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Metabolism of Arachidonic acid and formation of novel 3-Hydroxyoxylipins by *Candida albicans* and interaction of Hela cells-Candida albicans as a model for vulvovaginal candidiasis: redundancy of signaling pathways for activation of COX-2

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SUMMARY

Candida albicans is a pathogenic yeast responsible for causing infection in patients under immunosuppressive therapy, and is responsible for recurrent vulvovaginal candidiasis. Although, number of *C. albicans* derived intrinsic factors and the host factors have been found responsible for virulence and favouring the disease process, there is no antimycotic drug which proves out to be completely fungicidal, suppressing recurrent infections. This work deals with the study of *Candida albicans*-host cell interaction, to give an insight of the disease process, which could lead to new therapeutic interventions. Earlier studies on *D. uninucleata* revealed presence of a biologically active 3-HETE derived from exogenously fed Arachidonic acid. It was found, that it is not only important for its own life cycle, but also shows biological activity in human cells. We tried to find if a similar mechanism occurs in pathogenic fungi. It was found that although *C. albicans* does not contain arachidonic acid, it is able to grow on arachidonic acid as a sole carbon source as efficiently as it can utilise linoleic acid. Metabolism of arachidonic acid by *C. albicans* occurs both by mitochondrial dependent and independent pathways as shown by inhibitor studies. Arachidonic acid was shown to block the alternative oxidase pathway of energy generation in *Candida albicans*, and was efficiently metabolised to other fatty acids, carbohydrates, and proteins. Arachidonic acid metabolism by *Candida albicans* produced a novel compound 3,18-diHETE, which was closely related to previously discovered 3-HETE by Dr. S. Nigam's group. It was also observed that 3,18-diHETE was mainly associated with the cells bearing germ-tube and hyphael forms. This production was found to be aspirin sensitive. We found that *C. albicans* grown in aspirin showed diminished immunofluorescence with anti-3-OH-oxylipins antibody, indicating decreased amounts of 3,18 diHETE. Apart from this, there was diminished germ-tube formation, which is essential for infection, in *C. albicans* in presence of aspirin. Aspirin suppressed not only cell growth, but it also reduced the adhesion of *C. albicans* to the host cells. These observations prompted us to explore a therapeutic role for aspirin. When given along with clotrimazole, aspirin was found to reduce minimum inhibitory concentration (MIC) of clotrimazole against *C. albicans*. The action of aspirin was directed towards inhibiting 3-hydroxylation mechanism in *C. albicans* and of clotrimazole towards 18-hydroxylation mechanism and ergosterol synthesis, which occurs via cytochrome p450. This demonstrates a novel approach to potentiate the action of antimycotic drug clotrimazole. Moreover, aspirin is a cyclooxygenase-2 inhibitor, which inhibits formation of $PGE_{2,}$ and

thus the infectivity of *C. albicans*. Our results thus showed that aspirin may be a suitable drug for the therapy of recurrent Candidiasis.

It is known that host factors are also responsible for the establishment of infection. HDL, which is lowered in sepsis patients, and therefore substituted, was found to further increase virulence by inducing germtube formation in *C. albicans.*

The first step of infection includes adhesion of pathogen to host cells. *C. albicans* which adheres to host cells rapidly is found to be more virulent, and is able to proceeds further to establish the infection process. Our results demonstrate that aspirin is able to decrease the adhesion of *C. albicans* to HeLa cell, thus decreasing its virulence.

Upon infection with *C. albicans* signal transduction pathways are triggered in host cells (HeLa cells). We found upregulation of COX-2 in HeLa cells upon infection with *C. albicans* as well as increase in PGE_2 production. Not only *C. albicans* but also 3-HETE, upregulated COX-2, showing that 3-HETE was important compound which mediated cell signaling during infection. Using specific chemical inhibitors to various signal transduction pathways, it was established that Protein Kinase C (PKC) and p38 MAP kinase pathway were primarily involved in the COX-2 upregulation. In other systems, involving cytokines and LPS, control of COX-2 transcription involved ERK1/2 and JNK MAP kinase pathways. Further studies were performed to evaluate the exact sequence of activation of kinases involved. GF 203190X (PKC inhibitor) prevented the phosphorylation of p38 MAP kinase, while other inhibitors failed to have any effect. Thus, PKC acts as an upstream factor phosphorylating p38 MAP kinase. *C. albicans* induced p38 MAP kinase was also observed to be involved in modulating cytoskeletol changes host cell. This effect involved the activation, of phosphorylated HSP27, a factor involved in the modulation of actin, which was abrogated by p38 MAP kinase inhibitor SB 202190.We find that inhibition of free radicals, NAC inhibits COX-2 and PGE_2 upregulation suggesting involvement of reactive oxygen species and NFκB during the infection of HeLa cells with *C. albicans*.

The control of transcription occur by the various transcription factors binding to the concerned gene promoter. NFκB is one of the important transcription factors implicated in the COX-2 transcription. The role of NFκB was studied using a NFκB-dependent reporter plasmid and a dominant negative plasmid for IκB (prevents the release of active NFκB).

NFκB-dependent transcription was triggered upon infection with *C albicans*. However, this could be only partially abrogated by the IκB dominant negative plasmid, moreover PI3-kinase pathway is involved in COX-2 upregulation via NFκB pathway. Thus *C. albicans* brings about redundant signalling for the upregulation and control of COX-2 transcription.

Extensive programmed cell death (apoptosis) was observed after 24 h in Hela cells infected with *C. albicans* as shown by genomic DNA laddering and TUNEL assay. After 6 h postinfection, upregulation of caspase-3 activity was observed. Caspase-3 is an effector caspase which is involved in the cleavage of cellular proteins during apoptosis. Further more, using DNA microarray technology, we showed that *C. albicans* upregulated PI-3-kinase in HeLa cells. PI-3-kinase is a prominent mediator of anti-apoptotic functions via AKT and p65 NFκB. Its inhibition by wortmannin increased the caspase-3 activity in infected cells. IκB dominant negative transfected cells showed no caspase-3 activity upon infection. This result was surprising as p65 NFκB, whose release is prevented by the dominant negative plasmid, is an anti-apoptotic molecule. This paradoxical reaction was clarified by the observation of truncated forms of p65 NFκB in *C. albicans* infected HeLa cells. Carboxy terminal truncation of p65 NFκB by caspase-3 renders the molecule inactive thereby preventing its anti-apoptotic function. The pro-apoptotic subunits of NFκB, p50 and c-REL, however, were upregulated in *C. albicans* infected cells. Thus, the anti-apoptotic pathway, from PI-3-kinase via AKT to p65 NFκB is rendered ineffective by the cleavage of p65 NFκB by caspase-3.

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ABBREVIATIONS

3R-HETE	- 3R-Hydroxy-5,8,11,14-eicosatetraenoic Acid
3R,18-diHETE	- 3R,18-Dihydroxy-5,8,11,14-Eicosatetraenoic Acid
5-CFDA	- 5-Carboxy Fluorescein diacetate
AA	- Arachidonic acid
APS	- Ammonium Persulphate
BSA	- Bovine Serum Albumin
COX	- Cyclooxygenase
DABCO	- 1,4-DiAzaBiCyclo (2,2,2)-Octane
DMEM	- Dulbecco`s Modified Eagles Medium
DTT	- Di Thio Threitol
EDTA	- Ethylenediaminetetraacetic Acid
ELISA	- Enzyme Linked Immuno-Sorbent Assay
FCS	- Fetal Calf Serum
GC-MS	- Gas chromatography – Mass spectrometry
HDL	- High density lipoprotein
HPLC	- High pressure liquid chromatography
LA	- Linoleic Acid
MOI	-Multiplicity Of Infection
PAPC	- 1-palmitoyl-2-arachidonoyl phosphatidylcholine
PBS	- Phosphate Buffered Saline (Phosphate Buffer)
PCR	- Polymerase Chain Reaction
PMN	- Polymorphonuclear Leukocytes
RT-PCR	- Reverse Transcription – Polymerase Chain Reaction
SSC	- Sodium Chloride, sodium citrate
SHAM	- Salicylhydroxamic acid
TEMED	- N,N,N',N'-Tetramethylethylene-diamine
TLC	- Thin Layer Chromatography
Tris	- Tris(hydroxymethyl)aminomethane
UV	- Ultraviolet

INTRODUCTION

1.1. *Candida* and vulvovaginal infection

Candida is one of the most abundant fungal pathogens, belonging to phylum Ascomycetes; class Saccharomycetes, evoking fungal infections in humans. This fungus is found to colonise and infect a wide range of micro environments in the human body. Candida not only causes infections of skin, nails, oral or vaginal epithelium, but is also frequently involved in life-threatening infections. Candida is an opportunistic pathogen and causes nosocomial infections (disseminated candidiasis), in particular, in cancer patients under therapy or in immunocompromised individuals [1-6]. It also causes mucocutaneous infections, such as vulvovaginal candidiasis. Vaginal candidiais is found to be the most prevalent superficial fungal infection in women with AIDS, under oral contraceptives, antibiotics, corticosteroids, and with diabetes millitus. Symptomatic vaginal candidiasis presents with the symptoms, which include itching, burning, soreness, abnormal vaginal discharge, and dysparunia and signs that include vaginal and vulvar erythema [7]. Among the various species, *Candida albicans* is the most prevalent and accounts for approximately 75% of all infections in women during the child-bearing period [8]. Although *C. albicans* exists in the vagina of most of the women as an innocuous commensal organism with no apparent symptoms or clinical signs [9], in others, it causes a shear untreatable problem. Of the women diagnosed with an episode of sporadic vulvovaginal infection a significant percentage experience subsequent recurrent episodes of acute vulvovaginal infections.

Patients with recurrent infection are those having four episodes or more of infection per annum. The recurrent vaginitis occurs through frequent vaginal reinfection, or through vaginal relapse occurring due to incomplete clearance of organism caused by therapy with fungistatic antimycotics. The factors responsible for recurrent vaginal candidiasis are both organism and host-derived. Spontaneous changes occur in *C. albicans* either due to induction of antimicrobial resistance or due to induction of phenotypic switching along with dimorphism. These properties induce antigenic variation and make it more virulent in establishment of the disease. Change in commensal flora in vagina and changes in protecting mucosal immunity, makes host more susceptible to the infection due to changes in the vaginal environment. It has been known that infections of vagina by *C. albicans* are

dependent on the presence of reproductive hormones. Estrogen replacement therapy has been shown to enhance the susceptibility to infection. Fidel et al. demonstrated that estrogen plays an important role in the infection by enhancing on one hand the avidity of *C. albicans* to vaginal cells and on the other reducing the ability of vaginal cells to inhibit the growth of *C. albicans* [10]. Host defence mechanism to counteract Candida is mainly mediated by cells like PMNs, macrophage and natural killer cells. Other factors such as prostaglandins were found to be involved in candida infection. It was found that that mononuclear cells from the patients suffering with recurrent vaginal candidiasis produce higher PGE_2, compared to control women [11], Moreover, Kustimur demonstrated involvement of PGE_2 and leukotrine C_4 in the kidney damage induced by *C. albicans* [12]. These reports suggest an important role for host mediated response during infection.

1.2. Morphogenesis in *Candida albicans*

Morphogenetic changes in *C. albicans* are important virulence factors [8,13]. *C. albicans* is pleiomorphic, and undergoes reversible morphogenetic transitions between budding yeast form (blastospores), pseudohyphal, and hyphal forms [14, 15]. Besides the morphological heterogeneity at the cellular level, this pathogen exhibits variable colony morphologies, like “white” and “opaque” colonies [16, 17]. The pseudohyphae form ranges from relatively short to extended cells, while the hyphal forms possess constrictions at the septa [18, 19]. Hyphae are found during the early stage of tissue colonisation, where as yeast cells are found to be associated with diseased and necrotic tissues [8]. The formation of hyphae enhances adherence and tissue invasion. The asymptomatic carriers carry *C. albicans* in a small number and predominantly in a blastospore form rather then the hyphal forms. The ability to switch between these forms is thought to be important for *Candida's* virulence [20] and enables the pathogen to colonise different loci of the host, such as oral and vaginal tracts, and to invade the parenchyma of inner organs via the blood stream [21]. The enormous range of environmental factors have been shown to play a role in the phenotype switching. The factors which induce formation of hyphae include temperature >37°C, pH >6.5, high CO_2 to O_2 ratio, low inoculum (<10^6 blastoconidia per ml), presence of chemical factors like N-acetyl-D-glucosamine, glucose+glutamine, glucose+glycine, proline, low zinc concentration, dextrin and particular formulations of growth media include amino acid/salt medium, neopeptone-starch medium, TC199, and MEM medium. The preferred morphological phenotype thus can switch in response to some external

stimuli or stress conditions. No single environmental factor has yet been considered as the sole factor responsible for morphogenesis. The earlier studies showed that there are number of host derived factors *in vivo* like prostaglandin E_2 (PGE_2), gamma interferon [22, 11], estrogen [23], progesterone [24, 25], which are responsible for morphogenetic transformation in *C. albicans*. However, the exact mechanisms by which morphogenesis is regulated by host-derived mediators are far from clear [15] and is still a controversial and a stimulating area in fungal research. There are several aspects that support the role of Candida dimorphism, in virulence, adhesion, invasion and escape from phagocytic cells among others. Chaffin, et al. [26] showed that the different composition of the fungal cell wall in the yeast and the hyphal forms play a role in adherence to different cell surfaces. Moreover, the switch from to hyphal form of growth is important for the invasion of epithelial cells [27] and may provide a mechanism for draining blastospores into a major systemic bloodstream. Dimorphism also provides a pathway to efficiently escape from professional phagocytic cells, since the extrusion of hyphae damages the cellular membrane. The association between dimorphism and virulence has also been controversial. It was found that isolation of mutants deficient in their ability to switch on or switch off the dimorphic transition required different random mutagenic treatments. These mutants also differed considerably in there pathogenicity [28, 29, 30]. It was found that those mutants were less virulent than wild-type strains, however their phenotypes were very pleiotropic in nature. This demonstrated that several other phenotypic traits apart from morphogenetic change.

Figure (i). Morphogenesis in *C. albicans*

1.2.1. Regulation of morphogenesis at molecular level

Number of genes have been found to play an important role in the phenotype switching and in morphogenesis in *C. albicans.* Braun and Johnson [31] found that when the gene for both the copies of a transcriptional factor TUP1 is deleted in *C. albicans*, the cells grow exclusively in pseudohyphae form, showing the necessity of TUP1 transcription factor to maintain the yeast form. In addition, it was found that hyphal growth is blocked by inactivation of transcription factors belonging to mitogen-activated protein kinases (MAPK) and Ras-cAMP pathways (Cph1p and Efg1p) [32]. The switch to pseudohyphal growth requires a transcription factor encoded by the *STE12* gene, and proteins of mitogen-activated protein (MAP) kinase cascade including Ste7p (a homolog of MAP kinase kinase or MEK), Ste11p (a MEK kinase homolog), and Ste20p (a MEK kinase kinase) [33, 34]. The studies show that the serinie-threonine protein kinase Cst20p activates MAPK module containing the MAPK kinase HST7p and MAPK Cek1p, which in turn activate morphogenesis [35]. Stoldt, V. R et al. [36] demonstrated that the transcription factor Efg1p is involved in cell growth and morphogenesis and regulates development in fungi. *SIR2* gene shown to change the phenotype of colony of *C. albicans* [17]. In *C. albicans* the ability to change morphology from yeast to hyphae helps better establishment of infection and the variation in colony morphology helps to survive in uncertain environment.

1.3.1. Therapy

The etiology of recurrent vulvovaginal candidiasis is unknown. It is supposed to include treatment-resistant *Candida albicans*, frequent antibiotic therapy, contraceptive use, compromise of the immune system, hyperglycemia, as well as promiscuity. The present therapy includes the use of antimycotic topical agents such as nystatin and azoles. Imidazole antimycotics such as clotrimazole have been found to be effective in the treatment of Candidial vulvovaginitis. However, despite availability of antifungal drugs, the treatment is unsatisfactory due to frequent recurrence of infection and the drug resistance [37]. After the acute episode has been treated, subsequent prophylaxis (maintenance therapy) is important. Since, many patients experience recurrence once prophylaxis is discontinued, long-term therapy may be needed. Women with recurrent vulvovaginal infection despite avoiding potential causes of acute vaginitis, still experience repeated episodes of vaginitis. In women with idiopathic candidiasis, antifungal therapy

was found to be highly effective for individual attack. However, it failed to prevent future recurrence, and episodes of vaginitis have been seen to appear as early few days to 3 months after the cessation of therapy [38]. Species differences were also observed in the effectiveness of antifungal therapy, Polak [39] and Armstrong [40] demonstrated a significantly higher degree of success with *C. tropicalis* as compared with *C. albicans*.

1.3.2. Mechanism of action of antifungal drugs

Most of the antifungal drugs in common usage are directed in some way against ergosterol, which is the major sterol of the fungal plasma membrane. Ergosterol in the fungal membrane contributes to a variety of cellular functions like maintenance of fluidity and integrity of the membrane and the proper function of many membrane-bound enzymes, including chitin synthetase, which is important for proper cell growth and division [41,42]. The common class of antibiotics includes:

Polyenes:

The polyenes are a class of antifungal drugs which target membranes containing ergosterol. Amphotericin B and nystatin belong to this class of antibiotic. These drugs intercalate into membranes, forming a channel through which cellular components, especially potassium ions, leak and thereby destroying the proton gradient within the membrane [43, 44]. The specificity of amphotericin B for ergosterol-containing membranes may also be associated with phospholipid fatty acids and with the ratio of sterol to phospholipids. It has also been suggested that amphotericin B causes oxidative damage to the fungal plasma membrane [45]. Initially Amphotericin B was introduced for the systemic treatment of fungal infections. It can, however, be given both systemically and topically. It is found that infections with *C. albicans* resistant to flucytosine are treated effectively combination with Amphotericin B [46].

Ergosterol Biosynthesis Inhibitors

Several inhibitors of the ergosterol biosynthetic pathway have been developed for the use against clinically important fungi, which include allylamines and thiocarbamates, azoles, and morpholines. All of these drugs interact with enzymes involved in the synthesis of ergosterol from squalene. It is found that allylamines such as terbinafin bind to squalene epoxidase and inhibit ergosterol biosynthesis at an earlier level than azoles [47]. However,

the mode of action of these compounds is found to be more fungicidal rather then fungistatic. The allylamines (i.e., naftifine and terbinafine) and thiocarbamates (i.e., tolnaftate and tolciclate) inhibit the conversion of squalene to 2,3-oxidosqualene by the enzyme squalene epoxidase [48,49]. The azoles antibiotics, such as, ketoconazole, miconazole, fluconazole, itraconazole, voriconazole, are directed against lanosterol demethylase in the ergosterol pathway. This enzyme is a cytochrome P-450 enzyme containing a heme moiety in its active site [50, 51]. The morpholines, fenpropimorph and amorolfine, inhibit two enzymes in the ergosterol biosynthetic pathway, C-14 sterol reductase and C-8 sterol isomerase.

5-Flucytosine

5-Flucytosine (5-FC) has an entirely distinct mode of action from the azoles. 5-FC is taken up into the cell, and deaminated into 5-fluorouracil. 5-FU is apparentely converted by cellular pyrimidine-processing enzymes into 5-fluoro-dUMP (FdUMP). FdUMP is a specific inhibitor of thymidylate synthetase, an essential enzyme for DNA synthesis, 5-fluoro-UTP (FUTP) is incorporated into RNA, thus disrupting protein synthesis. 5-FC is fungus specific drugs, since mammalian cells have little or no cytosine deaminase [43,45].

1.3.3. Current antimycotics

Major chemical classes	Target/mechanism of action	Side effects
Polyenes	Complex with ergosterol, causes membrane disruption	Nephrotoxicity
Azoles	Inhibits cytochrome P450 51A1-mediated sterol 14-α–demethylation	Rare hepatoxicity, Gynaecomastia, adrenal insufficiency, teratogenicity.
Allylamines	Interference with RNA	Reduces renal toxicity of Amphotericin B
Morpholines	Inhibits δ–14-reductase and δ–7- δ–8–isomerase	Topical use of amorolfine can lead to transient burning sensation, erythema and pruritis
Benzofuranes	Inhibits guanine	Teratogenicity

Figure (ii) Current antimycotic drugs

Korting and Grundmann-Kollmann [42] suggested critical reduction of the availability of certain ions for the development of various antimycotics of the hydroxypyridone class, e.g encompassing ciclopiroxolamine and rilopirox. During the past few years Schaller, et al. [53] characterised secreted aspartic proteinase (Sap) as a major virulence factor of *C. albicans*. Sap plays a major role in both adhesion and penetration of *C. albicans* which is the first and most relevant step in pathogenesis. Drugs targeting to Sap, like pepstatin A, could be thus a potential antimycotic drug against *Candida.* Due to occurrence of resistance to antimycotics, *C. albicans* infections are difficult to treat. The current therapeutic strategy circumvents this problem by alternating cycles of treatment with different antimycotics, rather than treatment with a single antimycotic for prolonged period. This strategy has been found to retard the emergence resistances to some extent.

1.4.1. Role of lipids and Fatty acid metabolism in *C. albicans*

Lipids contribute toward important structural and functional molecules in *C. albicans*. Lipids constitute about 3.8-4.3 % of the dry weight of the fungal cells. Very little is known about the precise role of fatty acids for the growth and virulance of *C. albicans.* Fatty acids have been shown to modulate the fluidity and structure of plasma and other organelle membranes depending upon the composition of the phopsholipid head groups. Singh, et al [54] showed that alteration in lipid composition causes changes in the transport of amino acids like lysine, proline, glutamic acid and glycine. Number of cellular processes like cellular permeability, enzymatic activity, morphogenesis, and cell cycle are also influenced by fatty acids. Adherence, and virulence, which are important pathogenic factors, are found to dependent upon the lipid composition of *C. albicans* [55, 56, 57]. Goyal. and Khuller [58] showed variations in the lipid composition of yeast and mycelia forms in *Candida albicans*. They found that total lipid, phospholipid and sterol contents of log phase mycelial cells were significantly higher than in yeast cells. Moreover, the saturated fatty acid content is also much higher in the yeast forms. In general, yeasts are deficient in polyunsaturated fatty acids, but *Candida* species are rare exceptions as they contain often linoleic (18:2) and linolenic (18:3) acid.

