical scavenger, was found to prevent COX-2 upregulation and PGE_2 biosynthesis, suggesting an involvement of ROS and NF-κB. Infection with *C. albicans* triggered NF-κB-dependent transcription. Regulation of NF-κB transactivation function is controlled at several levels, including interaction with coactivator proteins. In the present study, we showed that the transactivation function of NF-κB is also regulated through interaction of p65 subunit of NF-κB with HDAC-1 corepressor protein. Our data indicated that HDAC-1 recruits directly p65 subunit of NF-κB and likely exerts its corepressor function. Strikingly, we found that the expression of Gam-1, an early gene product of the avian adenovirus CELO, which is essential for viral replication, increased the level of COX-2 transcription through the NF-κB in a similar way, as with the HDAC inhibitor TSA. We also showed that Gam-1 can effectively inhibit histone deacetylation by HDAC-1, and that it binds to HDAC-1 both *in vitro* and *in vivo*. The effect of Gam-1, having the specific function of binding and inactivating HDAC-1, suggests that these deacetylase complexes play an important role in limiting the early gene expression as a result of invading microorganisms. In support of this, we showed that transient transfection of HDAC-1 is able to repress *C. albicans*-induced NF-κB-mediated COX-2 gene activation. Moreover, TSA treatment drastically incrased the hyperacetylation of the *C. albicans*-induced histone H3. We observed these changes in nuclear transcription, thereby demonstrating that the fungal infection can induce *in vivo* chromatin remodeling events to stimulate the inflammatory genes.

Furthermore, *C. albicans* infection induced activation of the defence mechanism of the host. Thus, the host cells activate PLA_2 enzymes which lead to the production of AA. AA is converted

by eicosanoid-converting enzymes to eicosanoids that mediate the inflammation. We have shown that *C. albicans* possesses a potent active calcium-independent PLA_2 enzyme, which in cooperation with $cPLA_2$, is capable of releasing AA from membrane phospholipids of the host cell. This AA is subsequently converted by COX-2 to PGE_2. Seemingly, PLA_2 activity of *C. albicans* and not of host cell PLA_2 causes the initial cleavage of AA from membrane lipids of host cell. This process then leads to activation of host cell PLA_2. Confirmation of this pathway was obtained when inhibition of $iPLA_2$ enzyme by BEL or MAFP was performed. A complete inhibition of *C. albicans*-mediated NF-κB-regulated COX-2 activation, and PGE_2 production in HeLa cells was achieved. The inhibition of $iPLA_2$ also caused the marked decline of PKCα in HeLa cells infected by *C. albicans.* These findings imply that $iPLA_2$ not only plays a crucial role in the phospholipid remodeling, but also a key role in the signaling for AA release and PGE_2 production in *C. albicans*-infected HeLa cells.

ROS, including superoxide anion, hydroxyl radical, and hydrogen peroxide, have been known to trigger a variety of biological responses within cells. The cleavage of IκBα requires an oxidizing milieu and appears to be one of the mechanisms by which ROS activate NF-κB. ROS have been shown to potentate the $iPLA_2$ activity. It was reported that, in uterine stromal cells, H_2O_2 caused a significant release of AA and $iPLA_2$ activity, which is independent of intracellular Ca^{2+} concentration. Both AA and PLA_2 activity could be inhibited almost completely by BEL. We also found similar results demonstrating a significant inhibition of $iPLA_2$ by the free radical scavenger PDTC or by antioxidant NAC in HeLa cells infected by *C. albicans*. In order to obtain support for the observation regarding ROS-$iPLA_2$-NF-κB regulation, we investigated whether $iPLA_2$ and the NF-κB p65 subunit react not only functionally but also physically in *Candida*-infected HeLa cells. The coimmunoprecipitation experiments with monoclonal antibodies against $iPLA_2$ and NF-κB p65 subunit in untreated and *Candida*-infected HeLa cells showed that $iPLA_2$ may undergo a physical binding with the whole complex IκBα-p50/c-Rel-p65.

Next, we investigated the *C. albicans*-induced apoptosis of host cells. Several pathogens are known to interfere with host cell apoptotic control. Intracellular pathogens have evolved diverse strategies to induce or inhibit host cell apoptosis, aiding dissemination within the host or facilitating intracellular survival. Macrophages undergo apoptotic cell death after infection with *C. albicans* strains capable of hyphal formation. Activation of caspase-3 has been observed after endocytosis of *C. albicans* by neutrophils. In this study, we report that *C. albicans*-infected HeLa

cells undergo apoptosis under strict regulation of cellular $iPLA_2$ and TLR2. The caspase-3 activation, which mediates the cleavage of multiple substrates to cause characteristic alteration that occur during apoptosis, was maximally elevated almost 6 hours before the onset of apoptosis. Moreover, caspase-3-mediated apoptosis of HeLa cells followed the classical death receptor pathway in which TLR2 triggers the signaling by recruitment of FADD containing protein and caspase-8. Dissection of early and late responses to *Candida albicans* infection revealed that cellular $iPLA_2$-mRNA expression first increased for 6 hours, but subsequently declined until 18 hours and finally disappeared. Concomitantly, the onset of apoptosis was detected at 12 hours, and continued until 30 hours and then decreased. To confirm the role of $iPLA_2$, we transfected an $iPLA_2$ containing plasmid in HeLa cells and infected with *C. albicans*. No apoptosis was observed in cells overexpressing $iPLA_2$ until 24 hours. Conversely, when HeLa cells pretreated with $iPLA_2$ inhibitor BEL were challenged with *C. albicans*, an earlier onset of apoptosis after 8 hours was observed. Moreover, BEL caused upregulation of TLR2 mRNA and increased apoptosis in infected cells. We therefore conclude that $iPLA_2$ functions as a key regulator for tuning of TLR2-mediated apoptosis in *Candida*-infected HeLa cells.

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ABBREVIATIONS

AA	Arachidonic acid (5Z,8Z,11Z,14Z eicosatetraenoic acid)
Acetyl-CoA	Acetyl coenzyme A
AIF	Apoptosis inducing Factor
ATP	Adenosin 5` triphosphate
APS	Ammonium persulphate
BEL	Bromoenol lactone
BSA	Bovine serum albumin
cAMP	Cyclic adenosine 3`,5`phosphate
Caspase	Cysteine aspartyl-specific protease
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CHX	cycloheximide
3,18-DiHETE	3,18-dihydroxy-5Z,8Z,11Z,14Z eicosatetraenoic acid
DAPI	4'-6-Diamidino-2-phenylindole
DAXX	Death domain-associated protein 6
DMEM	Dulbecco’s modified eagles medium
Diablo	IAP-binding protein with low pI
DTT	Dithiothreitol
EET	Epoxy-eicosatetraenoic acid
EGF	Epidermal growth factor
EGTA	Ethylenglicol-tetraacetic acid
ELISA	Enzyme linked immuno-sorbent assay
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
FADD	Fas-associating protein with death domain
IL	Interleukin
IRAK	IR-1R associated ptotein kinase
3-HETE	3-Hydroxy- 5Z,8Z,11Z,14Z eicosatetraenoic acid
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HPLC	High performance liquid chromatography
LB	Luria-Bertani medium

LRRs	Extracellular leucine-rich repeats
MAPKs	Mitogen-activated protein kinases
MAFP	Methyl arachidonylfluorophosphonate
MOI	Multiplicity of Infection
NP-40	Nonidet P-40
ONPG	ortho-nitrophenyl-para-D-galactopyranoside
PAMPs	Pathogen-associated molecular patterns
PAP-1	Phosphatidate phosphohydrolase-1
PGE_2	Prostaglandin E_2
PGH_2	Prostaglandin H_2
PLA_2	Phospholipase A_2
PRR	Pattern recognition receptors
PAPC	1-palmitoyl-2-arachidonoyl phosphatidylcholine
PBS	Phosphate buffered saline (Phosphate Buffer)
RLU	Relative light units
RHD	Rel-homology domain
RIP	Receptor interacting protein
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulphate
SMAC	Second mitochondrial-derived activator of caspase
SIMPs	Soluble intermembrane proteins
TNFR	Tumor necrosis factor receptor
TRADD	TNFR-associated death domain
TRAF6	Tumor necrosis factor receptor-activation factor 6
TLC	Thin layer chromatography
TIR	Toll-interleukin-1 receptor
Tris	Tris(hydroxymethyl)aminomethane
YNB	Yeast nitrogen base
YPD	Yeast-Peptone-Dextrone rich medium
VVC	Vulvovaginal candidiasis

1. INTRODUCTION

1.1 *CANDIDA ALBICANS* AND ITS PATHOGENESIS

Fungi are eukaryotic organisms with approximately 300 000 different species. Of these, about 200 are potential parasites, with only a few of these affecting humans [1]. Fungal diseases of mammals, mycoses, range from the common mild cutaneous or subcutaneous skin infections, such as athletes foot, to the potentially lethal acute or chronic infection of deep tissues that are typically caused by *Candida* species. Of the *Candida* species afflicting humans, *Candida albicans* is by far the most common. *Candida albicans* belongs to the class Ascomycetes and the family, Saccharomycetaceae. This yeast can live as harmless commensal in many different body locations, and is carried in almost half of the population. However, in response to a change in the host environment, *C. albicans* can convert from a benign commensal into a disease-causing pathogen, causing infections in the oral, gastrointestinal and genital tracts [2]. The infection caused by *C. albicans* can be defined in two broad categories, superficial mucocutaneous [3] and systematic invasive, which involves the spread of *C. albicans* to the blood stream (candidemia) and to the major organs [4]. Systemic candidemia is often fatal. Superficial infections affect the various mucous membrane surfaces of the body such as in oral and vaginal thrush. The incidence of vulvovaginal candidiasis (thrush) has increased approximately 2 fold in the last decade. Approximately 75 % of all women experience a clinically significant episode of vulvovaginal candidiasis (VVC) at least once during the reproductive period [5]. VVC is a relatively benign condition that responds well to anti-fungal treatment. It is proposed that the infection is due to minor changes in epithelial conditions, such as pH, altered glucose/glycogen concentration or changes in epithelial integrity. During pregnancy the risk of vaginal thrush increases, possibly due to changes in hormone production, leading to increased glycogen content in the vagina [5, 6]. The pathogenesis of recurrent vaginal thrush involves a defect in the local immunity to candidiasis, possibly through inappropriate prostaglandin E_2 (PGE_2) production [7]. The role of prostaglandins during the infection is not very clear, however it has been demonstrated that mononuclear cells from the patients suffering from recurrent vaginal candidiasis produce higher levels of PGE_2 as compared with cells from control women, indicating the important role of PGE_2 during infection. Recurrent vaginal candidiasis is also common in female patients with acquired immune deficiency syndrome (AIDS), suggesting a role for depressed cell-mediated immunity in candidiasis [8, 9]. Factors responsible for recurrent vaginal candidiasis may

originate from both the microorganism and the host cells, tissues and organs [10]. Therefore, the severity of *Candida* infection often depends upon the status of the host's immune system. However, there are differences in the pathogenicity of *C. albicans* strains which suggests that strain related virulence factors may play a role in disease severity. Numerous virulence factors have been attributed to the pathogenicity of *C. albicans* [11, 12]. These include dimorphism, phenotypic switching and immune interference.

Dimorphism and phenotypic switching: *Candida albicans* is a diploid asexual and dimorphic fungus and depending upon environmental conditions, can exist as unicellular yeast (blastospores and chlamydospores) as well as in different filamentous forms (hypha, pseudo-hyphae). Several studies suggest that the ability of *C. albicans* to switch between the yeast and mycelial forms is an important virulence factor [13, 14]. Increased adherence to oropharyngeal surfaces has been observed for the mycelial form. Decreased adherence has been demonstrated by a non-germ tube producing variant in experimental vaginitis [14]. Moreover, *C. albicans* can not only change its cellular morphology in response to growth conditions, but can also irreversibly switch its cellular phenotype both *in vitro* and *in vivo* [15]. This switching is most easily observed in the morphology of colonies [16]. These phenotypic differences are a product of differences in surface protein expression [17]. The different surface protein expression results in differential adherence characteristics for the switch variants and differential sensitivities to neutrophil and phagocytic leukocyte killing [18]. These data suggest that differences in the phenotype may allow for increased resistance to immune attack or increased invasiveness. The process of morphogenesis and phenotype switching is found to be dependent upon the lipid composition of *C. albicans* [19-21]. Lipids constitute about 3.8-4.3 % of the dry weight of the fungal cell and are important structural and functional molecules in *C. albicans.* Goyal and Khuller [22] showed variations in the lipid composition of yeast and mycelia forms in *C. albicans*. They found that total lipid, phospholipid and sterol contents of log phase mycelial cells were significantly higher than in yeast cells.

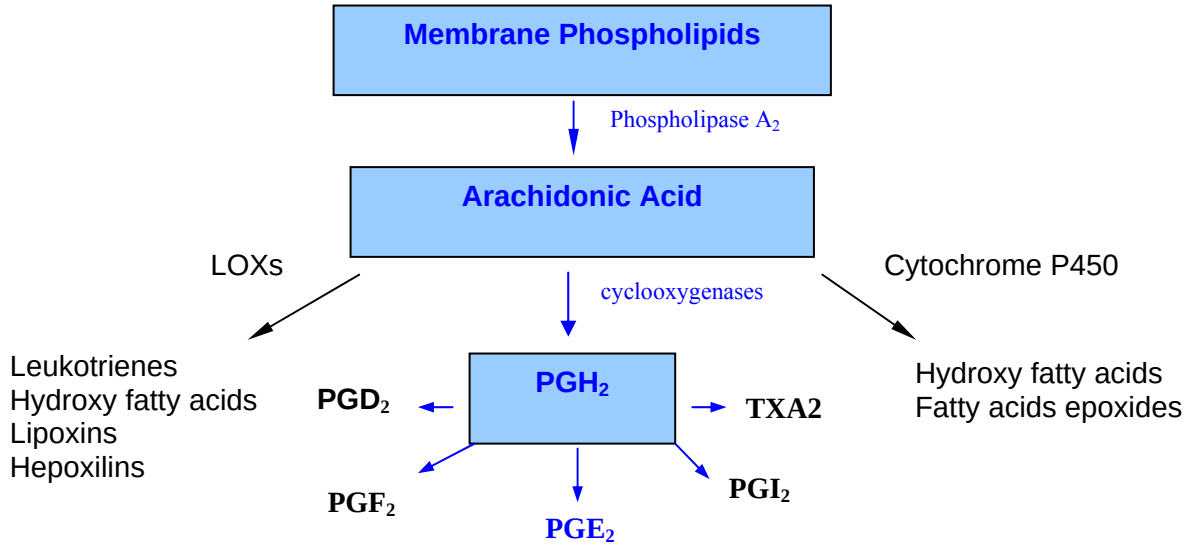
1.1.2 Lipid and fatty acid metabolism in *Candida albicans*

Candida albicans is able to utilise various monosaccharides, such as glucose and maltose but also other carbon sources like ethanol, saturated and lower unsaturated fatty acids [19, 23]. Fatty acid degradation in *C. albicans* is different from that of other eukaryotic organisms. Generally, *C. albicans* degrades fatty acids by peroxisomal beta-oxidation system, via a series of reactions

catalyzed by a multienzyme complex, MFE-2. The gene encoding the multifunctional protein of peroxisomal beta-oxidation was first discovered in *Saccharomyces cerevisiae* [24-26]. Fatty acid degradation occurs via different mechanisms, such as, alpha oxidation leading to formation of CO_2, beta-oxidation in mitochondria and peroxisomes and omega-oxidation carried out by cytochrome P450. The last two mechanisms lead to the formation of hydroxylated fatty acids [27]. Beta-oxidation is a cyclic oxidation system of fatty acids in beta position and requires four different enzymes, which are: acyl-CoA dehydrogenase, enoyl-CoA hydratase, β-hydroxyacyl-CoA dehydrogenase and acyl-CoA acetyltransferase (thiolase). In general, fatty acyl-CoA thioester undergo enzymatic dehydrogenation by acyl-CoA dehydrogenase to form transenoyl CoA. The double bond of trans-enoyl CoA is hydrated to form 3-hydroxyacyl-CoA by the enzyme enoyl-CoA hydratase and is further dehydrogenated to form 3-ketoacyl-CoA. 3-ketoacyl-CoA further undergoes cleavage by thiolase by interaction with a molecule of free acetyl-CoA resulting in a fatty acid shorter by two carbon atoms [28]. Earlier, Dr. Nigam's laboratory reported that fungi in general do not contain higher fatty acids, such as arachidonic acid (AA), eicosapentaenoic acid, docosahexaenoic acid etc., but *C. albicans* was shown to grow on AA as a sole carbon source for energy supply. In addition, they reported for the first time that AA is transformed via glyoxalate shunt to carbohydrates by *C. albicans* [29]. This incomplete beta-oxidation takes place in mitochondria, causing 3R-hydroxylation of AA, and leading to formation of 3-HETE, which is the oxygenated and hydroxy derivative of this long-chain fatty acid [30]. Further, 3-HETE acts as a growth factor for the fungus and is involved in different biological activities in host cells and so a vicious cycle is turned on. This implies that the infection process can be assumed to be a host-pathogen interaction, in which host cell release AA, which is converted to 3-HETE by the pathogen [29]. 3-HETE is finally converted by eicosanoid-converting enzymes to novel bioactive 3-hydroxyeicosanoids [31]. These data accentuate a prominent role of host cell-derived arachidonic acid in the inflammatory events induced by *C. albicans* infections.

The arachidonic acid cascade AA is a 20 carbon atom containing unsaturated fatty acid distributed throughout the lipid bilayer of all mammalian cells. It is derived directly from the diet or via modification of linoleic acid, and normally resides in cell membranes esterified to phospholipids. Phospholipids are major components of biological membranes and membrane-bound vesicles, and contribute to their structure and function. Among the natural phospholipids

phosphatidylcholine (PC, lecithin) is most common and a suitable substrate for phospholipase A_2. Other phospholipids are phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidylethanolamine (PE). In membrane glycerophospholipids, PC is enriched with long chain fatty acid including AA in the sn-2 position. Once liberated, free AA can be metabolised by one of three major enzymatic pathways: cyclooxygenase, lipoxygenase, or the cytochrome P450 (Scheme 1).



Scheme 1. Classic arachidonic acid cascade

1.2 HOST CELL RESPONSE TO FUNGAL INFECTION

Fungi have developed many mechanisms to colonize human hosts. The ability to grow at 37 °C is one of the most important. Production of keratinase allows dermatophytes to digest keratin in skin, hair and nails. Dimorphism allows many fungi that exist in nature in yeast form to change to a mycelial form in the host and thus become pathogenic. Thus, antiphagocytic properties of the *Cryptococcus neoformans* capsule and the adherence abilities of *C. albicans* allow pathogenic potential for these fungi. Fungi may spread locally such as dermatophytes on the skin or eumycotic mycetomas in subcutaneous tissue. *Sporothix schenckii*, another subcutaneous pathogen, spreads via local lymphatics. Most common opportunistic mycoses include invasive aspergillosis, cryptococcal meningitis, mucormycosis and candidiasis. The fungi producing

systemic mycoses may initially cause pulmonary infection. The fungi are phagocytosed by alveolar macrophages but are not destroyed. Instead, the fungi are spread hematogenously to distant sites in the body. An exception is *Cryptococcus neoformans* which disseminates without being phagocytosed. The pathogenesis of some fungi may be at least partly due to the host reaction, such as allergic reactions elicited by some fungi.

1.2.1 Adherence as an initial step of host-pathogen interaction

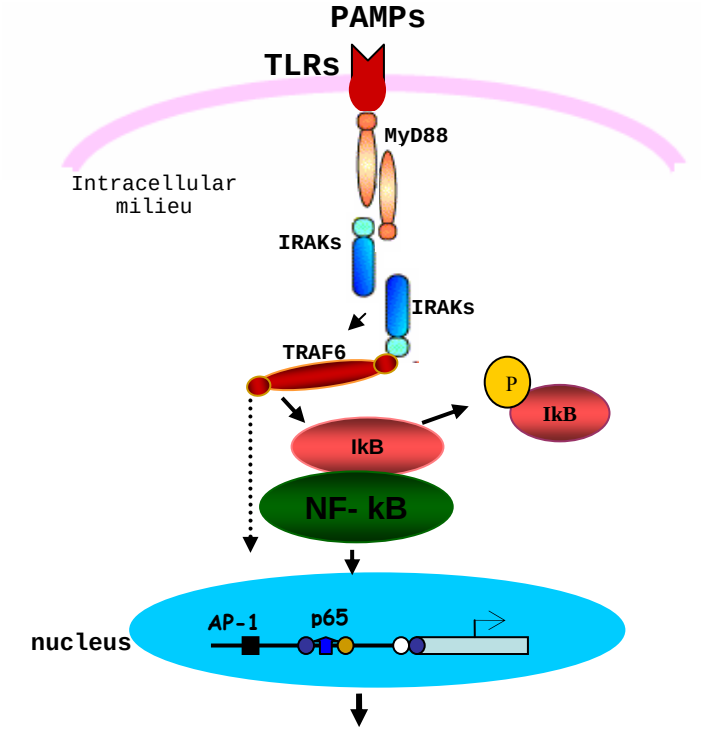
Adhesion of *Candida albicans* to host tissue is considered as an important virulence factor in the development of diseases. Poorly adherent strains of *C. albicans* are less virulent in animal models [32]. Invasive strains of *C. albicans* recovered from patients show greater adherence than strains recovered from asymptomatic carriers [33]. Attachment is believed to be due to a specific interaction of *C. albicans* cell wall components with cell surfaces. Infection of host cells by a pathogenic fungus is mediated through surface receptor [35, 36]. The nature of the components which mediate adhesion is controversial, but some findings indicate that mannan, mannanproteins or polysaccharides are responsible for the adhesion [34]. Factors that enhance adherence include fungal cell surface hydrophobicity, the phenotype of *C. albicans*, pH, temperature, pregnancy, diabetes and oral contraceptive usage [32, 33].

The ability of pathogenic fungus to establish a persistent infection is critically dependent on cellular signals that regulate release of factors from target cells responsible for host cell replication and establishment of the pathogen. Therefore, cell signaling is designed to serve the purpose of cell survival for both host and pathogen.

1.2.2 Toll-like receptors and cell signaling

TLRs have been identified in humans as important components 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 defence by the production of drosomycin, an antifungal compound [37]. Recognition of invading fungi by the innate immune system is the very first step to activate a rapid response and to ensure survival after infection. The targets of innate immune recognition are conserved molecular patterns, so called pathogen-associated molecular patterns (PAMPs) [38]. Accordingly, the receptors of the innate immune system that recognise PAMPs are called pattern recognition receptors (PRR). It is important to note that PAMPs are not unique to pathogens and are produced by both pathogenic and

non-pathogenic microorganism [39]. Toll-like receptors (TLRs) are essential PRRs and constitute a novel protein family of cellular receptors that mediates recognition of microbial challenges and the subsequent inflammatory response in vertebrates. TLRs, homologues of *Drosophila* Toll, are type 1 transmembrane proteins belonging to a protein family characterized by extracellular leucine-rich repeats (LRRs) and intracellular Toll-interleukin-1 receptor (TIR) domain [39, 40]. The LRR motifs are sequentially arranged in the ectodomain of the receptors. In mammalian species there are at least ten TLRs, and each seems to have a distinct function in innate immune recognition. In the past few years, dozens of TLR ligands have been identified. Ligands for TLR-1, 2, 3 and TLR- 4, 5, 6, - 9 have been identified, but the ligands recognized by the other TLRs are unknown [40, 41]. During ligand-binding the TLR recruits the adapter protein myeloid differentiation marker-88 (MyD88) to the receptor (Scheme 2). Thus, in several myeloid cell systems MyD88 links the IL-1 receptor to IR-1R associated protein kinase (IRAK), a serine-threonine kinase that is related to the Pelle kinase of *Drosophila*. Upon binding of ligand to IL-1R, IRAK is phosphorylated, subsequently dissociated from the receptor complex and associated with tumour necrosis factor receptor-activation factor 6 (TRAF6) (Scheme 2).



Scheme 2. Toll-like receptor signaling pathway

Activation of TRAF6 results in the activation of two different pathways involving the c-Jun NH2-terminal kinase (JNK) and p38 mitogen activated protein kinase ($p38^{MAPK}$) family and Rel family transcription factor NF-κB. Moreover, an additional adapter molecule called TRIP that has homology with MyD88 is required for MyD88–dependent signaling through TLR2 and TLR4 but not by the IL-1R or other TLR such as TLR-3, 5, 7 and 9 [40, 42]. In addition to their common activation of MyD88-IRAK-TRAF pathway, individual TLRs may activate different, alternative, signaling pathways. Characterization of the TLR signaling pathways should reveal the molecular mechanism that link the initial recognition of pathogens and elicitation of acquired immunity. A limited number of TLRs can respond to a broad spectrum of microbial products. The implication of TLR2 and TLR4 in the host defence to *C. albicans* was first described by Netea and co-workers [43]. Although growth of *C. albicans* was increased 10-fold in TLR4 mutant mice compared with wild type C3H/HeN mice, TLR4 did not affect the level of TNF-α and IL-1β production in mouse macrophages after stimulation with *C. albicans*. The data have implicated TLRs in the recognition of *C. albicans* cell wall PAMPs [39, 44]. The cell wall of *C. albicans* is mainly composed of chitin (a cellulose-like biopolymer consisting predominantly of N-acetyl-D-glucosamine), glucan (a β-linked branched chain polysaccharide of glucose), mannan (an α-linked polymer of mannose), mannoproteins and glycolipids. Recognition of 1, 2, 3-β-D-glucans by TLRs is dependent on MyD88 [44]. Furthermore, the discovery of Dectin-1 as a small type II transmembrane receptor for β-glucan containing particles, including *C. albicans* and zymosan, also emphasized the importance of other PRRs optionally interacting with TLRs [45]. Dectin-1 is involved in phagocytosis and is expressed on dendritic cells, monocytes and macrophages. It mediates immunological response, such as release of TNF-α and IL-12 in response to fungal derived β-glucans in cooperation with TLR2 [46, 47].

1.3 HOST CELL SURVIVAL

Earlier report from Castro *et al*. [48] showed that the stimulation by *C. albicans* of both rabbit alveolar macrophages and human monocytes caused a sizeable release of AA from pre-labelled cells, which was subsequently converted to cyclooxygenase and lipoxygenase-derived eicosanoids. It was also demonstrated that upon infection, *C. albicans* induced monocytes to

liberate proinflammatory cytokines like interleukin-6 (IL-6) and interleukin-8 (IL-8) [48, 49]. In human endothelial cells, the *in vitro* invasion of *C. albicans* has been reported to stimulate AA metabolism and the secretion of PGI_2 [50, 51].

1.3.1 Phospholipase A_2 (PLA_2) enzymes

The first step in the metabolism of AA is the cleavage of the *sn*-2 ester bond by the enzyme PLA_2 to release AA. A number of mammalian PLA_2 isotypes have been identified. These are divided into three major subfamilies: calcium dependent secretory PLA_2s ($sPLA_2$s), calcium dependent cytosolic PLA_2s ($cPLA_2$s), and Ca^{2+} -independent PLA_2s ($iPLA_2$s) [52, 53]. $sPLA_2$s are low molecular mass enzymes (14 kDa) that require millimolar concentration of Ca^{2+} for activation. The $cPLA_2$ family consists of three isozymes $cPLA_2\alpha$, $cPLA_2\beta$, and $cPLA_2\gamma$, which are classified into groups IVA, ICB and IVC, respectively [54, 55]. $cPLA_2$ (85 kDa) enzyme activity is regulated by submicromolar levels of Ca^{2+}. The activity of $iPLA_2$ is Ca^{2+}-independent. It is mainly considered to be a remodeling enzyme that maintains the composition of membrane phospholipids. Generally, $iPLA_2$s are 80-85 kDa proteins, which are not selective for AA containing phospholipids [52]. In the last decade at least two enzymatically active forms of the enzyme, termed $iPLA_2$ VIA-1 and $iPLA_2$ VIA-2, have been identified [56]. Structurally they contain eight ankyrin repeats at the N-terminus. $cPLA_2$ and $iPLA_2$ are intracellular enzymes, but $cPLA_2$s are located in the cytosol and translocated into membrane, while $iPLA_2$s are located both in the cytosol and in membrane fraction [57].

1.3.1.1 Function of PLA_2s in AA release and phospholipid remodeling.

One of the key roles of any cell is the regulation of PLA_2 activity for AA release. A myriad of agents that exert effects on cells via receptor-dependent or independent pathways elicit a series of signals that ultimately lead to increased PLA_2 activity. Most cell types contain several PLA_2 forms, and all of them may eventually participate in the signaling process.

1.3.1.1.1 Regulation of $sPLA_2$ activation

Generally, $sPLA_2$s (Groups I, II, III, V, IX, X, XI, XII), require millimolar levels of Ca^{2+} for activity, have low molecular masses, and lack specificity for arachidonate-containing phospholipids. $sPLA_2$s were initially found in reptile (group IA) and bee venoms (group III) and in mammalian pancreatic fluid (group IB). It was shown later that a variety of $sPLA_2$s can be

expressed and released in human tissue [58]. Depending on the stimulation conditions, the $cPLA_2$ modulation of $sPLA_2$ cellular activity may occur at a gene regulatory level (delayed responses) [59] or at the level of regulation of enzyme activity itself (immediate responses) [60]. In the latter case, a variety of cellular mechanisms may account for this activation, such as $cPLA_2$-induced rearrangement of membrane phospholipids that enables further $sPLA_2$ attack to more sophisticated mechanisms such as inactivation of endogenous $sPLA_2$ inhibitors or Ca^{2+} fluxes. Therefore, $cPLA_2$ appears to initiate the AA release process and plays primarily a regulatory role, whereas the $sPLA_2$ acts in a second wave to amplify the response by providing the bulk of the AA liberation [62]. Increased levels of extracellular $sPLA_2$s have been detected in the plasma of patients affected by systemic inflammatory diseases such as acute pancreatitis [62], septic shock [63], extensive burns [64], and autoimmune diseases [65].

1.3.1.1.2 Regulation of $cPLA_2$ activation

$cPLA_2$ has been reported to play a role in cellular proliferation, transformation and oncogenesis in certain cell types [66]. $cPLA_2$, especially $cPLA_2\alpha$ shows a remarkable selectivity towards phospholipids bearing AA at the *sn*-2 position. Thus, it was found that the Ca^{2+}-dependent translocation of $cPLA_2$ from cytosol to the perinuclear membranes (Golgi, endoplasmic reticulum (ER), and nuclear envelope) is an essential step for the initiation of stimulus-coupled AA release [67, 68]. Duration of Ca^{2+} increase stabilizes the association of $cPLA_2$ with the perinuclear membrane. This temporal perinuclear localization permits efficient functional coupling between $cPLA_2$ and downstream eicosanoid-biosythetic enzymes, COXs and LOXs [69, 70]. Moreover, $cPLA_2$ mutants exhibit poor PGE_2 biosynthetic ability, indicating that the perinuclear translocation of $cPLA_2$ is important, if not obligatory, for its functional coupling with COX enzymes [71]. The maximum activation of $cPLA_2$ requires sustained dual phosphorylation of Ser^{505} and Ser^{727} sites of the enzyme by mitogen-activated protein kinases (MAPKs) and by MAPK-activated protein kinases, respectively [72]. As PLA_2 is an active component of many venoms and toxins, the secretion of $cPLA_2$ by *C. albicans* could play a part in the invasion of host cell tissues in candidiasis lesions. Thus, $cPLA_2$ found at the site of bud formation suggested a role for this enzyme in cell growth and possibly germ tube formation by hydrolysing phospholipids of cell and intracellular membranes [73]. The *C. albicans* isolates, which adhered most strongly to buccal epithelial cells and were most virulent in mice, exhibited the highest PLA_2 activity [74].

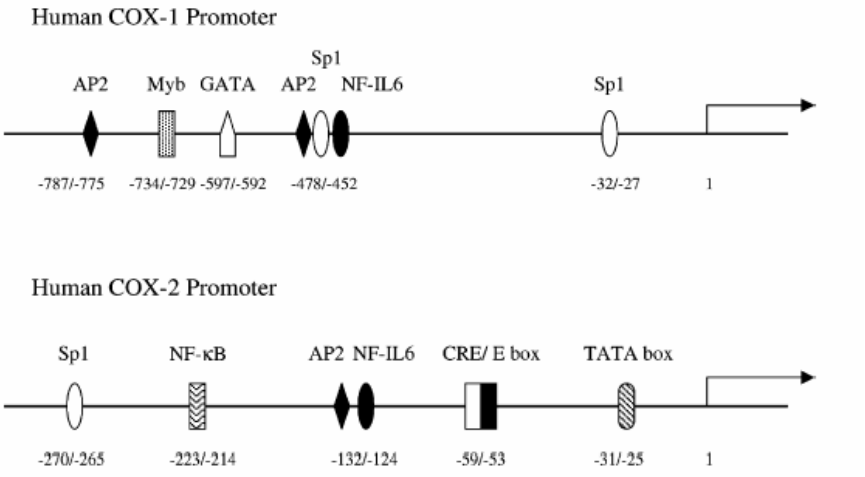
Isolates, which did not adhere and did not kill mice showed lower PLA_2 activity. Therefore, phospholipase activity of *C. albicans* is one of the most important pathogenic factors.

1.3.1.1.3 Regulation of $iPLA_2$ activation

$iPLA_2$ has been proposed to participate in fatty acid release associated with phospholipid remodeling and to play a major role in signal transduction [75]. Beyond the housekeeping role of $iPLA_2$–VIA in maintaining phospholipid homeostasis, several studies suggest that this enzyme is also involved in stimulus-coupled AA release [76, 77, 78]. For instance, agonist-induced PGE_2 production is attenuated by an antisense oligonucleotide for $iPLA_2$ [77], and the overexpression of $iPLA_2$ led to increased AA release in response to A23187 [76, 78]. Reactive oxygen species (ROS) have been reported as factors which potentiate the $iPLA_2$ activity. Birbes *et al*. [79] reported that in uterine stromal cells, H_2O_2 caused a significant release of AA and an increase in $iPLA_2$ activity, which is independent of intracellular Ca^{2+} concentration. These activations are almost completely inhibited by bromoenol lactone (BEL), a specific inhibitor of $iPLA_2$. Furthermore, it was demonstrated that PKCα is also involved in the $iPLA_2$-mediated AA release in response to zymosan [80]. In another study, the thrombin stimulation of vascular smooth muscle cells was found to increase the $iPLA_2$ activity, AA release, and DNA synthesis, which was, however, suppressed by BEL [81]. Moreover, the inhibitor of $p38^{MAPK}$ attenuated the thrombin responses in the cells, suggesting the implication of $p38^{MAPK}$ in thrombin-induced $iPLA_2$ activation [83]. Involvement of $iPLA_2$ has also been implicated in triggering apoptosis in various cell types [84, 85]. So, for instance, apoptosis of U937 cells by anti-Fas antibody is coupled with release of AA from membrane phospholipids which is independent of group IV and group II PLA_2s, but can be inhibited by BEL [84]. Furthermore, caspase-3-catalyzed cleavage of $iPLA_2$ has been reported during apoptosis of U937 cells [85]. The resulting 62 kDa immunoreactive product was found to be more active than the full-length $iPLA_2$ [84]. Interestingly, several reports presented also contradicting results. Thus, even though the $iPLA_2$ inhibitor, BEL, was found to suppress the binding of IgM and Annexin V to the cell surface membrane of peripheral blood mononuclear (PBMT) cells, it failed to inhibit the cleavage of caspase-3 substrates, Phi-phi Lux or PARP or to attenuate nuclear condensation [86].

1.3.2 Cyclooxygenases and its transcriptional regulation

Host cells provide one source of prostaglandins during fungal infection, however, another potential source of prostaglandins is the fungal pathogen itself [87]. Since AA is a major precursor of mammalian eicosanoids, a role for eicosanoids in the host-pathogen interaction seems to be crucial. Thus, PGE_2 has been shown to stimulate germ tube formation in *C. albicans* [88]. Upon stimulation by the invading hyphae of *C. albicans*, cells of the vaginal tract release significant amounts of PGE_2 [89]. The free AA released through the infection acts as a substrate for the cyclooxygenases (COXs) and is converted to unstable PGG_2, which is further converted to PGH_2 by the peroxidase activity of COXs. PGH_2 is the common intermediate for all prostaglandins and thromboxanes, which are formed by the action of the respective enzymes i.e. prostacyclin synthase, thromboxane synthase, PGE_2 synthase, and AA other isomerases [90]. COXs are rate-limiting enzymes in the synthesis of prostaglandins [91]. Two isomers of COX have been primarily identified. In general, cyclooxygenase-1 (COX-1) is constitutively expressed in many tissues and plays a major role in the cell and tissue homeostasis. Cyclooxygenase-2 (COX-2) is a primary response gene, not constitutively expressed in appreciable amounts by normal tissues, but, it is rapidly induced by proimflammatory cytokines, tumour promoters, oncogenes, and growth factors [92, 93]. The gene for COX-2 is approximately 8.3 kb long with 10 exons, and it is transcribed as 4.6, 4.0, and 2.8 kb mRNA variants [94]. The sequence analysis of the 5'-flanking region has shown several potential transcription regulatory elements, including a TATA box, NF-IL-6 motif, two AP-2 sites, three Sp1 sites, two NF-κB sites, a CRE motif and an E-box (Scheme 3) [95].



Scheme 3. Regulatory elements in the human COX-1 and COX-2 promoters.

Although, COX-2 was initially demonstrated in fibroblast cells [90, 92], the expression of the COX-2 gene is associated with several diseases. Overexpression of the COX-2 protein plays an important role in many pathophysiologic states, including inflammation, cancer, angiogenesis, and several forms of arthritis. The elevated COX-2 expression was associated with locally increased PGE_2 production, suggesting that COX-2 is responsible for the overproduction of PGE_2 [96]. The signaling pathways that mediate COX-2 expression are tissue-specific and depend on the stimulus. Therefore, the regulation of COX-2 gene expression can share other signaling pathways involving the NF-κB site, NF-IL6 motif, CRE and E box in the COX-2 promoter [97]. Previously, it has been demonstrated that hypoxia causes increased binding of NF-κB p65 to one out of the two NF-κB consensus elements in the COX-2 promoter, which is closest to the transcription start site of the COX-2 gene [98]. Thus, the intracellular signaling mechanism that leads to induction of COX-2 by hypoxia was found to include binding of p65 to the relatively 3' NF-κB consensus element in the COX-2 upstream promoter region in human vascular endothelial cells [98]. Recently, it was found that the high glucose treatment of human THP-1 monocyte cells increased the recruitment of NF-κB p65, CPB and p/CAF by the COX-2 promoter [99]. These authors showed that diabetic conditions can increase *in vivo* recruitment of NF-κB and HAT, as well as histone acetylation at the promoters of COX-2 gene.

1.3.3 Nuclear factor-κB and its activation

In order to survive, all organisms are able to respond rapidly and efficiently to changes in their environment. To accommodate to the changing micro environment, cells adjust the pattern of gene expression. This regulation occurs primarily at the level of transcription and is controlled by the transcriptional factors, which are translocated from the cytoplasm into the nucleus, and bind to their cognate site to activate or repress transcription. One of these transcription factors is nuclear factor-κB (NF-κB). This transcription factor was first described as a nuclear factor that binds to a specific decameric DNA sequence, GGG ACT TTC C, within the intronic enhancer of the immunoglobulin kappa light chain in mature B-and plasma cells but not in pre B-cells. [100]. For a long time the transcription factor NF-κB has attracted attention because of its unique activation pathway and its physiological importance as a key regulatory molecule of the immune response, cell proliferation and apoptosis. Endogenous activation of NF-κB is a cellular defence mechanism that protects cells by inducing survival genes, such as xIAP and $BCLx_L$ [101, 102].

There is also ample evidence for the independent upregulation of NF-κB to cause apoptosis [103, 104]. NF-κB is dimeric protein composed of various combinations of the five different DNA-binding subunits: NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), cRel, RelB and p65 (RelA), although the most frequently observed form of NF-κB is a p50–p65 heterodimer. All NF-κB family members have a conserved N-terminal Rel-homology domain (RHD), which is responsible for dimerization, DNA binding and interaction with IκBs (inhibitors of NF-κB) [105]. The precursor proteins, p105 and p100 can be processed by the proteasome to generate p50 and p52, respectively. Recently the three-dimensional structures of NF-κB IκB ternary complexes (composed of the RHDs of p50 and p65 and the ankyrin repeat core of IκBα) were solved [106]. It was found that p50 can be produced by an alternative pathway, which involves the cotranslational dimerization of the RHD of p50 with p105. In unstimulated cells, NF-κB is maintained in an inactive form in the cytoplasm by association with IκBs. Physical and chemical stresses, viruses, bacteria and pro-inflammatory cytokines like interleukins (IL) and tumour necrosis factor (TNF) activate NF-κB by inducing the rapid phosphorylation of IκBα at positions Ser32 and Ser36. Phosphorylated IκBα is then ubiquitinated on Lys21 and Lys22 to trigger the rapid degradation of the protein by the 26S proteasome [107]. Released p65 subunit of NF-κB then translocates to the nucleus, binds to its cognate DNA element and activates transcription of numerous target genes. The inducible phosphorylation of IκB is mediated by recently identified IκB kinases (IKKα, β, and ε) [108]. The catalytic subunits, IKKα and IKKβ and the regulatory IKKγ/NEMO (NF-κB essential modulator) subunit, form the prototypic core IB kinase complex (IKC) [109]. Importantly, this complex serves as an intracellular point of convergence for distinct signals that ultimately activate NF-κB. Therefore, activated NF-κB induces the expression of specific genes that encode negative regulators of NF-κB [110, 111]. One of these negative regulators induced by NF-κB is IκBα [112, 113]. Newly synthesized IκBα proteins can shuttle in and out of the nucleus and can physically remove NF-κB from DNA. Subsequent nuclear export of IκBα promotes the return of the new inactive NF-κB- IκBα complex to the cytoplasm and serves to terminate the NF-κB transcriptional response [114, 115].

1.3.4 Chromatin modification

Chromatin modification is an important event for any gene activation. In eukaryotes, DNA is packaged into chromatin that interferes with DNA metabolic processes such as transcription, replication and DNA repair. Two known mechanism that regulate chromatin structure are ATP-dependent chromatin remodeling, and histone replacement [116]. Whereas DNA is modified by cytosine methylation, histones are targets for acetylation (lysine), phosphorylation (serine and threonine), and methylation (lysine and arginine) [117, 118]. More than three decades ago, Allfrey and colleagues [119] found a correlation between increased histone acetylation and increased transcription. Since then, using both genetic and biochemical approaches, several mechanisms by which histone acetylation and deacetylation regulated the gene activity have been elucidated. Histone acetylation is catalysed by histone acetyl transferases (HATs), whereas the reverse reaction is performed by histone deacetylases (HDACs) [120, 121]. There are two main mechanisms that regulate the chromatin modification: a. post-translational modification, which is the chemical modification of a protein after its translation, b. protein-protein interaction [122].

1.3.4.1 Post-translational modification

Compared with methylation and phosphorylation, the acetylation of core histones is probably the best understood type of modification. Histone acetylation occurs at the ε amino groups of evolutionarily conserved lysine residues located at the N-termini. All core histones are acetylated *in vivo*. Modification of histone H3 and H4 are, however, much more extensively characterized than those of H2A and H2B [123, 124]. Important positions for acetylation are Lys^{9} and Lys^{14} on histone H3, and Lys^{5}, Lys^{8}, Lys^{12}, and Lys^{16} on histone H4 [125]. In general, increased levels of histone acetylation (hyperacetylation) are associated with increased transcriptional activity, whereas decreased levels of acetylation (hypoacetylation) are associated with repression of gene expression [124, 126]. Therefore, acetylation is a key component in the regulation of gene expression. On the other hand, histone deacetylases act as transcriptional repressors or silencers of genes, which can catalyse the removal of acetyl groups on amino-terminal lysine residues [123, 124].

1.3.4.2 Protein-protein interactions

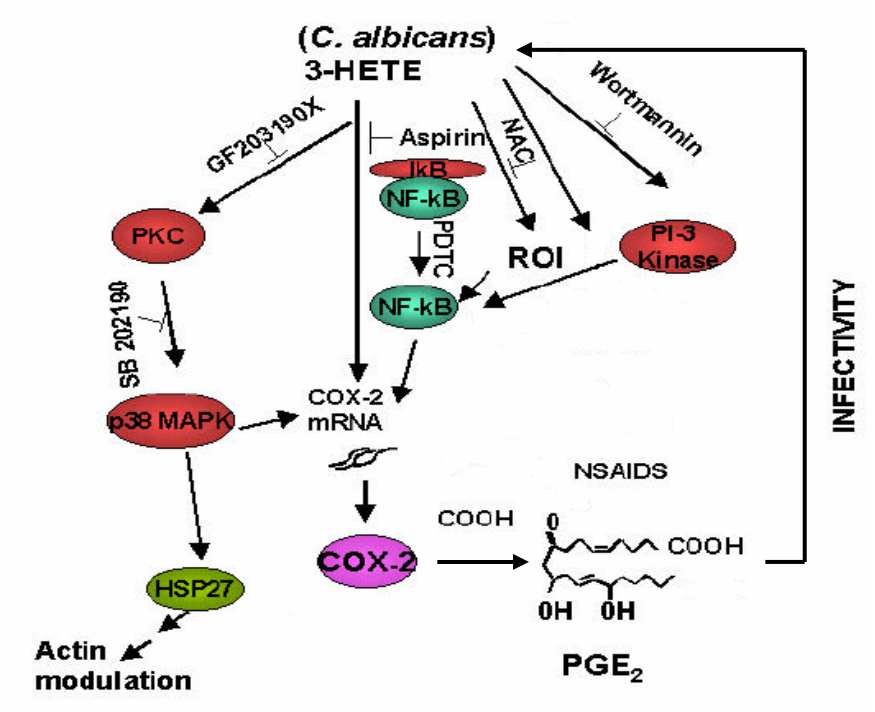
Both HATs and HDACs are usually part of large multimolecular complexes, which contain other components that are required for the enzyme activity. For instance, the activity of CBP (CREB-

binding protein) or the closely related p300 HAT has been shown to be stimulated in cells by a variety of sequence-specific transcription factors such as Sp1, NF-E2 and C/EBP [127]. Through this stimulation, these sequence-specific transcription factors are thought to increase the acetylation of histones or other transcription factors at their target promoters. Thus, binding of transcription factor to HAT can effect gene expression through other transcription factors. The activity of acetylation controlling enzymes can also be modulated through the recruitment to the complex of an enzyme that catalyzes the reverse reaction. Indeed, some complexes containing both HAT and HDAC activities have been characterized [128]. For example, p300 can interact with HDAC-6, and this interaction brings about transcriptional repression [128]. Therefore, it may be proposed that the HDAC enzyme seldom operates alone. Many proteins, transcription factors, with various functions such as recruitment, corepression or chromatin remodeling, are involved in forming a complex that results in the repressor complex.

1.4 Earlier studies regarding host factors, that affect the pathogenicity of *C. albicans*

During infection by *C. albicans*, AA derived from the host cells, can be converted by the fungus to a novel growth factor, 3-hydroxy eicosotetraenoic acid (3-HETE) [31]. This compound was first detected by its immunoreactivity towards an antibody specific to the 3-OH group. This implies that an infection process can be assumed as host-pathogen interaction, in which the host cells release AA, and the pathogen converts it to 3-HETE. This 3-HETE acts as a growth factor for the fungus, and is involved in different biological activities in host cells and so a vicious cycle is turned on. The possible biological role of the oxylipins produced by *Candida* species is unknown. The ability to morphologically switch from yeast cells (blastospores) to filamentous forms (hyphae) is an important virulence factor which contributes to the dissemination of *Candida* in host tissues and which promotes infection and invasion. The filamentous forms of the fungus appear to be more infectious than the yeast forms, however, both morphogenetic forms are apparently necessary for colonisation and invasion by the pathogen under different environmental conditions within the host organism. Approaching both hyphal and yeast forms for the treatment could be the ideal treatment since different forms are involved in spread and progress of infection. Thus, it was found that aspirin, which inhibited formation of 3-hydroxyoxylipins, also inhibited formation of filamentous forms as well as yeast cell growth. Thus, pharmacological intervention by aspirin in the fatty acid metabolism along with classical therapy would be a good approach for the treatment of infection. The exact mechanism of

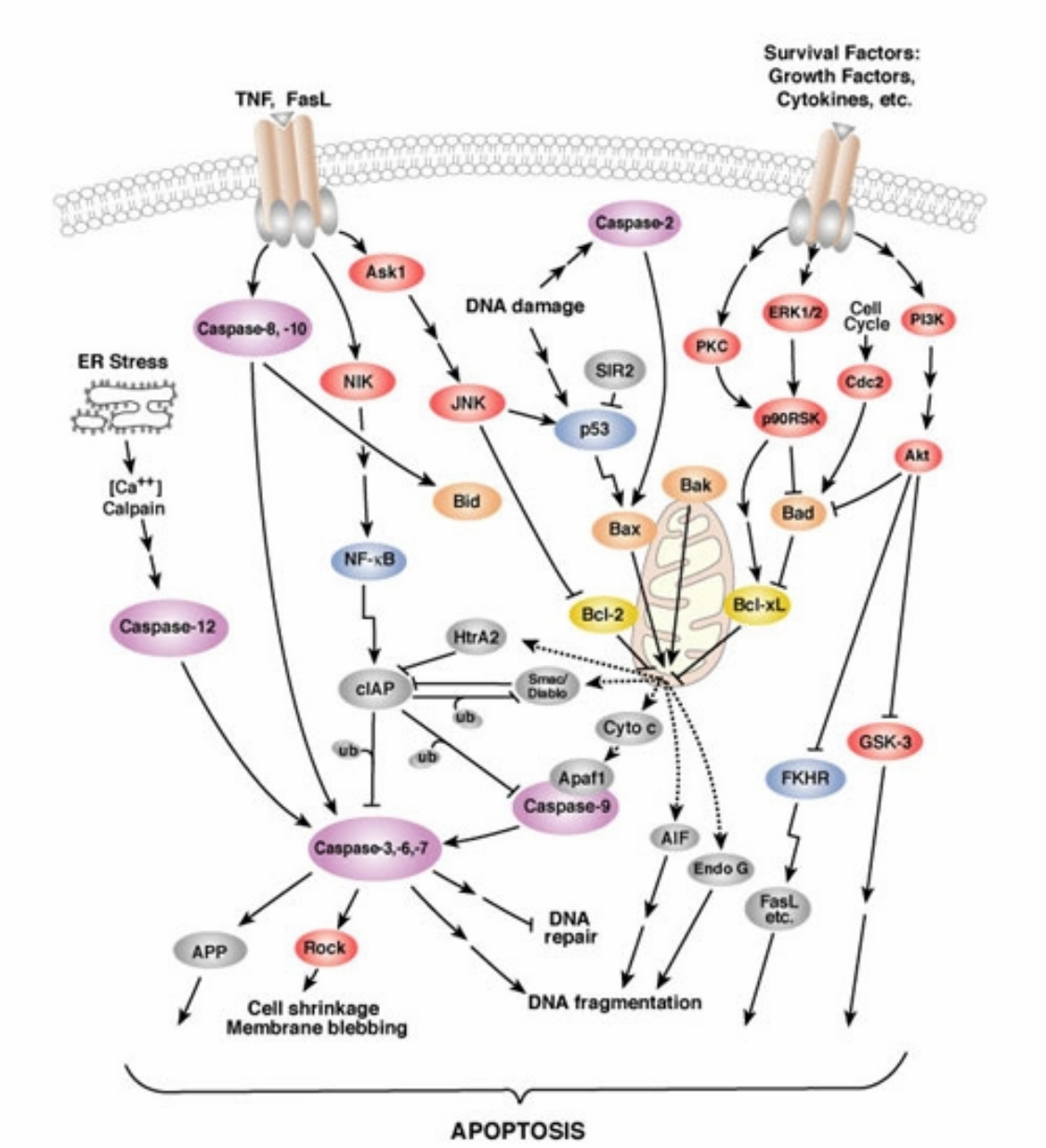
inhibition of 3-hydroxy oxylipins by aspirin is not yet known. It has been suggested that aspirin may inhibit beta-oxidation [29]. During the study of the infection process by *C. albicans* in Dr. Nigam's group Deva *et al*. [96] observed the ability of *C. albicans* to selectively upregulate the COX-2 gene in HeLa cells as well as the production of PGE_2 (Scheme 4). Deva *et al*. also found that the p38 MAP kinase activation by *C. albicans* was inhibited by the PKC inhibitor GF203190X, suggesting that the phosphorylation of $p38^{MAPK}$ is a downstream signal of PKC [96]. However, PD98059, ERK1/2 kinase inhibitor failed to modulate the phosphorylation state of $p38^{MAPK}$ suggesting that these are not involved in this pathway. These results suggest key roles for PKC as well as for p38MAP kinase cascades in the biosynthesis of PGE_2 by upregulating the induction of COX-2 in *C. albicans* infected HeLa cells. Moreover, they also investigated NF-κB activation. Remarkably, high levels of NF-κB binding activity were detected after *C. albicans* infection. The activation of COX-2 and NF-κB were inhibited by SB202190 and GF203190X [96]. This inhibitory effect was, however, significantly lower as compared to inhibitor-mediated repression of COX-2. This discrepancy suggested that PGE_2 production by *C. albicans* is not only dependent on the upregulation of COX-2, but may also involve COX-1 in the cell signaling.