1.4.2. Lipid composition in *C. albicans*

Lipids	Range (µg/mg of dry wt.)
Total lipids	105-175
Total Phospholipids	39-51
Total sterols	49-55
Triacylglyceride	9-11
Free fatty acids	1
Polar lipids (%composition)	
Phosphatidylcholine	25-56
Phosphatidylethannolamine	11-33
Phosphatidylinositol	5-20
Phosphatidylserine	9-24
Phosphatidic acid	9
Cardiolipin	8
Spingolipids	4-10
Sterols (% Composition)	
Ergesterol	14.6
Zymosterol	17
24, 28-Dihydroergosterol	42
3β-hydroxy 24- methylcholesterol	12
4,4-dimethylzymosterol	10
Fatty acids (% Composition)	
14:0	8-40
14:1	2-6
16.0	17-34
16:1	12-27
18:0	3-5
18:1	11-14
18:2	3-10
18:3	4-7

Figure (iii) Fatty acid composition of *c. albicans*

[Refs 58, 55]

1.4.3. Lipid and carbohydrate metabolism in *Candida albicans*

Candida albicans is able to utilise various carbon sources efficiently for the generation of energy. For instance, it utilises various monosaccharides, such as glucose, maltose but also other carbon sources like ethanol, saturated and lower unsaturated fatty acids. Fatty acid degradation by *C. albicans* is different from those in plants and mammals. It occurs via

different mechanisms: alpha oxidation leading to the formation of CO_2, beta oxidation leading to acetyl CoA, which is returned to citrate cycle [60, 61, 62], or omega-oxidation carried out by P450 cytochrome leading to the formation of fatty acids with a hydroxyl group as well as to the formation of dicarboxylic acid. Generally fatty acid in Candida species are degraded by peroxisomal beta-oxidation system. Beta-oxidation is a cyclic oxidation system of fatty acids in beta position. In general, initially fatty acyl CoA thioester undergo enzymatic dehydrogenation by acyl-CoA dehydrogenase to form trans-enoyl coA. The double bond of trans-enoyl coA is hydrated to form 3-hydroxyacyl-CoA by enzyme enoyl-CoA hydratase which 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 into fatty acid shorter by two carbon atoms. In yeast β–oxidation occurs in peroxisomes via series of reactions by a multienzyme complex MFE-2. The gene encoding the multifunctional protein of peroxisomal beta-oxidation was first discovered in Saccharomyces cerevisiae [59]. However, it is found that, non peroxisomal oxidation enzymes are induced when grown on alkanes [64]. This reaction occurs in endoplasmic reticulum and involes cytochrome P-450 monooxygenase [65]. This mechanism of fatty acid beta-oxidation involves FAD and FMN redox system, which catalyse insertion of one oxygen atom into the fatty acid.

1.5. Pathways for energy generation in *Candida albicans*

In mammalian cells glucose is metabolised by glycolysis followed by tri-carboxylic acid (TCA) cycle for the generation of ATP. However in lower organisms like yeasts there occurs enzymatic machinery for both respiratory and fermentative pathway for energy generation. In *C. albicans,* apart from all the key enzymes of citrate cycle, and pentosephosphate cycles, Lozinov, et al. found the activity of malate synthase, the enzyme which is involved in glyoxylate shunt in Candida [66]. Generally, glucose is broken down to pyruvate by glycolysis, and citric acid cycle for the generation of energy. However, when the culture is grown on complex substrates like alkanes, utilise the glyoxylate bypass. Isocitrate lyase cleaves isocitrate formed from citric acid to form succinate and glyoxylate. Succinate enters the pathway for biosynthesis of new sugar molecules. While Glyoxylate further condenses with another molecule of acetyl-CoA, to form malate by the action of malate synthase. Malate thus formed continues the cycle via oxaloacetate to citrate. In this way, by bypassing citric acid cycle, *Candida albicans* is able to utilise fatty acids or acetate in the form of acetyl-Co-A with net synthesis of carbohydrates.

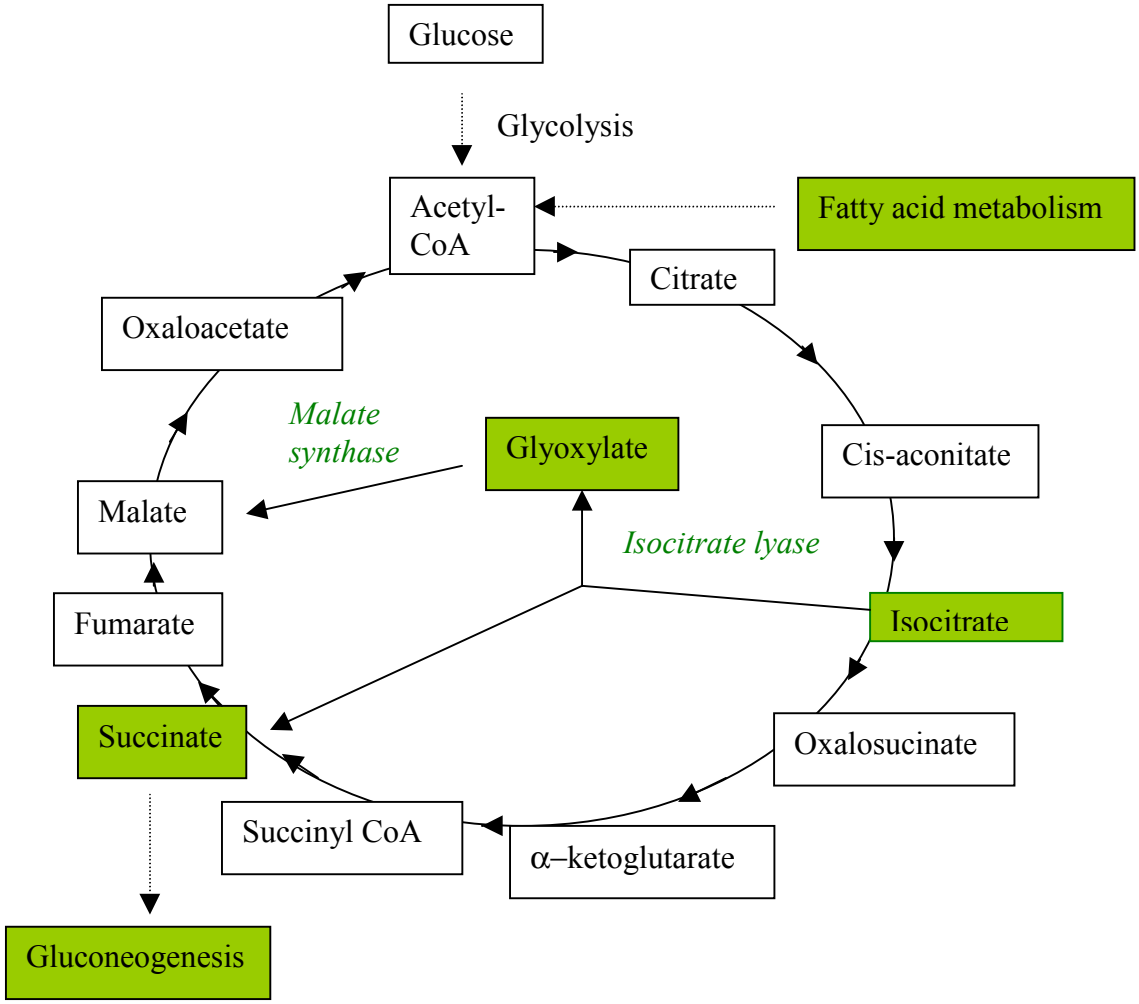


Figure (iv). The Glyoxylate cycle. The reaction shown are catalysed by the enzymes which efficiently utilise the breakdown product of fatty acid, Acetyl CoA, for the biosynthesis of sugar molecules.

For energy generation, *C. albicans* possesses the classical respiratory chain A sequence of biochemical reduction-oxidation reactions that effects the transfer of electrons through a series of carriers. An electron transport chain, also known as the respiratory chain, forms the final stage of aerobic respiration. In the mitochondria, NADH or $FADH_2$, generated by the Krebs cycle, transfer their electrons through a chain of carrier molecules, including ubiquinone and a series of cytochromes that undergo reversible reduction-oxidation reactions, accepting electrons and then donating them to the next carrier in the chain, a process known as electron flow. Cytochrome oxidase combines electrons and hydrogen ions with oxygen to form water. This process is coupled to the formation of ATP. It also possesses cyanide and antimycin A resistant pathway and is inhibited by salicylhydroxamic acid. Like components of the normal electron transport chain,

alternative oxidase, which reside in the mitochondrial inner membrane, transfers electrons directly to oxygen and in doing so bypasses the regular transportation through respiratory chain. The electron transfer reactions occur in mitochondria by transfer of electron from NADH to oxygen through various substrates and are coupled with ATP generation. The electrons are collected from many different cycles and transported to energy generation system through the action of NAD-linked dehydrogenases. These electrons are transferred form one substrate to another due to difference in oxido-reduction potentials which include complexes of cytochrome b and c_1, iron-sulphur proteins, and a complex of NADH dehydrogenase. Various inhibitors like antimycin A, rotenone and cyanide act by blocking the transfer of electrons through the respiratory chain.

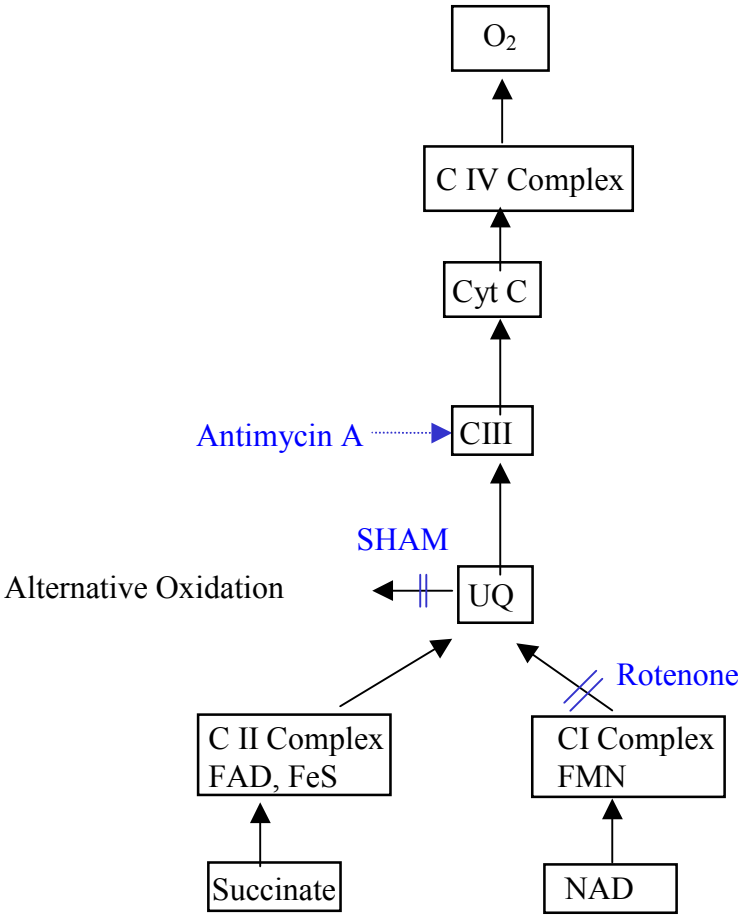


Figure (v). Schematic representation of electron transport chain

1.6. Host response to the infection

Infection by *C. albicans* is known to be accompanied by strong inflammatory reactions of host cells which are mediated via mannose and β-glucan receptors [67, 68, 69]. Castro et al. [70] studied that upon stimulation by *C. albicans* macrophages and human monocytes, release sizeable of arachidonic acid and are subsequently converted to lipoxygenase- and cyclooxygenase-derived eicosanoids. It was also demonstrated that upon infection, *C. albicans* induces monocytes to liberate proinflammatory cytokines like interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNFα), interleukin-6 (IL-6) and interleukin-8 (IL-8) [71]. In human endothelial cells subjected to *in vitro* invasion of *C. albicans*, stimulation of arachidonic acid metabolism leading to secretion of PGI_2 has been reported [72, 73]. Moreover, Kustimur [12] demonstrated that in infected mouse kidney a shift of the arachidonic acid metabolism to a preferential formation of LTC_4 via lipoxygenase pathway occurred. These examples accentuate a prominent role of host cell-derived arachidonic acid in the inflammatory events induced by *C. albicans* during infection. Although arachidonic acid does not occur in the lipids of *C. albicans* [55, 58], its release from host cells to a significant extent renders it to a potential exogenous modulator of cell growth and morphogenesis in *C. albicans.*

1.7. Cyclooxygenases (Prostaglandin synthases)

Prostglandins (PG) are shortlived substances derived from arachidonic acid that act as local hormones. Prostaglandins play an important in normal physiology by maintaining hemostatis acting as both vasodilatory agents and vasoconstrictors, renal function by altering both sodium excretion and water clearance and control of gastric functions. Using inhibitors of prostaglandin synthesis, it has also been shown that prostaglandins are involved in platelet aggregation (prostacylins and thromboxanes), pain, fever and respiratory functions such as, bronchoconstriction and bronchodilation. The role of prostaglandins in reproductive physiology is amongst the most important of its functions. Prostaglandins play a direct role in ovulation, lutenization, fertilization, fetal development and parturition as demonstrated in human and animal studies. [review 81]. In pathological conditions PGs are involved in diseases like arthritis, cancer, and inflammation mediating swelling and pain [review 76].

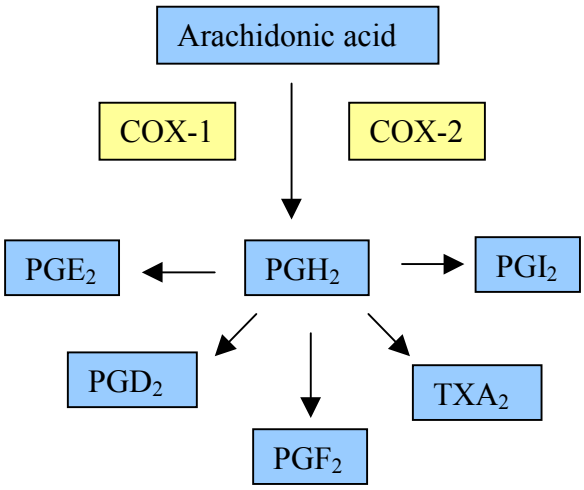


Figure (vi): The role of cyclooxygenase (COX) in arachidonic acid metabolism.
PG: Prostaglandin, Tx: Thromboxane.

First step in the production of prostaglandins is the liberation of arachidonic acid (AA) from phospholipids by phospholipase A_2. The free AA then acts as a substrate for the Prostaglandin synthases and is initially converted to unstable PGG_2, which is further converted to PGH_2 by the peroxidase activity of PGHS. PGH_2 is the common intermediate for all the prostaglandins and thromboxanes, which are formed by the action of the respective synthases [review 81]. Prostaglandin synthases, also known as cyclooxygenases (COXs) enzyme, occur in two forms: the inducible and the constitutive having similar affinity and capacity to convert arachidonic acid to prostaglandins [75, 76]. COX-1 the constitutive type is is expressed in all tissues under basal condition, in gastrointestinal tract, platelets, and endothelial cells [77] where as COX-2 is inducible. It is almost undetectable in most tissues but its expression is increased in pathological condition by external stimuli [78]. COX-2 (cyclooxygenase) was independently discovered by Herschman, H.R. and Simmons, D.L. [79, 80]. COX-2 is found to be induced by wide range of mitogens, tumor promoters and cytokines like interleukin 1β, IFNγ and TNFα [81]. Although, COX-2 was initially demonstrated in fibroblast cells [79, 80], it was found to be involved in the modulation of the inflammatory responses in monocytes and macrophage, associated with inflammatory bowel diseases, asthma, arthritis and human colorectal adenomas and adenocarinoma [81]. Under some circumstances modest

elevations of COX-1 could occur. For example, phorbol easter treatment can induce a modest elevation COX-1 along with substantial increase in COX-2 in bronchial epithelial cells [82, 83]. These data suggest that basal levels of COX-1 may differ as cell move through differentiation pathways, and may be altered as a consequence of molecular responses to cytokines and other agents modulating cell phenotype.

Upon stimulation, signals are initiated via both tyrosine kinase receptors and transmembrane receptors and activate a number of distinct signal transduction pathways mediated by protein kinase A, protein kinase C, JAK-STAT signalling mechanism etc. All these signaling pathways converge on the regulatory region of the COX-2 gene and influence the expression of COX-2 gene. Cloning and sequencing of the murine and human COX-2 promoter region demonstrated presence of binding sites for AP-2, SRE and NFκB, SP-1 transcription factors along with NFIL-6, CRE and E-Box sequences [84, 85]. There seems to exist a number of redundant pathways utilizing NFκB, ERK-2, p38 and JNK MAP kinase pathways in COX-2 upregualtion by endotoxins in monocytes [86]. In colon cancer cells, the expression of COX-2 was found to be regulated by p38 MAP kinase pathway, but not by ERK pathway was not found to be involved [87]. In murine astrocytes, Protein kinase C along with the MAP kinases upregulated COX-2 expression upon stimulation with IL-1beta [88]. Each cell system and type of stimulus appears to have a definite pathway for the expression of COX-2.

1.8. COX inhibitors

Non steroidal anti-inflammatery (NSAIDs) inhibit cyclooxygenase enzymes and thus are used as anti-inflammatory, analgesic, and anti-pyretic drugs. The pharmacological differences between COX-1 and COX-2 have been extensively explored and used for development of new drugs. Depending upon the ability of drugs to inhibit COX-2 and COX-1, these are classified into nonselective, selective and highly selective drugs. Aspirin, indomethacin, ibuprofen and diclofenec are nonselective COX inhibitors that inhibit both COX-2 and to an extent COX-1. Aspirin is a very potent inhibitor of COX-2 and is also able to inhibit at a low dosage COX-1 in platelets, is thus effective in cardiovascular medicine [89]. Treatment of patients with these drugs is found to reduce gastric perforations, ulcers, and gastrointestinal bleeding. Agents show great preference for COX-2 but are relatively ineffective towards COX-1. Animal studies show that these selective inhibitors are non-ulcerogenic and non nephrotoxic. Both acryl methyl sulfonyl DuP 697,

and NS398 inactivate COX-2 by binding to the enzyme in a noncovalent fashion that causes COX-2 induces a slow structural transition that results in its selective inactivation [91].

1.9. 3-Hydroxy-oxylipins

The oxygenated and hydroxy derivatives of long-chain fatty acids are collectively designated as oxylipins. A number of fungi and related organisms are found capable of synthesising sizeable amounts of arachidonic acid or other eicosanoid precursors [92, 93] and oxylipins. A large number of oxylipins have been discovered in fungal species and oomycetes [94-98]. Only a few groups in the world are dealing with fungal oxylipins. Oxylipins comprise not only of the eicosanoid but also the oxygenated compounds formed from other unsaturated fatty acids such as oleic, linoleic and linolenic acids. Since oxylipins seem to play a regulatory role for fungal growth, and the control of fungal diseases of plants and human is a basic problem in agriculture and medicine respectively, the research on these lipid mediators needs to be intensified.

In earlier work of the Berlin group together with a South African partner, a new arachidonic acid-derived metabolite has been uncovered, which is produced by the yeast *Dipodascopsis uninucleata*, and the structure of which has been established as 3(R)-hydroxy-5Z,8Z,11Z,14Z-eicosatetraenoic acid (3-HETE) [100]. Unlike the lipoxygenase-derived eicosanoids occurring in mammalian cells, such as 5-HETE, 12-HETE and 15-HETE, 3-HETE is a genuine hydroxylated derivative of arachidonic acid preserving its double bond positions and geometries. Later on, 3-HETE turned out to belong to a novel group of fungal oxylipins, the 3(R)-hydroxy-5Z,8Z-tetradecadienoic acid (3-HTDE) [100]. 3-HTDE is also formed from exogenous arachidonic acid by *Mucor genevensis*, which undergoes retroconversion to linoleic acid and finally oxidised to 3-HTDE [101]. It is found that the formation of 3-hydroxyoxylipins occurs primarily in the mitochondria of *Diposascopsois uninucleata* [107]. An immunofluorescence microscopic study provided ample evidence that in yeast *Dipodascopsis uninucleata* 3(R)-hydroxy-oxylipins serve as growth regulators during the sexual stage of the reproductive life cycle [109]. This conclusion follows from the fact that *in situ* these compounds selectively occurred in the gametangia, asci, as well as between the released ascospores [109, 110]. Moreover, acetylsalicylic acid, which suppresses fungal synthesis of 3(R)-hydroxy-oxylipins, also prevented the onset of the sexual stage of the life cyle of this yeast as well as the

concomitant release and the subsequent self-assembly of ascospores in orderly clusters [110]. Regulatory functions in the life cycle of fungi have also been proposed for other oxylipins [105].

In Berlin, also preliminary evidence was obtained for a putative role of 3(R)-hydroxy-oxylipins in fungal diseases of humans. Using a polyclonal antibody raised against 3-HETE a novel arachidonic acid metabolite was detected in the fungal pathogen *Candida albicans* and identified as 3,18-dihydroxy-5Z,8Z,11Z,14Z-eicosatetraenoicacid (3,18-diHETE) [102]. This observation merits particular attention owing to the fact that 3-HETE was shown to cause chemotaxis and to modulate cell signaling of human neutrophils [104]. Neutrophils and other phagocytosing cells constitute the main defense system of the organism against fungal pathogens. Therefore it is reasonable to assume that 3(R)-hydroxy-oxylipins may play a role in the host-pathogen interaction during the infection process.

Oxylipins play an important role in vegetative growth and sexual reproduction of yeast [100, 108, 109]. Dr. Nigam's group in Berlin and South Africa, screened a few hundreds strains of yeast and have demonstrated that 3(R) hydroxy-oxylipins are specifically observed in sexual reproductive stage of life cycle of *D. uninucleata.*

3-hydroxy oxylipins have also been investigated for potential biological activity in mammalian cells. 3-HETE has diverse spectrum of biological activities. 3-HETE has been observed to be a proinflammatory mediator Moreover, 3-HETE was found to be a strong chemotactic agent, the potency of which is comparable to LTB_4 or fMet-Leu-Phe. 3-HETE, however does not exert chemokinesis and exocytosis. It augments the release of arachidonic acid and platelet-activating factor (PAF) via. activation of phospholipase A_2. Cell signaling by 3-HETE seems to imply a G-protein-dependent process [112].

1.10. Adhesion

Attachment of microorganism to the human tissue is primary step for the establishment of infection and the development of disease. Adhesion of *Candida albicans* to the host tissue is considered and virulance factors in the development of disease and is one of the [113, 114]. The nature of the component which mediates adhesion is controversial. Some findings indicate that mannan, mannanproteins or polysaccharides are responsible for the adhesion. Other findings show aspartyl proteases and phospholipases relevant for adherence and invasion of host structures by pathogenic yeasts [115]. It is believed that the type of disease and the host response depend in part on the invasiveness of the strain of *C.*

albicans that is causing disease [56, 116]. Thus, phosphatidylinositol 3-kinase of *Candida albicans* has been demonstrated to affect the adhesion of *C. albicans* to the host tissue. The mutants depleted of PI-3 kinase gene have been shown to have decreased adherence and thus less pathogenic [117].

1.11.1 MAP kinase signaling pathway

Mitogen-activated protein kinases (MAP kinases) are enzymes which transmit signals from cell surface receptors to critical regulatory targets in the cells. These are activated by various environmental signals, such as growth factors and cytokines, and physical stress and play an important role in cell survival and adaptation to the new environment. These are also found to be involved in cell growth, and in the co-ordination of cell functions. The basic arrangement of the cascade includes a G-protein working upstream of a core module consisting of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK kinase (MAPKK), which in turn activates MAPK, this set-up provides not only the signal amplification, but, more importantly, additional regulatory interfaces that allow the kinetics, duration and amplitude of the activity to be tuned precisely.

The distinct classes of MAPKs, which have been identified include p42-p44 extracellular signal-regulated kinases (ERK MAPKs), Stress Activated Protein Kinases (SAPK) which include NH_2-terminal jun kinases (p46-p54 JNKs), and p38 MAPKs α, β, γ and δ. These kinases are induced by growth factors and cytokines. Each MAPKs are induced by different stimuli, and trigger specific biological response, whereby the specificity is maintained throughout the whole cascade. Each MAPK is specifically recognised and phosphorylated by map kinase kinase (MKKs). The MKK protein kinase has specificity towards Ser/Thr and Tyr residues on exogenous substrates. MKK themselves get activated by phosphorylation at Ser/Thr residues by various MAP kinase kinase kinases (MEKKs), which include Raf family members, c-Mos, MEK kinase (MEKKs), and multilineage protein kinase (MLKs). Thus, the diversity in the activation of MAPK pathway resides upstream of MKK, and the transmission of signal occurs via sequential protein kinases regulated by dual phosphorylation [118]. The overall sequence identity among JNK/SAPK, p38 MAPK, and ERKs is 40-45%, and all three enzymes have a common mechanism of activation through phosphorylation of Thr-X-Tyr motif [119].

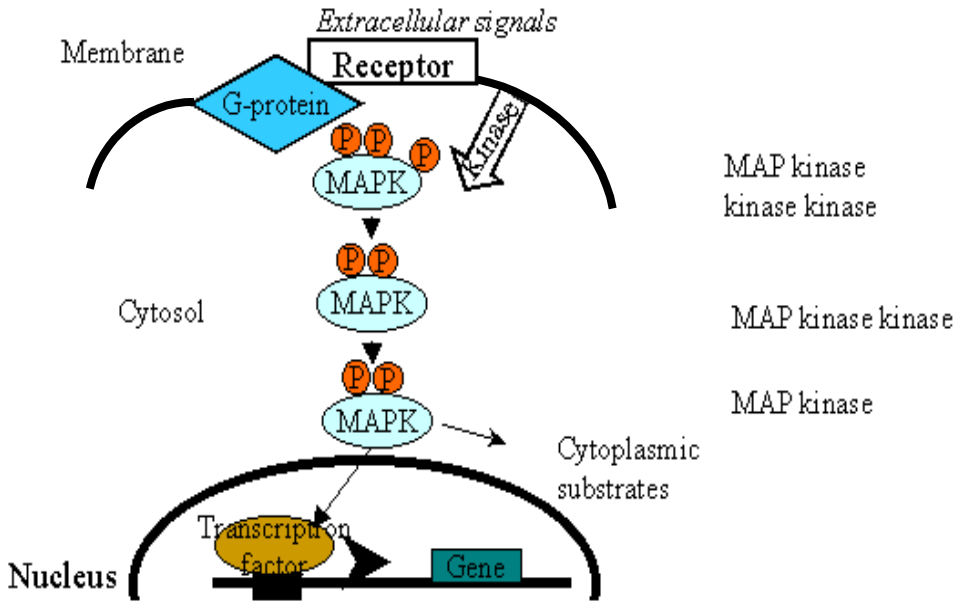


Figure (vii). Schematic representation of the structure of MAPK pathways

1.11.2. Specificity in MAPK activation and function

The control of diverse cellular function in response to extracellular stimuli by MAPKs implies a specificity for MAPKs activation and function. Activation of MAPK occurs by three different mechanisms, where mammalian protein physically intracts with ERK1 potentiating the ERK1 activation [120], or as suggested by Xia et al. [121] where MAPK activation occurs depending upon physical interactions between members of given cascade, for example JNK1/2 is bound by the N-terminal extension of MEK4 (JNKK1). Third mechanism found for signal amplification is based on the ability of MAPKs to regulate directly the expression of both ligands and inhibitors for cell surface receptors that feed into MAPK cascades [122].

1.12. ERK1/2 and MKK1/2 Pathway

Two components of ERK1/2 pathway, Ras and Raf, are proto-oncogenes and have a major role related to growth control in all its facets, including cell proliferation, transformation, differentiation and apoptosis. ERK1 and ERK2 and their upstream regulators MKK1 and MKK2 are stimulated by receptors for tyrosine kinases. Most of these stimuli activate Ras proteins by inducing the exchange of GDP with GTP, which converts Ras into its active conformation. Activated Ras functions as an adapter that binds to Raf kinases with high

affinity and causes their translocation to the cell membrane, where Raf activation takes place [123]. Mammals possess three Raf proteins: Raf-1, A-Raf and B-Raf. All three Raf isoforms share Ras as a common upstream activator and MEK as the only commonly accepted downstream substrate [124]. MEK is activated by phosphorylation of two serine residues in the activation loop. Although other kinases such as MEKK-1 (MEK kinase-1), mos or Tpl-2 can phosphorylate the same serines. It is predominantly done by most cell types by Raf kinases. Raf can activate both MEK-1 and MEK-2 (also called MKK-1 and MKK-2) with similar efficacy *in vitro*. MEK in turn activates ERK-1 and ERK-2 (also called p44 and p42 MAPK) via phosphorylation of a -Thr-Glu-Tyr- motif in the activation loop. Although biochemical and transfection experiments suggest that ERK-1 and ERK-2 are functionally equivalent, it is still unclear as to why two ERK genes exist.

1.13.1. Stress activated protein kinases and Stress-activated MAP Kinase Kinases

The stress activated protein kinases include NH2-terminal c-jun kinase (JNK) and p38 MAP kinase, which are ubiquitously expressed, and get activated in response to cellular stress, inflammation growth factors and lipopolysaccharides. Four different MKKs have been identified, which phosphorylate MAP kinases. Derijard et al. [125] and Raingeaurd et al. [126], found that MKK3 selectively phosphorylates p38 MAPK, while MKK6 which is closely related to MKK3, also phosphorylates p38 MAPK, although with a higher basal activity [127]. MKK4 and MKK7 are the regulators of JNK Stress activated protein kinases, while MKK1 and MKK2 phosphorylate ERK at N-terminus. The activation of these enzyme occurs by a dual phosphorylation at serine and threonine residues [128].There are two classes of Map kinase kinase kinases which include: MEK Kinase Kinase (MEKK) and Mixed Lineage Kinases (MLKs). Out of nine MKKs, JNK/SAPK pathways are regulated through MKK4. *In vitro* MEKK2 phosphorylates MKK1 and 4 while MEKK3 phosphorylates MKKs 1, 3, and 4 [129, 130].

1.14. Nuclear factor-κB

Upon infection or stress cells, try to adjust to the environment by bringing about the changes in the pattern of gene expression. Sixteen years ago Sen & Baltimore [131], first described NF-κB as a B-cell nuclear factor that bound a site in the immunoglobulin enhancer. These changes are controlled by the transcriptional factors, which are translocated from cytoplasm into the nucleus, and bind to there cognate site to activate or repress the transcription. Nuclear factor-κB NFκB is one such transcription factor. For a

long time the transcription factor nuclear factor κB (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 [132,133,134]. Endogenous activation of NF-κB is a cellular defence mechanism that protects cells by inducing survival genes, such as xIAP and $BCLx_L$ [133, 134]. There is as well the evidence for the independent upregulation of NFkB in apptosis [132]. NF-κB is a 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), c-Rel, 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) [135]. Direct phosphorylation of NF-kappaB itself is essential for its transcriptional activity [136]. 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 repeat core of IκBα) have been solved [137, 138]. In most cell types, 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 and tumour necrosis factor (TNF) activate NF-κB by inducing the rapid phosphorylation of IκB and its subsequent ubiquitination and proteolytic degradation. Released 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). The catalytic subunits, IKKα and IKKβ, and the regulatory IKKγ/NEMO (NF-κB essential modulator) subunit, form the prototypic core IB-kinase complex (IKC). Importantly, this complex serves as an intracellular point of convergence for distinct signals that ultimately activate NF-κB.

Although NF-κB is generally considered to be cytoplasmic in most cell types until stimulation with an inducer. Many cell types appear to have moderate levels of the p50 (NF-κB1) homodimer in nucleus. A role for this factor in constitutive-type transcription is not very clear, but it has been shown to serve to induce transcription of transcriptional repressor proteins [135, 137].

However, there is accumulating evidence that NF-kB is also subject to an IkB-independent level of regulation, as implicated by the earlier finding that the p38 mitogen-activated protein (MAP) kinase inhibitor SB203580 does not interfere with induced nuclear translocation and DNA binding of NF-κB, but significantly inhibits NF-κB-dependent gene expression. Modulation of transcription factor function by regulatory phosphorylations of the DNA-binding subunits is also observed in other inducible transcription factors, such as activating protein 1 (AP-1) and cyclic AMP-responsive-element binding protein (CREB).

1.15. Apoptosis

Apoptosis is a term which describes programmed cell death in vertebrates. The control of cell death could help physicians control ageing process and in diseases like ischemic heart disease and brain diseases [139]. The induction of apoptosis could help fight autoimmune disease and cancer [140]. Apoptosis involves number of proteins like Bcl-2 family, caspases, and mitochondrial proteins like cytochrome C. Bcl-2 protein blocks apoptosis and acts as survival agent [141], which is triggered by stumulus like growth factor deprivation and, irradiation. It is found that Bcl-xl, Bcl-2 and Bax can form channels in lipid bilayers [142, 143] and thus control the movement of other regulators of apoptotis that reside inside mitochondria. These proteins exerts pro or anti-apoptotic function by regulating the release of mitochondria to cytosol initiating cell death, however the exact mechanism of control of cytochrome C by the proteins of Bcl-2 family is not very clear [144]. A similar mechanism is observed with Bcl-xL which interacts with human CED-4 homologue Apaf-1 and suppresses its pro-apoptotic activity [145,146]. Proteases involved in apoptosis are collectively termed as caspases. Caspases are found to be involved in both the initial signaling events and downstream protolytic cleavages that characterise the apoptotic phenomenon [147]. These enzymes mediate cell death by cleavage of proteins are the effectors of apoptosis [148]. Apoptosis is marked with disruption of mitochondria and is a feature of apoptotic cell death. Disruption of mitochondria leads to the release of cytochrome c, from the intermembrane space after disruption of outer membrane [149]. Cytochrome c brings about activation of caspases.Caspase-9 is processed into an active enzyme when it combines with Apaf-1 in presence of cytochromec and either ATP or dATP [150]. However all apoptotic pathways are not cytochrome c dependent [151]. TNF-family death receptors directly induce apoptosis by by activating caspases without involving mitochondria [152]. But since caspases trigger changes in mitochondrial

permeablity, leading to release of cytochrome c, it indirectly involves mitochondria for apoptosis. Mechanism of induction of cell death also involves, cell surface death receptors [153]. Fas, the receptor for Fas ligand (FasL) is a member of TNF (tumor necrosis factor) family receptor. Binding of FasL to Fas induces apoptosis of Fas bearing cell [154].The Fas mediated pathway is important in T-cell selection and perephiral clonal deletion. Trail is found to be the member of TNF ligand family, and is shown to induce apoptosis.

One enigmatic molecule is NF-kB, which is a DNA-binding dimer of nuclear factor κB (NF-κB) is retained in the cytoplasm by interaction with the inhibitor of NF-κB protein (IκB) [156]. It has been shown to be an important transcription factor playing a role in and apoptosis [157]. Levkau et al demonstrated that caspase-3 brings about cleavage of p65 at COOH terminus and thus inactivating NFκB by generating truncated p65 which can still bind to the DNA, thus potentially acting as a dominant negative inhibitor by competing with the intact p65 [132]. There is ample data suggesting the therapeutic role of the study of cell death. It has been shown that the gene transfer in tumor cells initiate apoptosis, which could be the basis of therapy [158].

2. AIMS AND OBJECTIVE OF STUDY

The production of 3-HETE, an eicosanoid first reported by Dr. Nigam's group in collaboration with Dr. Kock's group in South Africa from spore bearing fungus *D. uninucleata*. fed with exogenous arachidonic acid was the beginning of a novel feild of research, so called 3-hydroxyoxylipins. Most striking aspect of synthesis from *D. uninucleata* was that it had to be supplied with AA exogenously, since most of the fungi do not contain fatty acid higher then LA (18:2) and its formation was aspirin sensitive. While screening of 3-OH-oxylipins in numerous fungi using antibody raised against 3-HETE, which recognised only only fatty acids which contain hydroxyl group at position C-3, Dr. Nigam's group demonstrated the presence of 3-OH oxylipins in *C. albicans*, which was later identified by us (section 4. Results) as 3,18-diHETE. The inflamed host tissue in vulvovaginal candidiasis has been shown to release huge amount of AA. We therefore hypothesized that this AA can be taken up by *C. albicans* and transformed to 3-OH-oxylipins, which in turn may have effects on one side related to morphogenesis of the attaching pathogen and on the other side host muscle cells to leading to immunomodulatory activation. With this concept of host–pathogen interaction

Objectives were:

1. to find whether there occurs release of arachidonic acid by the host cells during *Candida* infection, taking HeLa cells as model
2. to study the metabolism of arachidonic acid by *Candida albicans* and its ability to convert arachidonic acid into 3-hydroxyoxylipins or related compound
3. to study the role of 3-hydroxyoxylipins on the growth and morphology of *C. albicans*.
4. to investigate the biological effects of 3-hydroxyoxylipins on HeLa cells
5. to study the upregulation of *Candida albicans*-induced eicosanoid production
6. to study the mechanism of signal transduction in HeLa cells mediated by *C. albicans*
7. to use microarray analysis to find the pathways triggered by *C. albicans*

3. EXPERIMENTAL PROCEDURES

3.1. Materials

Fatty acids and Eicosanoids

Arachidonic acid, linoleic acid and PAPC- Sigma (Germany).

Inhibitors

Antimycin A- Sigma, SHAM- sigma, Rotenone SB212190, GF203190X, PD98059 – Calbiochem (Germany); NS398 – Cayman (USA), LY294002, Wortmannin- Biomol (Germany), Pyrrolidine Dithiocarbamate (PDTC) – Sigma (Germany).

Culture reagents

FCS, DMEM, Trypsin/EDTA, Streptomycin/Penicillin – Seromed (Germany); CHROMagar – Mast diagnostica, (Germany), Yeast Nitrogen Base, Yeast extract, Peptone, Malt Extract, Agar, Tryptone – Difco (USA).

Separation media

Agarose – Roth (Germany); Silica gel-60 TLC plates – Merck (Germany); Rotiphorese acrylamide Gel 30 – ROTH (Germany); C8 reverse phase HPLC column, C18 RPLC column – VDS Optilab (Germany).

Kits

RNeasy Mini Kit and QIA shredder - Qiagen (Germany); PGE_2 ELISA - Cayman Chemical Company (U.S.A); Protein Determination kit – Biorad (USA), ECL Detection kit – Santa Cruz (California, USA); Atlas Microarray kit – Clontech (USA), Luciferase Reporter Assay – Promega (Germany), PolyFect Transfection Kit- Qiagen (Germany).

Antibodies

Actin, phosphoserine – Sigma (Germany), COX-2 – Cayman (USA); phosphotyrosine – ICN (USA) and Upstate, Germany; JNK, p38 MAP Kinase, p42/44 Kinase, PI-3 Kinase, HSP-27, COX-1, BCL-2, anti-mouse, anti-rabbit, anti-goat secondary antibodies conjugated with horse raddish peroxidase Anti mouse-FITC – Santa Cruz, (Heidelberg, Germany)

RT-PCR reagents

Expand reverse transcriptase – Roche Biochemicals (Germany), Taq DNA Polymerase – AB Technologies (Germany)

Radiochemicals

^{14}C-Arachidonic acid (55 mCi/mmol) - Amersham (England).

Primers

All primers were purchased from TIB Biomol (Germany).

All organic solvents used in the experiments analysed by HPLC and GC-MS were of LiChrosolv or SupraSolv quality, all other reagents were of analytical grade.

Other reagents

5-CFDA- Calbiochem, Aspirin, Clotrimazole, High density lipoprotein (HDL), estrogen, progesterone, Gram staining reagents – Sigma, Scintillation fluid- dithiothreitol, bromophenol blue, glycerol, Ponceau were from Sigma, nitrocellulose membrane (Schleicher & Schuell), Luminol reagent-Amarsham, Protein A-Agarose- dianova, Germany), Reporter lysis buffer -Promega)

Enzymes

Proteinase K- Roche Biochemicals, Germany T4 DNA ligase-Gene Craft, Germany

Electrophoresis material

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

Solvents

All organic solvents used for TLC were of analytical grade and for HPLC and GC-MS analysis were of LiChrosolv or SupraSolv quality.

MEDIA COMPOSITIONS: All chemicals from GIBCO BRL.

Universal medium for yeast: YEAST EXTRACT 0.3%

Malt extract 0.3%

Peptone 0.5%

Glucose 1%

Agar 1.5%

pH 5.6

Sabourauds glucose broth :

Peptone 1%

Glucose 4%

pH 7.4

Inorganic salt medium:

KH_2PO_4 0.3%

$(NH_4)_2SO_4$ 0.025%

$CaCl_2$ 0.025%

$MgSO_4$ 0.025%

Biotin 0.01%

LB broth:

Tryptone 1%

Yeast extract 0.5%

NaCl 1%

pH 6.5

Tissue Culture Medium

DMEM 90ml

FCS 10 ml.

Penicillin

CHROM^R agar - Mast diagnostica, Reinfeld, Germany

3.2. Organisms and cell culture

Candida albicans 1386 (a clinical isolate from bronchomycosis) was obtained from "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (DSMZ), and a culture of *Candida albicans,* isolated and identified as *C. albicans* from the vaginal swab of a 34 year old women infected with recurrent severe vaginal candidiasis was used for all our studies. The cultures were maintained on universal medium for yeast containing 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 1% glucose in 1.5% agar, pH 5.6. The chemicals used for cell cultures were purchased from Difco Laboratories. HeLa cells were used as model, grown in DMEM supplemented with 10 % FCS and incubated at 37°C with 5% CO_2. The cells were split regularly, with a split ratio of 1:3 or 1:4, and passaged every 2-5 days. The cells were not grown over 100 % confluency. For most of the experiments cells were used just when they reached confluency. For microscopic examination the cells were grown on coverslip to 60-70 % confluency.

3.3. Isolation and identification of vaginal *C. albicans* from patients

Swabs from the vagina of the patients suffering form Candidiasis were collected in a tube containing 1% peptone and were immediately used for the isolation. The swab was swirled in the tube and the suspension was spun at 2000 X g for 20 min. The supernatant was discarded and the pellet was suspended aseptically in 50 µl sterile PBS. The suspension thus made was streaked on the Sabouraud agar plate. The colonies formed were examined microscopically for the yeast forms and the probable colonies of *C. albicans* were restreaked on CHROMagar^{R}. Green colonies, which could possibly be *C. albicans,* as recommended by the company, were picked up to check the capability for germ tube formation at 37°C in human serum within 4-6 hr. A loopful of culture from the isolate was inoculated in medium containing 1% peptone either with 1% glucose, sucrose, lactose or maltose in presence of phenol red as indicator to see the fermentation capability of the isolate and to confirm the isolate as *C albicans* biochemically.

3.4. Basal culture conditions for *C. albicans*

C. albicans was suspended in Sabouraud glucose broth containing 1% peptone giving an optical density of 0.165 at 490 nm corresponding to a protein concentration of approximately 0.05 mg/ml (Lowry method, Biorad kit), and grown in a rotary shaker at

100 rpm for 36 h at 37°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.

3.5. Preparation of Blastospores

The blastospores were used for inoculation into the medium for the study of morphogenesis or for inducing infection to HeLa cells. Blastospores were prepared by inoculating a single colony from the plate in Sabouraud glucose broth pH 5.6, and incubated for at 27°C for 48 hr followed by centrifugation at 2000 X g for 10 min.

3.6. Preparation of hyphal form

C. albicans was induced to form hyphae by inoculation in a tube containing 10 ml Dulbecco's Minimal Essential Medium (DMEM) in 15 mM HEPES buffer and incubation at 37°C for 48 hr. The cells were centrifuged at 3000 X g and the supernatant was replaced with the fresh medium and used for the experiments.

3.7. Determination of cell growth for the study of metabolism of different fatty acids as carbon source

Cells (10^3) were inoculated in 1 ml of carbon source free inorganic salt medium (ISM) containing 0.3% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.025% $CaCl_2$, 0.025% $MgSO_4$, and 0.001% biotin. To medium, various concentrations of arachidonic (1, 3, 10, 30 μM) or linoleic acids (1, 3, 10, 30 μM) were added followed by incubation at 37°C for 24 h under shaking. The cell number was determined by serial dilution of the culture and plating them out on Sabouraud agar plates. The number of colony forming units (CFU) was counted after 24 h. To study whether 3-HETE (1, 10 μM) stimulates the cell growth, similar experiments were carried out on the microtitre plate containing inorganic salt medium and 3-HETE as a sole carbon source. Medium (100μl) was inoculated with 10^3 *C. albicans.*

3.8. Metabolism of [1-^{14}C]-arachidonic acid

C. albicans was grown in 1 ml Sabouraud broth made in 100mM phosphate buffer pH 7.4. The cells were washed by centrifugation and suspended in 1.0 ml Sabouraud broth

containing 1% peptone without glucose followed by addition of 10 μM [1-^{14}C]-arachidonic acid (specific radioactivity = 50 μCi/mmol) and incubation at 37°C for 2, 4 and 6 h. Cells and supernatant were separated by centrifugation and were subjected to Bligh and Dyer [159] extraction at pH 3, with equal amount of chloroform: methanol (9:1) mixture. The lipid extracted was dried under inert nitrogen atmosphere and were subjected to thin layer chromatography (TLC) in solvent n-hexane: diethyl ether: acetic acid (50:50:1). The distribution of radioactivity was quantified by radioscanning (automatic TLC linear analyser, Berthold, Berlin, Germany) and scraping off of the labelled spots from the plates. Radioactivity was measured by addition of liquid scintillation cocktail (Packard) in a liquid scintillation counter (Beckman). The TLC Plates were also subjected to autoradiography using X-ray films.

3.9. Distribution of radioactivity derived form arachidonic acid into different biomolecules

C. albicans was grown for 36 h at 37°C in buffered Sabouraud broth. The cell suspension was transferred to the main compartment of a Warburg vessel, the inset of which contained 0.1 ml 5 M KOH. The closed Warburg vessel was shaken at 105 rpm for 5 h at 37°C and various components were separated and analysed for the presence of radioactivity. For determination of labelled CO_2 formation, the liquid of the inset was directly transferred to scintillation counting cocktail and measured in scintillation counter. The liquid of the main compartment was subjected to Bligh-Dyer extraction. The organic phase was evaporated to dryness under nitrogen and dissolved in a small volume of chloroform. The lipid extract was separated by thin-layer chromatography on pre-coated silica gel plates (Merck, Darmstadt, Germany) using the solvent systems n-hexane–diethylether–glacial acetic acid 60 : 40 : 1 or 50 : 50 : 1 (by vol.). For separation of phospholipids and glycolipids the solvent system chloroform–methanol–water 65 : 25 : 4 (by vol.) was applied. The aqueous phase of the Bligh-Dyer extraction was subjected to precipitation of proteins by 10% trichloroacetic acid (TCA). After centrifugation of the denatured proteins, the supernatant fluid was extracted with diethylether to remove TCA and used for analysis of carbohydrates, mono-, di- and oligosaccharides. Other low-molecular compounds were separated from the polysaccharides (mainly glucans and mannans) by extensive dialysis. The carbohydrates were concentrated under vacuum and separated by TLC on boric acid-impregnated silica gel plates using the solvent n-butanol–glacial acetic acid–diethylether–

water 9 : 6 : 3 : 1 (by vol.) and visualised with either 4-methoxybenzaldehyde–H_2SO_4 or N-(1-naphthyl)-ethylenediamine–H_2SO_4 reagents. Authentic trehalose, glucose and mannose were run as standards. The distribution of radioactivity was estimated in identically as described above for the lipids.