Scheme 4. Signaling mechanism by *C. albicans* and 3-HETE in HeLa cells.

1.5 HOST CELL APOPTOSIS

Apoptosis, or programmed cell death, is considered as a normal physiological process and a major form of cell death that is used to remove damaged or infected cells throughout the life [129]. Apoptosis is also a mechanism by which the organism deals with stress, injury and factors threatening its integrity such as infection. Apoptosis is therefore important in normal cell development, occurring during embryogenesis as well as in the maintenance of tissue homeostasis [129].



Scheme 5. Overview of apoptotic signaling pathways (designed by Digizyme, Cell signaling technology, Beverly, MA 01915, USA).

1.4.1 General mechanisms of apoptosis

Apoptosis is a regulated physiological process characterized by cell shrinkage, membrane blebbing and DNA fragmentation [130]. Caspases, a family of cysteine proteases, are central elements of apoptosis. Initiator caspases (including 8, 9, 10 and 12) are closely coupled to pro-apoptotic signals. Once activated, these caspases cleave and activate downstream effecter caspases (including 3, 6, and 7), which in turn cleave cytoskeletal and nuclear proteins and induce apoptosis [131]. Two pathways initiate the signaling cascade that results in apoptosis as shown in scheme 5**:**

a. ***Receptor-mediated apoptosis*****:** One major pathway for the induction of apoptosis is the receptor-mediated or extrinsic pathway. External signals such as FasL (produced by cytotoxic T cells on their surface) and TNFα can activate pathway specific receptors of the TNF-R family known as death receptors (TNF-R1, Fas or CD95, TRAIL R1 and R2) which contain a cytosolic death domain (DD) [132, 133]. The receptor oligomerization results in the recruitment of specific adapter proteins such as FADD, TRADD, RIP and DAXX via their DDs [134, 135]. FADD in addition contains a death effector domain (DED), to which the DED of pro-caspase-8 can bind. This complex is designated as DISC (death- inducing signaling complex). The formation of DISC leads to the recruitment and cleavage of pro-caspase-8 and 10 to their active forms. Caspase-8 and 10 function as initiator caspases leading to the activation of effecter caspases.

b. ***Intrinsic pathways*****:** This pathway is triggered in response to DNA damage, oxidative stress, chemotherapeutic agents and other types of stress [136]. It involves the Bcl-2 family of proteins which consists of pro- and anti-apoptotic members [137]. The anti-apoptotic members such as Bcl-2 and Bcl-X_L exist in the mitochondria [138, 139]. Upon receiving the stress signal, the proapoptotic members (bax, bid, bak, PUMA) translocate to the mitochondria and neutralize the anti-apoptotic members by oligomerisation [140]. This results in the permeabilisation of the membrane, resulting in the release of apoptogenic factors such as SMAC/Diablo, cytochrome c, SIMPs and AIF. Cytochrome c binds to Apaf-1 to form the apoptosome resulting in the cleavage of caspase-9 and progression of the cascade *via* the effecter caspases [141, 142].

2. AIMS AND OBJECTIVES

C. albicans is an important pathogen for those afflicted with AIDS and is also of great concern for those undergoing immunosuppressive therapy for cancer or organ transplants. The ability of *C. albicans* to establish a persistent infection is critically dependent on cellular signals that regulate the release of factors from target cells responsible for replication of pathogen. The inflamed host tissue in vulvovaginal candidiasis has been shown to release huge amount of AA, which can be taken up by *C. albicans* and transformed to 3-OH oxylipins. These compounds can have effects on one side related to morphogenesis of the attaching pathogen and on the other side to immunomodulary activation in host muscle cells. During host cell signaling, significant release of PGE_2 was observed. Because COX-2 is an inducible enzyme, it was highly activated by *C. albicans* infection. Although it was known that COX-2 activation was mediated via NF-κB dependent mechanism, the exact nature of the influence these factors in the pathogenesis of inflammatory fungal diseases are not clear. HeLa cells were chosen because in a previous study we found enhancement of PGE_2 synthesis in HeLa cells upon infection with *C. albicans*. In addition, this model HeLa cell/*C. albicans* closely represents the VVC. Therefore, this study was performed to clarify the host cells signaling by fungal infection at the cellular and molecular level.

Aims and objectives of this study are as follows**:**

I. To define the regulatory network for COX-2 gene expression by *C. albicans* infection in the cervical carcinoma cell line, HeLa. Experiments were aimed at elucidating the signal transduction pathways involved. We focussed on understanding the changes occurring in the chromatin structure, especially histone modifications and the interaction between the various transcription factors and co-activators required for the induction of COX-2 mRNA synthesis.

II. To determine the role of PLA_2 enzymes in the host-pathogen-interaction process. We investigated whether the host cell infection requires *Candidal* PLA_2 or host cell PLA_2 or both. We aimed to show the activity and ability of candidial PLB gene (PLA_2 gene analogue in mammalian cells) in the host cells.

III. To investigate the nature of the host-pathogen-interaction process with respect to host cell apoptosis during fungal infection. The critical role of $iPLA_2$ and TLR2 were examined during the host cells apoptosis.

3. MATERIALS AND METHODS

3.1 MATERIALS

Culture reagents

Fetal bovine serum (FBS), Dulbecco's modified eagles medium (DMEM), Trypsin/EDTA, Streptomycin/penicillin – Seromed (Germany); Yeast nitrogen base, Yeast extract, Tryptone, Peptone, Agar– DIFCO Co (USA).

Separation media

Agarose, Silica gel-60, Rotiphorese acrylamide Gel 30 - ROTH (Germany); TLC plates - Merck (Germany).

Kits

RNeasy mini kit and QIAshredder - Qiagen (Germany); Protein BCA kit – PIERCE (USA); Chemiluminiscent system - Santa Cruz (Germany); Luciferase reporter assay – Promega (Germany); Polyfect transfection kit- Qiagen (Germany); Cell death detection ELISA – Roche (Germany); PGE_2 ELISA kit - Cayman (USA) Chromatin immunoprecipitation assay kit - Upstate (USA).

Inhibitors

Forskolin, Trichostatin A and Pyrrolidine Dithiocarbamate (PDTC) – Sigma (Germany); BEL, MAFP and AACOOCF3 - Cayman (USA); H89 and SB203580 - Calbiochem (Germany); NS398 – Cayman (USA); Wortmannin - Biomol (Germany); Propranololhydrochlorid (PAP-1) - AstraZeneca GmbH (Germany).

Antibodies

Actin – Sigma (Germany); histone H3 and H4 and acetyl histone H3 or H4 – Upstate (Germany); TLR2 - eBioscience (UK); HDAC-1, MyD88, COX-2, p65, cRel, $iPLA_2$ (T-14), $cPLA_2$, Protein A agarose, anti-mouse, anti-rabbit, and anti-goat secondary antibodies

conjugated with horse radish peroxidase – Santa Cruz (Germany); $iPLA_2$ antisera- Cyaman (USA); PKCα-Transduction Laboratories, Inc (USA).

RT-PCR reagents

Expand reverse transcriptase – Roche Biochemicals (Germany); Taq DNA polymerase - Invitrogen (Germany); Pfu turbo polymerase – Stratagene (USA); Platinum Taq polymerase – Invitrogen (Germany); TaqMan assays - Applied Biosystems (Germany).

Caspase substrate and inhibitor

Caspase-3 substrate Ac-DEVD-pNA and inhibitor Z-VAD-FMK – Calbiochem (Germany).

Enzymes

Proteinase K- Roche Biochemicals (Germany); RNase A was from Fermentas (USA).

Radiochemicals

^{14}C-Arachidonic acid (55 mCi/mmol); 1-palmitoyl-2-[1-^{14}C]-palmitoyl-*sn*-glycero-3-phosphorylcholine) and α^{32} P- dCTP was from NEN (Germany).

Primers

All primers were purchased from TIB Biomol (Germany).

3.2 MEDIA AND BUFFERS

LB medium

10 g/L Tryptone

5 g/L Yeast extract

5 g/L NaCl

The pH was adjusted to 7.2 and autoclaved.

Universal medium for yeast: (dl)

Yeast extract	0.3
Malt extract	0.3
Peptone	0.5
Glucose	1.0
Agar	1.5

pH 5.6

YPD medium: (dl)

Peptone	2.0
Yeast extract	1.0
Glucose	4.0

pH 7.4

3.3 METHODS

3.3.1 Mammalian cell culture

HeLa (DSMZ, Braunschweig, Germany) were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 50 U/ml penicillin, and 50 µg/ml streptomycin and incubated at 37 °C with 5 % CO_2. Before starting experimental procedure cells were incubated in DMEM medium containing maximum 1 % FBS for over night. For microscopic examination the cells were grown on cover slips to 60-70 % confluence.

3.3.2 Basal culture conditions for *C. albicans*

C. albicans was suspended in YPD containing 2 % peptone and grown in a rotary shaker at 150 rpm for 12 h at 37 ^{o}C. The cells were separated from the medium by centrifugation at 3000 × g for 5 min, washed three times with phosphate-buffered saline (PBS) and finally resuspended in 1 ml fresh medium of proper composition for the respective experiments.

3.3.3 Infection of HeLa cells with *C. albicans*

C. albicans 1386 was grown at 37 ^{o}C for 12 h. Cells were centrifuged at 3000 x g and washed three times with PBS and suspended in DMEM containing 1 % FBS. HeLa cells (2×10^6) were taken as a monolayer in 25 mm^2 in DMEM medium containing 1 % FBS and infected by above prepared *Candida* cells. For the inhibitor studies, a stock solution was diluted in the culture medium and preincubated with HeLa cells for one hour with each inhibitor at appropriate concentrations.

3.4 Phospholipase A_2 assay

Pure substrates, 1-Palmitoyl-2-arachidonoyl phosphatidyl choline (PAPC) and PAPC-OH were prepared as described previously by Chaitidis *et al*. (1998). Each substrate at a concentration of 25 µM, was dissolved in the assay buffer containing 10 mM Tris-HCl, pH 8, 180 mM NaCl, and 5 mM $CaCl_2$ by sonication for five minute. *C. albicans* cells were broken down mechanically using protein lysis buffer containing aluminium or glass beads with sonication on ice, and centrifuged at 5000 x g for 10 min. to remove the cell debris. 50 µg protein of *Candida albicans* lysate was added to the assay buffer and was incubated for one hour, the pH was adjusted to 3.5

and extracted using ethyl acetate. Products were analysed by reverse-phase HPLC using solvent system, methanol: acetonitrile: water 90:6:4 with 20 mM choline chloride. The column used was a Nucleosil 1005 C8 column (250 x 4.6 mm, Macherary and Nagel, Germany). The detection was performed with a diode array detector SPD-M10A (Shimadzu, Japan) at wavelengths 210 and 235 nm.

3.5 iPLA$_2$ enzyme activity assay

iPLA$_2$ activity was assayed using established methods [143]. Briefly, cell homogenates were prepared in 10 mM HEPES (pH 7.4), 0.34 M sucrose, 1 mM EDTA, 1 mM DTT, 10 µg/ml leupeptin, 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (assay homogenization buffer) by sonication on ice. Each assay contained 400 µM Triton X-100, 100 µM dipalmitoylphosphatidylcholine (DPPC) (containing 400,000 cpm 1-palmitoyl-2-[1-^{14}C] palmitoyl-*sn*-glycero-3- phosphorylcholine), 100 mM Hepes, pH 7.5, 5 mM EDTA, and 0.1 mM ATP in a final volume of 500 µl. Assays were started by adding cell homogenates (250 µg) and incubating at 40 ^{o}C for 45 min with agitation. The released [^{14}C] fatty acid was extracted according to the method of Dole [144] and quantified by reading radioactivity on a scintillation counter. Control reactions lacking enzyme were routinely carried out and subtracted from the reported activities.

3.6 Release of Arachidonic acid by *Candida albicans*

HeLa cells were grown in 75 cm^2 flask and were labelled with 1 µCi [1-^{14}C] AA for 18 h. Cells were washed three times with PBS, and lysed with RIPA buffer on ice for 30 min as whole cell lysate. *C. albicans* cells were washed with PBS and lysed using a lysis buffer containing 1.2 M sorbitol, 10 mg/ml lyticase and aluminium. To the HeLa cell lysate, 0.04 mg of *C. albicans* protein was added and incubated for 1 h. After incubation Bligh and Dyer extraction of lipids was performed. The Lipids were separated by TLC using the solvent diethyl ether: n-hexane: acetic acid 50:50:1 and analysed by radio TLC (automatic TLC linear analyser, Berthold, Berlin, Germany) for the liberated labeled arachidonic acid.

3.7 Determination of PGE$_2$

HeLa cells were grown as a confluent monolayer in 6-well plate and pretreated with inhibitors. Infection by *C.albicans* was performed as described above. The reaction was stopped by keeping

the culture on ice. The plate was centrifuged at 2000 x g for 10 min and the supernatant was stored at –80 °C until analysis for PGE_2 by ELISA (Cayman, Ann Arbor, MI). ELISA analysis was performed in triplicate.

3.8 Transient transfection of cell lines

Transient transfection of DNA plasmids and their mutants were performed using polyfect reagent as described earlier [96]. 5 x 10^5 cells were seeded into 6-well plates. The experiment was performed after at least 24 hours of culture. 1.5 µg of plasmid DNA was mixed with 12 µl of polyfect reagent in 100 µl antibiotic and serum free medium and DNA-liposome complexes were allowed to form at room temperature for 15 minutes. The complexes were gently seeded onto the cells and incubated at normal conditions for 24 h. The cells were used for further experiments at the end of this period. For the cotransfection study the ratio of DNA was 9:1. The cells were cotransfected with 0.1 µg of control plasmid pRSVLACZ to normalise for transfection efficiency. Transfection efficiency was determined by performing β-galactosidase assay.

3.8.1 Plasmids

Plasmids for FADD-DN vector was a kind gift from Dr. Schmitz, M.L (Cancer Research Center, Heidelberg, Germany). IKK-DN plasmid was kindly provided by Dr. Ghosh, S (Yale University, New Haven, USA). TLR2 - DN was kindly provided by Dr. Kirschning, C.J (Immunologie und Hygiene Technical University of Münich, München, Germany) and $iPLA_2ß$ was kindly provided by Dr. Ma, A.Z (Mount Sinai School of Medicine, New York, USA). *C. albicans* wild type and mutants were kind gift from Dr. Agabian, N (University of California, S. Francisco, USA), COX-2 plasmid and promoter constructs were kindly provided by Dr. Prescott, S (Univiversity of Utah, Salt Lake city, USA), E1A and mutant oncoprotein were provided by Dr. Hecht, A (University of Freiburg, Germany), Expression vectors for HDAC-1 and Gam-1 and their mutants were generously provided by Dr. Seiser, C (University Institutes at the Vienna Biocenter, Vienna, Austria) and Dr. Chiocca, S (European Institute of Oncology, Milan, Italy), respectively. Other plasmids were constructed in our laboratory by cloning required fragments. All the fragments and the cloning procedure were confirmed by DNA sequencing (SeqLab, Germany).

3.9 Western blotting

3.9.1 Preparation of total protein from cells

Cells were washed 3 times with ice cold PBS and lysed directly on the plates or flasks after addition of RIPA protein lysis buffer [50 mM TRIS-HCI pH 7.4, 150 mM NaCI, 1 % nonidet P-40 (NP- 40), 0.5 % sodium deoxycholate, 0.1 % SDS, 1 mM EDTA, 1 mM PMSF, 10 µM sodium orthovanadate 10 mM, 20 µg/ml, leupeptin, 10 µg/ml pepstatin A, 20 µg/ml of aprotinin]. The cells were scraped and lysed by sonication for 20 seconds on ice using UW70 type dounce homogenizer and kept on ice for 20 min and centrifuged at 10.000 x g for 12 min. The protein concentration was determined using BCA protein assay kit (PIERCE).

3.9.2 SDS-Polyacrylamide gel electrophoresis and immunoblotting

10-12 % SDS Polyacrylamide gel was prepared according to the following recipe: for separating gel of 10 ml volume: 4 - 3.3 ml of distilled water, 2.5 ml of 1.5 M Tris-HCl (pH 8.8), 3.3 – 4.0 ml of 30 % Polyacrylamide mix (37.5 : 1 ratio of mono and bis acrylamide), 100 µl of 10 % SDS, 100 µl 10 % ammonium persulphate (APS) and 4 µl N, N, N', N' tetramethylethylenediamine (TEMED). For 5 ml of stacking gel: 3.4 ml of distilled water, 630 µl of 1 M Tris-HCl (pH 6.8), 830 µl 30 % polyacrylamide mix, 50 µl 10% SDS, 50 µl 10 % APS and 5 µl TEMED. The gel was cast in MiniProtean III apparatus (BioRad, Germany). The protein samples were mixed with SDS sample solution (Rotiload, Roth, Germany) and boiled for 5 minutes for complete denaturation. This mixture was loaded onto the gel and electrophoresed. Immunoblotting was performed utilising the semi-dry transfer method. Nitrocellulose membrane (Schleich and Schuell, Germany) and 6 layers of Whatman 3 paper were cut exactly to the size of the gel and soaked in transfer buffer along with the gel. A stack was made with 3 sheets on the top and bottom of the gel and membrane and placed in between the graphite plates of the transfer apparatus (Biometra, Germany). Transfer was performed at 0.8 mA/cm^2 current for 1 hour. The efficiency of the transfer was confirmed by staining the membrane with a 0.1 % solution (containing 1 % acetic acid) of Ponceau S (Sigma, Germany). The excess stain was removed with water. After visualisation of the protein, the blot was completely washed off the stain with water and put into blocking solution (5 % nonfat milk powder in PBS containing 0.05 % tween 20) for 1 h at room temperature or over night at 4 ^{o}C. The blots were then incubated in the blocking solution containing 1: 500 –1:1000 dilution of the primary antibody for 1 h at RT. After washing

with wash buffer (PBS containing 0.05 % tween 20) for 10 minutes, the blot was incubated with the secondary antibody (against the species of the primary antibody and linked to horse radish peroxidase) (1:2000 dilution) for 1 h at RT. After 3 washes of 5 minutes each with wash buffer, the specific bands were developed using Chemiluminiscent system (Santa Cruz, Germany). Equal volumes of solution A and B were mixed and the blot was incubated in this solution for 1 minute. The blot was immediately exposed to an X-Ray film in dark for varying periods of time ranging from 5 sec to 2 min and developed automatically. The specific bands were scanned and quantified densitometrically using TINA version 2.09 g. All immunoblots were sequentially incubated with anti-β-actin as control, and specific signals adjusted in relation to the expression of this house-keeping gene.

3.9.3 Nuclear extract preparation

Cells were washed with ice cold PBS, scraped and pelleted at 10.000 x g, 4 ^{o}C. The pellet was resuspended in 400 µl of hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0,5 mM DTT, 1 mM PMSF) and incubated on ice for 15 minutes. Simultaneously, cells were lysed by addition of 100 µl of 2.5 % NP40. Nuclei were pelleted for 1 minute at 2000 x g at 4 ^{o}C. The nuclear pellet was resuspended in 50 µl extraction buffer (20 mM HEPES, pH 7.9, 0.48 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25 % glycerol) and incubated for 30 minutes on ice. Nuclei were pelleted by centrifugation for 10 min at 12000 x g. For long term storage, the supernatant was mixed with 50 µl of freeze buffer (20 mM HEPES, pH 7.9, 0.1 mM KCl, 0.2 mM EDTA, 20 % glycerol) and stored at –70 ^{o}C

3.10 Immunoprecipitation

Immunoprecipitaions were performed by incubating the protein extracts (500 µg) or 500-1000 µl whole cell lysate [which should be precleared with protein A/G agarose (in 1 ml cell lysate + 100 µl A/G agarose slurry 50 % beads) for 30 min at 4 ^{o}C], with 2 – 4 µg of the primary antibody for 2 hours or overnight at 4 ^{o}C. The immune complexes were captured with protein-A agarose (Santa Cruz, Germany) for 1-2 hours at 4 ^{o}C. The beads were spun down (2500 x g, 2 min, 4 ^{o}C), washed 3 times with RIPA buffer (1 % NP-40, 0.5 % deoxycholate, and 0.1 % SDS in PBS) and the immune complex released by boiling in SDS sample solution. The complex was then electrophoresed. The proteins were eluted from the beads with 2 x SDS sample buffer and western blot analysis was performed.

3.11 Chromatin immunoprecipitaion

Formaldehyde was added to the cells at a final concentration of 1 % and incubated for 30 minutes at 37 ^{o}C. The reaction was stopped by the addition of glycine to a final concentration of 0.125 M. The cells were washed with cold PBS containing protease inhibitors and harvested. The nuclear extract was prepared according to Dignam *et al*. [145] and sonicated on ice at maximal power for 30 seconds twice to shear the genomic DNA. Immunoprecipitations were performed with various antibodies. Crosslinking was reversed in the immunoprecipitated complexes by the addition of NaCl to a final concentration of 200 mM and incubation at 65 ^{o}C for 6 hours. The DNA was purified by proteinase K treatment (150 µg/ml) for 1 h followed by phenol/chloroform extraction chloroform washing and precipitation by ethanol. The presence of specific promoters was detected by PCR with specific primers. The extract aliquoted prior to the immunoprecipitaions was used to prepare control input genomic DNA, which was also used for PCR analysis.

3.12 RNA preparation

Total RNA was prepared using RNAsy kit, from Qiagen, Germany. Cells were washed 3 times with PBS and lysed in 350 µl RNA lysis buffer and sonicated at maximum wattage for 30 seconds. The lysate was mixed with equal volumes of 70 % ethanol and loaded onto the column. The column was spun at maximum speed for 15 seconds at RT. The column was then washed with RW1 and RPE buffer and RNA was eluted in 30-50 µl RNase free water by centrifugation and stored at –80 ^{o}C.

3.12.1 Reverse transription and polymaerase chain reaction (RT-PCR)

Reverse transcription was performed using 2-4 µg of RNA, which was denatured at 65 ^{o}C for 10 minutes. 50 pmoles of random hexamer primer and 1 µl of AMV reverse transciptase (Roche, Germany) was added to reaction buffer (1.5 mM $MgCl_2$, 1.0 mM dNTPs, 10 mM DTT). The reaction was performed at 42 ^{o}C for 1 h. 1/20th of the resultant cDNA was used in the subsequent PCR reaction. PCR was performed with 5 pmoles of forward and reverse primer, 200 µM of each dNTP and 1 U of Taq polymerase. GAPDH primers were used to perform control reactions to quantitate the product formed. Negative and positive controls were used with each set of PCR reaction. The primers and reaction conditions are listed below:

Homo sapiens phospholipase A_2, group VI (cytosolic, calcium-independent) gene:

5‘AATACCTTCAGTGGCGTCAC 3‘

5‘CTGATGATACGGCTGTGATG 3‘

94 ^{o}C 3 min; 94 ^{o}C 1 min, 54 ^{o}C 1 min, 72 ^{o}C 1 min for 30 cycles; 72 ^{o}C for 7 min; 4 ^{o}C.

Human phospholipase A_2, group IVA (cytosolic, calcium-dependent) gene:

5‘CCAAAGTGACAAAGGGGGCC3‘

5‘GCTACCACAGGCACATCACG3’

94 ^{o}C 3 min; 94 ^{o}C 1 min, 59 ^{o}C 1 min, 72 ^{o}C 1 min for 25 cycles; 72 ^{o}C for 7 min; 4 ^{o}C.

Human cyclooxygenase-2 gene:

5‘GTCACAAGATGGCAAAATGCTG3‘

5‘TAAGATAACACTGCAGTGGCTC3‘

94 ^{o}C 3 min; 94 ^{o}C 1 min, 52 ^{o}C 1 min, 72 ^{o}C 1 min for 30 cycles; 72 ^{o}C for 7 min; 4 ^{o}C.

Human cyclooxygenase-2 promoter:

5’ CAAGGCGATCAGTCCAGAAC3’

5’ GGTAGGCTTTGCTGTCTGAG3’

94 ^{o}C 3 min; 94 ^{o}C 1 min, 55 ^{o}C 1 min, 72 ^{o}C 1 min for 35 cycles; 72 ^{o}C for 10 min; 4 ^{o}C.