3.10. Alkaline hydrolysis of lipids

The total lipid extracts, obtained by the Bligh-Dyer method or single lipid fractions, separated by TLC, scraped off and re-extracted with methanol, were hydrolysed with 1 ml 5 M KOH–methanol 4 : 1 for 45 min at 60°C under inert nitrogen atmosphere. The mixture was then acidified to pH 3 and extracted with ethyl acetate. After drying the residue was analysed by TLC as described above.

3.11. Preparation of methyl esters of lipids

The diazomethane apparatus was kept on ice. Two ml of diethyl ether was poured into the bottom of the outer glass tube, and 133 mg of N-methyl-N'-nitro-N-nitrosoguanidine (sigma) in the inner flask. The reaction was started by addition of 1ml 10 M KOH dropwise through rubber septum. The diazomethane evolved got dissolved in diethyl ether within 15 min. This solution was stored at –20° C and used for methylation.

3.12. Preparation of lipid methyl esters and silyl derivatives of hydroxylipids

The fatty acid to be methylated was dried under nitrogen and methylation was carried out by addition of 50µl of methanol, and 300µl of etherial diazomethane solution, and incuabated in dark for 15 min at room temperature. Esterification was confirmed by a significantly higher migration distance of the corresponding lipid fraction in TLC than the parent compound. Arachidonic acid was used as positive control to check the end of the reaction. After the completion of the reaction, the solvent was evaporated and silylation was carried out using 30 µl BSTFA, 60°C for 5 min. The methyl-silyl derivative was used for GCMS analysis.

3.13. GC-MS analysis

The GC-MS system comprised a Varian Saturn 4D MS-MS equipment with a Supelco SPB-5 fused silica capillary column (30m × 0.25 mm inner diameter; 0.25 µm coating thickness) and a SPI injector. Helium was used as a carrier gas at a head pressure of 1×10^5 Pa. Other operating conditions were: ion trap 220°C, electron impact energy 70 eV and the multiplier voltage 2.0 kV. GC temperature parameters were: injector temperature programmed from 80° C to 270° C at 300° C × min^{-1}; initial column temperature, 130°C; initial time, 1 min; ramp rate, 6°C × min^{-1}; final temperature, 250°C; final time, 15 min; injection volume 1 µl.

3.14. Immunofluorescence microscopy of 3(R)-hydroxy-oxylipins

Antibody raised against 3-HETE was prepared as described previously [109] and used for the detection of 3(R)-hydroxy-oxylipins by means of immunofluorescence microscopy both *in situ* and in isolated lipid fractions of *C. albicans* in a similar way as described earlier for the studies with *Dipodascopsis uninucleata*. The cell smear was made on the lysine coated slides (DAKO) using cytospin at 200 rpm for 5 min. The cells were fixed by inserting the slides in ice cold methanol for 5 min followed by immersion in ice cold acetone for 30 sec. Cells were then treated with 30µl 3-OH antibody (1:200 diluted) for 30 min. in a humidified chamber at room temperature followed by three washes with PBS containing 0.05 % BSA. The slides were incubated in dark at room temperature with secondary antibody anti-rabbit-FITC for 30 min and washed three times with PBS containing 0.05% BSA, two times with PBS and once with saline. The coverslips were mounted in 0.21 M 1,4-diazobicyclo-[2.2.2]octane (DABCO) solution and visualised by immunofluorerescent microscopy.

3.15. Measurement of cell respiration

Oxygen uptake was recorded at 37°C with an Oxygen Meter Model 781 (Strathkelvin Instruments, Glasgow, UK) equipped with a micro Clark electrode 1302 in a thermostat-jacketed chamber of 400 µl. For the measurements cells were suspended in ISM (as described earlier) up to a concentration of 10^7 cells/ml and kept at room temperature while shaking throughout to avoid anaerobiosis before starting measurement. Cells (10^6) were

added to the chamber before inserting the Clark electrode and compounds dissolved in 2-methoxyethanol or methanol were injected by a micro-syringe.

3.16. Effect of HDL on germ tube formation in *C. albicans*

The morphogenesis of *C. albicans* was studied in the charcoal stripped serum as discribed by White and Larsen [160]. The serum was prepared from the blood of healthy donor and was treated with 10% activated charcoal at 37°C for 1 hr followed by centrifugation at 50,000 g for 30 min to remove charcoal. The experiment was performed in a 96-well plate. serum (100 µl) was added to each well together with HDL at concentrations of 10, 30, 100, 300 µM along with 10^6 *Candida albicans* blastospores and incubated for 4 h. Percent germ tube formation was determined using Neubauer's chamber.

3.17. Synergistic effect of aspirin on the minimum inhibitory concentration of *Candida albicans*

To determine the effect of aspirin on the minimum inhibitory concentration (MIC), each vaginal strain of *Candida albicans* was isolated using the double dilution method. The *Candida albicans* cells were grown in DMEM in 15mM HEPES (Gibco BRL). Clotrimazole was taken from the stock of 100 µg/ml using double dilution technique up to 0.75 µg/ml, followed by a serial dilution to obtain the final concentration of 1, 2, 3, 4, 6 µg/ml in 10 ml DMEM. To study the effect of aspirin on the inhibition of different isolates, along with clotrimazole 1mM aspirin was added. To each tube containing 10 ml of DMEM with and with out aspirin and clotrimazole, 10^6 Candida cells were added and incubated for 36 hr. The minimum concentration required for complete inhibition of *Candida albicans* was considered as minimum inhibitory concentration (MIC).

3.18. Effect of aspirin on germ tube formation in *C. albicans* and adhesion of *C. albicans* to HeLa cells

To study the effect of aspirin on germ tube formation, *C. albicans* was grown with and without aspirin in Sabouraud broth at 28°C for 36 hr. The yeast forms were then incubated in DMEM medium at 37°C for 6 hr. Cells were then microscopically observed and counted in Neubauer's chamber.

To study the effect of aspirin on adhesion of *C. albicans* to HeLa cells, HeLa cells were grown on coverslips in DMEM containing 10% FCS. Before starting the experiment, cells were washed with PBS, and fresh DMEM containing 1% FCS was added with or without 1mM aspirin in the wells containing coverslips. *C. albicans* of MOI 200 were added to it, and incubated for 1h. at 37°C in a humidified chamber with 5% CO_2. Cells were washed several times with PBS. For better visualisation, coverslips were treated with 1% periodic acid for 10 min and with Schiffs reagent for 10 min, and counterstained with haematoxylin for 10 mins. Number of cells per field were counted, with total 5 fields per coverslip.

3.19. Effect of *Candida albicans* on the release of arachidonic acid from HeLa cells

HeLa cells were grown in DMEM supplemented with 10 % FCS up to 70% confluence in 6 well plate. Confluent monolayer HeLa cells were labeled with 1 μCi [1-^{14}C]-AA for 18 h in DMEM supplemented with 1% FCS. Cells were washed and inoculated with *C. albicans* with MOI 5 for 6 h. After centrifugation at 2000 X g, the supernatant was collected and an aliquot was counted in scintillation counter (Beckman) for the release of radioactive arachidonic acid.

3.20. Release of Arachidonic acid by *Candida albicans* cell extract

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 lysis buffer (1.2M sorbitol, 10 mg/ml lyticase) at 37°C for 30 min. *C. albicans* cells were washed with PBS and broken mechanically using alumina. 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 seperated 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 labelled arachidonic acid.

3.21. Phospholipase A_2 assay

Pure substrates, 1-Palmitoyl-2-arachidonoyl phosphatidyl Choline (PAPC) and PAPC-OH prepared as described previously by Chaitidis et al. [161]. Each substrate at a concentration

of 25 μM, was dissolved in the assay buffer containing 10mM tris-HCl, pH 8, 180 mM NaCl, and 5mM $CaCl_2$ by sonication for one minute. *C. albicans* cells were broken down mechanically using alumina, and spun 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 incubated for one hr. Following extraction with ethyl acetate, fatty acids were analysed by HPLC using solvent methanol: acetonitrile: water 90:6:4 with 20 mM choline chloride on a reverse phase Nucleosil 100-5 C_8 column (Macherary and Nagel, Germany) by detection at 210nm for (AA), and 235nm for (15-HETE) using a diode-array detector (SPD-M10A, Shimazu, Japan).

3.22. Infection of HeLa cells with *C. albicans.*

C. albicans 1386 was grown as blastospores at 30°C for 36 h. Cells were centrifuged at 3000 X g and washed three times with PBS and suspended in DMEM in such a way that the MOI was 5. HeLa Cells (3×10^6) were taken as a monolayer in a 25 mm^2 flask and washed with PBS. Blastospores suspended in the DMEM were added to the HeLa cells and incubated for 6, 12, or 24 hr. The cells were washed three times with PBS and lysed using ice cold RIPA buffer containing PBS, 1% nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 1% SDS, 10 mM sodium orthovanadate, and 2mM, PMSF. The buffer lysed only HeLa cells leaving *C. albicans* unlysed. For the inhibitor studies, a stock solution was prepared and diluted appropriately. Preincubation with Hela cells was carried out for one hour with SB202109 at concentrations of 0.01-10 μM for inhibition of p38 MAPK, with GF203190X 10 μM, for inhibition of PKC, with PTDC 0.1-10μM, for the inhibition of free radicals, and NFκB, and with PD98059, 10μM for the inhibition of ERK1/2. COX-2 inhibitors NS398 and Indomethacin were used at a final concentration of 10 μM while dexamethasone at a final concentration of 1 μM was used. After preincubation, cells were infected with *C. albicans* for 6 hr. Cells were then lysed using RIPA buffer at 4°C and spun at 12000 X g The supernatant was collected and PMSF (2mM) sodium orthovanadate (10 mM) 20μg/ml, leupeptin, 2μM pepstatin A 20 μg/ml of aprotinin were added.

3.23. SDS-PAGE and Western blotting

The protein was resolved on 10 or 12% SDS-Polyacralymide gel electrophoresis (PAGE) [162]. For this, protein was denatured by heating at 100°C for 10 min in SDS loading

buffer containing 100 mM Tris-Cl (pH 6.8), 200 mM dithiothreitol, 4% SDS, 0.2 % bromophenol blue, 20 % glycerol (all chemicals from Sigma). Gel (12 %) were made by addition of 3.3 ml water, 4 ml 30% acrylamide solution containing 0.8% bisacrylamide (Roth, GmbH.), 2.5 ml Tris-HCl pH 8.8 (Sigma), 100 µl 10 % SDS, 100 µl 10 % ammonium persulphate (APS), and polymerized using 25 µl N, N, N´,N´-tertamethyl ethylenediamine (TEMED). The electrophoresis was peformed on a mini gel appratus (Biorad Inc.) at 20 mA in SDS PAGE buffer (0.1% SDS (Sigma), 25 mM Trizma Base, (Sigma), 250 mM glycine (Sigma), and was transferred on to nitrocellulose membrane (Schleicher & Schuell, Germany) in Tris-glycine buffer (39mM glycine, 48 mM Tris base, 0.037% SDS and 20% methanol) at 0.65 mA per cm^2 for 1h. The membrane were then stained in 0.1 Ponceau S. (Sigma) in 1% acetic acid (MERCK, Darmstadt), and washed with water to visualise the protein transfer on the membrane. The western blot was blocked overnight in 5% fat free milk powder in 0.5% tween-20, incubated with primary antibody COX-1 (Santa Cruz), COX-2 (Santa Cruz), p38 (Santa Cruz), ERK1, p42/44 (Santa Cruz) for 1-2 h, and rinsed with water 2 times followed by PBS containing 0.5% PBS for 15 min. The blots were then incubated with the appropriate anti-rabbit-HRP (Santa Cruz, Germany) or anti mouse-HRP (Santa Cruz) secondary antibody (1:2000 or 1:3000) for 45 min. After incubations with primary or secondary antibody, blots were washed three times, 15 min each using PBS containing 0.5% Tween 20. The blots were developed and visualised by Luminol reagent (Amersham, Braunschweig, germany).

3.24. Preparation of Nuclear protein

HeLa cells were infected with *C. albicans* for 6 h as described earlier. Nuclear protein was prepared as described by Dignam et al [163]. After the incubation, the cells were washed with ice cold PBS, scraped and pelleted at 1000 X g. The cells were resuspended in 400µl of hypotonic buffer (10mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10mM KCl, 1mM PMSF). The cells were lysed with addition of 100µl 2.5% NP40 on ice for 15 min. Nuclei were pelleted for 30 sec at 2000 X g and resuspended in 50 µl extraction buffer containing 20mM HEPES, pH 7.9, 0.48M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol and incubated on ice for 30 min. Following centrifugation for 10 min at 12000 X g, the supernatant was mixed with 50 µl of 20mM HEPES, pH 7.9, 0.1 mM KCl, 0.2 mM EDTA and 20% glycerol for further use.

3.25. Analysis of PI-3 kinase pathway during infection of HeLa cells with *C. albicans*

HeLa cells were grown as a monolayer in 25 cm^2 flask incubated with *C. albicans* for 6 hr in DMEM without FCS. Flasks were preincubated for 1 hr with PI-3 kinase Inhibitor wortmannin (100nM) (Biomol) and LY294002 (50 μM) (Biomol) before infection with *C. albicans*. After incubation cells were lysed using RIPA buffer and the protein was resolved on 8% SDS-PAGE. The Protein was transferred on the nitrocellulose membrane and probed with anti-PI-3 kinase antibody (Biomol, Germany), and secondary antimouse-HRP (Santa Cruz, Heidelberg). Proteins were visualised by Luminol reagent (Amersham, Germany).

3.26. Immunoprecipitation

Cells were infected with *C. albicans* as described earlier. After incubations, cells were washed with PBS and lysed with 1 ml ice cold RIPA buffer. The cellular debris was pelleted by centrifugation at 10,000 X g for 10 minutes at 4° C. Subsequently, PMSF (2mM), sodium orthovanadate (10 mM) 20μg/ml, leupeptin, 2μM pepstatin A and 20 μg/ml of aprotinin were addd. To 50μg of cellular protein, 2 μg of primary antibody against phosphotyrosine (Upstate biotechnology, USA and ICN, Germany) was added and incubated for 1 h at 4°C, followed by addition of 20 μl of Protein A-Agarose (Dianova, Germany) 4°C on a rocker platform or rotating device for 1 hour. Immunoprecipitates were collected by centrifugation at 1,000 X g for 5 minutes at 4°C. The aspirate was carefully removed and discarded. The pellets were washed thrice with RIPA buffer and after the final wash the protein was lysed in SDS buffer and analysed by western blotting using antibody against p38 MAPK and p42/44 ERK kinases (Santa Cruz, Germany).

3.27. Determination of PGE_2

HeLa cells were grown as a confluent monolayer in 6 well plate. Blastospores of *C. albicans* were added in such a way that multiplicity of infection (MOI) was 5. To study effect of inhibitors of MAPK, ERK1/2, PKC, free radical scavengers were preincubated for 30 min. Arachidonic acid (1μM) was added as a positive control and incubated for 4h. 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 –20°C till analysis of PGE_2 by ELISA

(Cayman). Three separate experiments were performed with three determinanations for each set up.

3.28. Isolation of RNA and identification of COX-2 by RT-PCR.

HeLa cells were incubated with or without *C. albicans* with MOI of 5 for 6, 12 and 24 hr at 37°C in a CO_2 incubator. Cells were lysed using RTL buffer and the total RNA was extracted using RNAeasy extraction kit (Qiagen). RNA (5 µg) was denatured at 65 ^{0}C for 10 min and immediately cooled on ice. The reverse transcription was performed in presence of 10 mM DTT, 1 mM dNTP, 2.5 µM $(dT)_{18}$ primer, 2.5 U RNAsin. The reaction was performed at 42 ^{0}C for 60 minutes. PCR amplification of 1 µl cDNA was carried out using forward primer 5' GTC ACA AGA TGG CAA AAT GCT G 3' and reverse 5' TAA GAT AAC ACT GCA GTG GCT C 3' in 1.5 mM $MgCl_2$, 200µM dNTPs and 1 unit of Taq polymerase (AB Technologies, Berlin, Germany) on Unoblock thermo cycler (Biometra, Göttingen, Germany). Initially the DNA was denatured at 94°C for 3 min, and 30 cycles of 94°C for one min, 52°C for one min and, 72°C for one min were carried out, followed by a final extention for 7 min. The PCR products were separated on 1% agarose gel and the DNA was stained using ethidium bromide.

3.29. Effect of *C. albicans* on actin cytoskeleton

The HeLa cells were grown on coverslips till 60% of them were confluent. Coverslips were then subjected to infection with *C. albicans* in DMEM with MOI of 5 in presence and absence of 1mM aspirin to study effect of SB 202109 on *C. albicans* induced changes in actin cytoskeleton. Cells were incubated with 10µM SB 202109 for 1h prior to infection with *C. albicans*. After 6 h of incubation at 37°C in humid 5% CO_2 atmosphere, cells were fixed using 4% paraformaldehyde for 20 min. The cells were then washed with PBS two times and incubated with 1:50 diluted monoclonal anti-actin antibody (Sigma Che Co.) in a humid chamber for 1 hr. The coverslips were washed three times with PBS containing 0.05 % BSA, followed by incubation with 1:2000 diluted secondary anti mouse-FITC antibody. After washing coverslips were mounted in 30 µl of DABCO and viewed under fluorescence microscope for changes in actin.

3.30. Effect of *C. albicans* on actin cytoskeleton

3.30.1 Labeling of *C. albicans* cells with 5-CFDA : *C. albicans* was grown in Sabouraud broth at 28°C for 24 h. Cells were washed by centrifugation and suspended in 25µM 5- Carboxy fluorescein (5-CFDA) in PBS pH 6.5 and incubated at 28°C for 10 h. Cells were then washed, centrifuged and resuspended in DMEM for further experiment.

3.30.2. Infection and assay for phagocytosis: HeLa cells were grown on heat sterilised coverslips in DMEM supplimented with 10 % FCS, till 60 % confluency was achieved.. The cells were washed 3 times. *C. albicans* at the MOI of 5 was added to the HeLa cells and was incubated for 6 h. The cells attached to the surface were removed by washing the cells with PBS without Ca^{++} and Mg^{++} with 50mM EDTA three times followed by three washes with PBS. The coverslips were visualised by immunofluorescence microscopy.

3.31. cDNA array

RNA was prepared from the cells using Qiagen RNeasy kit. The total RNA was then converted to the corresponding cDNA and labeled with Atlas Pure total RNA labeling system (Clontech, USA) using [α-^{32}P]dATP. Approximately 5 µg of total RNA was denatured at 65 ^{0}C along with CDS primer mix for 10 minutes. The primers were allowed to bind to the RNA by incubating the reaction mix at 50 ^{0}C for 2 minutes. After the completion of the 2 minute incubation, a master mix containing dNTPs (A labelling mix), [α-^{32}P]dATP (3,000 Ci/mmol), DTT and MMLV reverse transcriptase were added to the reaction mixture and the production of the labeled cDNA was performed at 50 ^{0}C for 25 minutes. The reaction was stopped using the termination mix provided with the kit. The labeled cDNA was purified from the unincorporated nucleotides using NucleoSpin columns (Macherey-Nagel, Germany). The labeling efficiency was determined by liquid scintillation counting. Probes with labeling efficiency of 5 x 10^8 cpm /µg were used for the hybridization.

The atlas array membrane was hybridized to the cDNA as per instructions of manufacturer. The membranes were pre-hybridized for 1 hour at 60 ^{0}C. In the meantime the cDNA probe was denatured at 100 ^{0}C for 5 minutes and snap cooled on ice. The denatured cDNA probe was added to the hybridization mix and hybridization with the membrane was performed at 60 ^{0}C for 16 h. Membranes were washed in the following order :

2x Sodium Chloride, sodium citrate (SSC) for 5 minutes at room temperature.

2x SSC + 0.1 % SDS at 60 ^{0}C for 15 minutes, 3 times.

0.5x SSC + 0.1 %SDS at 60 ^{0}C for 10 minutes.

The blot was then washed with 2x SSC (0.03M sodium citrate, 0.3M NaCl) for 5 minutes at room temperature and sealed in a plastic bag. The membranes were them exposed to Hyperfilm (Amersham, UK) at -70^{0}C. The spot intensities were quantified using TINA program and were normalised with the housekeeping gene spot intensities in the same membrane. The genes corresponding to the spots on the membrane were identified by matching the X-ray film with the provided template.

3.32. COX-2 Promoter construct

3.32.1 Designing of primers. Primers were designed for the promoter for Human prostaglandin H synthase (Cox-2) [85, 159] with minor modification in insertion of cleavage sites. The primers used were

GG**GGTACC**ACATTTAGCGTCCCTGCA (Kpn I site) (forward)

GG**AAGCTT**CGGACGTGCTCCT (HindIII). (reverse)

Kph1 and HindIII sites were inserted as underlined. The two sites were chosen for the insertion since they could be cloned directly into pGL3 Luciferase Reporter Vector.

3.32.2. PCR amplification

PCR amplification of the promoter region of Cox-2: PCR amplification was carried out using the primers as described above. The PCR was performed using 0.5 µl of genomic DNA in 1.5 mM $MgCl_2$, 200µM dNTPs and 1unit of Taq polymerase (AB Technologies, Berlin, Germany) on Unoblock thermo cycler (Biometra, Göttingen, Germany). Initially the DNA was denatured at 94°C for 3 min, and 35 cycles of 94°C for one min, 54°C for one min and, 72°C for one min, followed by a final extension for 7min. The PCR product were separated on 1% agarose gel, and the DNA was stained using ethidium bromide. The 1 Kb fragment was cut from the gel and the DNA was extracted from the gel using Nucleospin extract kit from Macherey Nagel (Germany).

3.32.3. Cloning into Luciferase Reporter Vector (pLUC)

The DNA was eluted and digested along with the vector pLUC, with KpnI (Gene Craft, Germany) in 50 mM Tris-HCL (pH 8.0), 10 mM $MgCl_2$ for 1 h followed by digestion with HindIII (Gibco BRL) in the reaction buffer 50 mM Tris-HCL (pH 8.0), 10 mM $MgCl_2$, 50

mM NaCl for 1 h and precipitated using $1/10^{th}$ volume of 3M sodium acetate, and 0.65 volumes of isopropanol. The DNA was precipitated by centrifugation at 4°C at 12000 x g for 20 min, washed with 80% ethanol and air dried. The DNA was resuspended in water and ligation was performed in a ligase buffer containing 1mM ATP and 10 Weiss units of T4 DNA ligase (Gene Craft, Germany) at 14°C for 16 h.

3.32.4. Transformation and screening of clone

The ligated plasmid DNA was subjected totransformation into chemically competent E. coli DH5α (Gibco BRL), and plated on LB plates containing ampicillin 50µg/ml and was incubated at 37°C for 24 hr. Positive colonies were picked up and cultured into fresh LB broth for plasmid extraction. For plasmid extraction, 3 ml of culture was grown overnight, centrifuged for 1 min. and suspended in 100 µl distilled water. Cells were lysed by boiling for 2 min. in 100 µl lysis buffer (1% SDS, 10mM EDTA, 0.1N NaOH), followed by addition of 50 µl of 1M $MgCl_2$, and 50µl 5M potassium acetate, mixed and kept on ice for another 2 min. Cells were then centrifuged for 2 min at 12000 X g. The supernatant was collected and the DNA was precipitated with 600 µl Isopropanol at 4°C. The pellet was washed with 70% ethanol, dried and resuspended in 50 µl Tris-EDTA containing 20µg/ml of RNAse A.

3.33. Transfections

3.33.1. Transient transfection of HeLa cells with COX-2, pNFκBp-Luc promoter and effect of *C. albicans* on the promoter activity

The day before transfection, $8X10^5$ HeLa cells were grown per well in 6 well plate, in DMEM supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (100 units/ml). Cells were incubated at 37°C in a humid 5% CO_2 incubator till the cells were about 60% confluent. Plasmid construct at a concentration of 1.5 µg dissolved in TE buffer, pH 8.0, was mixed by vortexing with DMEM without FCS to a volume of 100 µl. To this, 12 µl of PolyFect transfection reagent (QIAGEN) was mixed and vortexed for 10 sec. Samples were incubated for 10 min at room temperature for complex formation. While complex formation took place, the growth medium was gently aspirated from the wells and washed with PBS before addition of 1.5 ml DMEM containing 10% FCS. Six hundred µl of DMEM with 10 % FCS was then mixed with the complex and added to the cells in each

well. Cells were then incubated at 37°C and 5% CO_2 for 24-48 h for the gene expression. To investigate effect of *C. albicans* on HeLa cells, HeLa cells were infected with *C. albicans* (MOI=5) for 2 hr. Cells were then washed and lysed with reporter lysis buffer (Promega) The promoter activity was measured in a liquid scintillation counter.

3.33.2. Transfection of HeLa cells with NF-kB dominent negative plasmids

HeLa cells were grown per well in 6 well plate in DMEM supplemented with 10% FCS till 60% confluency was achieved. For transfection of pNFκB-Luc plasmid (CLONTECH) or with NFkB dominent negative DNA (kindly provided by Ghosh, S., Yale University, Connecticut, USA), plasmid at a concentration of 1.5µg was transfected into the cells using Polyfect reagent according manufacturer's instructions (QIAGEN). Cells were then incubated at 37° C and 5% CO_2 for 24 h. The cells were then infected with *C. albicans* with a multiplicity of infection (MOI) of 5 in 1% DMEM for a period of 6h. Luciferase activity was measured using luciferase substrate (Promega) to measure the upregulation of NF-κB activation by *C. albicans*. For investigating the effect of IKK dominant negative plasmid on COX-2, a ratio of 6:1 of dominant negative and reporter plasmids, was used to get a final concentration of 1.5µg of DNA, for each transfection.

3.34. TUNEL assay for apoptosis

HeLa cells were grown on coverslips and were infected with *C. albicans* for 6h with a MOI of 5 in DMEM containing 1% FCS. Cells were fixed using 4% Paraformaldehyde for 20 min at RT, washed with PBS, and permeabilized using 0.5% saponin in PBS at RT for 30 minutes. Coverslips were washed once and were incubated for 2 minutes in 1x cacodylate buffer containing 100 mM cacodylate, pH 6.8, 0.1 mM DTTg 150 U/ml TdT (terminal deoxynuclueotide transferase) and 0.5 µM dUTP-Biotin in a humidified chamber at 37°C for 30 min. Coverslips were washed 3 times with PBS and blocked by incubation in 2% BSA in PBS for 10 min at room temperature. The coverslips were then incubated with streptavidin-HRP conjugate in blocking buffer for 20 min at RT, followed by PBS wash and staining with TrueBlue (KPL labs) for 10 minutes at RT. After another wash cells were observed under microscope.

3.35. DNA Ladder assay for apoptosis

HeLa cells ($1.5x10^7$) were incubated with *C. albicans* for 6h. with a MOI of 5. Cells were then lysed by addition of 500µl hypotonic lysing buffer containing 10mM Tris, 1mM ethylenediaminetetraacetate (EDTA), 2%Triton X-100. pH 7.4) for 10 min. *C. albicans* was removed by centrifugation at 12,000 X g for 20 min. The supernatant was treated with RNAse (20µg/ml) at 50°C, followed by the treatment with proteinase K (100µg/ml) at 37°C. DNA was extracted using phenol-chloroform-isoamyl alcohol (50:49:1) three times followed by a chloroform wash. DNA seperation was performed by electrophoresis in 3% agarose gel. 1µM storosporin was added to HeLa cells as a positive control.

3.36. Caspase-3 assay

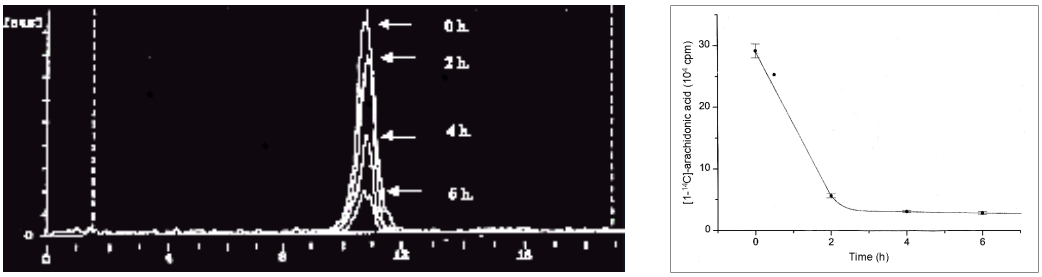
HeLa cells were incubated with *C. albicans* for 6h or for different time periods with a MOI of 5 in DMEM containing 1% FCS. Cells were lysed using lysis buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0,1 % CHAPS, 1 mM DTT, 100 µM EDTA) for 10 min on ice. The cells were then scraped and incubated in ice for 10 minutes, spun at 10,000 X g for 10 min. 100µg of the protein was used for the assay. The assay was performed using microtitre spectrophotometric measurement at 405 nm by incubation of cell lysates with 2 mM of AcDEVD-pNA as a substratein an assay buffer containing 50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 % CHAPS, 10 mM DTT, 100 µM EDTA. 10 % glycerol at 37°C. To see the effect of wortmannin, the cells were preincubated with 10µM of wortmannin for 1h prior to infection.

4. RESULTS

4.1 *C. albicans* is able to metabolize exogenous arachidonic acid.

Rationale : On the basis of previous experiments with D. *uninucleata*, it was known that fungi are capable of metabolizing exogenous arachidonic acid [99]. We, therefore, implicated that upon infection HeLa cells produce arachidonic acid which could be used by *C. albicans* as substrate for the metabolism.

Candida albicans was incubated with [1-^{14}C]-AA and the utilisation was studied by determining the reduction of [1-^{14}C]-AA with time.



(a) (b)

Figure. 1. Clearance of [1-^{14}C]-AA by *C. albicans*. The cells were spun down at 3000 × g for 5 min and resuspended in a Warburg vessel in 1.0 ml Sabouraud medium without glucose containing 10 µM [1-^{14}C]-AA and incubated for the time indicated at 37°C under shaking. Thereafter, the lipids were extracted according to Bligh and Dyer, and separated by TLC using the solvent system n-hexane–diethylether–acetic acid 60 : 40 : 1 (by vol.). The AA fraction was scraped off and quantified by liquid scintillation counting. The values are indicated as mean ± SD of three experiments.

As shown in Fig. 1a and 1b, the labelled fatty acid was cleared from the medium in a time-dependent manner. The reduction of arachidonic acid to a large extent was found within 6 h. of incubation with *Candida albicans.*

4.2. Metabolism of arachidonic acid by *C. albicans* occurs independent of mitochondrial pathway.

Rationale: Since *C. albicans* possesses different modes of energy generation, via mitochondria, via peroxisomes and by alternative oxidase pathway [63,165,166], and it was found to metabolize arachidonic acid (see above), the experiment was carried out to find the biochemical pathways involved in the metabolism of arachidonic acid.

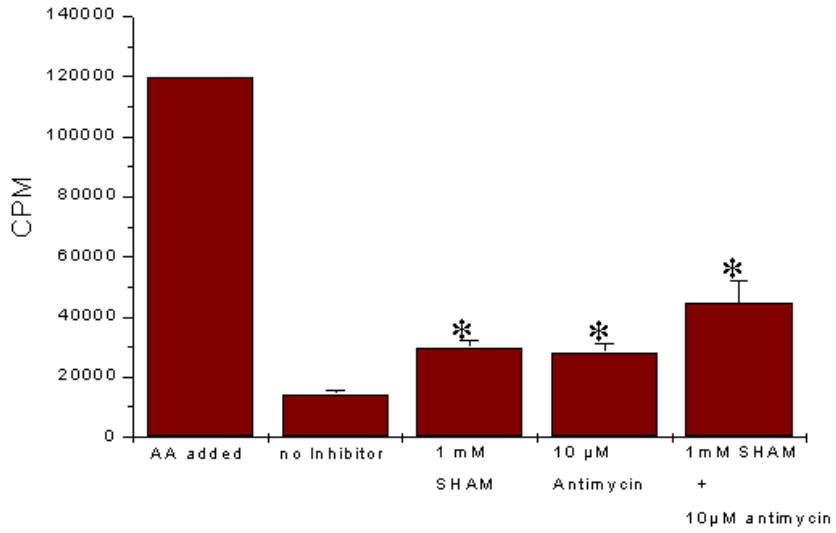
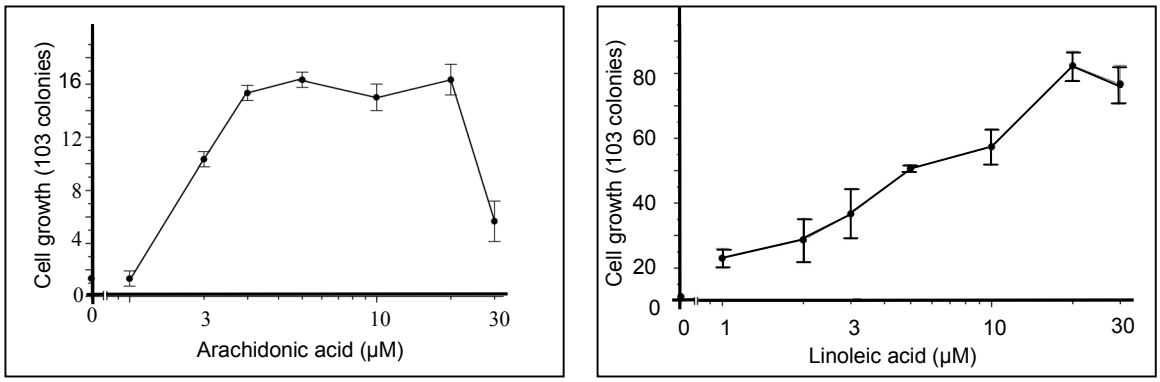


Figure 2. Effect of respiratory inhibitors on the metabolism of arachidonic acid by *C. albicans*. The Cells were fed with radioactive arachidonic acid in presence of 10µM antimycin A, a mitochondrial respiration inhibitor, or 1mM SHAM the inhibitor of alternative oxidase, or in combination of both. Data represent mean ± Standard Deviation of three separate experiments. Significance is given when P < 0.05 in comparision with control.

Candida albicans was grown in Sabouraud broth and incubated with (AA) for 6h. at 37°C in absence and presence of mitochondial respiratory inhibitors. The utilisation of [1-^{14}C]-AA by *C. albicans* was found to be partially inhibited by mitochondrial respiratory inhibitors antimycin and by the inhibitor Salicylhydroxamic acid (SHAM) for the alternative oxidase pathway as shown in Fig. 2. It is implicated that arachidonic acid is metabolised by *C. albicans* to a large part by non-mitochondrial pathways. This result is in line with the fact that in contrast to mammalian cells, fatty acids are degraded in yeasts to a large extent via the peroxisomal β-oxidation [63].

4.3. *C. albicans* is able to grow on arachidonic acid or linoleic acid as sole carbon source

Rationale: Since arachidonic acid was found to be metabolised by *C. albicans*, the experiment was carried out to see if *C. albicans* can grow in a carbon free medium containing only (AA) or (LA) as sole carbon source.



(a) **(b)**

Figure 3. Arachidonic acid (a) or linoleic acid (b) retains cell growth of *Candida albicans* when present as a sole carbon source. The yeast batches were spun down at 3000 × g for 5 min, and 10^3 cells were inoculated in inorganic salt medium in the presence of varying concentrations of arachidonic or linoleic acids. Cell growth was estimated by counting of colony forming units. The values are expressed as mean ± SD of three experiments.

To study the effect of AA on cell growth of *C. albicans*, the pathogen was grown as described in Sabouraud broth containing 1% peptone and 1% glucose till it reached the stationary phase in 36 h. It was then transferred to fresh broth containing inorganic salt medium with varying concentrations of AA as a sole carbon source, and incubated for another 24 h. No significant increase in the number of cell colonies on the plates occurred when AA was absent in the second culture period. Presence of arachidonic acid, however, enhanced the number of colonies by one order of magnitude in a dose-dependent manner (Fig. 3a). The growth-inducing effect of AA was observed above a threshold concentration of 1 μM and was found to be maximal at 3 μM. At concentrations higher than 20 μM, an inhibition of cell growth was observed, which might be due to the known cell-toxic action of free fatty acids on yeasts [167]. Cell growth was also induced when linoleic acid instead of AA was used as substrate (Fig. 3b). Linoleic acid, which is, in contrast to AA, also endogenously produced, was already active at a concentration as low as 1 μM and exhibited comparable effects with arachidonic acid up to 3 μM. Maximal stimulation of cell growth was, however, achieved at concentrations of linoleic acid as high as 20 μM. From these experiments we conclude that polyenoic fatty acids at micromolar concentrations are capable of maintaining cell growth of *C. albicans* independent of the presence of any other carbon source.

4.4 Arachidonic acid selectively inhibited the alternative oxidase of *C. albicans*

Rationale: The experiment was carried out to see the effect of exogenous arachidonic acid on the respiration, which leads to the generation of energy in *C. albicans*.

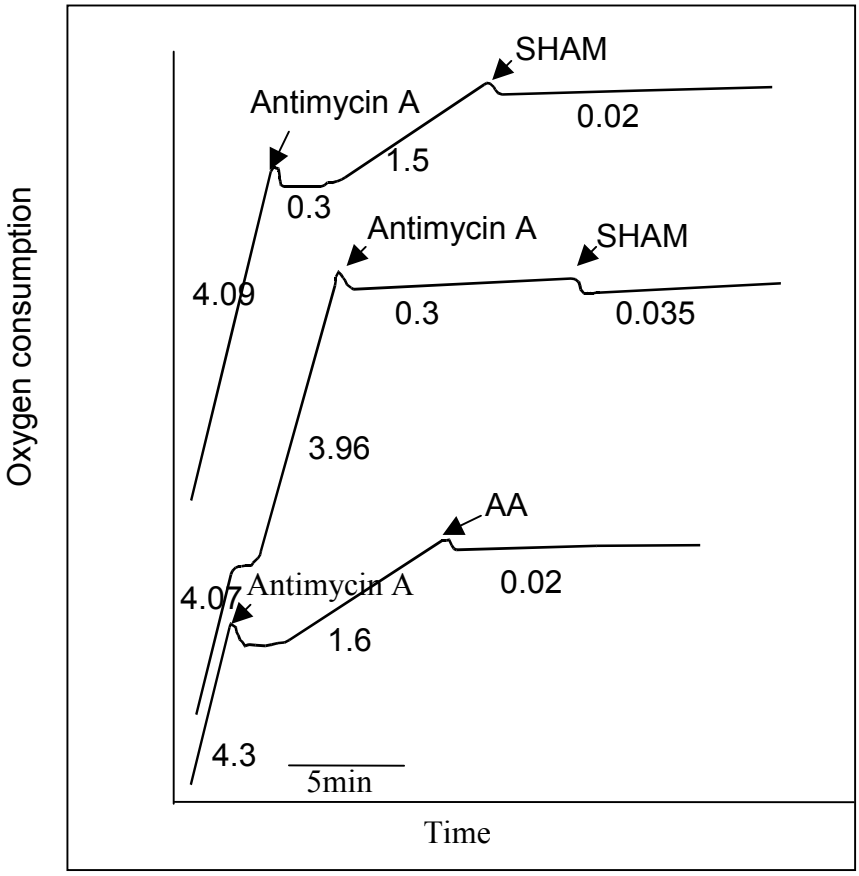


Figure 4. Action of arachidonic acid on cell respiration of *C. albicans*. The fungal batches were spun down at 3000 × g for 5 min and resuspended in inorganic salt medium (ISM) as described. For oxygraphic measurement, 0.1 ml cell suspension was added to 0.3 ml air-saturated ISM at 37°C, and the measuring chamber was closed with the Clark electrode. At time points indicated by the arrows, the agents (10 µM antimycin A, 1 mM salicylhydroxamic acid – SHAM, or 10 µM arachidonic acid – AA, final concentrations) were added. The values denoted on the traces refer to the slopes of oxygen uptake (expressed in nmol/10^6 cells × min) corrected for the base line. Traces A and B show the effects of antimycin A and SHAM in the absence (A) and presence (B) of AA, respectively. Trace C demonstrates the SHAM-like effect of AA on the antimycin A-resistant alternative oxidase.

To see the effect of exogenous AA on cell respiration of *C. albicans* by means of a micro Clark electrode, the cells were resuspended in an inorganic salt medium without exogenous substrate. The fungus at 37°C showed a high endogenous oxygen uptake of 4.1 ± 0.3

nmoles/10^6 cells × min (n = 3) which was linear for more than 10 min in the oxygraphic measuring chamber, and was retained for several hours when stored at room temperature under gentle shaking. AA, at concentrations up to 10 μM, did not affect this oxygen uptake which suggests that the mobilisation of substrates from endogenous sources is adequate for the full capacity of the respiratory system of the fungus. Addition of antimycin A, a specific inhibitor of the main pathway of the mitochondrial electron transfer chain, caused an instantaneous stop of oxygen uptake, which was partially reactivated after a short lag period (Fig. 4). This induction of antimycin A-resistant respiration in *C. albicans* has been reported earlier and is attributed to the ”alternative oxidase” (AOX) pathway [168], which by-passes the cytochrome chain of mitochondria and is inhibited by salicylhydroxamic acid (SHAM). It was observed that the antimycin A-resistant respiration of *C. albicans* was completely inhibited not only by 1 mM SHAM but also by 10 μM AA (Fig. 4). This inhibitory effect of AA was detectable at 2 μM and was complete at 5 μM . It occurred independent of whether AA was added before or after antimycin A (traces B and C in Fig. 4). Together with the lack of any effect on cell respiration in the absence of antimycin A, we conclude that AA selectively inhibits the alternative oxidase.

4.5. Analysis of the distribution of radioactivity from arachidonic acid by *C. albicans*

Rationale: The distribution of arachidonic acid which is metabolised by *C. albicans* was determined to study the metabolism pattern and the biochemical pathways involved during the metabolism of AA.

As shown in the table 1, the distribution of radioactivity between various compound classes formed from [1-^{14}C]-AA was carried out, For this purpose, the reaction samples were fractionated by Bligh-Dyer extraction for lipids followed by trichloroacetic acid precipitation in the aqueous phase to separate proteins from other polar compounds. In addition, the formation of labeled carbon dioxide was measured. The data are compiled in Table 1. Notably, only one-seventh of the radioactivity metabolised was found in CO_2, whereas two-thirds occurred in the fraction containing water-soluble compounds other than proteins.

Table 1. Distribution of radioactivity in various fractions following metabolism of [1-^{14}C]-AA by *C. albicans* [a].

Fraction	Radioactivity recovered (%) [b]
Carbon dioxide	13.1 ± 0.8
Proteins	13.4 ± 0.7
Carbohydrates	60.2 ± 2.4 [c]
Arachidonic acid	13.3 ± 1.6
Lipid fraction A	0.53 ± 0.07
Lipid fraction B	0.50 ± 0.06

[a] after 5 h incubation at 37°C; other conditions as in the experiments related to Fig. 3.

[b] mean values ± SD of three separate experiments

[c] more than 60% of the radioactivity of this fraction were found to permeate through a dialysis membrane

4.6. Analysis of radioactivity derived from arachidonic acid in the carbohydrate fraction of *C. albicans*

Rationale: Since most of the arachidonic acid was found to be incorporated into carbohydrate fraction of *C. albicans*, analysis was carried out to find out as to which carbohydrates are formed from the metabolism of arachidonic acid.

After extraction of lipids by Bligh and Dyer and removal of proteins, analyses showed that a major part of radioactivity was mainly attributed to carbohydrates. Extensive extraction of the fraction with diethylether after acidification to pH 3.0 failed to remove significant amounts of radioactivity. This observation ruled out a significant contribution of labelled acetate originating from β-oxidation of AA as well as of hydrophobic amino acids to the final reaction products. It was found that more than one-half of it was found to permeate through a dialysis membrane and consisted of mono- and oligosaccharides. In thin-layer chromatography (TLC) followed by autoradiography, defined labelled peaks were found which co-migrated with authentic trehalose and glucose plus mannose, respectively. These

carbohydrates have been reported to occur in *C. albicans*. Another part of the radioactivity of the aqueous non-protein fraction turned out to be non-dialysable, did not migrate in TLC and is believed to be due to glucans and mannans. The distribution of radioactivity after TLC separation of the aqueous non-protein fraction is shown in Table 2. The data in Table 2 and the subsequent experiments showed that, unlike in mammalian cells, only a minor part of AA is oxidised via citrate cycle and respiratory chain, whereas the major part is converted to carbohydrates via β-oxidation followed by the glyoxalate shunt of the citrate cycle. A significant portion of radioactivity was also found in the proteins which could arise from glycoproteins, peptide-bound glucoplastic amino acids or covalently acylated proteins.

Table 2. Distribution of radioactivity among major carbohydrates after TLC separation of the aqueous non-protein fraction following metabolism of [1-^{14}C]-AA by *C. albicans*.

Fraction	Radioactivity recovered (%)[a]
Polysaccharides	25.0 ± 4.0
Trehalose	42.3 ± 4.5
Glucose/mannose	17.7 ± 3.2
Unidentified (sum)	15.0 ± 4.4

[a] related to the sum of carbohydrates and other compounds in this fraction; mean values ± SD of three separate experiments.

4.7. Analysis of lipid fraction

Rationale: The AA is converted into various bioactive lipids by mammalian cells [75, 81]. It was previously discovered by our group that micro-organisms are able to convert AA into bioactive 3-hydroxy-oxylipins [99, 100, 109]. The lipid analysis was therefore carried out to see if similar products were formed by *C. albicans* from arachidonic acid

A small but significant amount of radioactivity recovered (approximately 1%) was formed in lipids other than non-metabolised AA. Separation of the lipids in various TLC solvent systems revealed that there was no incorporation of AA into either triacylglycerols or phospholipids. However, two other labeled fractions could be detected by autoradiography (Fig.5).