Human Toll-Like receptor-2 gene:

5' GCCAAAGTCTTGATTGATTGG3'

5' TTGAAGTTCTCCAGCTCCTG3'

94 ^{o}C 2 min; 95 ^{o}C 40 s, 54 ^{o}C 40 s, 72 ^{o}C 1 min for 24 cycles; 72 ^{o}C for 7 min; 4 ^{o}C.

Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene:

5' TCCATGACAACTTTGGTATCGTGG3'

5' GACGCCTGCTTCACCACCTTCT3'

95 ^{o}C 2 min; 94 ^{o}C 30 s, 62^{o} C 30s, 72 ^{o}C 30 s for 40 cycles; 72 ^{o}C for 10 min; 4 ^{o}C.

The PCR mixture was mixed with DNA loading buffer and electrophoresed in 2 % agarose gel in 1 x TBE at 7 volts/cm of gel. DNA was visualised under UV after staining the gel with 0.5 μg/ml ethidium bromide.

3.12.2 Real-time quantitative PCR

Relative levels of mRNA were quantified with real-time, quantitative RT-PCR using fluorescent TaqMan technology. The TaqMan assay ID for $iPLA_2$ (Hs 00185926), for COX-2 (Hs 00153133) or for TLR2 (Hs 00152932) from Applied Biosystems were used . Equal amounts of total RNA

were reverse transcribed with the TaqMan Reverse Transcription Reagents (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions using random hexamers. All samples that were compared were reverse transcribed from the same reverse transcription master mix in order to minimize differences in reverse transcription efficiency. Negative control reactions omitting the reverse transcriptase were also performed. The fluorogenic hybridization probes (i.e., TaqMan probes) were labelled with the quencher dye TAMRA attached to the 3′ end and the reporter dye FAM (6-carboxyfluorescein) covalently linked to the 5′ end of the oligonucleotide (Applied Biosystems). 18s RNA was used as an endogenous control to normalize the amount of sample RNA (TaqMan Ribosomal RNA Control Reagents, Applied Biosystems). The PCR was performed in the GeneAmp 5700 sequence detection system (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. Each reaction (total volume 25 µl) for the quantification of $iPLA_2$ contained 50 ng RNA equivalents as well as 400 nM forward primer, 400 nM reverse primer and 200 nM TaqMan probe. For the quantification of 18S RNA, 25 ng RNA equivalents, 100 nM forward primer, 100 nM reverse primer, and 50 nM TaqMan probe were used. The reactions were performed in MicroAmp 96-well plates capped with MicroAmp optical caps (Applied Biosystems). The reactions were incubated at 50 °C for 2 min, at 95 °C for 10 min followed by 45 cycles of 15 s at 95 °C and 1 min at 62 °C. Each sample was measured in triplicate. The comparative Ct method was used for relative quantification. The amount of target, normalized to 18S RNA and relative to a calibrator, is given by $2^{-\Delta\Delta Ct}$, where Ct is the cycle number at which the fluorescence signal of the product crosses an arbitrary threshold set within the exponential phase of the PCR, and $\Delta\Delta Ct = (Ct_{\text{target (unknown sample)}} - Ct_{\text{18S (unknown sample}}) - (Ct_{\text{target (calibrator sample)}} - Ct_{\text{18s (calibrator sample)}})$ the ΔΔCt calculation for the relative quantification could be used without running standard curves [146].

3.13 Reporter assay

The cells were washed twice with ice-cold PBS and lysed with 200 µl luciferase lysis buffer (Promega, Germany). The lysis was completed by freezing and thawing. After centrifugation at 12000 x g for 2 min at 4 ^{o}C the supernatant was assayed for luciferase and β-galactosidase activity according to manufactures instruction.

3.13.1 Luciferase assay

The luciferase assay was performed by vortexing 5 µl of the extract with 50 µl of luciferase assay solution (Promega, Germany). The bioluminescence was measured by scintillation counting.

3.13.2 β-galactosidase assay

For quantification of β-galactosidase activity, 50 µl of the above prepared extract was incubated at 37 ^{o}C for 10 min, then 100 µl of pre-warmed 4 mg/ml o-nitrophenyl para-D-galactopyranoside (ONPG) in 0.1 M sodium phosphate buffer (pH 7.5) was added. After a suitable (2-3 h) reaction time, 300 µl of 1 M Na_2CO_3 was added, and the absorbance of the solution was measured at 420 nm with a spectrophotometer. The β-galactosidase activity was expressed as ONPG unit (1 unit: the activity producing 1 mmol o-nitrophenol per minute normalised against the inoculated cell number). The β-galactosidase activity was determined as the average value from triplicate dishes under the same transfection conditions.

3.14 ASSAYS FOR APOPTOSIS

3.14.1 Nuclear staining

HeLa cells were grown on coverslips and infected with *C. albicans* as described before. After washing by PBS, the cells were fixed by 4 % paraformadehyde for 15 min at room temperature and washed again with PBS. The solution containing DAPI (1:1000 dilution in PBS) was added and incubated for 2 min at RT. After washing the cells with PBS and water, the nuclei were observed under fluorescence microscope at 350 nm.

3.14.2 Cell Death Detection ELISA

The Cell Death Detection ELISA kit (Roche Biochemicals, Germany) was used to quantify the apoptosis in cells. 4 x 10^5 cells were plated on 6-well plates in DMEM medium. The plates were first centrifuged for 10 min at 2000 x g and after removing supernatant the cells were lysed for 30 min by a hypotonic solution added directly onto the 6-well plate. The cytoplasmic extract was incubated with anti-histone and anti-DNA antibodies. The complexes bound to the ELISA plate were quantified using horse radish peroxidase enzyme and DTNB reagent. Effectors or inhibitors

of apoptosis were added, and apoptotic cell death was determined after incubation for various time periods.

3.14.3 DNA ladder fragmentation assay

HeLa cells (1.5 x 10^7) in 10 ml medium were incubated with *C. albicans* at a MOI of 5. After incubation the cells were lysed by addition of 500 µl hypotonic lysis buffer containing 10 mM Tris, 1mM EDTA, 0.25 % NP-40 (pH 7.4) for 20 min on ice. The cell debris was removed by centrifugation at 12.000 x g for 15 min. The supernatent was treated with RNAse (100 µg/ml) at 50 ^oC for 1 h, followed by the treatment with proteinase K (100 µg/ml) at 37 ^oC for 1 h. DNA was extracted using phenol-chloroform-isoamyl alcohol (50:49:1) three times followed by a chloroform wash. DNA was precipitated with 100 % ethanol containing 1/10 % 3 M sodium acetate and separated by electrophoresis in 2 % agarose gel. In a parallel experiment 1 µM staurosporin was added to HeLa cells for 12 h as a reference positive control.

3.14.4 Caspase-3 assay

The Caspase-3 assay was performed using Ac-DEVD-pNA as substrate. Cells were lysed with lysis buffer (50 mM HEPES, pH7.4, 100 mM NaCl, 0.1 % CHAPS, 1 mM DTT, 100 µM EDTA, 1 mM PMSF, 20 µg/ml leupeptin, 20 µg/ml aprotinin) and centrifuged at 10.000 x g for 10 min. 100 µg of protein were incubated with 200 µM solution of Ac-DEVD-pNA (Calbiochem, Germany) in a reaction buffer (lysis buffer with 10 mM DTT and 10 % glycerol) at 37 ^oC. The development of yellow colour at 405 nm indicated caspase-3 activity. The reaction was monitored periodically for 1-3 hours. The rate of reaction was calculated as the difference in the absorbance at 405 nm per unit time. The results were represented as fold increase in caspase-3 activity over the control reactions. Z-VAD-FMK (Calbiochem, Germany) was used as inhibitor of caspase-3 at the concentrations of 100 µM an hour prior to standard treatment.

3.16 Statistics

All experiments were repeated at least three times and only the representative pictures are shown. The data are presented as mean ± SD of after three separate experiments. Statistical comparisons between groups were made using Student's test for paired observations. Significance was achieved at $p<0.05$ level. All calculations were performed using www.graphpad.com.

4. RESULTS

4.1 Activation of COX-2 gene

4.1.1 *C. albicans* induces transcriptional activation of COX-2 in HeLa cells.

In a recent report from Dr. Nigam's laboratory Deva *et al*. [96] showed that *C. albicans* can selectively inducing COX-2 mRNA and protein expression. However, they did not find any change in the COX-1 expression. Interestingly, these authors reported enhancement of mRNA expression upto 24 hours, whereas the protein levels reached maximum at 6 hours. This discrepancy between COX-2 protein and COX-2 mRNA expressions was attributed to posttranscriptional regulation of the COX-2 gene, in which RNA-binding proteins were presumably bound to the 3'-untranslated region of the COX-2 mRNA to abrogate its translation. Since it is known that semiquantative RT-PCR in certain cases may not reflect the true mRNA expression, we verified the observation of Deva *et al*. [96] with quantitative real-time PCR. Upon infection of HeLa cells with *C. albicans* at MOI 5 for various periods of time, we found a gradual time dependent decrease in COX-2 mRNA expression (Fig. 1), which is consistent with several reports cited in the literature.

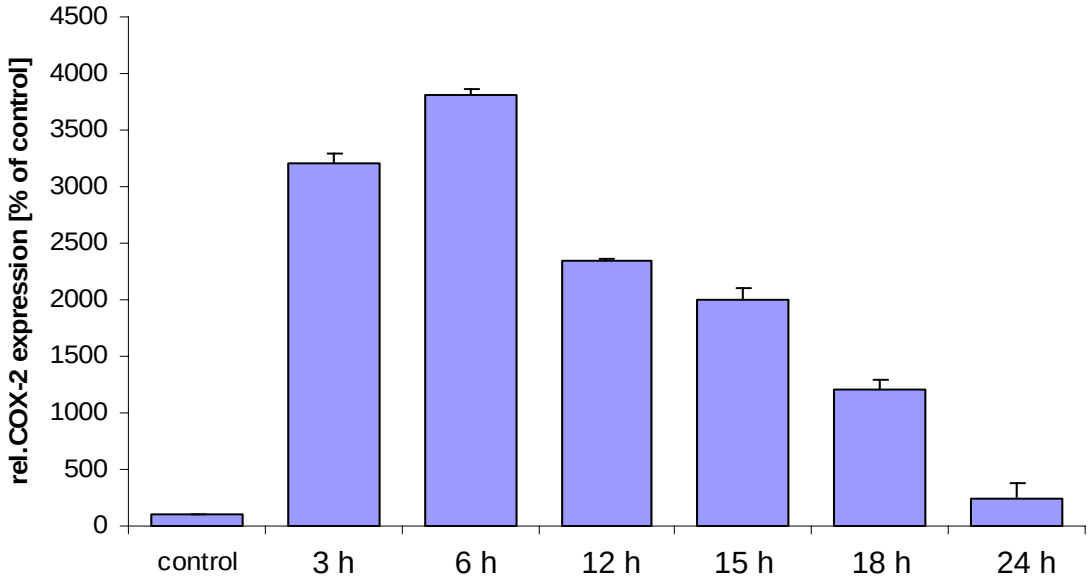


Figure 1. Relative level of COX-2 mRNA expression induced by *C. albicans*. HeLa cells were infected with *C. albicans* for different time periods and total RNA was extracted. 2 µg of RNA was reverse transcribed and quantified using real-time PCR the fluorescent TaqMan technology. 18S RNA was used as an endogenous control to normalize the amount of RNA. For further experimental details, see materials and methods. Values represent the mean ± SEM of 3 separate experiments.

Following infection the COX-2 mRNA reached maximum at 6 hours, which was almost 32 fold of the basal level. As can be seen in Figure 1 the COX-2 mRNA declined in dependence of time reaching a steady level at 24 hours (2.5 fold) of the control. This clarifies the divergence between PCR -based reports on COX-2 mRNA and protein expression by Deva *et al*. [96].

4.1.2 Is COX-2 activation mediated by PGE_2?

Deva *et al*. [96] reported earlier that initially synthesized PGE_2 by *Candida*-infected HeLa cells may exert an autocrine affect and function as trigger for further induction of COX-2. To investigate this we pre-and coincubated HeLa cells with 100 nM PGE_2 prior to infection with *C. albicans.* No additional upregulation of the COX-2 gene was observed by PCR (Fig. 2). To investigate if PGE_2 receptors on activation by PGE_2 participate in transactivation of EGF receptors, we also coincubated HeLa cells with 100 ng/ml of EGF and measured the upregulation of COX-2 by PCR.

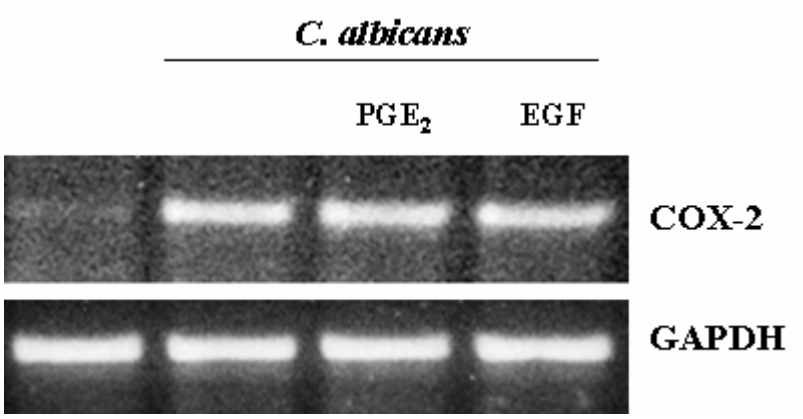


Figure 2. The expression of COX-2 was not influenced by PGE_2 and EGF. Cell were exposed to PGE_2 (100 nM) or EGF (100 ng/ml) along with infection by *C. albicans* for 6 hours. Total RNA was extracted and 2 μg RNA was assayed by RT-PCR for COX-2 mRNA detection.

In our hands, we were unable to detect any additional induction of COX-2 gene by cross talk between PGE_2 and EGF receptors.

4.1.3 Activation of COX-2 is dependent from cAMP expression.

COX-2 gene promoter is known to contain cAMP responsive elements, NF-κB and NF-IL-6 [95, 97]. Therefore, we investigated whether increasing cAMP levels would enhance the COX-2 expression. HeLa cells were preincubated with forskolin, a direct activator of adenylyl cyclase, before challenging with *C. albicans*. Cells were harvested after 6 hours, and total proteins were extracted and immunoblotting was performed. As Figure 3 shows, forskolin has slightly increased COX-2 expression when compared with unstimulated cells. When cells were pretreated with PKA inhibitor H89 (10 μM) and forskolin, *C. albicans* did not increase the COX-2 expression, which is indicative of important role of cAMP-mediated activation of COX-2 gene.

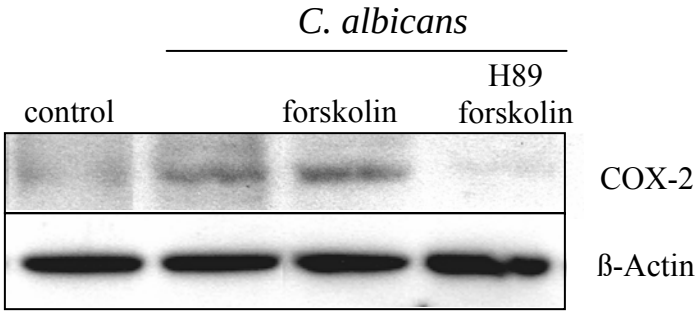


Figure 3. The COX-2 activation is dependent on the activation of cAMP. Cells were exposed to forskolin (20 μM) alone or together with H89 (10 μM) along with infection *C. albicans* for 6 hours. COX-2 expression was assayed by western analysis as described in material and methods.

4.2 NF-κB activity in HeLa cells infected by *C. albicans*.

Inasmuch as PDTC, a free radical scavenger, has been shown to suppress the activation of COX-2 gene, it was implicated that NF-κB is involved in the upregulation of COX-2 gene [96]. This role of NF-κB in the activation of COX-2 gene was confirmed by transfection of pNF-κB Luc plasmid and mutants of IKK and IκBα [96]. After challenging transfected cells with *C. albicans* for 6 hours significant inhibition of NF-κB and COX-2 promoter activities were observed. To dissect and verify these data we performed western analysis specifically of the p65 subunit of NF-κB and cRel. After stimulation with *C. albicans*, IκBα is cleaved and phosphorylated by IKK complex and then degraded by the 26S proteasome [106, 107]. The p65 subunit of NF-κB translocates to the nucleus, where it docks to DNA binding sites within the

promoter region of the target genes. Upon infection of HeLa cells by *C. albicans* (MOI 5) p65 NF-κB rapidly increased in about 3 hours and its level was almost maintained till 12 hours, then markedly decreased to below the basal level at 18 hours. Moreover, the cRel subunit of NF-κB, which is responsible for the growth of the cell and whose deficiency in various cell types may lead to apoptosis [103, 104], increased significantly after 3 hours and remained stable till 18 hours. These data suggest the stimulatory role of NF-κB in the activation of COX-2 gene. Since cRel has been shown to be stimulated by catalytic subunit of PKA (PKA-Cβ), possibly through the interaction with p300/CBP, we can assume a vital role of cRel in cell proliferation and COX-2 activation in *Candida*-infected cells.

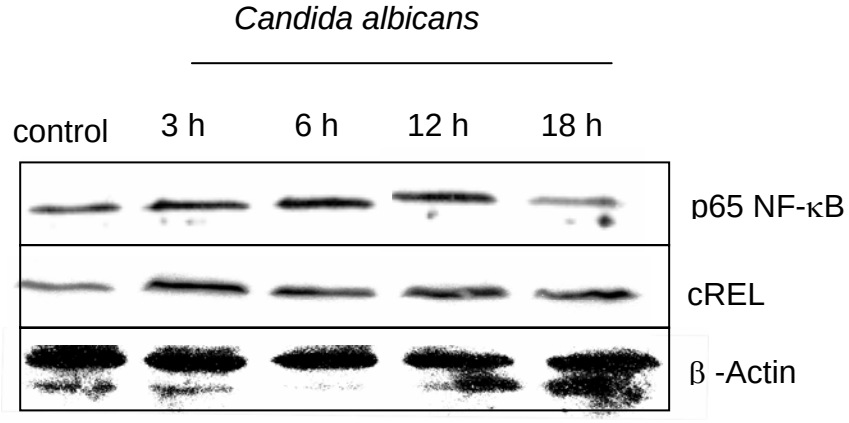


Figure 4. Time course analyse of NF-κB. HeLa cells were infected with *C. albicans* for indicated periods of time. Whole cell lysate was prepared and 50 μg proteins were used for western blotting. Immunoblotting was performed as described in materials and methods.

4.3 Suppression of HDACs upregulate the COX-2 and NF-κB expression in *Candida*-infected HeLa cells.

Regulation of nuclear histone acetylation is emerging as a crucial mechanism for controlling gene expression. However, the degree of histone acetylation depends on the activity of histone deacetylases. That means, acetylation of histones is controlled by HAT and HDAC activity. In stimulated cells thus exists an equilibrium state of acetylation and deacetylation processes. Any disturbance by inhibitor or coactivator of either process may shift the equilibrium in either direction. To investigate if histone acetylation induced by coactivator p300/CBP, which displays

strong HAT activity, is important for *C. albicans* induced COX-2 expression, we performed experiment with viral oncoprotein E1A, which has been identified as an endogenous inhibitor of p300/CBP, and its mutant E1AmCBP. Figure 5 shows that acetylation of histone by p300/CBP was completely inhibited by the E1A. However, cells transfected with E1AmCBP did not restore completely the COX-2 promoter activity.

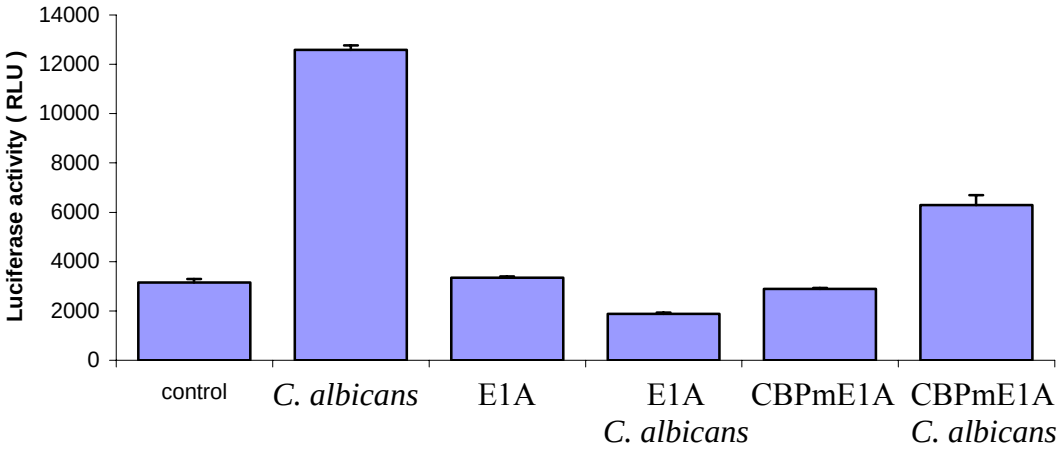


Figure 5. Involvement of HAT on COX-2 promoter activity. HeLa cells were transfected with E1A and COX-2 or CBPmE1A and COX-2 promoters as described before. After 24 hours of transfection cells were infection by *C. albicans* for 6 hours. Cell lysate were analysed for luciferase activity of COX-2 promoter, and values reported as mean RLU ± SD (n=3)

But, suppressors of HDAC activity showed likewise that they act synergistically with *C. albicans* or may even induce COX-2 expression without fungal infection. To verify this, HeLa cells were transfected with COX-2 promoter or NF-κB Luc plasmid ligated to luciferase vector, and treated with HDAC inhibitor trichostatin A (TSA), a specific inhibitor of multiple HDACs, and luciferase activity for both NF-κB and COX-2 promoters was measured after infection with *C. albicans*.

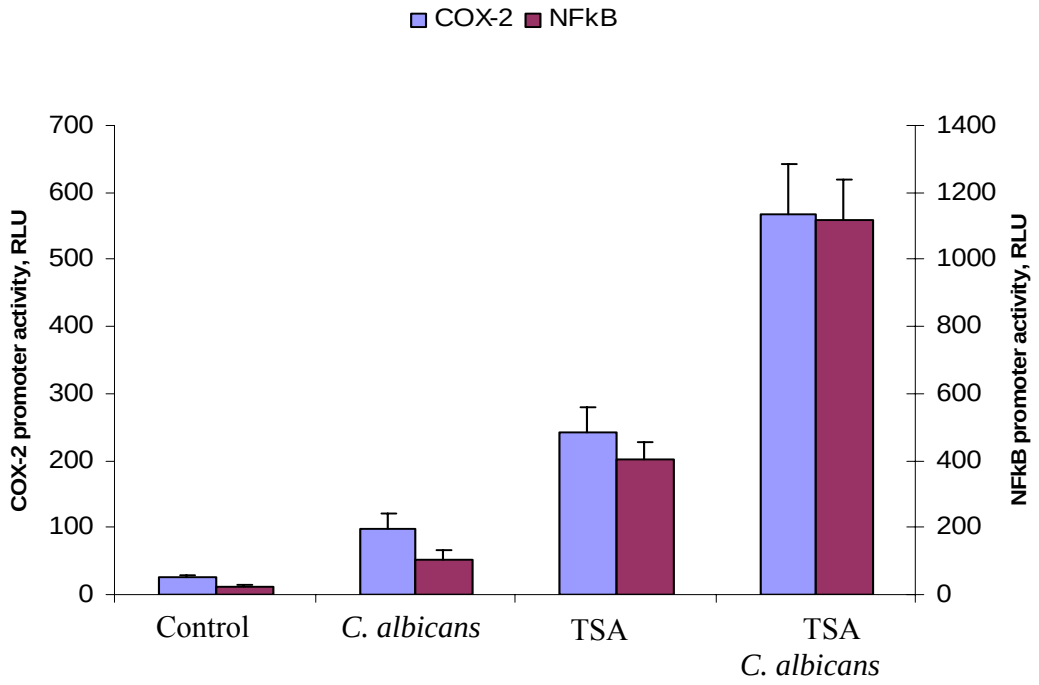


Figure 6. Inhibition of HDAC activity caused an increase of COX-2 and NF-κB promoter activity. HeLa cells were transfected with COX-2 and NF-κB promoters as described before. After 24 hours of transfection cells were treated with TSA (200 nM) for 18 hours before infection by *C. albicans* for 6 hours. Cell lysate were analysed for luciferase activity of COX-2 und NF-κB promoters, and values reported as mean RLU ± SD (n=3)

Figure 6 shows that TSA alone induced both promoter activities, almost 10-fold for COX-2 and 20-fold for NF-κB over the basal value. The stimulation of promoter activities in *Candida*-infected cells was found to be almost 2.5 fold for COX-2 and about 4-fold for NF-κB. Moreover, inhibition of HDAC by TSA in *Candida*-infected cells increased COX-2 5.5-fold and NF-κB more than 10-fold. These findings suggest that repression of the inducible enzyme COX-2 in unstimulated cells maybe due to the dominance of deacetylation process over the acetylation process.

4.3.1 Specific role of HDAC in the activation of NF-κB promoter by *C. albicans* is dependent on the upregulation of IKK activity.

In Figure 6 we showed that TSA stimulated COX-2 activity in infected cells was increased by 5.5- fold but NF-κB activity was increased by 10-fold. This discrepancy led us to investigate if IKK activation in unstimulated and stimulated cells is caused by TSA. Therefore, we cotransfected HeLa cells with NF-κB and IKK dominant negative plasmid and measured NF-κB promoter activity in cells preincubated with TSA before challenging with *C. albicans*. Indeed, *Candida*-stimulated NF-κB expression was completely abolished in IKK-DN-transfected cells (Fig. 7). Moreover, TSA treatment did not cause the gene upregulation in these cells. This finding clearly pinpoints the specific role of IKK in relation to HDAC.