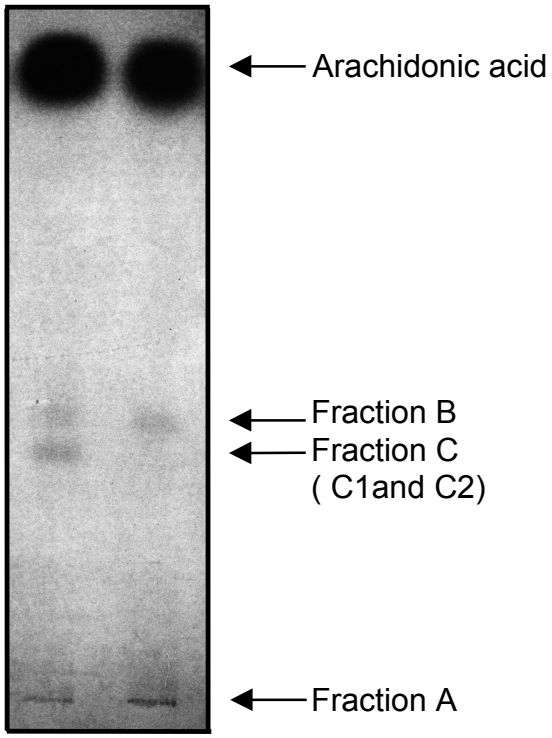


Figure 5. Autoradiography of the TLC of lipids formed from [1-^{14}C]-AA in *C. albicans*. The cells were treated in an identical manner as in the experiments in Fig. 3, except that the solvent system n-hexane–diethylether–acetic acid 50 : 50 : 1 (by vol.) was used. Representative separations are shown for the metabolism of AA in the presence (lane 1) and absence (lane 2) of 1 mM salicylic acid. For characteristics of the fractions A, B and C, see Table 3.

These fractions were characterised with respect to their stability against alkaline hydrolysis and to their ability to react with diazomethane in order to test for the presence of a free carboxylic group. Moreover, the fractions were examined for immunoreactivity with an antibody raised against 3-HETE that revealed group-specificity against 3(*R*)-hydroxy fatty acids of different structures [109]. The data are summarised in Table 3. It was found that the non migrating fraction A showed a strong immunofluoresence with anti 3-OH antibody as shown in Fig. 6. while other fractions did not show the strong immunoreactivity. Fraction B was found to be cleaved upon alkaline hydrolysis and did not react with

diazomethane, where fraction C was found to consist two compoounds one of which was stable to alkaline hydrolysis while the other got cleaved. Both fraction B and C did not show immunoreactivity with antibody against 3-OH group.

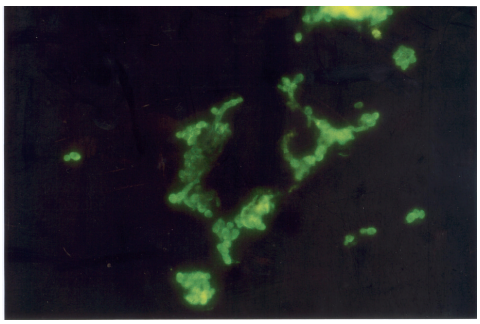


Figure. 6. Immunofluoresence with fraction A. *C. albicans* was incubated with arachidonic acid. Lipids were extracted by Bligh and Dyer method and seperated on TLC. The lipid were scraped form TLC plate re-extracted and were stained using anti 3-HETE antibody and secondary antibody conjugated with FITC.

Remarkably, the pattern of lipophilic AA metabolites was affected by salicylic acid, an inhibitor of 3(*R*)-oxylipin synthesis in yeasts (Table 4), which is consistent with earlier report for formation by *C. albicans* of a metabolite of this compound class.

4.8. Carbon derived from arachidonic acid, gets incorporated into monoacylglycerols in *C. albicans*

Rationale: To analyse the arachidonic acid derived salicylic acid- resistant lipid fraction in *C. albicans* and its identification.

The salicylic acid-resistant part of the radioactivity was found to be due to the formation of monoacylglycerol(s), as shown in Fig. 7. The compound was found to be cleaved by alkaline hydrolysis. When it was subjected to HPLC in solvent methanol: water: acetic acid 80:20:0.02, it was found to have the same retention time with authentic monoacylglycerol, for further confirmation co-chromatography was performed with the standard.

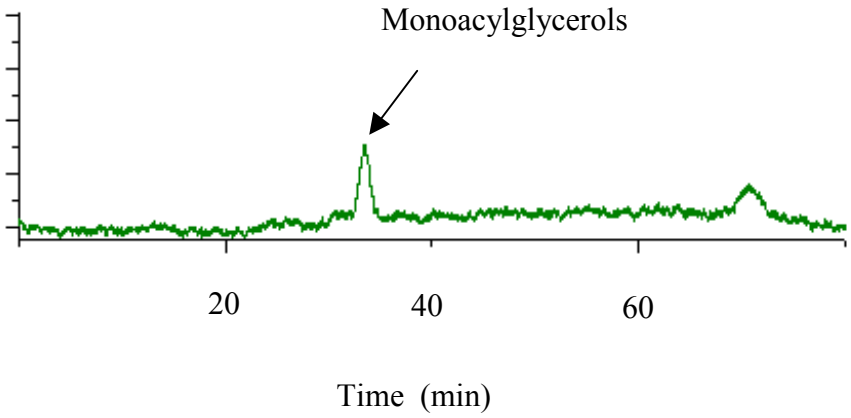


Figure. 7. HPLC profile of arachidonic acid metabolite formed by *Candida albicans*. The Compound B was subjected to analysis on HPLC using solvent methanol: water: acetic acid 80:20:0.02

Table. 3. Characteristics of lipophilic arachidonic acid (AA) metabolites of *C. albicans*

Fraction on (TLC)[b]	Rf value[b]	Alkaline hydrolysis	Reaction with diazomet-hane[c]	Reaction with antibody against 3-HETE[d]	Effect of salicyl-ate	Propose structure
A	0.00	Stable				3,18-diHETE
A	0.00	Cleaved to AA and a polar fraction	-	-	-	Glycolipids (phospholip-ids)
B	0.38	Cleaved to AA and a polar fraction	-	-	-	Monoacylglycerols[e]
C	0.34	Stable		-		A metabolite.
C	0.34	Stable	-	-		

[a] according to Fig. 5, [b] solvent: n-hexane–diethylether–acetic acid 50:50:0.1 (by vol.)

[c] as judged from shift to higher migration distance, [d] assessed by immunofluorescence assay, [e] identified by co-chromatography with an authentic standard in TLC and reverse phase HPLC (methanol–water–acetic acid 80:20:0.02, by vol.)

Table 4. Effect of salicylic acid on the incorporation of radioactivity from [1-^{14}C]-AA into lipid metabolites other than free AA

Presence of salicylic acid (1mM)	Alkaline hydrolysis of total lipid extract	Radioactivity dpm×10^6	%
-	-	4.86 ±0.96	100
-	+	1.93 ±0.21	39.7 ±4.0
+	-	1.73 ±0.51	35.5 ±3.5
+	+	0.28 ± 0.13	6.5 ±4.3

[a]mean values ±SD of three separate experiments corrected for blank

AA was metabolised by *C. albicans* and was seen in two fractions (Fig. 5). To characterize these compounds, *C. albicans* was grown in presence of 1mM salicylic acid. The non migrating fraction separated on TLC in solvent n-hexane: diethylether: acetic acid (50:50:1) was found to be aspirin sensitive, (Table 4) upon alkaline hydrolysis of this fractions, it was found to contain two classes of compounds one that was could be alkaline hydrolised and the other which was resistant to it as shown in table 4. The aspirin-sensitive non migrating fraction reacted with diazomethane and was identified as 3, 18-diHETE. This fraction also consisted of phospholipids which could be alkaline hydrolysed. (as shown in table 3). The aspirin sensitive nature of compound was in consistent with earlier bioactive compound 3-HETE identified in *D. uninucleata* [99].

4.9. Presence of alternative carbon source does not significantly alter the pattern of products formed form AA.

Rationale: The experiment was carried out to determine whether a simple carbon source like glucose, which is readily taken up by *C. albicans,* has influence on the uptake of the arachidonic acid and the formation of the lipid metabolites.

C. albicans was incubated with [1-^{14}C]-arachidonic acid in Warburg's chamber for 6h and the incorporation of radioactivity into various biomolecules was analysed. A parallel experiment was performed with a similar set up with an addition of glucose along with arachidonic acid. As shown in table 5 presence of glucose in the medium did not significantly alter the pattern of products formed from [1-^{14}C]-AA except that the formation of $^{14}CO_2$ was diminished by about 50%.

Table. 5. Radioactivity distribution from arachidonic acid in presence and absence of glucose.

	Carbon-dioxide	Protein	Non Protein	AA	Fraction A	Fraction B
Glucose -	12%	15%	57%	15%	0.5%	0.5%
Glucose +	6%	13%	68%	12%	0.67%	0.3%

After 6 h. of incubation at 37°C.

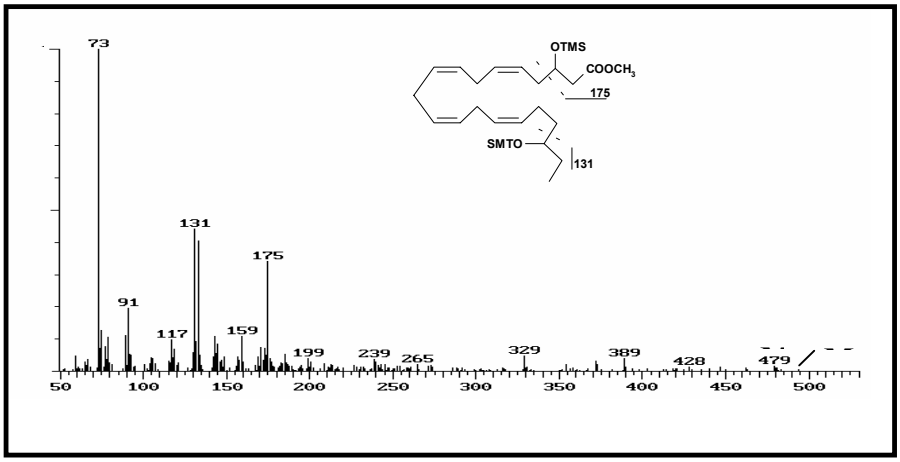


Figure. 8. Electron impact mass spectrum of the arachidonic acid metabolite produced by *Candida albicans.*

4.10. Identification of 3,18-diHETE as arachidonic acid metabolite of *C. albicans*

The positive immunoreactivity of the lipid fraction A (Table 3) and its sensitivity towards salicylic acid (Table 4) prompted us to analyse this fraction by GC-MS for the presence of 3-hydroxy fatty acids. After conversion to the methyl-trimethylsilyl derivatives, the GC separation scanning the partial total ion program revealed a major compound with a retention time of 26 min (Fig. 8), which was the only peak that exclusively occurred when the fungus was grown in the presence of 10 µM arachidonic acid but not in its absence. Addition of 1 mM salicylic acid to the fungal culture strongly diminished the amount of this metabolite. The main ions produced from this compound were 175 [$CH_3O{\cdot}CO{\cdot}CH_2{\cdot}CHO{\cdot}TMS$], which is characteristic for 3-hydroxy fatty acids, and 131 [$(CH_3)_3{\cdot}SiO^+{=}CH{\cdot}CH_2{\cdot}CH_3$], the loss of which is characteristic for a hydroxyl group at C-18. Other major ions observed were 479 [M–15] showing loss of CH_3 group, 389 [M–15–90] and 239 [329–90] showing losses of both OTMS groups. The UV spectrum of the compound did not reveal an absorbance maximum at 235 nm showing that the isolated double bounds of the AA molecule were not converted to a conjugated diene. Taken together, we present ample evidence that the AA metabolite of *C. albicans* is a 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid (3,18-diHETE).

4.11. Location of Compound

The *Candida albicans* was incubated with arachidonic acid and was incubated for different time periods and the pellet and the supernatant was analysed for the location of the compound identified using TLC. It was found that most of the compound was present in the supernatant of the culture as shown in the Fig. 9. The presence of the compound in the supernatant was also confirmed by GC-MS analysis.

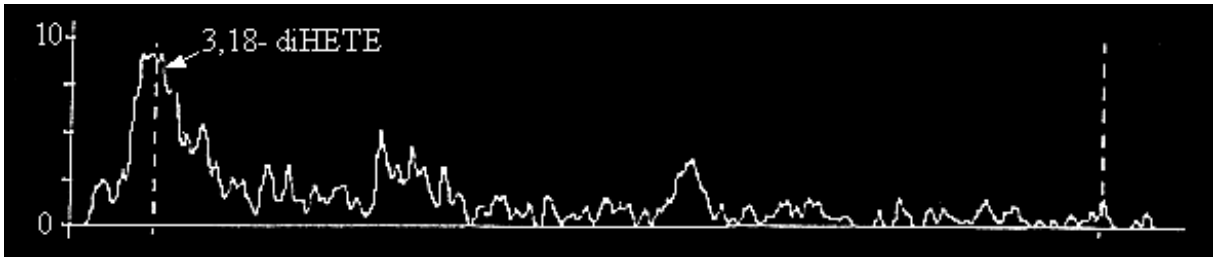


Figure 9. TLC chromatogram of lipids from the supernatant of *C. albicans* fed with C^{14} arachidonic acid. *C. albicans* was fed with arachidonic acid, and incubated for 5h. Cells were separated from supernatant.ipids were extracted using Bligh and Dyers method and separated by thin layer chromatography using solvent n-hexane: methanol: water (50:50:1).

4.12. Utilisation of 3-HETE as a carbon source by *C. albicans*

Rationale: 3-HETE was observed to be associated with the life cycle of *D. uninucleata,* [109] therefore, it was of interest to determine whether 3-HETE, which is similar to 3, 18-diHETE, is able to influence and stimulate the growth of *C. albicans*.

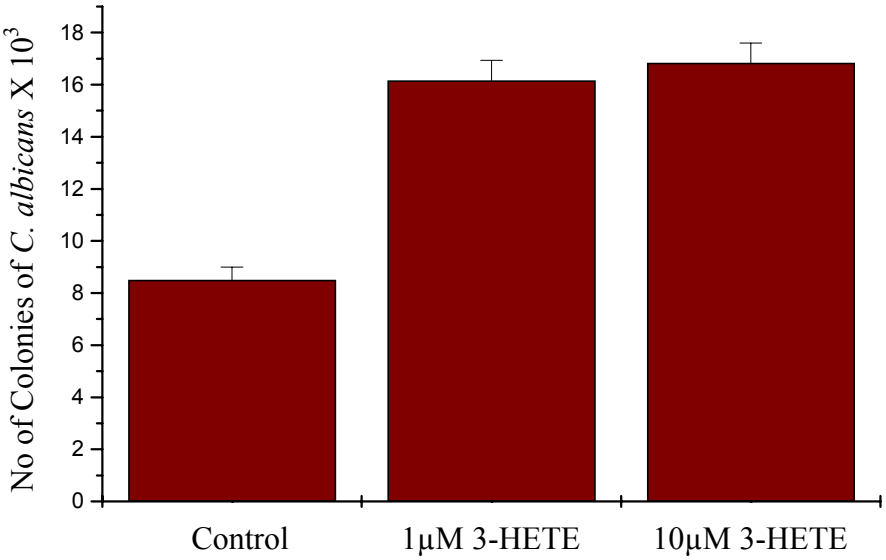


Figure 10. Effect of 3-HETE on cell growth of *C. albicans*. Cells were grown in the presence of 3µM and 10 µM 3-HETE as a sole carbon source and incubated for 48 h. at 37°C. The number of cells were counted using Neubauer's chamber. Data represent means ± Standard Deviation of three experiments $P < 0.05$ compared to the control.

To see the effect of 3-HETE on *C. albicans,* it was incubated with 3-HETE at the concentration of 3 µM and 10 µM. It was found that 3-HETE was able to stimulate the

growth of *C. albicans*. In presence of 3-HETE, number of *C. albicans* cells were significantly increased.

4.13. The cell extract of *Candida albicans* is able to release arachidonic acid from Hela cells

Rationale: Since *C. albicans* interacts with human cells during infection, the study was carried out to see whether *C. albicans* possessed phospholipid cleavage enzymatic system to release the arachidonic acid from host cells.

To see the capability of *C. albicans* to liberate arachidonic acid from the host cells, the cell free extract of *Candida albicans* was added to lysate prepared from HeLa cells previously labeled with [1-^{14}C]-AA and was analysed by radioscanning. As shown in Fig. 11. it was found that *C. albicans* is capable of releasing esterified arachidonic acid from phospholipids.

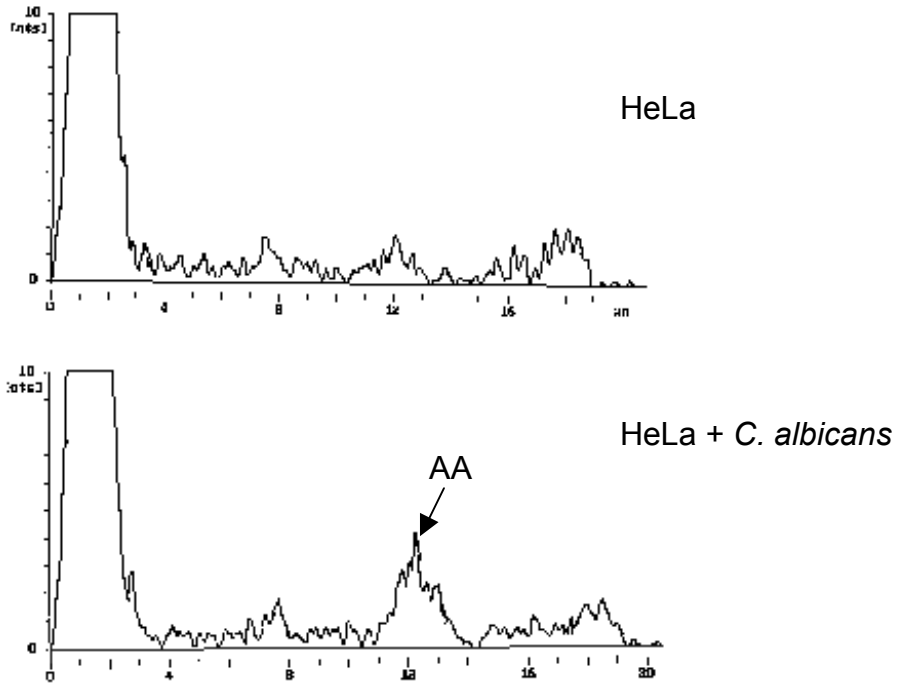


Figure 11. Arachidonic acid release by C. albicans. HeLa cells were incubated with 1 µCi [1-^{14}C]-Arachidonic acid for 18 h. To the HeLa cell lysate 0.04 mg of Candida albicans protein was added and was incubated for one hr, followed by Bligh and Dyer extraction of lipids. The extracted lipids were separated by TLC in the solvent system containing diethylether:n-hexane:acetic acid (50:50:1) and analysed by TLC reader.

4.14. *Candida albicans* shows Phosholipase activity

Rationale: The experiment was carried to see whether *C. albicans* possess phospholipase A_2 activity to release arachidonic acid in a significant amount from HeLa cells.

To determine the phospholipase A_2 activity, 25µg protein lysate of *C. albicans* was incubated with pure substrates PAPC and PAPC-OH which are specific for PLA2 activity for one hr. After Bligh and Dyer extraction lipids were analysed by HPLC for AA and 15-HETEs. It was found as shown in Fig. 12 a and b that *Candida albicans* is able to release AA from phospholipids. Both 1-Palmitoyl-2-arachidonoyl phosphatidylcholine (PAPC), and PAPC-OH released arachidonic acid and 15-HETE, respectively, demonstrating phospholipase in *C. albicans.*

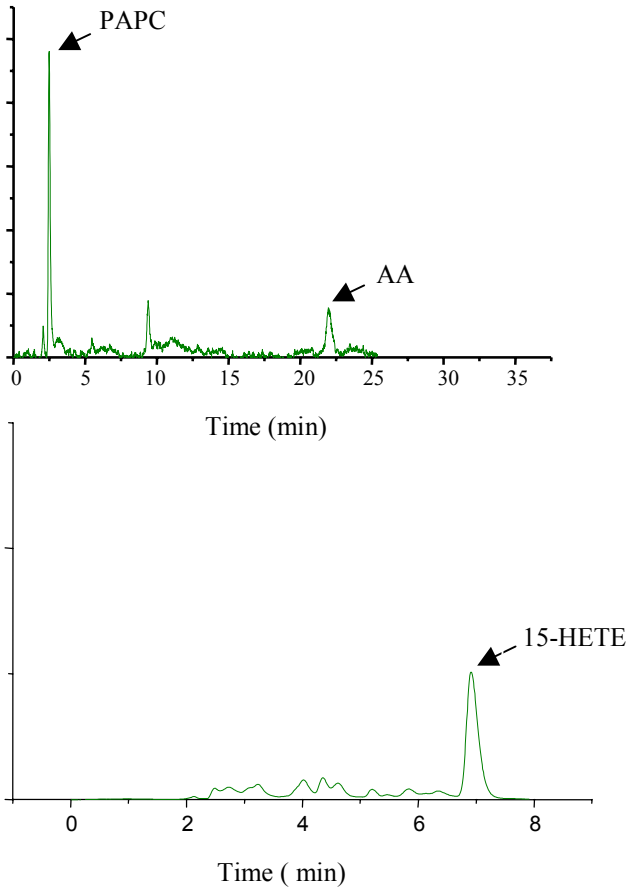


Figure 12. Phospholipase activity in *C. albicans.* Cell free extract of *C. albicans* was incubated with 1-Palmitoyl-2-arachidonoyl phosphatidyl Choline (PAPC) and PAPC-OH and incubated for 1h. After incubation the lipids were extracted by Bligh and Dyer and was analysed by Reverse phase HPLC in solvent methanol: acetonitrile: water 90:6:4 with 20 mM choline chloride.

4.15. Different morphological forms found in *Candida albicans*

When blastospores of *Candida albicans* are inoculated in DMEM, the cells undergo morphogenesis. The blastospores germinate to form germtube followed by formation of pseudohyphae, hyphae and chlamydospores. As shown in Fig. 13. cells stained with crystal violet (a) hyphal form, (b) blastospores, (c) pseudohyphae, (d) cluster of blastospores attached to the hyphae, (e) chlamydospores.

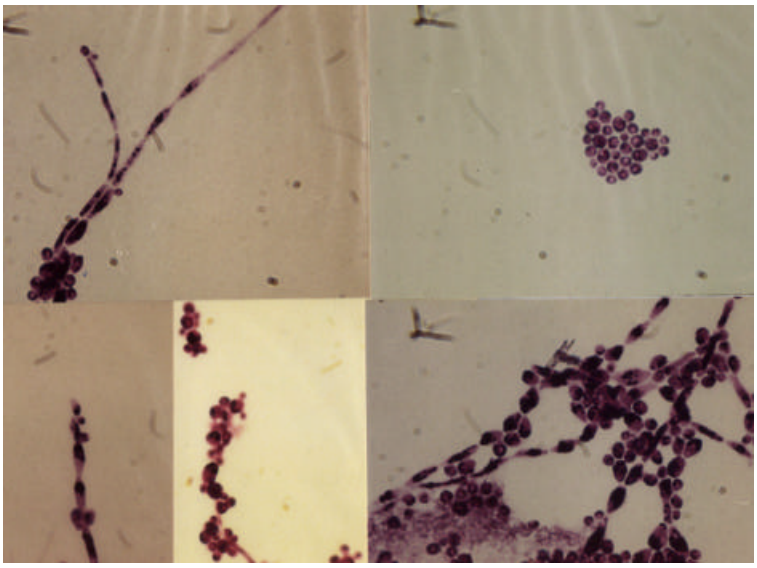
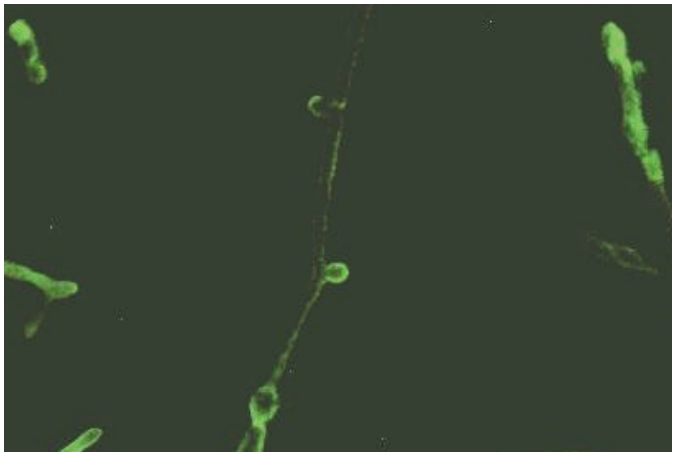


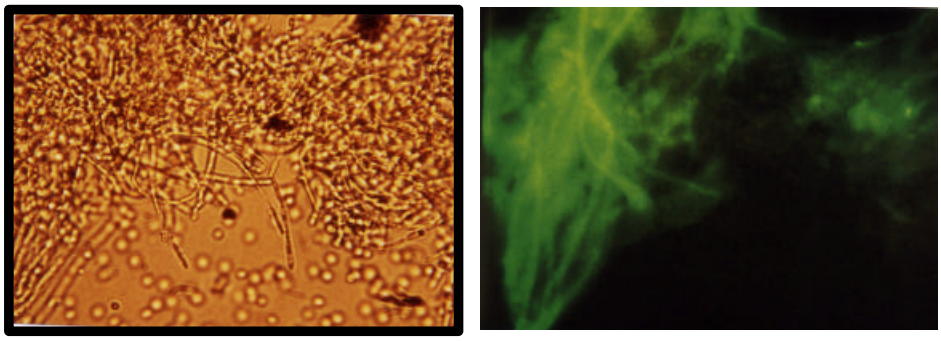
Figure 13. Different morphological forms of *C. albicans* stained with Crystal violet. Various forms of multicellular structures formed by *Candida albicans*.

4.16. Evidence for the presence of 3(R)-hydroxy-oxylipins in *C. albicans in situ*

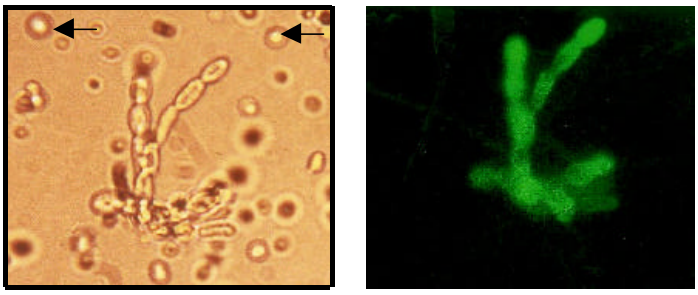
Rationale: 3-HETE, which was discovered by Dr. S. Nigam together with Dr. Kock from South Africa in *D. uninucleata*, was shown to have a strong biological action [99, 109, 169]. This prompted the search of similar compounds in the pathogenic yeast *C. albicans.* Polyclonal antibody raised against 3(*R*)-HETE was used for *in situ* detection of oxylipins by immunofluorescence microscopy [109]. This antibody, which shown to react with a number of 3-hydroxy fatty acids but not with the corresponding fatty acids lacking the 3-hydroxy group [109]. As shown in Fig.14. positive immunofluorescence staining was observed selectively in the hyphae but not in budding cells representing the yeast form of the pathogen, demonstrating higher amount of 3-OH in the hyphael forms compared to the yeast form. The immunofluorescence in the hyphae occurred independently of whether AA was present in the cell culture or not.



(a)



(b)



(c)

Figure 14. Light and Immunofluorescence microscopy of *C. albicans* for detection of 3-hydroxy-oxylipins.

(a) immunofluorescence microscopy of different morphological forms of *C. albicans*. (b) and (c) light microscopy of cultures having blastospores and hyphae of *C. albicans* along with the immunofluorescent microscopic view of the same slide immunostained with anti 3-OH antibody.

4.17. Effect of Aspirin on cell growth of *C. albicans*

Rationale: Since formation of 3, 18 diHETE was abolished in presence of aspirin, the *in vivo* effect of aspirin on *C. albicans* was investigated.

Table 6: Effect of aspirin on cell growth of *C. albicans* grown in DMEM at 37°C [a] for 48h.

Treatment with aspirin (mM)	Single yeast cells	Hyphae and other multicellular structures
0	100[b]	100c
0.1	56.7±7.0	56.7±7.4
1.0	31.3±8.0	29.0±9.6

a The cells were cultured at 37°C for 48 h. in presence or absence of aspirin. The percentages represent mean ±S.D of three independent experiments.

b Controls: (15.9±1.0) x 106 cells (n = 3).

C Control: (3.5±0.5) x106 structural elements (n = 3).Hyphae, pseudohyphae and other multicellular structures were counted as single structure element.

C. albicans was grown in DMEM, which has been shown to induce growth [19] in presence and absence of aspirin at the concentration of 0.1 mM and 1mM. It was found that cell growth was inhibited by aspirin concentration as low as 0.1mM. as shown in the Table 6. It was found that 1mM aspirin was able to inhibit growth of yeast formation as well as the formation of multicellular forms of *C. albicans.* With 1 mM Aspirin, 70% reduction was found in both multicellular as well as in number of cells.

4.18. Effect of Aspirin on germ-tube formation of *C .albicans.*

Rationale: Since we found that 3-hydroxyoxylipins are associated with the hyphal forms and the multicellular structures, the effect of aspirin on germ-tube formation in *C. albicans* was investigated.

To study the effect of aspirin on the germ tube formation of *C. albicans*, the blastospores were inoculated in DMEM in absence and presence of 0.1, 0.3, 0.5 and 1mM Aspirin and incubated for 6 h. Aspirin was found to reduce the formation of multicellular structures in a dose-dependent manner, and the IC_{50} was found to be approximately 0.7 mM (Fig.15).

Moreover, the formation of germ tube was found to be reduced by 15% on incubation with 1 mM aspirin as shown in Fig 15a.

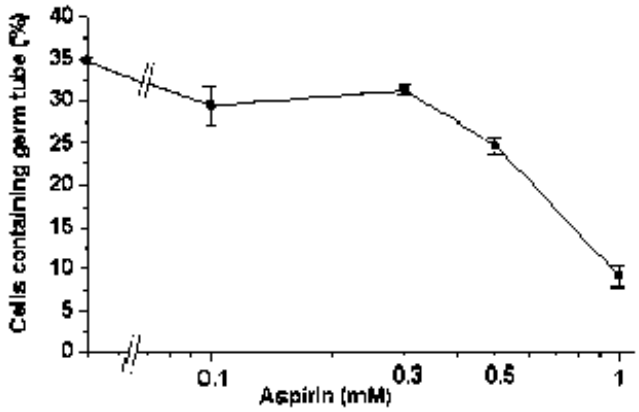
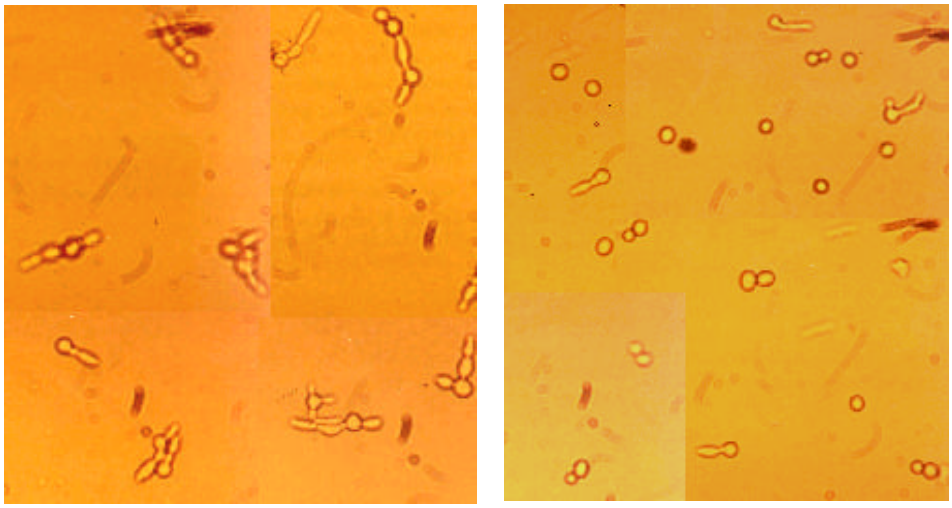


Figure 15. Aspirin diminishes the percentage of germ tubes formed by *C. albicans* after incubation for 6 h. in DMEM in absence and presence of various concentration of Aspirin. Number of cell containing germ tubes were counted using neubauers chamber. The values expressed is the mean ± S.D of three independent experiments.



(a) **(b)**

Figure 16. Light microscopie of demonstration of the effect of aspirin on *C. albicans*. Cells were incubated in DMEM for 6h in presence (a) and absence of 1mM (b) aspirin. (400 X magnification).

4.19. Effect of aspirin on the minium inhibitory (MIC) of clotrimazole on the vaginal isolates of *C. albicans*

Rationale: It was found that 3-hydroxylation occurs via β–oxidation pathway and 18-hydroxylation through cytochrome P-450. Therefore, we used clotrimazole a common antifungal antibiotic inhibiting cytochrome p450 [51] and affecting ergosterol synthesis in *C. albicans* and aspirin, an inhibitor of β–oxidation, in combination on the growth of *C. albicans*.

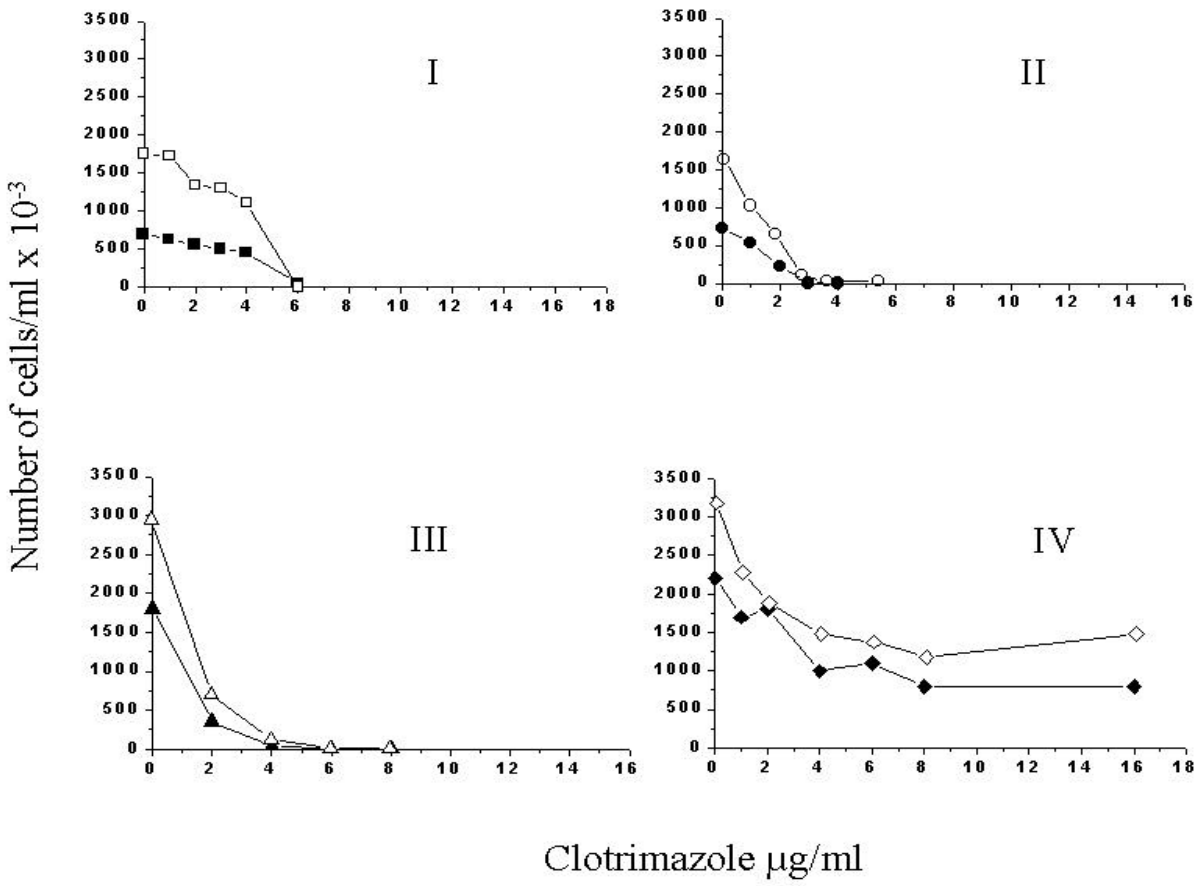


Figure 17. Combined effect of clotrimazole and aspirin on four different vaginal isolates of *C. albicans*. The culture showing susceptibility to clotrimazole were grown at 25°C for 36 h. Tubes containing 10 ml of DMEM bufferred by 25 mM hydroxyethylenepiperazinesulfonate along with varying concentrations of clotrimazole in absence (open symbols) and presence of 1mM aspirin (full symbols) were inoculated with 10^4 *C. albicans* cells. The cultures were incubated at 37°C for 40h. before counting the cells. The values represent the mean of two separate experiments for each culture.

To see the effect of aspirin on the inhibition for four different strains of *C. albicans* in combination with clotrimazole, the minimum inhibitory concentration was determined using two-fold dilution method for clotrimazole with and without addition of 1mM aspirin.

It was found that aspirin could decrease the MIC of *C. albicans* irrespective to the individual difference in strains for the minimum concentration of clotrimazole required for the complete inhibition. The aspirin was found to inhibit both clotrimazole sensitive and resistant strains of *C. albicans*. The pathway for inhibition by clotrimazole and aspirin seems to be different from each other. Where as clotrimazole is a cytochrome p450 inhibitor, aspirin inhibits β–oxidation pathways [170] as well as NFκB [171].

4.20. Aspirin inhibits the adhesion of *C. albicans* to HeLa cells

Rationale: Adhesion is known as an important factor inducing virulence to *C. albicans* [116]. Hence we investigated the effect of aspirin on *C. albicans* infected HeLa cells.

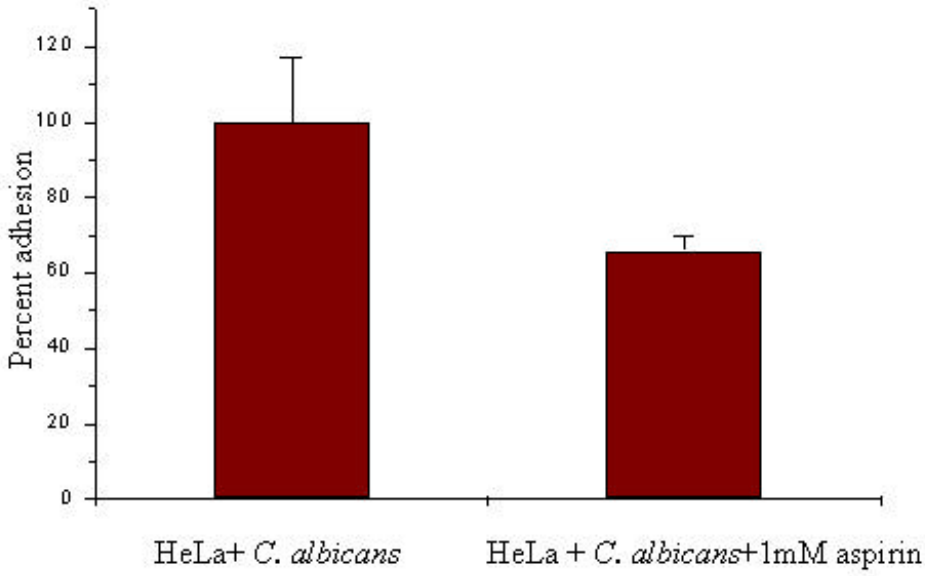


Figure 18. Aspirin inhibits adhesion of *C. albicans* to HeLa cells. HeLa cells were grown on coverslips in 6 well plates. *C. albicans* (MOI=5) was added to each well in DMEM +1% FCS with or without 1mM aspirin. Cells were incubated for 1h and washed with PBS several time and were viewed under microscope. Five arbitrary fields of each coverslip were counted. The figure shows three determinants of 3 experiments.

To study the effect of aspirin on adhesion of *C. albicans*, coverslip cultures of HeLa cells were made. These cultures were infected with *C. albicans* in presence or absence of 1mM aspirin. Aspirin was found to inhibit significantly the adhesion of *C. albicans* to HeLa cells as shown in (Fig. 18).

4.21. High density lipoprotein (HDL) induces formation of germ-tube in *C. albicans*

Rationale: Reconstituted high-density lipoprotein (rHDL) is administered to sepsis patients for their neutralising effect on lipopolysaccharide. However, it has been demonstrated [172] that this therapy enhances growth of *C. albicans* in patients. This led us to investigate the effect of HDL on morphogenesis of *C. albicans*.

To study the effect of HDL on germ-tube formation in *C. albicans*, human serum was stripped using activated charcoal to adsorb all the hormones and other organic molecules. To of stripped serum 1 $X10^6$ *C. albicans* cells were incubated with various concentrations of HDL. It was found that HDL induced germ-tube formation in the *C. albicans* in a dose-dependent manner. As shown in Fig. 19., at a concentration as low as 10µM, HDL induced significant enhancement in germ-tube formation.

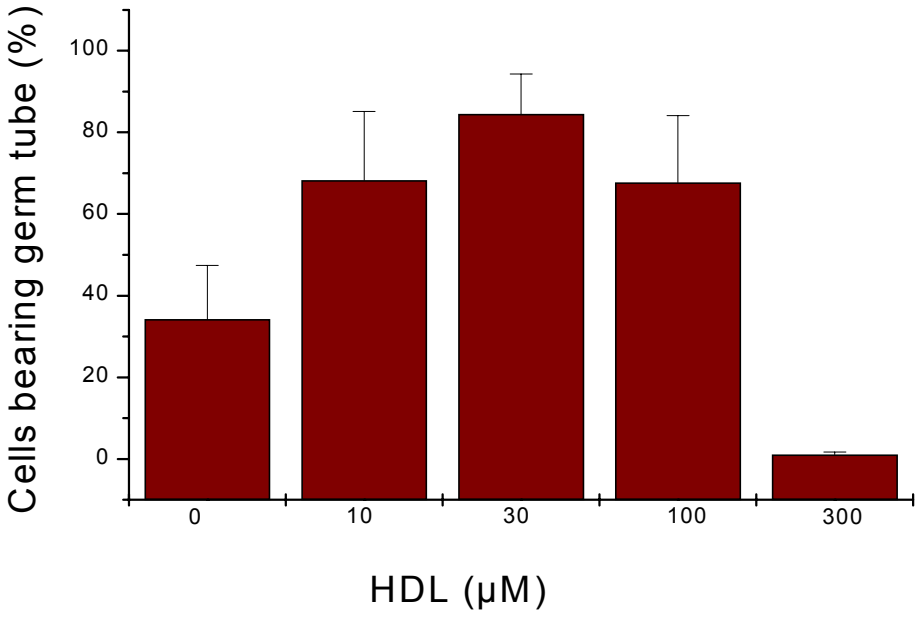


Figure 19. HDL induces germ-tube formation in *C. albicans*. Blastospores of *C. albicans* ($1X10^6$) were incubated in charcoal stripped serum in presence of 10, 30, 100 and 300µM HDL for 4h. The cells containing germ-tube were counted using Neubauer's chamber. Data represent means ± Standard deviation of three experiments $P < 0.05$ compared to the control.

4.22. *C. albicans* upregulates selectively COX-2 in HeLa cells

Rationale: Infection with *C. albicans* has been shown to be associated with production of prostaglandins [72, 12]. Since, cyclooxygenase is the rate-limiting enzyme that catalyzes the oxygenation of AA to prostaglandin endoperoxides, which are transformed to prostaglandins, we studied COX-2 production by HeLa cells upon infection with *C. albicans*.

HeLa cells were infected with *Candida albicans* with a multiplicity of infection 5 and incubated for 6 h in DMEM for different time periods. Total protein extract of HeLa cells was prepared and analysed by Western blotting. Upon infection with *C. albicans* COX-2 protein was found to be upregulated as shown in Fig 20a. The RNA from infected cells was extracted and was subjected to reverse transcription and analysed for COX-2 mRNA level using RT-PCR. The RNA levels were quantified and the COX-2 levels were compared with the levels of glyceraldehyde-6-phosphate dehydrogenase (GAPDH) by RT-PCR as shown in Fig. 20b. An upregulation of COX-2 mRNA in *C. albicans* was observed within 6 hr. of infection of HeLa cells. This is in agreement with COX-2 protein results by western blotting (Fig. 20a) demonstrating that *C. albicans* induces upregulation of COX-2 in HeLa both at the transcriptional and translational level.

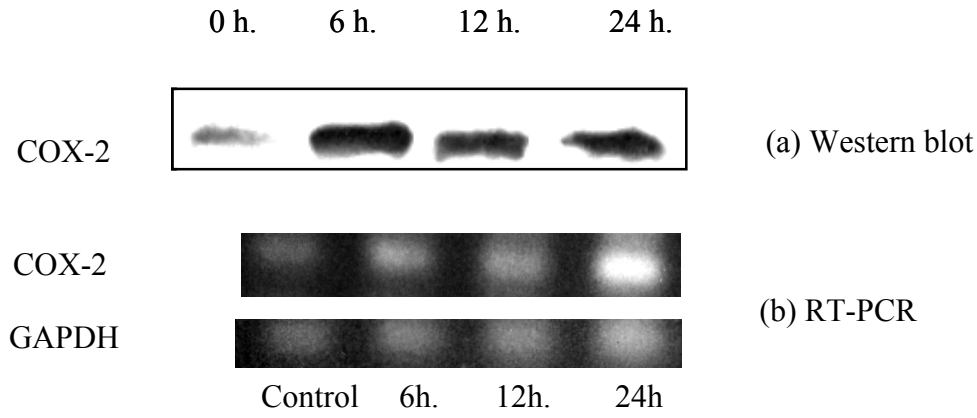


Figure 20. Upregulation of COX-2 in HeLa cells by *C. albicans*. HeLa cells were infected with *C. albicans* (MOI=5) and incubated for different time, the protein was prepared and was analysed by Western blotting for the levels of COX-2 as shown in (a). In a parallel experiment RNA was extracted and was subjected to RT-PCR for the analysis of the COX-2 mRNA level at different time period along with standerd of GAPDH as shown in (b).

4.23. *C. albicans* does not upregulate COX-1 in HeLa cells.

Rationale: Prostaglandin synthesis is mediated by both COX-1 and COX-2. COX-1, which is responsible for basal prostanoids synthes, is a constitutive enzyme and has been found to be upregulated under certain conditions [77,83]. Involvement of COX-1 in infection of host cells with *C. albicans* was studied.

HeLa cells were infected with *C. albicans* with multiplicity of infection of 5 for different time periods. The protein prepared was analysed using Western blotting for COX-1. As shown in Fig. 21, *C. albicans* did not upregulate COX-1 protein levels in HeLa cells.