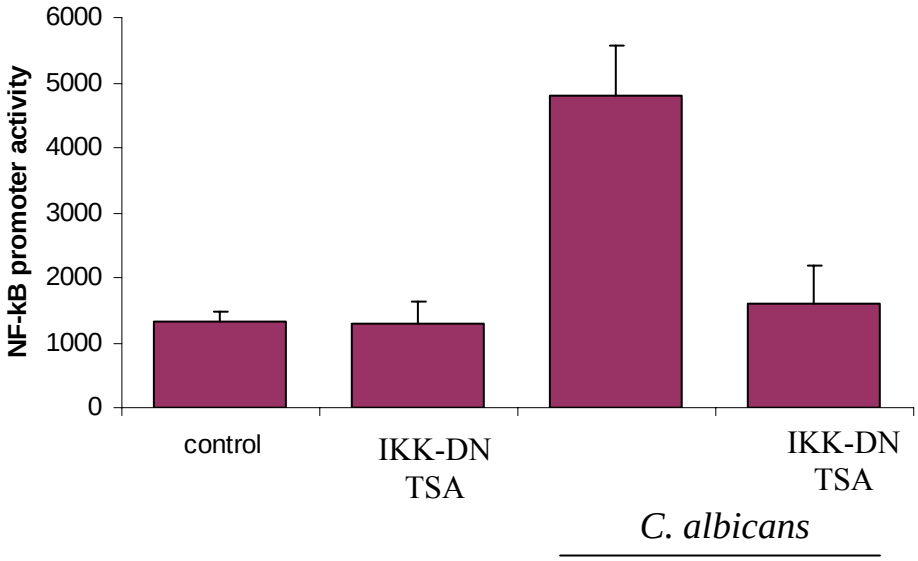


Figure 7. Inhibition of IKK activity causes a decrease of NF-κB activity. HeLa cells were cotransfected with IKK-DN and NF-κB promoters as described before. After 24 hours of transfection cells were treated with TSA (200 nM) for 18 hours before infection by *C. albicans* for 6 hours. Cell lysate were analysed for luciferase activity of NF-κB promoter, and values reported as mean RLU ± SD (n=3)

4.3.2 Gam-1 inhibits COX-2 and NF-κB activity by HDAC-1 in infected cells.

Gam-1 is an avian CELO adenovirus early gene product, which does not show any homology to known proteins, and has been reported to be an important inhibitor of HDAC [147]. Gam-1 has been shown to stimulate several target genes by inactivating HDAC-1 [147]. To assess Gam-1

mediated transcriptional upregulation of COX-2 and NF-κB genes, we transfected HeLa cells with Gam-1 or Gam-1 mutant plasmids and challenged with *C. albicans*.

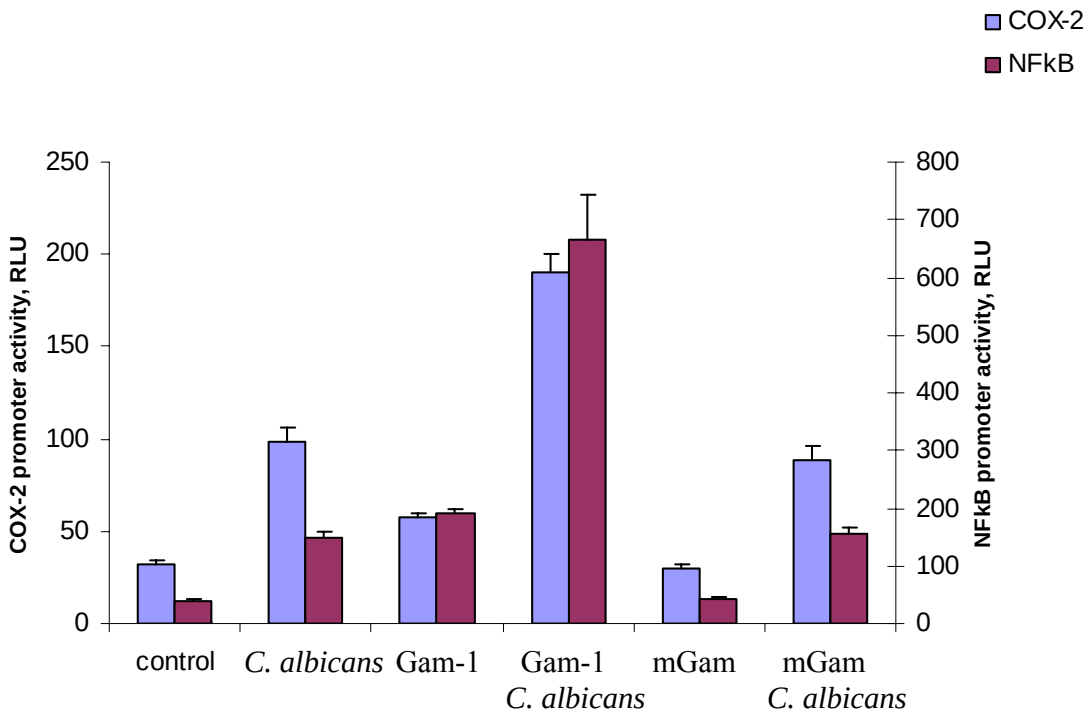


Figure 8. Inhibition of HDAC-1 activity causes an increase of COX-2 and NF-κB activity. HeLa cells were cotransfected with COX-2 and Gam-1 or NF-κB and Gam-1 and after 24 hours of transfection cells were infected by *C. albicans* for 6 hours at MOI 5. Cell lysate were analysed for luciferase activity of COX-2 und NF-κB promoters, and values reported as mean RLU ± SD (n=3)

Figure 8 shows a significant elevation of Gam-1-mediated expression of COX-2 and NF-κB. COX-2 promoter activity was increased by 2-fold over the infected cells, whereas NF-κB promoter activity was augmented 4-fold (Fig. 8). Although both HDAC inhibitors, TSA and Gam-1, enhanced HAT-mediated COX-2 and NF-κB promoter activities, TSA was found to be more potent transcriptional activator than Gam-1. When both inhibitors Gam-1 and TSA were incubated together, we did not observe any additional transcription by Gam-1, which suggests an identical mechanism of action for Gam-1. Furthermore, to check whether Gam-1 directly interacts with HDAC-1, we transfected Gam-1 mutant protein, which has been reported to express reduced binding to HDAC-1, into HeLa cells before infection with *C. albicans*. Whereas wild type Gam-1 strongly stimulated COX-2 and NF-κB promoter activities Gam-1 mutant

protein was almost ineffective. These data are consistent with previous report of Chiocca *et al*. [147] and suggested that Gam-1 directly binds to HDAC-1.

4.3.3 Overexpression of HDAC-1 suppressed NF-κB mediated COX-2 activation.

As discussed in 4.3.1- 4.3.3 we can predict regulatory role of HDAC-1 inhibition in the *C. albicans*-mediated transcriptional activation of NF-κB and COX-2. Thus, overexpression of HDAC-1 in cells should suppress the NF-κB and COX-2 expression. To verify this, we transiently transfected HDAC-1 plasmid as well as COX-2 promoter into HeLa cells before challenging with *C. albicans*. Empty vectors for HDAC-1 and COX-2 were transfected into cells to give the controls. This treatment of cells yielded a 4.5-fold induction of the COX-2 promoter activity in the cells transfected with only COX-2 promoter alone. However, the cells overexpressing HDAC-1 reduced the *C. albicans*-mediated induction of the COX-2 gene by more than 60 %. Strikingly, the COX-2 promoter activity in HDAC-1 overexpressed cells was still two-fold over the basal level. This implicates that the regulation of the COX-2 is supported by factors other than HDAC-1.

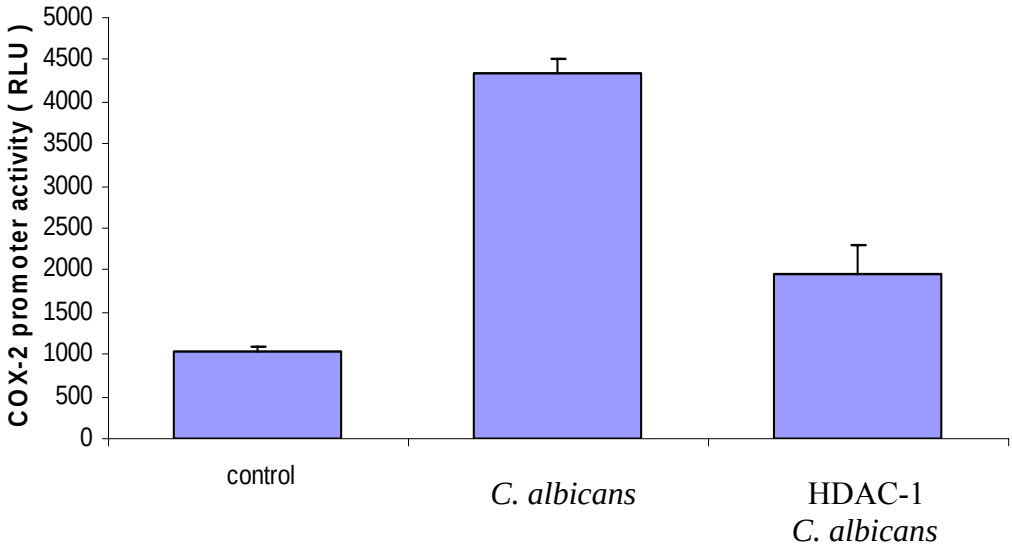


Figure 9. Overexpression of HDAC-1 inhibits C. *albicans* indused COX-2 activation. HeLa cells were transiently coransfected the HDAC-1 reporter gene and COX-2 promoter. After 24 hours of transfection HeLa cells were challenged with *C. albicans* for 6 hours. Cell lysate were analysed for luciferase activity of COX-2 promoter. Values represent the mean ± SEM of 3 separate experiments.

4.3.4 Inhibition of HDAC in HeLa cells infected with *C. albicans* caused increase of hyperacetylation of histone H3.

Intracellular balancing of histone acetylation and deacetylation during gene expression and cellular differentiation is crucial event for histone modification [148]. Our previous results showed that inhibition of HDACs caused stimulation of NF-κB-mediated COX-2 expression. It is known that increased histone acetylation increased the transcription of genes.

Therefore, we focussed to identify histone modification, such as acetylation in our system. HeLa cells were treated with TSA (200 nM) for 18 hours and followed by infection with *C. albicans* for 6 hours. Nuclear proteins were extracted as described in materials and methods and were examined by western blot analysis using specific antibodies against acetyl-H3 and acetyl-H4.

As expected, we found that C. *albicans*-infection enhanced the histone H3 acetylation in HeLa cells. Moreover, TSA treatment also resulted a dramatic increase of acetylation of histone H3 as compared with untreated cells. When we checked for acetyl-H4 protein, we did not find any expression of this protein, indicating that no acetylation of histone H4 protein occurred in HeLa cells upon infection with *C. albicans*.

Figure 10. Treatment by TSA caused a dramatic increase in histone H3 acetylation. HeLa cells were infected by *C. albicans* with or without preincubation with TSA**.** Acetyl-H3 was detected by western blotting in nuclear extracts (see materials and methods).

4.3.5 Recruitment of p65 subunit of NF-κB by HDAC-1 in HeLa cells infected by *C. albicans.*

In order to determine if NF-κB p65 and HDAC-1 undergo physical binding in *Candida*-infected HeLa cells we transfected expression plasmids for NF-κB p65 and HDAC-1 into HeLa cells upon infection by C. *albicans* at MOI 5, and coimmunoprecipitations were performed. As shown in Figure 11A, NF-κB p65 was immunoprecipitated with HDAC-1 in cells cotransfected with HDAC-1 and NF-κB p65. From the results, we observed that the p65 subunit of NF-κB was recruited by HDAC-1 upon infection by *C. albicans*. This interaction was dependent if both NF-κB p65 and HDAC-1 were present, because HDAC-1 was not immunoprecipitated with p65 in control cells or in cells transfected with NF-κB p65 or HDAC-1 alone. As a control, an immunoprecipitation and western blotting for HDAC-1 (Fig. 11A, *right panel*) or NF-κB p65 (Fig. 11B, *right panel*) from cells transfected with HDAC-1 or NF-κB p65 were performed. Figure 11B shows that HDAC-1 was coimmunoprecipitated with NF-κB p65 in cells transfected with p65 subunit of NF-κB and HDAC-1. The previous observation from the result A was confirmed by this coimmunoprecipitation experiment. Thus, HDAC-1 and NF-κB p65 can interact mutually with each other upon infection for 6 hours by *C. albicans* in HeLa cells.

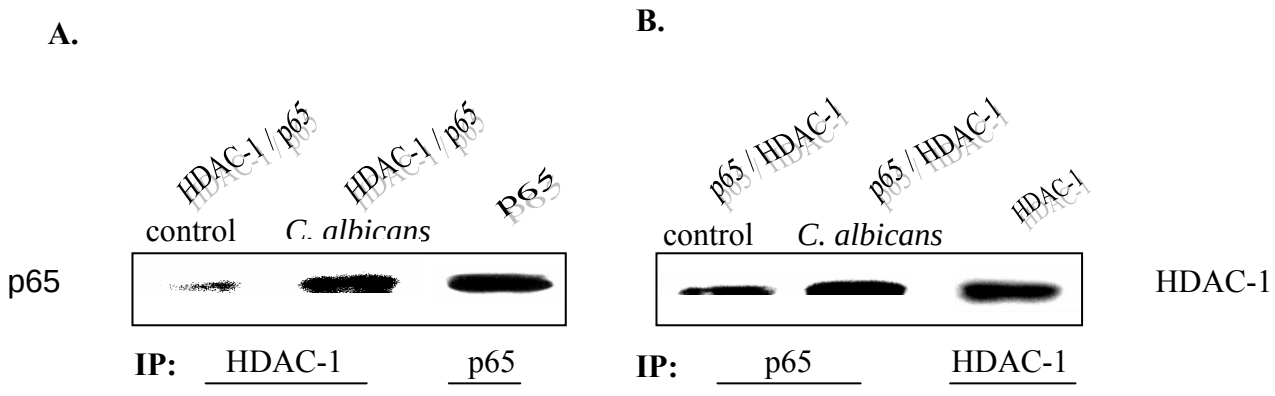


Figure 11. Mutual interaction of HDAC-1 and NF-κB p65 upon infection by *C. albicans.* Initial steps for coimmunoprecipitation experiment with HeLa cells were transfection of NF-κB p65 and HDAC-1 plasmids into cells after 48 hours of transfection, cells were infected with *C. albicans* for 6 hours. Cells were lysed and protein concentration was determined. Total 300 µg proteins were used for immunoprecipitation as described in materials and methods.

4.3.6 Binding study of p65 subunit of NF-κB and HDAC-1 to COX-2 promoter in HeLa cells infected by *C. albicans*.

From Figure 11, it was concluded that HDAC-1 and NF-κB p65 can interact with each other in *Candida*-infected cells. It was reported already that COX-2 activation is mediated by NF-κB upregulation. Therefore, the next experiments were aimed at addressing the question of whether or not of these factors can bind to COX-2 promoter *in vivo*. For this purpose we used chromatin immunoprecipitation (ChIP) assay, a powerful technique to determine true *in vivo* finding of transcription factors and other nucleosomal proteins to chromatin in response to an agonist [149, 150]. After the ChIP protocol, gene for COX-2 promoter region was amplified by semiquantitative PCR using specific primers. Figure 12 showed the time-course of *C. albicans* infection effect and it indicates that p65 subunit of NF-κB is recruited to the COX-2 promoter upon 6 hours of infection by *C. albicans.* One interesting result observed is that transcriptional repressor protein HDAC-1 is associated with the COX-2 promoter, and this association was higher in infected cells than in non-infected cells.

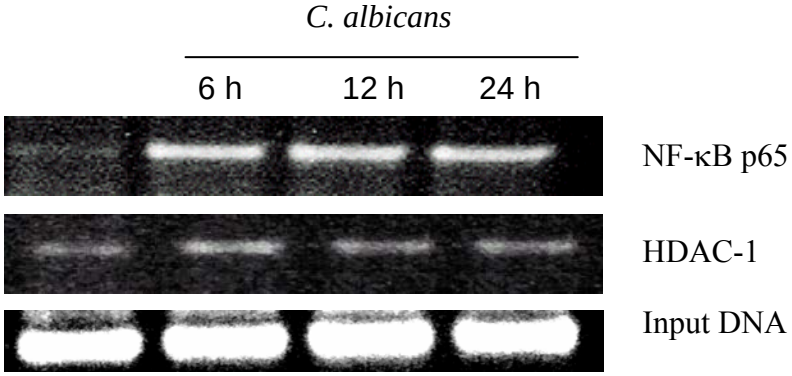


Figure 12. Time course analyse of *C. albicans* mediated binding of p65 NF-κB and HDAC-1 to COX-2 promoter. HeLa cells were cultured with or without infection *C. albicans* for various time periods. The protein-nucleic acid complexes were immunoprecipitated with anti-p65, or anti-HDAC-1 antibodies. The cross-linked DNA was purified and analysed by PCR for the presence of COX-2 promoter DNA. An aliquot of the complexes was removed before the immunoprecipitations and was similarly processed and used as a control for the PCR reaction**.** This DNA was referred to as input chromatin.

4.4 Regulation of PLA_2 enzyme activity in *C. albicans*-infected HeLa cells.

4.4.1 Activation of COX-2 gene was dependent on candidial PLA_2 gene

Microbial pathogens use a number of genetic strategies to invade the host and cause infection. The secretion of enzymes, such as phospholipase A, has been proposed as one of the virulence factors which is used by pathogenic fungi [53]. It was demonstrated that the phospholipase B gene is essential for candidial virulence [53]. In this study, we investigated if *C. albicans*, in which the PLA_2 gene was knocked out, can induce the activation of COX-2 gene in host cells. We used in our study the *Candida albicans* knock-out strains: ATCC 44-808 (PLB3/ PLB3), KH 44-13 heterozygous mutant (plb3/ PLB3), KH44-91 homozygous mutant (plb3/plb3), and KH44-KL complementary PLB3-Δplb3/plb3, kindly provided by Dr. Nina Agabian (UCSF, California, USA). HeLa cells were infected with the mutant *C. albicans* cells at MOI 5 for 6 hours. Total RNA was extracted and RT-PCR was performed to see the mRNA level of COX-2 gene.

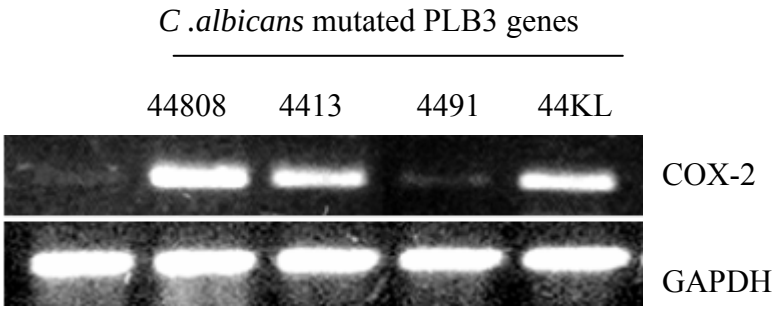


Figure 13. Involvement of candidial PLA_2 gene in the induction of COX-2 gene expression. HeLa cells were cultured and challenged with different *C. albicans* strains as mentioned above. COX-2 mRNA was determined with specific primers by semi-quantitative PCR.

From the results, we observed that the heterozygous mutant (KH4413) significantly induced the COX-2 mRNA. But this induced COX-2 mRNA level was lower than the wild type (44808) *C. albicans*. In contrast, the homozygous mutant (KH4491) failed to induce any COX-2 gene expression, whereas the complementary strain KH 44-KL regained PLA_2 activity to induce the COX-2 activity to heterozygous level.

4.5 Host cell PLA_2 activity during infection by *C. albicans*

It is known, that the first event in the production of prostaglandins is the release of arachidonic acid from membrane phospholipids by PLA_2 [50, 51]. Therefore, it was important to study PLA_2 expression in host cells. $cPLA_2$ and $iPLA_2$ were studied by determination of mRNA levels in HeLa cells infected by *C. albicans* for various time periods (Figs. 14A and B). Total RNA was extracted from HeLa cells and mRNA was analysed by semiquantitative and real time RT-PCR.

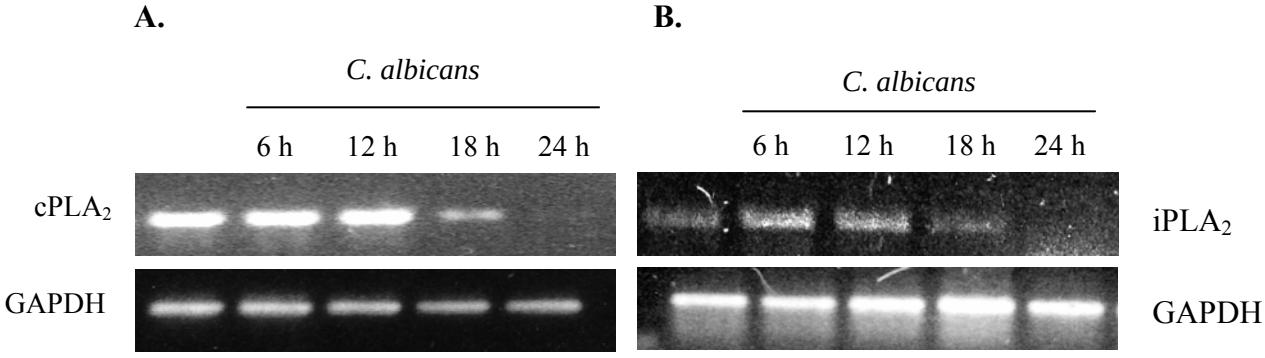


Figure 14A and B. The expression of $cPLA_2$ and $iPLA_2$ mRNA were decreased by *C. albicans* infection. HeLa cells were infected with *C. albicans* for indicated periods, cells were harvested and total RNA was extracted. Semi quantitative RT-PCR was performed to detect the **A**. $cPLA_2$ and **B**. $iPLA_2$. GAPDH RT-PCR was used to normalize $cPLA_2$ expression.

Strikingly, as shown in Figure 14B $iPLA_2$ mRNA level increased after 6 hours of infection by *C. albicans*, while the $cPLA_2$ expression remained constant. However, $cPLA_2$ and $iPLA_2$ expression in HeLa cells decreased markedly after 18 hours of infection by *C. albcans* and disappeared after 24 hours. This $iPLA_2$ mRNA decrease could be verified by real-time quantitative PCR as shown in Figure 14C.

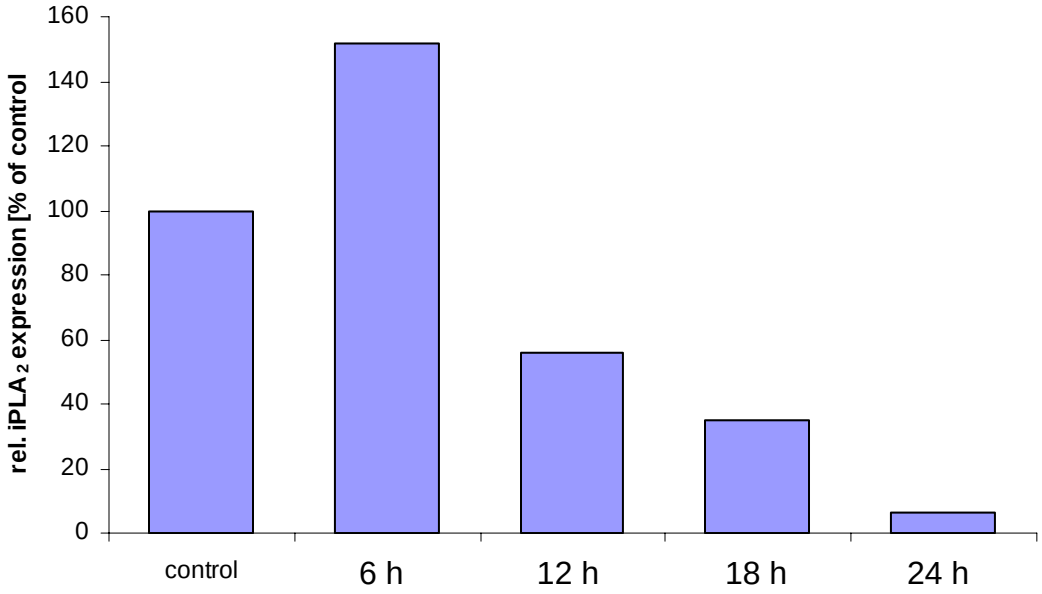


Figure 14C. The expression of $iPLA_2$ mRNA was decreased by *C. albicans* infection. HeLa cells were infected with *C. albicans* for indicated periods, cells were harvested and total RNA was extracted. Real-time RT-PCR was performed to detect the $iPLA_2$ as described in materials and methods. 18S RT-PCR was used to normalize $iPLA_2$ expression. Values represent the mean of 2 separate experiments.

4.5.1 PGE_2 production from host cells infected by *C. albicans* was dependent on $iPLA_2$ activity.

Earlier reports from Dr. Nigam's group demonstrated that the enhancement of COX-2 protein synthesis coincided with increased PGE_2 formation [96]. Therefore, the very first question to be answered was to find the relation of PLA_2 on PGE_2 production. PGE_2 production was measured in supernatants of HeLa cells-*C. albicans* incubation mixture in the presence or absence of MAFP (inhibitor of both $cPLA_2$ and $iPLA_2$) and BEL, a widely used selective inhibitor of $iPLA_2$. The PGE_2 production was completely inhibited by treatment with BEL and MAFP, exhibiting the important role of the PLA_2, especially $iPLA_2$, in the signaling of PGE_2 production (Fig. 15).

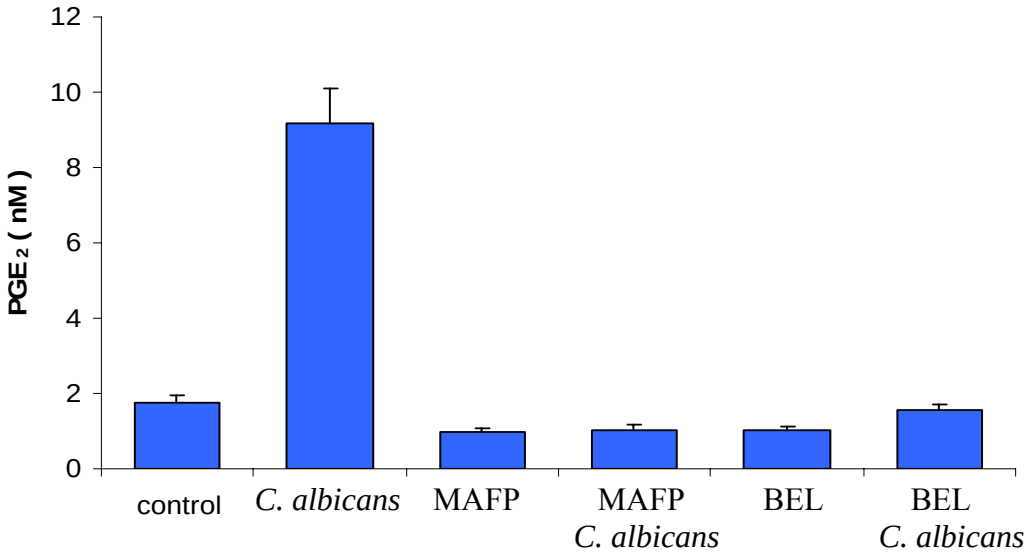


Figure 15. Inhibition of PLA_2 blocked the PGE_2 production in HeLa cells infected by *C. albicans*. HeLa cells were treated with selective $iPLA_2$ inhibitor BEL (20 μM) or $cPLA_2$ and $iPLA_2$ inhibitor MAFP (25 μM) for 1 hour before challenging with *C. albicans* for 6 hours at MOI 5. The supernatant was collected, and the PGE_2 production was determined by ELISA as described in material and methods. Values represent the mean ± SEM of 3 separate experiments.

4.5.2 Inhibition of $iPLA_2$ causes reduced activity of PKCα.

Protein kinase C (PKC) is an enzyme which transduces the cellular signals that promote lipid hydrolysis. The key role of PKC as well as $p38^{MAPK}$, which are upstream signals for COX-2 activation and biosynthesis of PGE_2, is to rapidly phosphorylate several proteins, such as $iPLA_2$ and $cPLA_2$. This is in line with a previous report in which it was demonstrated that $iPLA_2$-mediated AA liberation could be downstream of PKC activation in P388D1 cells [77]. Therefore, we examined the effect of BEL, a relatively selective inhibitor of $iPLA_2$, on PKCα expression. From the results, we observed that infection by *C. albicans* resulted in a continuous decline of PKCα protein level (Fig. 16). Moreover, the BEL treatment inhibited significantly expression of PKCα even in the control cells and *C. albicans* infection caused a marked decline in infected cells as compared with non-infected cells. This suggests that $iPLA_2$ activation is mediating PKCα activation and subsequently activation of $cPLA_2$ and COX-2.

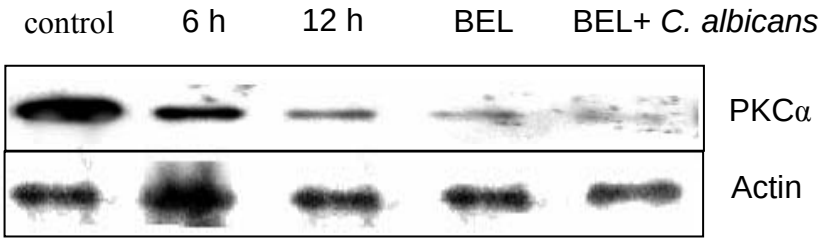


Figure 16. iPLA$_2$ inhibition caused decrease in the PKC activity in HeLa cells infected with *C. albicans*. HeLa cells were pretreated with BEL (20 μM) for 1 hour before infection by *C. albicans* for 12 hours. In addition, untreated cells were challenged directly with *C. albicans* for 6 and 12 hours. The cell lysates were then analyzed for PKCα protein expression using a specific antibody. The amount of protein was normalized by western blotting with β-actin antibody.

4.5.3 iPLA$_2$ is a key regulator of cPLA$_2$ and COX-2 activity in HeLa cells challenged with *C. albicans.*

From previous results, it was concluded that the calcium-independent PLA$_2$ enzyme is an important regulatory element in host cell signaling induced by *C. albicans*. The inhibition of iPLA$_2$ blocked of PGE$_2$ production by host cells. Moreover, inhibition by BEL of iPLA$_2$ decreased the PKCα activity. Therefore, the next series of experiments were aimed at addressing the question of whether or not cPLA$_2$ and COX-2 expression are dependent on iPLA$_2$ expression. For this purpose, HeLa cells were cultured and were exposed to *C. albicans* for 6 hours in the presence of iPLA$_2$ inhibitor BEL (20 μM) for 1 hour. Levels of mRNA for cPLA$_2$ and COX-2 were analyzed by semiquantitative RT-PCR with specific primers.

Interestingly, COX-2 activation and cPLA$_2$ expression were completely blocked by the inhibition of iPLA$_2$ with the selective inhibitor BEL (Fig. 17).

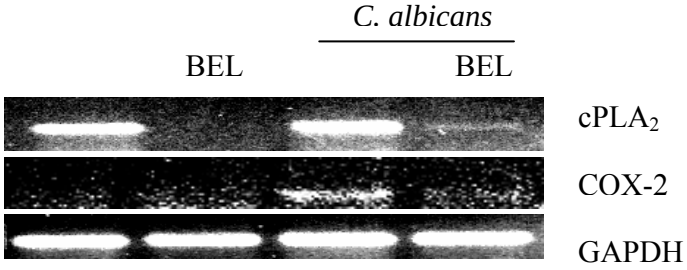


Figure 17. BEL treatment inhibits the activation of cPLA$_2$ and COX-2. Cells were harvested and total RNA was extracted. Semi-quantitative RT-PCR was performed to detect cPLA$_2$ and COX-2. GAPDH was used to normalize cPLA$_2$ and COX-2 expression.

4.5.4 Involvement of reactive oxygen species in the activation of $iPLA_2$.

Reactive oxygen species (ROS) are also called "free radicals" and are extremely unstable. Radicals quickly react with other molecules or radicals to achieve the stable configuration of 4 pairs of electrons in their outermost shell. Increased ROS formation is the common consequence of many pathologies, including infection and inflammation, and provides a link between signaling pathways and transcriptional elements regulating a large number of genes [151]. The previous report from Dr Nigam's group regarding intracellular glutathione content in HeLa cells, revealed that *C. albicans* infection alone caused a drastic depletion of glutathione, thus enhancing the oxidative stress and consequently remarkably enhancing COX-2 promoter activity [96]. They showed that antioxidants NAC and PDTC strongly inhibited the COX-2 activity [96]. Moreover, we observed that $iPLA_2$ is an important regulator in host cells signaling with infected by *C. albicans*, it was important to investigate the involvement of ROS in the expression of $iPLA_2$. $iPLA_2$ mRNA was determined by mRNA levels in cells pretreated with NAC (200 μM) or PDTC (25 μM) for 1 hour. These experiments indicated that PDTC and NAC treatment caused inhibition of $iPLA_2$ mRNA after 6 hours of infection by *C. albicans* (Fig. 18).

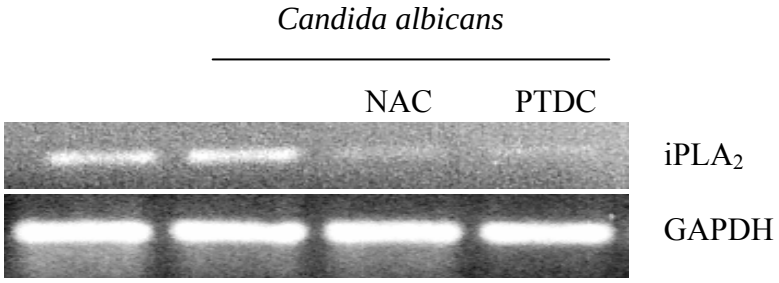


Figure 18. The depletion of ROS and inhibition of oxidative stress suppressed the activation of $iPLA_2$. Cells were harvested and total RNA was extracted. Semi-quantitative RT-PCR was performed to detect the $iPLA_2$. GAPDH was used to normalize $iPLA_2$ mRNA expression.

4.5.5 In vitro binding of NF-κB p65 and $iPLA_2$ in *Candida*-infected cells.

From the findings so far we can conclude that the inhibition of $iPLA_2$ represses the expression of $cPLA_2$, COX-2 and PKC and therefore, the production of PGE_2. We have already demonstrated that COX-2 activity is mediated via NF-κB activation. Next, we asked whether $iPLA_2$ and NF-κB p65 not only functionally but also physically interact in HeLa cells. Therefore, we

investigated the association of $iPLA_2$ and NF-κB p65 in untreated and *Candida*-infected HeLa cells by immunoprecipitation experiments. $iPLA_2$ or NF-κB p65 subunit was precipitated from the lysate of cells with or without *Candida*-infection with specific monoclonal antibodies, and were analysed by immunoblotting for $iPLA_2$ and NF-κB p65. As can be seen in Figure 19A iPLA2 was found in the precipitate obtained by NF-κB p65 antibody in *Candida*-infected cells (*left panel*). Such an association was absent in uninfected cells. Similarly, NF-κB p65 was detected in the immunoprecipitate obtained by $iPLA_2$ antibody in *Candida*-infected cells. However, uninfected cells also displayed the physical association of $iPLA_2$ with NF-κB p65 (*left panel*).

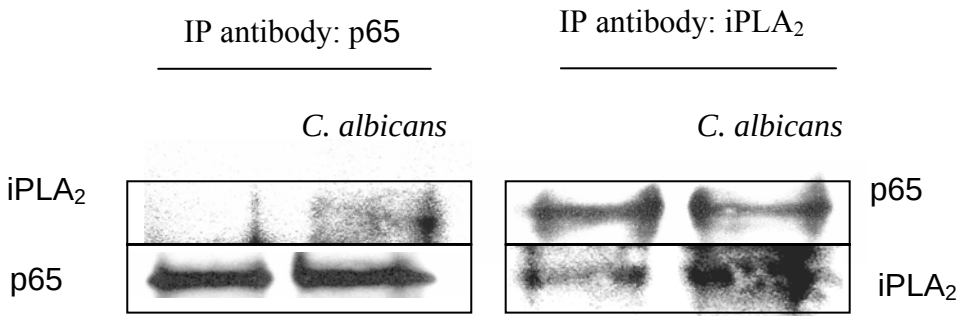


Figure 19A. Association of $iPLA_2$ with p65 subunit of NF-κB. HeLa cells were infected by *C. albicans* for 6 hours. Immunoprecipitation of $iPLA_2$ or p65 was carried out as described in materials and methods. Samples were subjected to immunoblot analysis with anti $iPLA_2$ or p65 antibodies.

From these results we assume that $iPLA_2$ protein may undergo binding with the whole IκBα/p65 complex. To verify this we coimmunoprecipitated IκBα and detected $iPLA_2$ and thus confirmed our conception (Fig. 19B).

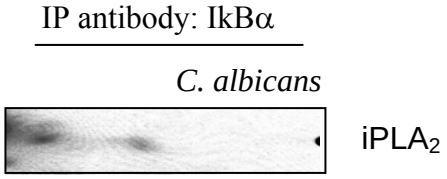


Figure 19B. Association of $iPLA_2$ with IκBα. HeLa cells were infected by *C. albicans* for 6 hours. Immunoprecipitation of IκBα was carried out as described in materials and methods. Samples were subjected to immunoblot analysis with anti $iPLA_2$ antibody.

4.6 *Candida albicans* and host cell apoptosis

4.6.1 *Candida albicans* causes apoptosis in HeLa cells.

An earlier report from Ibata-Ombetta *et al*. [152] showed that *C. albicans* causes macrophage death by inducing changes specifically in the MEK-ERK signal transduction pathway. Moreover, it has been demonstrated that *C. albicans*, which was isolated from a HIV-infected individual, induced early apoptotic changes in macrophages that phagocytosed them *in vitro* [153]. In order to see if *C. albicans* can induce apoptosis in human epithelial cells, we infected HeLa cells with *C. albicans* at MOI 5 and cells were assessed for apoptosis by nuclear staining as described in materials and methods. The results showed that HeLa cells underwent significant morphological changes after 24 hours of infection by *C. albicans* (Fig. 20). This method was performed to discriminate between apoptosis and necrosis. Necrosis is a process considered as unprogrammed cell death caused by injury, infection, infarction or inflammation. The basal frequency of apoptosis in control cells was around 1 %, which increased to more than 50 % in *C. albicans*-infected cells.

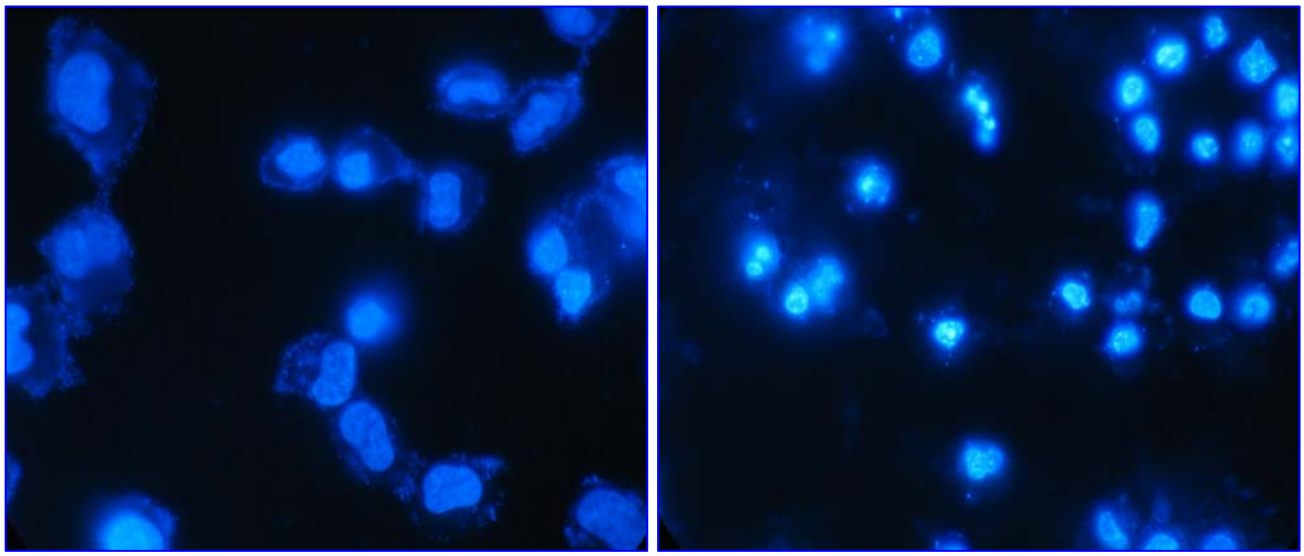


Figure 20. *C. albicans* causes nuclear shrinkage in HeLa cells. Nuclear staining was performed with DAPI after 24 hours of infection by *C. albicans*. The *right panel* shows apoptotic nuclei of HeLa cells as compared with nuclei of uninfected cells (*left panel)*.

4.6.2 A kinetic analysis of apoptosis induced by *C. albicans*.

One of the hallmarks of apoptosis is the digestion of genomic DNA by an endonuclease, generating a ladder of small fragments of double-stranded DNA. Therefore, *C. albicans* induced apoptosis was confirmed by a kinetic analysis of genomic DNA ladder fragmentation.

For this HeLa cells were challenged with *C. albicans* for 12 hours, 18 hours, 24 hours and 30 hours periods of time. Although after 12 hours of infection with *C. albicans*, an apoptotic change was observed (Fig. 26A), at this time period no DNA ladder fragmentation was detected (Fig. 21). Apoptosis continued to increase through 30 hours, and DNA fragmentation was detected after 18 hours of infection by *C. albicans* (Fig. 21).

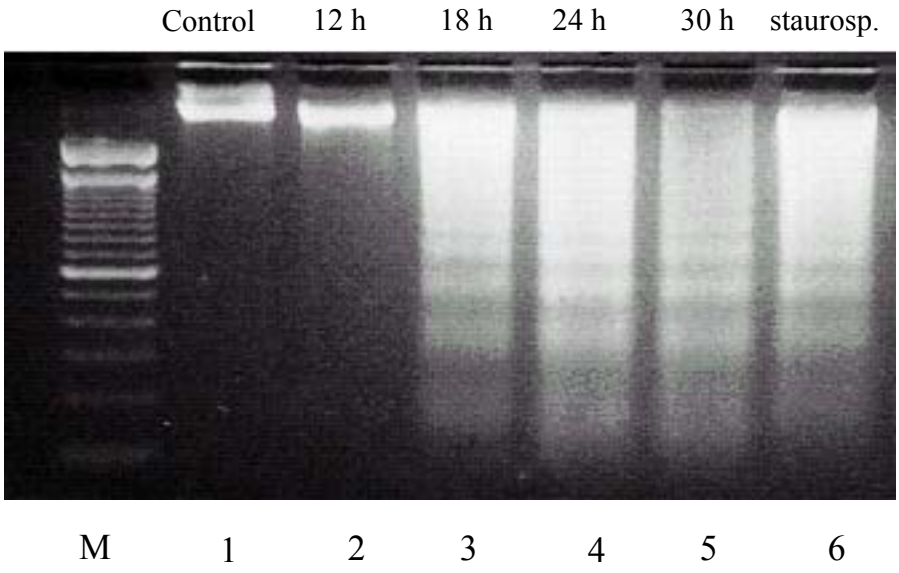


Figure 21. *C. albicans* induces apoptosis in HeLa cells. HeLa cells (10^7) were treated with *C. albicans* at MOI 5 for 12, 18, 24 and 30 hours. DNA was extracted and electrophoresed on 2 % agarose gel. Bands displayed after infection for 18 hours (line 3) and longer (lanes 3-5) are indicative of apoptosis, whereas DNA from uninfected and 12 hours infected cells (lanes 1 and 2) remained intact. Stauroporine (1 μM) was used as a positive control for 12 hours treatment (lane 6).

4.7 *C. albicans*-induces caspase-3 activation.

Caspase-3 activation, which is fundamental to the execution of apoptosis, was investigated. Caspase-3 mediates the cleavage of multiple substrates to cause the characteristic changes that occur in apoptotic cells. Upon incubation with peptide DEVD-pNA as substrate, increased activity of caspase-3 was found in *Candida*-infected HeLa cells (Fig. 22). This effect was, however, abrogated when cells were preincubated with 100 μM Z-VAD-FMK, a specific peptide inhibitor of caspase-3 (Fig. 26C).

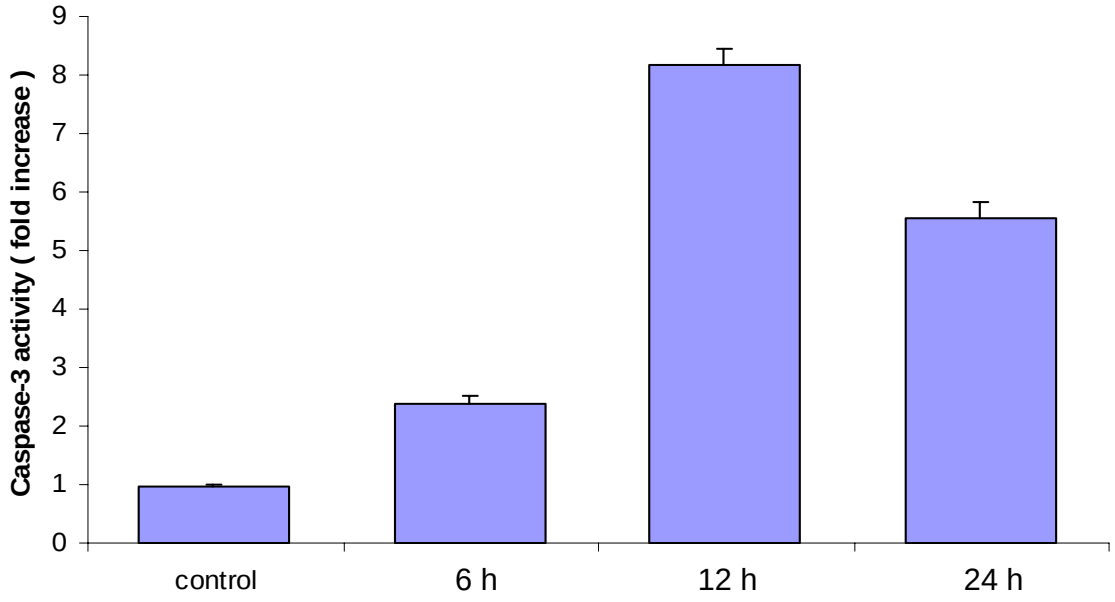


Figure 22. *C. albicans*-induced apoptosis via activation of caspase-3. The caspase-3 activity of cells was measured using Ac-DEVD-pNA as substrate. Cell lysate (100 μg protein) was incubated with 200 μM Ac-DEVD-pNA and the reaction was monitored for 1-3 hours at 405 nm. Values represent the mean ± SEM of 3 separate experiments.

4.8 TLR2 in HeLa cells are activated by *C. albicans* infection.

4.8.1 *C. albicans*-induced TLR2 activation was not mediated via COX-2, p38 or PI-3 kinases.

Earlier, it was shown that toll-like receptors are involved in the antifungal defence mechanism in *Drosophila* as well as in antibacterial defence in humans [154, 155]. In a previous report from

Dr. Nigam's group, the crucial role of TLR2 in mediating COX-2 gene activation by *C. albicans* was described. Neutralization of TLR2 by mAb specific for human TLR2 completely abolished the COX-2 activation [96]. Therefore, we studied the activation of TLR2 by *Candida* infection in HeLa cells. An increased TLR2 mRNA expression was observed in HeLa cells after 6 hours of infection by *C. albicans*. In order to determine whether this TLR2 activation could be involved in the *Candida*-triggered signaling cascades such as PI-3 and $p38^{MAPK}$, cells were pretreated with inhibitors of PI-3-kinase, $p38^{MAPK}$ and COX-1/2, namely wortmannin, SB203190, NS398 and aspirin, respectively, before challenging with *C. albicans.* We found that TLR2 mRNA expression was not affected (Fig. 23).

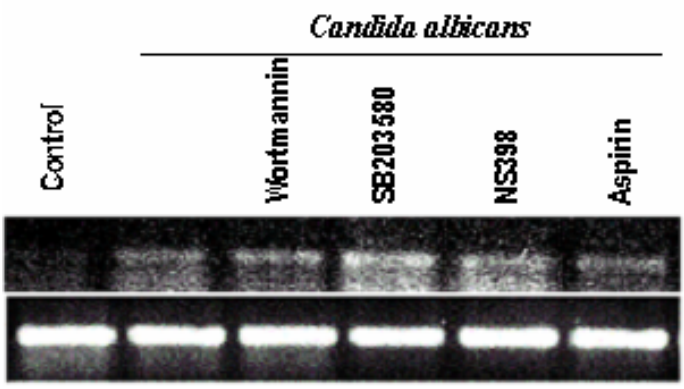


Figure 23. *C. albicans* induced TLR2 activation was not mediated via COX-2, p38 or PI-3 kinases. Cells were pretreated with 10 μM wortmannin, 20 μM SB203, 10 μM NS398 or 1 mM aspirin for 1 hour before challenging with *C. albicans* at MOI 5 for 6 hours. Total RNA (2 μg) was extracted, and RT-PCR analysis was performed. PCR was done for TLR2, as well as for GAPDH as a control to ensure that RNA amounts were equal.

4.8.2 TLR2 mRNA activation does not require *de novo* protein synthesis

Since we found TLR2 activation by *C. albicans* infection, it was probable that *de novo* synthesis of additional regulatory factors was required. To test this hypothesis, HeLa cells were challenged with *C. albicans* in presence of cycloheximide (0.07 mM), a protein synthesis inhibitor. Activation of TLR2 mRNA was measured by PCR. As shown in Figure 24 cycloheximide did not affect the activation of TLR2 gene.

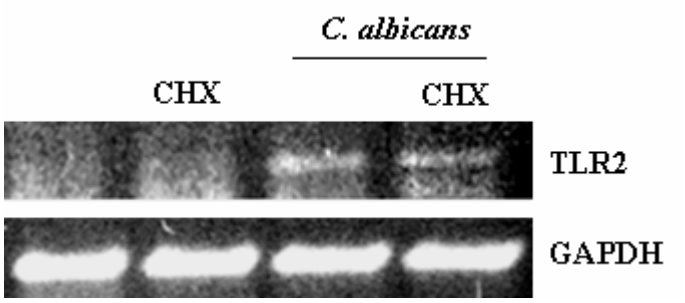


Figure 24. *De novo* protein synthesis was not required for the induction of TLR2 mRNA. Cells were exposed to cycloheximide (0.07 mM) along with *C. albicans* for 6 hours. TLR2 expression was assayed by RT-PCR.

4.8.3 Downstream signaling activation of TLR2 in HeLa cells infected by *C. albicans*.

The MyD88 adapter protein links members of the TLRs and IL-1R superfamily to the downstream activation of NF-κB and MAPKs. Although originally identified as a myeloid-differentiation marker, MyD88 is now known to play an essential role in the innate immune response of insects and mammals [40, 42]. Therefore, in our study we aimed to determine downstream signaling of TLR2 activation. We performed immunoblotting with specific antibody for activation of MyD88 expression by HeLa cells infected by *C. albicans*. MyD88 activation was detected after 3 hours of infection and was expressed continuously over the period studied (Fig. 25).

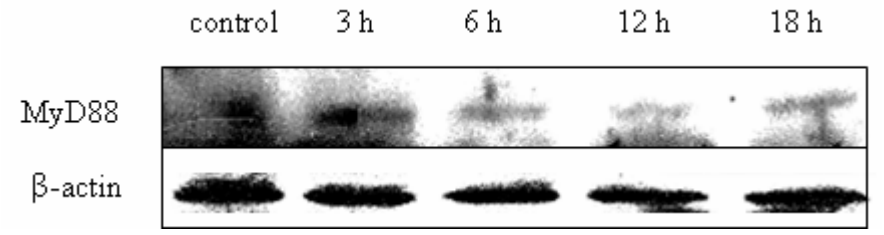


Figure 25. *Candida* infection caused activation of adapter protein MyD88. HeLa cells were infected with *C. albicans* for various periods. The 50 μg cell lysates were analysed by western blotting for the MyD88 and β-actin.

4.9 TLR2 mediates *C. albicans*-induced apoptosis

Candida-induced apoptosis in HeLa cells was also measured using cell death detection ELISA. This method assays the presence of DNA-histone nucleosomal complex, which is an indicator of apoptosis, in the cytoplasm. HeLa cells were infected by *C. albicans* at MOI 5 for various times and DNA-histone complexes were detected as early as 12 hours after exposure to *C. albicans* and continued to increase through 24 h and finally declined to basal levels at 48 hours (Fig. 26A). This decrease in apoptosis may result from the recovery of a subpopulation of HeLa cells insensitive to *C. albicans* after 48 hours.

A.

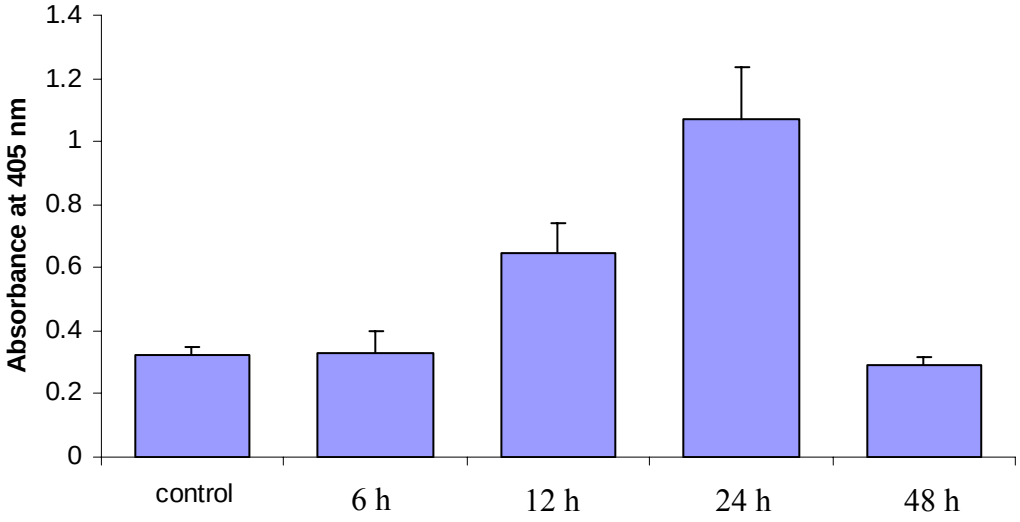


Figure 26A. *C. albicans* induces apoptosis in HeLa cells. *C. albicans* was added at MOI 5:1 for various time periods. Cell death detection ELISA experiments were performed with the cell lysates. Significant apoptosis was detected as early as 12 hours and continued to increase until 24 hours. Values represent the mean ± SEM of 3 separate experiments.

Furthermore, we examined the specific role of TLR2 in apoptosis. Surface TLR2 were blocked by neutralizing with a monoclonal Ab before infection of cells by *C. albicans* at MOI 5. As shown in Figure 26B, when compared with untreated controls, *C. albicans*-induced apoptosis was significantly inhibited within 24 hours as measured by cell death detection ELISA. The abrogation of apoptosis was also observed when HeLa cells were transiently transfected with TLR2-DN plasmid prior to infection by *C. albicans* (Fig. 26B).

B.

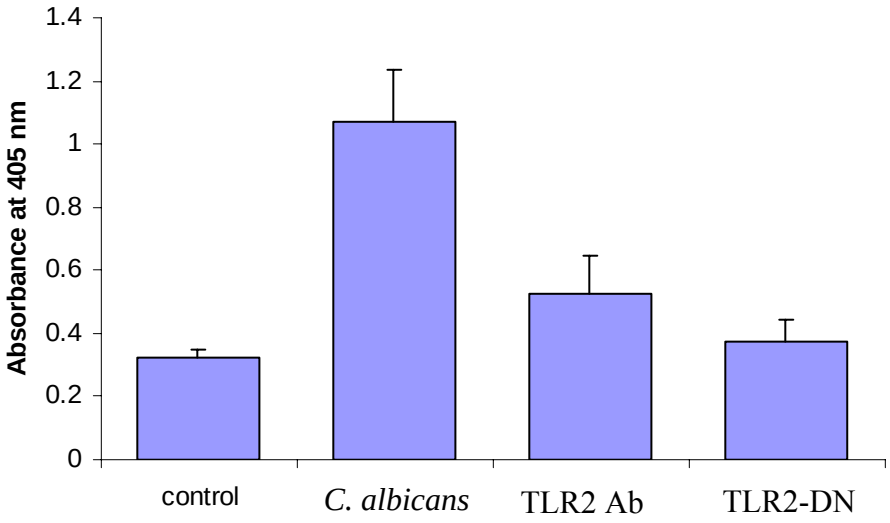


Figure 26B. TLR2 mediated *C. albicans* induces apoptosis in HeLa cells. HeLa cells were transfected with TLR2-DN or were preincubated for 1 hour with 20 μg/ml monoclonal antibody against TLR2. *C. albicans* was then added at MOI 5:1 for 24 hours. Cell death detection ELISA experiments were performed with the cell lysates.

Moreover, activation of Caspase-3 in *Candida*-infected HeLa cells (Fig. 22) was, however, abrogated when cells were treated with 100 μM of a specific peptide inhibitor of caspase-3 Z-VAD-FMK for 1 hour (Fig. 26C). The activation of caspase-3 requires the recruitment of Fas-associating death domain (FADD) containing protein and Apaf-1 [156]. FADD in turn recruits caspase-8 and –9 to activate downstream effecter caspase-3, -6 and –7. To check if FADD is involved in *C. albicans*–mediated apoptosis of HeLa cells, apoptosis was studied in cells transfected with FADD-DN plasmid. The results (Fig. 26C) showed complete inhibition of *C. albicans*-induced apoptosis in FADD-DN transfected cells.

C.

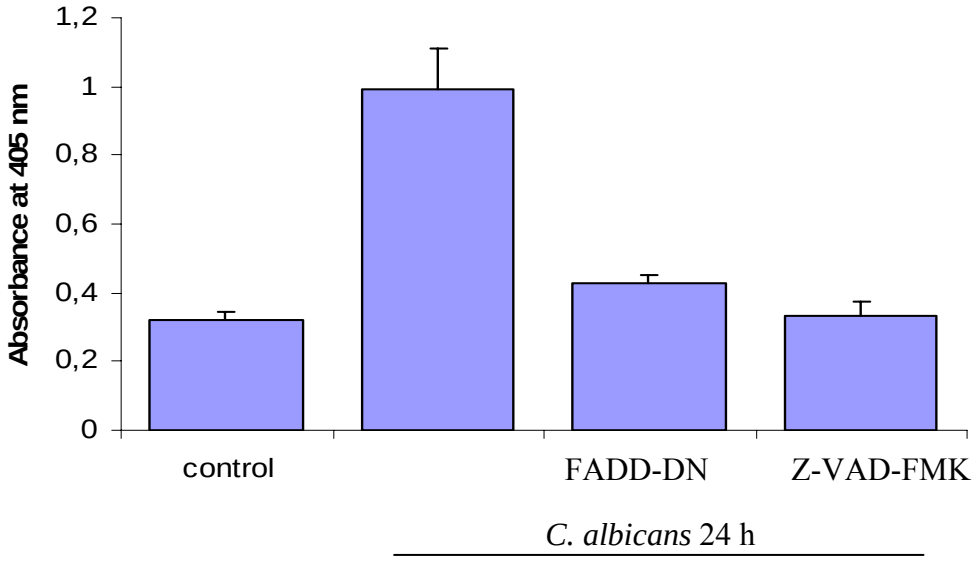


Figure 26C. Involvement of FADD in *Candida*-induced host cells apoptosis. HeLa cells were transiently transfected with FADD-DN plasmid or were preincubated for 1 hour with 100 μM Z-VAD-FMK, a caspase-3 inhibitor. *C. albicans* was then added at MOI 5:1 for 24 hours. Cell death detection ELISA experiments were performed with the cell lysates.

4.10 Overexpression of iPLA$_2$ inhibits apoptosis in *Candida*-infected HeLa cells.

Previous observations of iPLA$_2$ mRNA in HeLa cells over various periods showed an initial increase with a maximum at 6 hours after infection by *C. albicans*, which then subsequently declined in a time-dependent manner and disappeared after 24 hours (Fig. 14B and C). We determined the iPLA$_2$ protein expression for the same periods and observed that protein expression followed the same pattern as mRNA levels, i.e. an initial increase followed by a subsequent decline and disappearance after 24 hours of infection by *C. albicans* (Fig. 27).

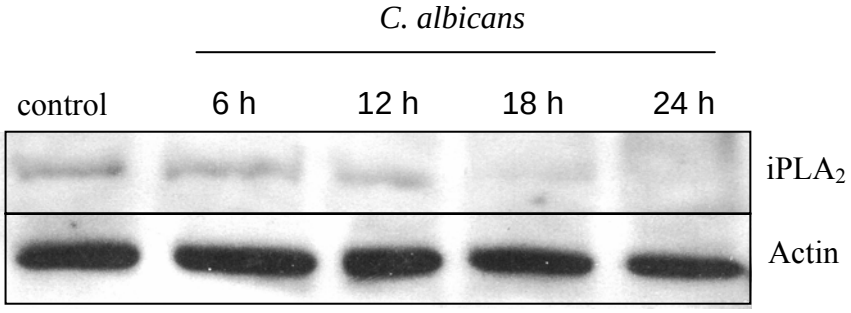


Figure 27. Time-course analysis of $iPLA_2$ protein expression in HeLa cells infected with *C. albicans*. Equal amount of HeLa cell lysates were analysed by western blotting with specific antibody against $iPLA_2$. Anti-actin antibody was used as a control to ensure that equal amounts of proteins were loaded.

Since $iPLA_2$ expression declined after infection, we overexpressed $iPLA_2$ in HeLa cells by transfection with $iPLA_2$ plasmid. $iPLA_2$ protein expression was then determined in overexpressed cells after infection with *C. albicans* at various time points. Transfection with the $iPLA_2$ plasmid prevented decline and disappearance of $iPLA_2$ expression till 24 hours of infection (Fig. 28).

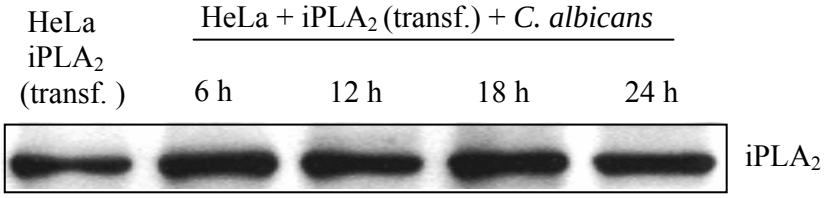


Figure 28. Time-course analysis of overexpressed $iPLA_2$ protein expression in HeLa cells infected with *C. albicans*. Equal amount of lysate of HeLa cell, transfected with and without $iPLA_2$ plasmid and were analysed for western blotting with specific antibody against $iPLA_2$.

Next, we investigated the $iPLA_2$ enzymatic activity in $iPLA_2$- transfected and non- transfected cells challenged with *C. albicans* at various time points. Activity assay as shown in Figure 29 indicated that the overexpression of $iPLA_2$ activity was increased at 6 hours of infection. The activity then remained constant till 24 hours. However, the $iPLA_2$ activity in non-transfected cells showed a similar initial increase at 6 hours, which however decreased to basal level over the time period studied (Fig. 29).

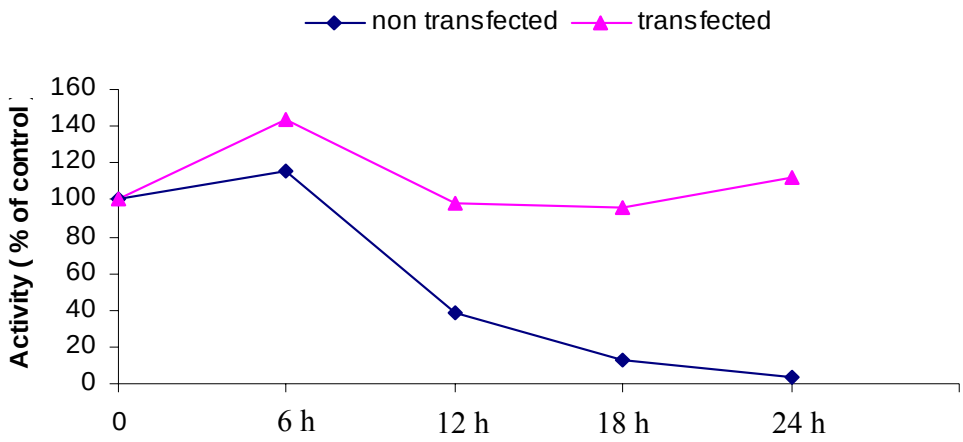


Figure 29. $iPLA_2$ enzymatic activity. Transfected or non-transfected HeLa cells were infected with *C. albicans* for indicated periods and homogenizates were assayed for $iPLA_2$ activity as described in materials and methods. The enzyme activity is plotted as percentage of the control non infected cells.

Assuming that the activation of $iPLA_2$ protects *Candida*-induced apoptosis, cells overexpressing $iPLA_2$ were studied for *Candida*-induced apoptosis by DNA ladder fragmentation assay. Strikingly, no apoptosis of HeLa cells was observed up to 24 hours after infection with *C. albicans*. These results pinpoint clearly the regulatory role of $iPLA_2$ in the execution of apoptosis (Fig. 30).

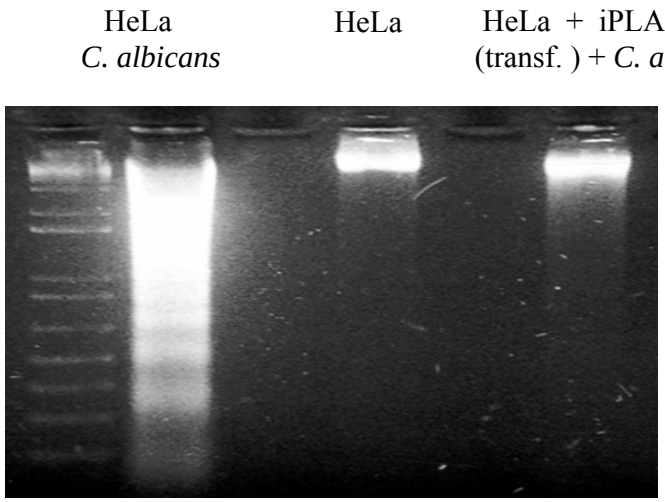


Figure 30. Overexpression of $iPLA_2$ inhibits *C. albicans*-induced apoptosis in HeLa cells. HeLa cells were transfected with $iPLA_2$ plasmid. After 24 hours cells were challenged with *C. albicans* for 24 hours and total DNA was electrophoresed for DNA ladder fragmentation.

4.10.1 Inhibition of $iPLA_2$ and not PAP-1 induces apoptosis in *C. albicans*-infected cells

From the above study we presumed that this dynamic expression of $iPLA_2$ activity is related to the progression of cellular apoptosis and if so, BEL should shorten the time for induction of apoptosis. To confirm this, cells were pretreated with BEL, MAFP or AACOCF3 before challenging with *C. albicans* for 8 hours. DNA was extracted and separated by agarose gel electrophoresis. Indeed, measurement of apoptosis in the absence and presence of the inhibitors BEL and MAFP (lane 6 and 7) revealed that inhibition of $iPLA_2$ accelerates the onset of apoptosis to less than 8 hours (Fig. 31). But AACOCF3, the inhibitor of $cPLA_2$, did not accelerate the onset of *C. albicans* induced apoptosis (lane 8). These results clearly indicate that $iPLA_2$ alone is playing a key role in host cells programmed death.

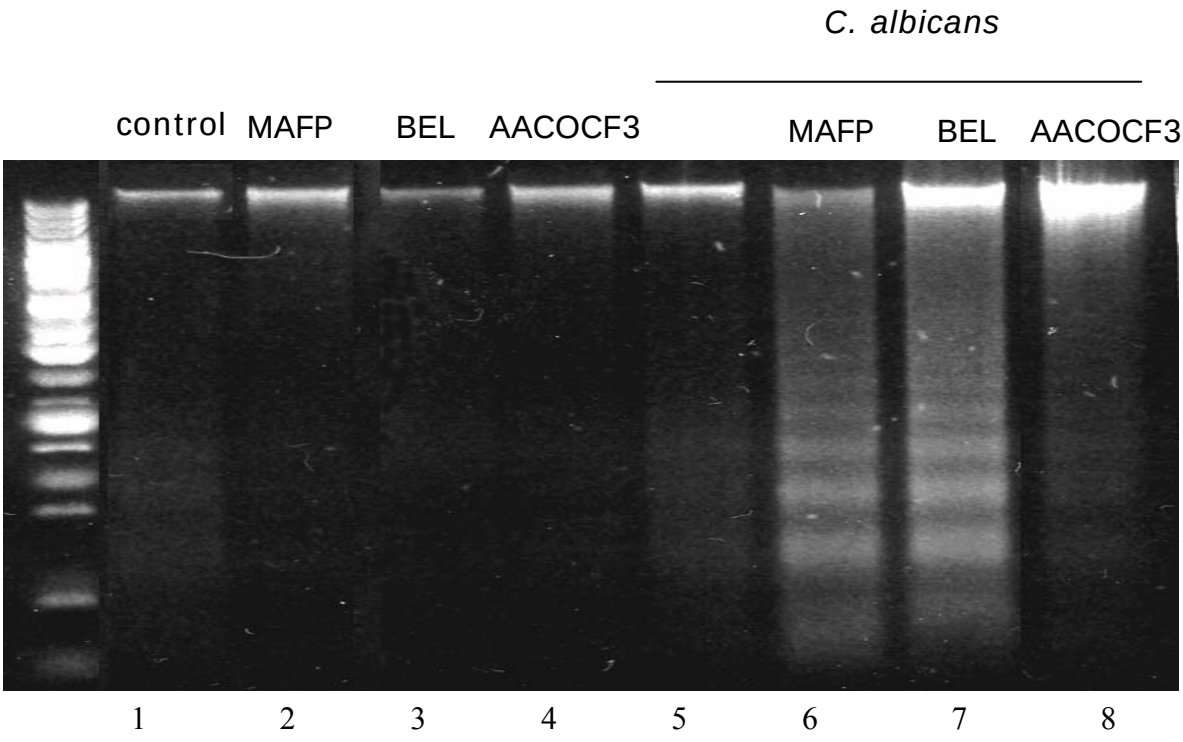


Figure 31. Inhibition of PLA_2 by BEL and MAFP but not by AACOCF3 triggers *C. albicans*-induced apoptosis in HeLa cells. DNA ladder fragmentation assay was performed after 8 hours of infection with *C. albicans* in HeLa cells pretreated with 20 μM of BEL, MAFP and AACOCF3 for 1 hour. DNA was extracted and electrophoresed on 2 % agarose gel. Apoptosis can be seen in cells pretreated with BEL or MAFP and *C. albicans* infected cells (lane 6 and 7), whereas DNA from uninfected cells or from cells only infected with *C. albicans* for 8 hours or treated with inhibitors alone remained intact.

In addition, the apoptosis was confirmed by nuclear staining with DAPI. For this cells were pretreated with BEL before challenging with *C. albicans* for 8 hours. The results showed clear changes in the nucleus of the BEL treated cells (Fig. 32).

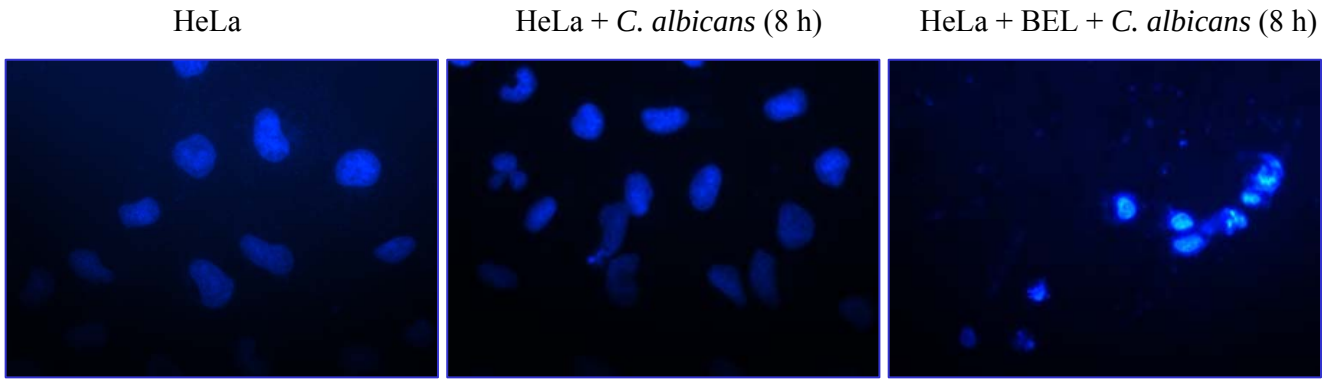


Figure 32. Nuclear staining of cells pretreated with BEL before infection with *C. albicans*. Cells were pretreated by BEL at 20 μM for 1 h and infected by *C. albicans* as mentioned above and nuclear staining performed with DAPI.

To determine if this apoptosis is caused by the inhibition of phosphatidate phosphohydrolase-1 (PAP-1) as reported by Fuentes *et al*. [157], we pretreated one set of cells with propranolol, an inhibitor of PAP-1. Apoptosis was assayed after 8 hours by DNA ladder fragmentation. No proapoptotic effect of propranolol was observed, and the time point for the onset of apoptosis remained unaltered (Fig. 33). We can therefore rule out the possible involvement of PAP-1 in apoptosis.

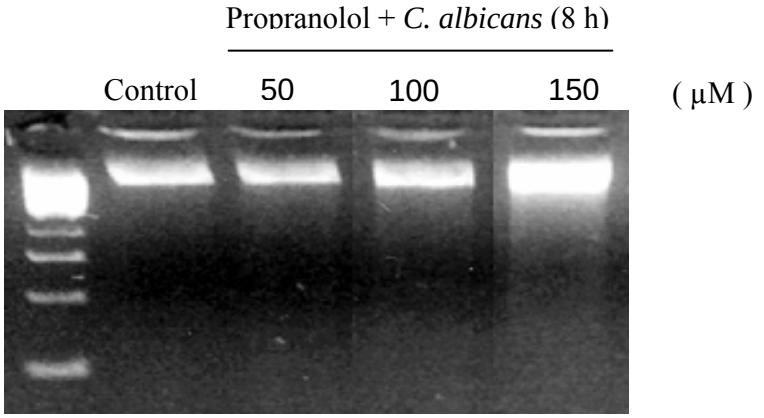
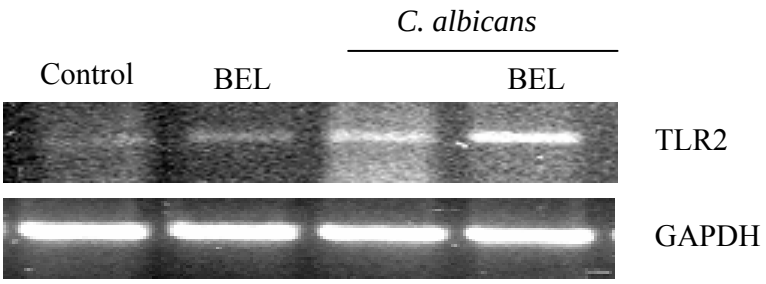


Figure 33. PAP-1 treatment did not affect the onset of apoptosis induced by *C. albicans*. DNA ladder fragmentation assay was performed on HeLa cells which were pretreated with 50-150 μM propranolol (PAP-1 inhibitor) before infection with *C. albicans* for 8 hours.

4.11 Inhibition of $iPLA_2$ promotes TLR2-mediated apoptosis by *C. albicans*

Concluding from the above study that the inhibition of $iPLA_2$ accelerated the onset of apoptosis, we expected that the inhibition of $iPLA_2$ by BEL should also upregulate the TLR2 mRNA expression to promote apoptosis. As shown in Figure 34A, treatment of cells with BEL upregulated the TLR2-mRNA expression significantly. Moreover, the overexpression of $iPLA_2$ completely inhibited the activation of TLR2 (Fig. 34B). This effect on TLR2 on the cell membrane is clearly attributed to attenuation of $iPLA_2$. In conclusion, these results strongly suggest that endogenous $iPLA_2$ is involved in the upregulation of TLR2.

A.



B.

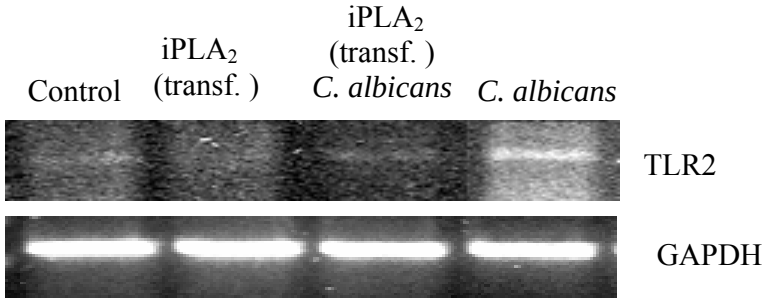


Figure 34. The inhibition of $iPLA_2$ promotes TLR2 mediated apoptosis. Cells were pretreated with 20 μM BEL for 1 hour (A) or transfected with $iPLA_2$ plasmid for 24 hours (B) before infection with *C. albicans*. Total RNA was extracted, and RT-PCR analysis was performed. GAPDH was used as a control to ensure that RNA amounts were equal.

5. DISCUSSION

5.1 *C. albicans* infection mediated host cell signaling.

COX-2 enzyme expression is elevated in numerous reproductive tract carcinomas, including cervical carcinoma, endometrial adenocarcinoma and ovarian adenocarcinoma [96, 159, 160]. In cervical and endometrial carcinomas, enhanced expression of COX-2 enzyme is associated with elevated synthesis of eicosanoids in neoplastic epithelial and endothelial cells [161], suggesting that COX enzyme products such as PGE_2 may be involved in neoplastic cell transformation and reproductive tract tumourgenesis. Moreover, host cells are a source of eicosanoids during fungal infection. As a pathogenic yeast, *C. albicans* is able to release arachidonic acid from HeLa cells and subsequently convert it into eicosanoids [29], such as PGE_2, which plays a major role in morphogenesis of *C. albicans* [88], and candidiasis [162]. The primary observation from our data is that *Candida albicans* able to upregulate the activation of COX-2 enzyme in HeLa cells and the production of PGE_2. COX enzymes convert AA to PGG_2, which is further converted to PGH_2 by the peroxidase activity of prostaglandin H synthase. The significant upregulation of COX-2 was clearly evident within 6 hours of infection with *C. albicans* (Fig. 1). The *C. albicans*-induced upregulation of COX-2 is related to a drastic increase of activation of NF-κB and MAPKs. $P38^{MAPK}$cascade is also critical to the magnitude and duration of COX-2 expression. It is known that $p38^{MAPK}$ and cAMP/PKA pathway interact to transactivate the COX-2 promoter in response to IL-1β in human synoviocytes [97]. The data presented in this study confirmed that *C. albicans* induced COX-2 activation was through the cAMP/PKA activation (Fig. 3).

As a signaling event it plays a pivotal role in cytokine, LPS, or stress induced gene expression [96, 163]. In addition, to regulate cellular responses to cytokines, oxidative stress and pathogens, NF-κB play also an important role in inflammation [110] and tumour proliferation. Several members of the Rel/NF-κB family have been implicated in the control of cell proliferation and differentiation [110, 111]. Therefore, markedly high levels of NF-κB promoter activity as well as activation of cRel and Rel A subunit of NF-κB are detected in HeLa cells following infection with *C. albicans* (Fig. 4 and 7). This activity was abolished by sodium salicylate, an inhibitor of COX-2 [96]. The NF-κB promoter construct and dominant negative plasmid of IKK kinase showed that COX-2 activation is mediated through $p38^{MAPK}$ and NF-κB pathways, whereas NF-κB upregulates the phosphorylation of p38 protein [96]. Several consensus sequences for NF-κB binding sites of the COX-2 gene have been reported as regulatory sequences in COX-2

induction by various agonists in different cell types [164]. Activation of transcription by NF-κB has been shown to require a number of different coactivator proteins including CBP/p300, p/CAF, and SRC-1 [165]. These activator proteins possess histone acetyltransferase activity and are capable of acetylating the histone proteins. Acetylation of proteins is a common principle to modify their biological activity. It impacts protein chemical properties and, thus may alter protein-protein interaction, DNA recognition and protein stability. Histones were the first proteins that have been identified as targets for protein acetylation. Although there are several lines of experimental evidence suggesting the importance of histone acetylation in the transcription of a variety of genes, its precise role in nucleosome remodeling is still elusive. The data presented in this study clearly indicate that *C. albicans* induced transcription of the COX-2 gene requires upregulation of histone H3 acetylation (Fig. 10), which is mainly due to activation of the acetyltransferase activity of CBP/p300. This conclusion may be drawn form the following experimental data that the viral oncoprotein wtE1A, an inhibitor of acetyltransferase activity of CBP/p300, prevented histone H3 acetylation, and expression of the functional enzyme (Fig. 5). In contrast, its non-inhibitory mutant E1AmCBP was unable to do so (Fig. 5). It should, however, be stressed that the acetylation degree of cellular proteins is a resultant of acetylating and deacetylating processes. Thus, an increase in the acetylation degree of histone H3 can either be achieved by activation of acetyltranferases (CBP/p300) or by inhibition of deacetylases [122-125]. Deacetylases have been shown to occur in the nucleus and appear to play an important role in transcriptional repression [123]. Whether they are recruited by nuclear hormone receptors bound to certain nuclear corepressors, is not clear. Recently, the data demonstrated that treatment the cells with TSA caused an increase in TNF-induced expression of NF-κB regulated IL-8 gene [166]. In addition, TSA treatment resulted in hyperacetylation of the IL-8 promoter. Moreover, increased PGE_2 production can stimulate the activation of IL-8 gene in human colonic epithelial cells [167]. In agreement with these data, we observed a drastic increase of COX-2 and IL-8 production in HeLa cells under the fungal infection. TSA treatment showed drastic increase of IL-8, as well as NF-κB and COX-2 production (Fig. 6) suggested an important role of HDAC complex in *C. albicans*-induced signaling cascade. This activation by HDACs inhibitors occurred in a relatively straightforward manner: the inhibition of HDACs led to histone hyperacetylation and the opening of the promoter region for basal transcription machinery. Recent results from gene microarray data showed that HDAC inhibition does not trigger global gene activation [168]. In fact, most genes remain unaffected by HDAC inhibitors. Our results showed that treatment of

cells with HDACs inhibitors TSA or Gam-1 before challenging with *C. albicans*, led to increase in both basal and *C. albicans* induced activation of COX-2 and NF-κB genes (Fig. 6 and 8). Gam-1 expression increased the level of transcription of COX-2 in a similar way as potent HDAC inhibitor, TSA. Therefore, Gam-1 can binds to HDAC-1 both in vitro and in vivo and effectively inhibit histone deacetylation by HDAC-1. The effect of Gam-1 having the specific function of binding and inactivating HDAC-1 suggests that deacetylase complexes play an important role in limiting early gene expression by invading microorganisms. In support of this, we showed that transient transfection of HDAC-1 is able to repress *C. albicans* induced NF-κB mediated COX-2 gene activation (Fig. 9).

Acetylation may induce conformational changes of the histone octamer, which then may provide access to transcription factors for binding to the promoter of relevant genes. In addition, we showed that, *C. albicans* infection specifically leads not only to an increase in histone H3 acetylation, but is also associated with the activation of specific HDACs. Therefore, TSA and Gam-1 alone is capable of inducing COX-2 and NF-κB expression in HeLa cells suggest that transcriptional repression of the COX-2 and NF-κB gene in resting cells may be due to a preponderance of deacetylating processes over acetyltransferases. Using both in co-immunoprecipitation and western blot analysis, we showed that HDAC-1 interacts directly with the p65 subunit of NF-κB and likely exerts its corepressor function (Fig. 11). The increased basal expression of the COX-2 gene as well as NF-κB, in presence of HDAC-1 inhibitor, shows that HDAC-1 protein represses COX-2 expression through the direct interaction with NF-κB. These factors enable to increase recruitment of p65 and HDAC-1 to the promoter of key *C. albicans* induced NF-κB dependent gene, COX-2. We used ChIP assays (Fig. 12) effectively to study these changes in the nuclear transcription. Thereby, we conclude that fungal infection can induce *in vivo* chromatin remodeling events to induce inflammatory genes.

5.2 Host cell PLA_2 enzyme and its role in the activation of COX-2 gene.

In the classification based upon the Ca^{2+} requirement for their activities, PLA_2s can be divided into three categories: $sPLA_2$ that require millimolar concentration of Ca^{2+}; $cPLA_2$ that require micromolar concentration of Ca^{2+}; and $iPLA_2$, which do not require Ca^{2+} for the activity. In many cases, several of these enzymes are present in the same cell. For example, Group II, IV, and Ca^{2+}

independent enzymes are present in P388D1 macrophage-like cells [143], Group II, Group IV, and a phosphatidylserine-specific, Ca^{2+}-dependent PLA_2 are all present in rat mast cells.

AA, the precursor of eicosanoids, that are the mediators of inflammation, is released from membrane phospholipids by the catalytic action of PLA_2 in cells exposed to stimuli. Cellular expression of specific PLA_2 enzymes is dependent on cell type and state of differentiation. For instance, specific expression of $cPLA_2$ (group IV) enzymes include: human primary monocytes [169], U937 [170], and THP-1 cells [171], while group VI $iPLA_2$ is reported in murine P388D macrophages [143]. Both $cPLA_2$ and $iPLA_2$ enzymes are expressed in HeLa cells. Infection of HeLa cells with *C. albicans* induces activation of a defense mechanism, in which the host cells produce AA by the activation of PLA_2 enzymes, which is converted into eicosanoids that mediate inflammation. Previous studies from Dr. Nigam's group showed that AA released from host cells, can be converted by *C. albicans* to 3-HETE (a growth factor), although these fungi do not contain AA. This implies that an infection process can be assumed as host-pathogen interaction. Thus, AA is an appropriate substrate for the human fungal pathogen *Candida albicans* [29], and that *C. albicans* at MOI as low as 5 can selectively upregulate the COX-2 gene in HeLa cells and produce PGE_2. PGE_2 has been shown earlier to trigger morphogenetic changes in *C. albicans* [88]. Since inflamed and infected tissues release large amount of prostanoids including PGE_2, the above process plays an important role in vulvovaginal candidiasis. Although PLA_2 enzymes are important catalysts of the AA release process, it is unclear whether *Candida* or host cell PLA_2 is the effecter for AA release. Dr. Nigam´s laboratory has investigated the role of the candidial PLA_2 gene, PLB3, in *C. albicans* strain 44808 and its PLB3 knockout (KO) mutants in cleavage of AA from synthetic substrates 1-palmitoyl-*sn*-2-arachidonoyl phosphatidylcholine (PAPC) and 1-palmitoyl-*sn*-2 (15-OH)-arachidonoyl phosphatidylcholine (PAPC-OH). The heterozygous mutant maintained half of the PLA_2 activity as compared with the wild type *C. albicans* cells (manuscript in preparation). Moreover, they found that the AA release process from host cells was totally Ca^{2+} independent (unpublished observations). In this study we found that PLB gene mutated *C.albicans* could not induce the COX-2 activity from host cells (Fig. 13). Therefore, *C. albicans* seems to possess a potent active calcium-independent PLB enzyme capable of releasing the AA from membrane phospholipids of the host cell. This suggests that the PLB activity of *C. albicans* and not host cell PLA_2 causes the cleavage of AA from membrane phospholipids of host cell (unpublished observations). This process then leads to activation of host cell PLA_2. A previous study from Dr. Nigam's group demonstrated that *C. albicans* isolated

from the vaginal tract of a patient with VVC when cocultured with HeLa cells caused significant release of PGE_2 [29]. In addition, they also observed significant activation of COX-2. $P38^{MAPKs}$ are member of the MAPK superfamily and can be activated in response to cellular stress [172], endotoxins and inflammatory cytokines [173]. Thus, *C. albicans*-induced activation of COX-2 was found to be mediated by $p38^{MAPK}$ pathway. The $p38^{MAPK}$ pathway was not only suppressed by $p38^{MAPK}$ inhibitor SB202190, but also by GF203190X, a PKC inhibitor, which suggested that the PKC activation occurs upstream of $p38^{MAPK}$ and is an obligatory event in *C. albicans*-mediated COX-2 induction in HeLa cells [96]. Strikingly, our data in this study demonstrated that inhibition of $iPLA_2$ enzymes by BEL or MAFP resulted in complete inhibition of *C. albicans*-induced COX-2 activation and PGE_2 production in HeLa cells (Fig. 17 and 15). Moreover, the inhibition of $iPLA_2$ also resulted in the marked decline of PKCα in HeLa cells challenged with *C. albicans* (Fig. 16). These results are in agreement with a recent report regarding the involvement of PKCα as a regulatory factor in the $iPLA_2$-mediated AA release in response to zymosan [77]. Contrastingly, the PGE_2 production in human embryonic kidney cell was found to be dependent on $cPLA_2$ and $sPLA_2$ enzyme activities, $iPLA_2$, however, failed to activate COX-2 [174]. To sum up, we can conclude that $iPLA_2$ not only plays a crucial role in phospholipid remodeling, but also a key role in the signaling of AA release and PGE_2 production in HeLa cells infected by *C. albicans*.

ROS, including superoxide anion, hydroxyl radical, and hydrogen peroxide, have been known to trigger a variety of biological responses within cells exposed to stimuli. Cleavage of IkB requires an oxidizing milieu and appears to be one of the mechanisms by which ROS activate NF-κB [175]. Recent findings have suggested that ROS potentate $iPLA_2$ activity. So, it was demonstrated in uterine stromal cells, that H_2O_2 caused a significant release of AA, which is independent of intracellular Ca^{2+} concentration. Both AA release and $iPLA_2$ activity could be inhibited almost completely by BEL [79]. We also found similar results with the free radical scavenger, PDTC and NAC (Fig. 18). This is in line with the report of Xu *et al*. [176], who have shown the involvement of the both $iPLA_2$ and $cPLA_2$ in H_2O_2-induced AA release in murine astrocytes. In order to gain support for our observation regarding ROS-$iPLA_2$-NF-κB regulation, we asked whether $iPLA_2$ and NF-κB p65 subunit not only functionally but also physically interact in HeLa cells. Therefore, we investigated the *in vivo* association of $iPLA_2$ and NF-κB p65 subunit in untreated and *Candida*-infected HeLa cells by immunoprecipitation experiments. As can be seen in Figure 19A, $iPLA_2$ was found in the immunoprecipitate obtained by NF-κB p65 antibody in

Candida-infected cells (*left panel*). Such an association was absent in uninfected cells. Similarly, NF-κB p65 was detected in the immunoprecipitate obtained by $iPLA_2$ antibody in *Candida*-infected cells. However, uninfected cells also displayed the physical association of $iPLA_2$ with NF-κB p65. The latter observation (Fig. 19B) pinpoints that $iPLA_2$ may undergo physical binding with the whole IκBα-p50/c-Rel-p65 complex.

5.3 *Candida albicans* induced host apoptosis

Various pathogens have been shown to control host cell apoptosis in order to promote their own survival [177, 178]. Although the exact mechanism for apoptosis is far from clear, COX-2-mediated upregulation of mcl-1, a Bcl-2 family member, has been defined as a dominant antiapoptotic factor for following reasons: **(a)** it inhibits apoptosis induced by c-Myc overexpression [179], **(b)** it interacts directly with proapoptotic factor Bak inhibiting its capability to induce apoptosis [180], and **(c)** its upregulation is promoted by the COX-2-mediated activation of phosphatidyl-inositol-3-kinase (PI3K)/Akt pathway [181]. In the present study, however, we show that *C. albicans* induces apoptosis in HeLa cells after 12 hours infection. In contrast to a few previous studies in which apoptosis of host cells by *C. albicans* has been primarily attributed to the cell wall component phospholipomannan [156], we describe here a mechanism, which pinpoints the regulatory role of $iPLA_2$ in the TLR2-mediated programmed death of *Candida*-infected HeLa cells. Upon infection of HeLa cells with *C. albicans* our data demonstrate a time-dependent apoptosis of cells as determined by DNA ladder fragmentation, DAPI-nuclear staining and cell death detection ELISA (Figs. 20, 21 and 22). Apoptosis was evident after 12 hours of infection and continued to increase through 30 hours. Caspase-3 activation, which mediates the cleavage of multiple substrates to cause characteristic alteration that occur during apoptosis, was maximally elevated almost 6 hours, which means 6 hours before the onset of apoptosis (Fig. 22). Moreover, caspase-3-mediated apoptosis of HeLa cells followed the extrinsic pathway in which TLR2 triggers the signaling by recruitment of FADD containing protein (Fig. 26) and caspase-8.

The central observation in the host-pathogen interaction was initiated from host cell survival by inducing the inflammatory genes such as COX-2 [96] to host cell apoptosis. The time-course for COX-2 stimulation and apoptosis in HeLa cells were surprising. This led us to investigate various factors such as PLA_2s and TLR2, which also played a pivotal role in the COX-2 activation. During the investigation of caspase-dependent apoptosis, the most striking observation was the

time point for the onset of *Candida*-mediated apoptosis of host cells and the concomitant alterations in the expression of $iPLA_2$. Several lines of evidence suggested the involvement of $iPLA_2$ in triggering apoptosis. Thus, induction of apoptosis of U937 cells by anti-Fas antibody or by IL-1ß through the activation of inducible nitric oxide synthesis (iNOS) and 12-lipoxygenase (12-LOX) was suppressed by $iPLA_2$ inhibitor BEL [84, 182]. Moreover, $iPLA_2$ and $cPLA_2$ both have been shown to be activated and cleaved by caspase-3 during apoptosis [86]. Whereas the cleaved form of $cPLA_2$ was reported to act as dominant negative inhibitor of apoptosis, cleaved $iPLA_2$ activity was found to cause increased phospholipid turnover [85]. However, our data revealed primarily a time-dependent decrease in $iPLA_2$ expression (Fig. 14B and C and 27). Decrease of $cPLA_2$ expression was also observed in HeLa cells (Fig. 14A), which, however, did not affect the apoptotic process when inhibited by AACOCF3. In contrast, the inhibition of $iPLA_2$ by BEL abrogated the apoptosis (Fig. 31). The decline in apoptosis to basal level after 48 hours may be a result of the proliferation of a subpopulation of HeLa cells insensitive to *C. albicans*. Furthermore, gradual decline of $iPLA_2$ upto 18 hours was coupled with a concomitant increase in apoptosis for the same period. In order to verify if $iPLA_2$ is a genuine inhibitor of apoptosis, we preincubated cells with BEL, a specific inhibitor of $iPLA_2$, and followed apoptosis (Fig. 31 and 32). Indeed, time for the onset of apoptosis was reduced by 4 hours. Conversely, HeLa cells overexpressing $iPLA_2$ failed completely to exhibit any apoptosis upto 24 hours (Fig. 30). These findings present a clear-cut evidence for the regulatory role of $iPLA_2$ in *Candida*-induced apoptosis in HeLa cells. Recently, it was claimed that BEL-induced apoptosis in various cell types is not due to inhibition of $iPLA_2$, but due to inhibition of phosphatidic acid phosphohydrolase-1 (PAP-1) [157]. Our observations are in disagreement with this report, since BEL-induced apoptotic effect was not imitated by propranolol-treated cells, so that the involvement of PAP-1 as causative agent for apoptosis can be ruled out (Fig. 33).

The correlation between the diminution of $iPLA_2$ expression and increased caspase-3 like protease activity in apoptotic cells is intriguing. Recently, Ramanadham *et al*. [183] reported that the endoplasmic reticulum stress-induced apoptosis is associated with activation of $iPLA_2$ and caspase-3 activities, and that the latter protease cleaves $iPLA_2$ to a 62 kDa product that couples with nuclei. In this report the induction of apoptosis is attributed to activation of $iPLA_2$. Interestingly, the data presented in our study showed clearly the down-regulation of $iPLA_2$ and not its activation during apoptosis. In order to determine if $iPLA_2$ protein is cleaved by caspase-3 like protease activity to a protein fragment with increasing catalytic activity, we determined the

time-course of $iPLA_2$ activity in normal HeLa cells as well as in cells overexpressing $iPLA_2$ before challenging with *C. albicans*. As shown by $iPLA_2$ activity assay and western analysis in Figures 27, 28, and 29 neither catalytic activity could be detected in degradation products nor was a truncated form of $iPLA_2$ protein visible with western blot analysis.

TLRs have been identified in humans as critical component of innate immunity against microbial agents. For macrophages, antimicrobial defences are primarily based on recognition of TLR2, TLR4 and TLR6, depending upon the ligand nature [184]. Whereas deletion of TLR4 and TLR6 genes have been shown to alter a limited response to *C. albicans* in induction of TNFα [185], deletion of the TLR2 gene or neutralization with TLR2 antibodies of TLR2 receptors completely abolished the cell response [96, 185]. Induction of the TLR2 gene by *C. albicans* in our experiments was relatively specific, because the use of inhibitors of downstream signaling cascade, such as PI-3-K, $p38^{MAPK}$ or COX-1/2 failed to abolish the expression of TLR2 (Fig. 23). Moreover, neutralization of TLR2 by monoclonal antibodies against TLR2 prior to infection significantly inhibited apoptosis. To determine whether TLR2 gene activation is affected by *Candida albicans* infection, we transfected cells with TLR2-DN plasmid before infection. A complete inhibition of apoptosis was observed (Fig. 26B).

Next, we investigated the relationship between $iPLA_2$ activity and TLR2 gene expression. The $iPLA_2$ inhibitor BEL has been shown to attenuate significantly cell surface membrane changes [86]. It inhibited the binding of both IgM and annexin V to the cell surface membrane. Because neither cleavage of caspase-3 substrates nor nuclear condensation was attenuated, a general inhibition of apoptosis by BEL as a cause can be excluded [86]. On contrary, inhibition of $iPLA_2$ by BEL should be capable of upregulating TLR2 gene expression and promote apoptosis in *C. albicans*-infected cells. Indeed, in our experimental set-up, BEL caused an early onset of apoptosis (Figs. 31 and 32) and a significant increase in TLR2 mRNA expression (Fig. 34A). Conversely, overexpression of $iPLA_2$ abrogated TLR2 gene expression (Fig. 34B). Taken together, our results indicate that the downregulation of $iPLA_2$ activity is inevitably required for the transcriptional activation of TLR2 expression following infection with *C. albicans*. It seems unlikely that $iPLA_2$ would directly interact with TLR2 in *C. albicans*-infected cells. However, if we look at the mycobacterial infection of macrophages, we find that the induction of TLR2 transcription requires not only NF-κB and Sp1 elements within the TLR2 promoter region, but also chromatin remodeling [186, 187].

6. REFERENCES

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My curriculum vitae is not published for privacy reasons in the electronic version of my thesis.

8. LIST OF PUBLICATIONS

- **Nigam, S., Patabhiraman, S., Ciccoli, R.,** Ishdorj, G., **Schwarz, K., Petrucev, B., Kuhn, H., and Haeggstrom, J.Z. 2004. The rat leukocyte-type 12-lipoxygenase exhibits an intrinsic hepoxilin A3 synthase activity.** *J. Biol. Chem.* 279**: 29023-29030.**

- Ishdorj, G., **Deva, R., Singh, S., Ciccoli, R., and Nigam, S. Calcium-independent PLA_2 as key regulator of toll-like receptor 2-mediated apoptosis in *C. albicans*-infected HeLa cells. Submitted.**

- **Ciccoli, R., Sahi, S., Singh, S., Prakash, H., Zafiriou, M.P.,** Ishdorj, G., **Kock, J.L., and Nigam, S. 2005. Oxygenation by cyclooxygenase-2 (COX-2) of 3-Hydroxyeicosa-tertraenoic acid (3-HETE), a fungal mimetic of arachidonic acid, produces a cascade of novel bioactive 3-hydroxy-eicosanoids.** *Biochem. J.* 390**: 737-747. .**

- Ishdorj, G., **Singh, S., Deva, R., and Nigam, S. Transcriptional activation of TLR2 gene expression by *C.albicans* in HeLa cells is regulated by the interaction of p65 subunit of NF-κB and Histone Deacetylase. MS in preparation.**

- Ishdorj, G., **Urtnasan, D., and Badgaa, D. 1999. Breast milk fatty acid composition: a comparative study between breast milk and white goat milk.** *Scientific Report Chemistry and Chemical Technology Institute.* 10**: 72-75.**

POSTER PRESENTATION:

- Ishdorj, G., **Rybak, A.M., Ciccoli, R., Köhler, G., Agabian, N., and Nigam, S. Activation of COX-2 gene in vulvovaginal candidosis is triggered by *Candida* PLA_2: specific role of 14-3-3 protein. 2nd International Conference on Phospholipipases A_2 and 8^{th} International**

Congress on Platelet-Activating Factor and Related Lipid Mediators. Berlin, Germany. 6-9 October, 2004. Abstract No: P-30.

- **Nigam, S.,** Ishdorj, G., **Deva, R., Rüter, H., Ciccoli, R., Agabian, N. Role of candidal PLA_2 on the activation of TLR2 and COX-2 genes in HeLa cells infected with *C.albicans*. 2nd International Conference on Phospholipipases A_2 and 8th International Congress on Platelet-Activating Factor and Related Lipid Mediators. Berlin, Germany. 6-9 October, 2004. Abstract No: L-28.**

- **Theiss, S.,** Ishdorj, G., **Ketschmar, M., Nichterlein, T., Murillo, L.A., and Nigam, S., Agabian, N., and Köhler, G.A. Ablation of PLB5 gene in Candida albicans reduces cell-associated phospholipase A_2 activity and attenuates pathogenicity. 2nd International Conference on Phospholipipases A_2 and 8th International Congress on Platelet-Activating Factor and Related Lipid Mediators. Berlin, Germany. 6-9 October, 2004. Abstract No: P-08.**

- **Nigam, S., Deva, R.,** Ishdorj, G., **Ciccoli, R., Singh, S., Zafiriou, M.P., Lui, S., Baran, A., Dewitz, J., Sahi, S., Kothekar, V., Ivanov, I., Groza, N., Venter, P., Bareetseng, A., Kock, L., Van der Merwe, R.R., Stapelton, E.H., and Agabian, N. 3-hydroxyoxylipins, novel fungal fatty acid metabolites, in the analysis of candidiasis: host-pathogen-interactions. 9th International Conference on Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Related Diseases. San Francisco, California, USA. 11-14 September, 2005. Abstract No: L-82.**

- **Ciccoli, R., Sahi, S., Singh, S., Deva, R., Zafiriou, M.P.,** Ishdorj, G., **Kock, L., and Nigam, S. 3-hydroxyeicosanoids: novel bioactive compounds obtained from the interaction of 3-HETE, a fungal mimetic of AA, with COX-2. 9th International Conference on Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Related Diseases. San Francisco, California, USA. 11-14 September, 2005. Abstract No: P-149.**