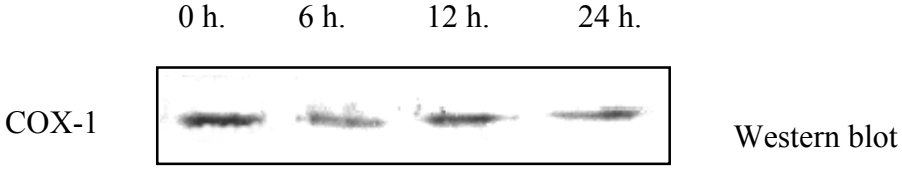


Figure 21 *C. albicans* does not upregulate COX-1 protein. HeLa cells were infected with *C. albicans* (MOI=5) for 6 h and the protein lysate was analysed for COX-1 protein by Western blotting.

4.24. 3-HETE is capable of inducing COX-2 in HeLa cells.

Rationale: 3-HETE was found to be a growth factor for *C. albicans* (Fig 22), and *C. albicans* was found to upregulate COX-2 in HeLa cells. Therefore, it was of interest to check if the upregulation of COX-2 by *C. albicans* in HeLa cells, is partly contributed by 3-HETE or its derivative 3,18-diHETE.

HeLa cells were grown to near confluency and incubated with various concentrations of 3-HETE in DMEM without FCS for a period of 6h. The protein lysate was resolved by electrophoresis and analysed by Western blotting. 3- HETE at a concentration of 5µM was found to induce COX-2 in HeLa cells. This result is comparable with that of *C. albicans* demonstrating the importance of 3-Hydroxy oxylipins in the infection process.

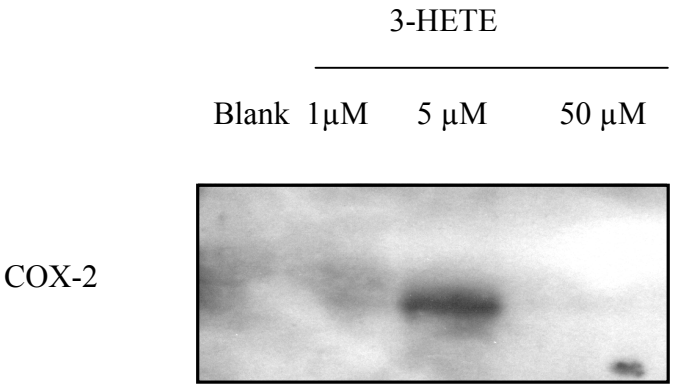


Figure 22. Upregulation of COX-2 in HeLa cells by 3-HETE. HeLa cells were incubated with 3-HETE at a concentration of 1, 2, and 50 μM in DMEM and was incubated for 6h. The protein lysate was prepared and was analysed by Western blotting for the levels of COX-2 .

4.25. *C. albicans* upregulates PGE_2 in HeLa cells

Rationale: PGE_2, an important inflammatory mediator of inflammation during infection, induces morphogenesis in *C. albicans* and contributes to the infectivity of the pathogen [22]. AA is metabolised by cyclooxygenases and subsequently converted to bioactive prostaglandins. Hence we investigated, if *C. albicans* is able to induce the production of PGE_2 from host cells.

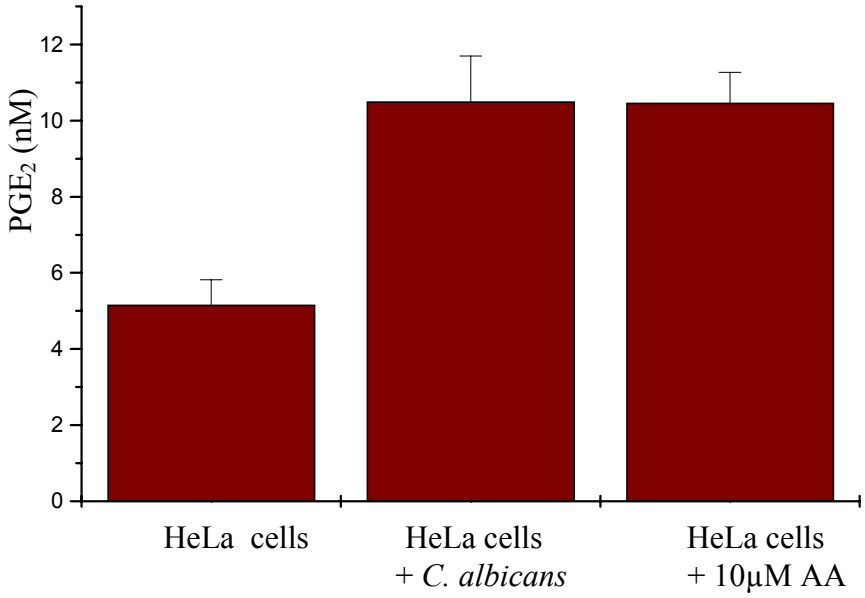


Figure 23. *C. albicans* upregulates PGE_2 upon infection with *C. albicans*. HeLa cells were infected with the blastospores (MOI=5) and incubated for 6h. The cells were spun down and supernatant was

analysed for PGE_2 by ELISA. Data represent means ± Standard deviation of three experiments P < 0.05 compared to the control.

Upon infection of HeLa cells with *C. albicans* with MOI=5, after 6 h of incubation the cells were spun down and supernatant was analysed for the PGE_2 levels. For comparison, HeLa cells were incubated with 5μM of arachidonic acid. As shown in Fig.23. *C. albicans* enhances PGE_2 levels in HeLa cells. The enhancement was 2.5 times of the control and was comparable to that obtained for arachidonic acid.

4.26. PI-3-Kinase is upregulated upon infection with *C. albicans.*

Rationale: In search of genes upregulated by *C. albicans* in HeLa cells, microarray analysis of mRNA from HeLa cells infected with *C. albicans* was performed

To find out the possible mechanism of upregulation of COX-2 RNA analysis by micro array was performed to see the genes upregulated upon infection by *C. albicans*. As shown in Fig. 24. PI-3-kinase mRNA levels were found to be upregulated by *C. albicans*. The micro array analysis did not show any significant difference for other genes, included in the commercial kit.

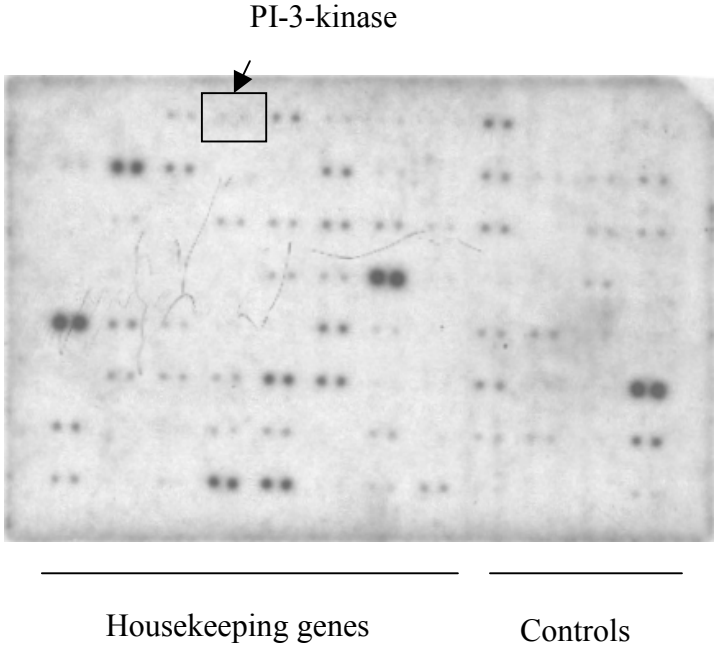


Figure 24. Analysis of upregulation of PI-3 kinase RNA by Micro array.

4.27. *C. albicans* upregulates PI-3-kinase in HeLa cells (Western analysis)

Rationale: To confirm the results of microarray results at the protein level, Western blotting was performed with PI-3-kinase antibody.

To support the results from the microarray experiment, HeLa cells were infected with *C. albicans* (MOI=5) for 6 hr., the protein lysate was prepared and analysed by Western blotting for the PI-3 Kinase protein. *C. albicans* was found to upregulate the PI-3 kinase levels.

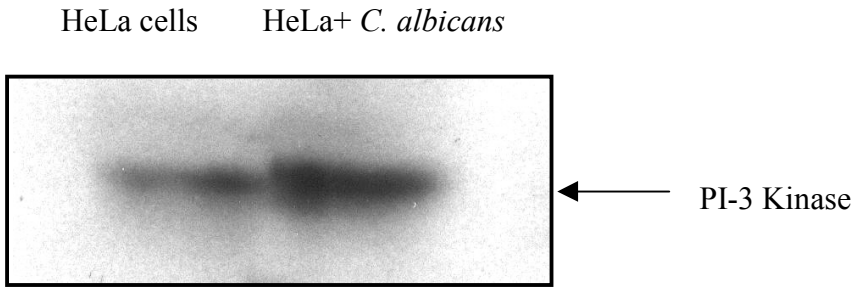


Figure 25. Upregulation of PI-3-kinase by *C. albicans* in HeLa cells. The cells were infected with *C. albicans* with multiplicity of infection 5 for 6 h. The cell lysate was prepared and analysed using Western blottting for PI-3 Kinase protein.

4.28. *C. albicans* upregulates COX-2 via p38 MAP kinase pathway

Rationale: p38 MAPK and ERK1/2 has been shown to be activated by endotoxins, inflammatory cytokines and environmental stresses and growth factors [173, 174, 175, 176]. To elucidate the mechanism of upregulation of COX-2, by *C. albicans*, specific inhibitors of p38 MAP kinase, ERK1/2 and PKC pathway were used in *C. albicans*-HeLa cell system.

To evaluate the role of p38 MAP kinase activation by *C. albicans* in COX-2 upregulation, we pre-treated HeLa Cells with SB 202190, an inhibitor of p38 MAP kinase at the concentration of 10 μM, followed by infection with *C. albicans* (MOI=5) for another 6 hr. The protein was analysed by Western blotting for COX-2 protein. PD 98059, an inhibitor of ERK 1/2 failed to inhibit *C. albicans* induced upregulation of COX-2 (Fig. 26), however, SB 202190 and GF 203190X, inhibitors for p38 MAPK, and PKC inhibited to a great extent *C. albicans* induced upregulation of COX-2, suggesting the involvement of p38 MAP kinase, and PKC in the induction of COX-2 by *C. albicans.* These results are in line with an earlier report demonstrating upregulation of COX-2 via p38 MAPK [177].

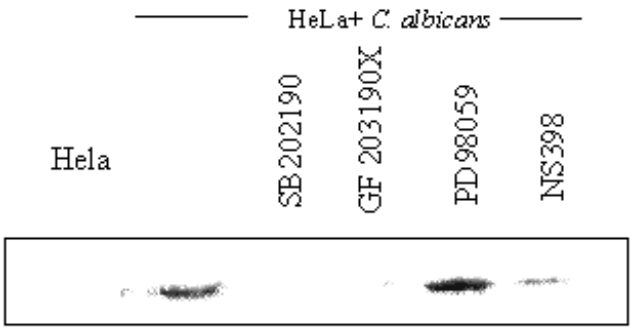


Figure 26. COX-2 is upregulated in HeLa cells via p38 MAPK and PKC pathway and is independent of ERK1/2 pathway. HeLa cells were infected with *C. albicans* (MOI=5) for 6 h. Prior to incubation the cells were treated with 10 µM of SB 202190, GF 203190X or PD 98059 and 1µM of NS398 taken as a positive control.

4.29. The Upregulation of PGE_2 is mediated via p38 MAP kinase and PKC pathway.

Rationale: PGE_2, a mediator of inflammation, is formed by the action of cyclooxygenase on AA. PGE_2 was found to be involved in infection of HeLa cells by *C. albicans*. Investigation was done to reconfirm the observation that COX-2 mediated enhancement of PGE_2 is via p38 MAP kinase and PKC.

Various MAP kinases, and Protein kinases C have been shown to be involved in the regulation of COX-2 [178, 179, 180]. To see which pathway is involved in the upregulation upon infection by *C. albicans,* HeLa cells were infected with *C. albicans* with MOI 5 and incubated for 6 h with or without preincubation with various concentrations (0.1-10 µM) of SB202190, p38 MAP kinase inhibitor and PKC inhibitor GF 203190X for 1 h.. The cells were spun down and the supernatant was analysed for PGE_2 by ELISA. We found that inhibition of p38 MAP kinase, or Protein kinase C leads to decrease in PGE_2 in a concentration dependent manner, as shown in Fig 27. This demonstrates that the upregulation of PGE_2 was mediated via p38 MAP kinase and Protein Kinase C.

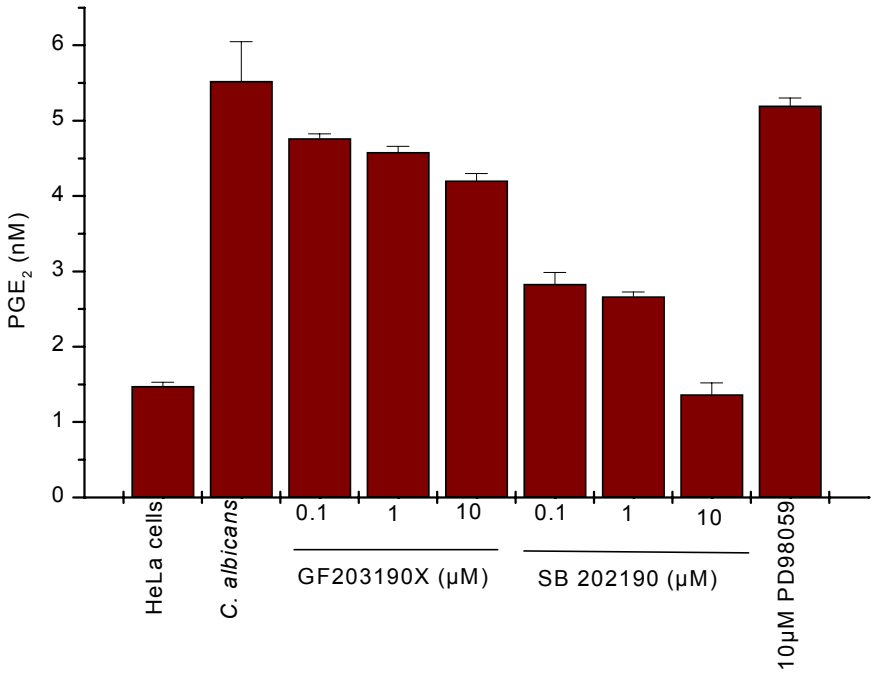


Figure 27. Involvement of p38 MAPK and PKC in *C. albicans* induced PGE_2 upregulation. HeLa cells were infected with *C. albicans* (MOI=5) for 6 h. with or with out preincubation of different inhibitors for 1h. The cells were spun down and supernatant was analysed for PGE_2 by ELISA. Data represent means ± Standard deviation of three experiments $P < 0.05$ compared to the control.

4.30. ERK1/ERK2 pathway is not involved in the upregulation of PGE_2 by *C. albicans.*

Rationale: ERK1/2 MAP kinase has also been observed to influence the expression of COX-2 and PGE_2 [174]. ERK1/2 pathway was studied for its involvement in the signal transduction by *C. albicans.*

To find whether ERK1/ERK2 were involved in *C. albicans* induced upregulation of PGE_2, the cells were infected in presence of specific ERK1/2 MAP kinase inhibitor PD 98059. After incubation cells were centrifuged and the supernatant was analysed for PGE_2. It was found that the inhibitor was unable to inhibit *Candida albicans* induced PGE_2 production (Fig. 27). This observation rules out the involvement of this particular pathway in the *Candida albicans* induced upregulation of COX-2 and PGE_2.

4.31. Upregulation of COX-2 in HeLa cells by *C. albicans* is mediated by reactive oxygen species.

Rationale: Reactive oxygen intermediates (ROIs) induce activation of intracellular stress kinases. ROIs have also been observed to upregulate COX-2 expression [181]. We, therefore, wanted to study the involvement of ROIs in upregulation of COX-2 by *C. albicans* infection in HeLa cells.

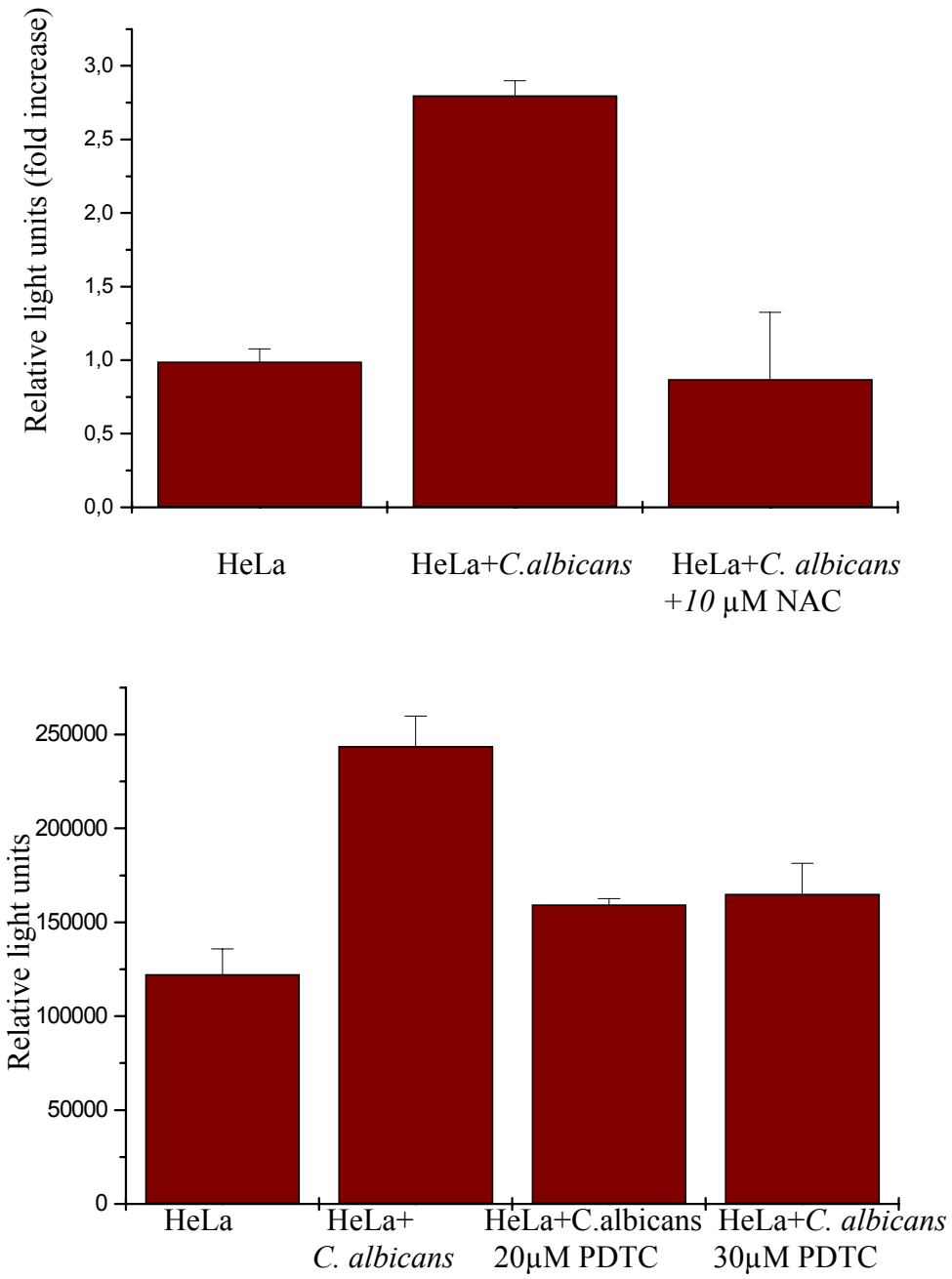


Figure 28. Involvement of Reactive Oxygen Intermidiates during *C. albicans* infection. HeLa cells were transfected with COX-2 reporter plasmid and after 24 h incubation experiments were performed. Cells were preincubated with 10mM N-acetyl L-cysteine for 18h or 20 or 30µM PDTC for 1h followed by infection with *C. albicans* for 2h. The lysate was analysed for COX-2 activity by scintillation counting.

To study the involvement of ROIs for the upregulation of COX-2 in *C. albicans*. Hela cells were transfected with COX-2 promoter plasmid followed by the infection with and without preincubation with N-acetyl L-cysteine or PDTC. It was found that *C. albicans* induced upregulation of COX-2 was inhibited to a great extant by free radical scavenger PDTC and glutathione precursor N-acetyl L-cysteine (Fig28).

4.32. *C. albicans* induced upregulation of PGE_2 is inhibited by PDTC

Rationale: The previous result demonstrates the involvement of ROIs in the upregulation of COX-2. PGE_2 production in the cell is directly controlled by the COX-2 activity and has been observed to be influenced by ROIs [182]. Therefore, we investigated the role of ROIs in PGE_2 upregulation utilizing PDTC, a free radical scavenger.

To study the effect of free radical scavengers on the *Candida albicans* induced upregulation of PGE_2, HeLa cells were preincubated with varying concentration of PDTC for 1h, followed by infection with *C. albicans*. PGE_2 was found to be inhibited by PDTC in a concentration dependent manner as shown in Fig. 29, demonstrating the involvement of free radicals in the upregulation of COX-2 by *C. albicans*.

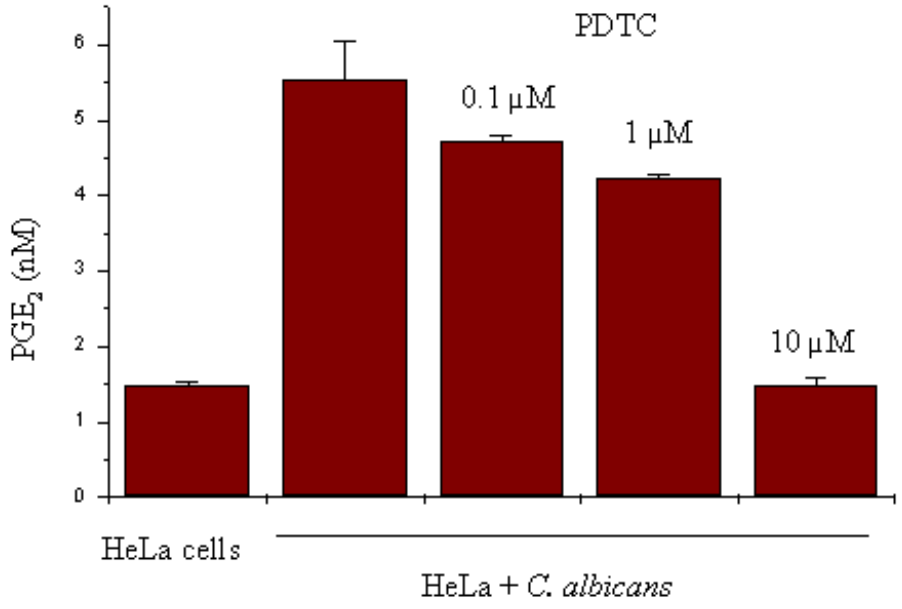


Figure 29. *C. albicans* induced upregulation of PGE_2 is inhibited by PDTC. HeLa cells were infected with *C. albicans* (MOI=5) for 6h. with preincubation with PDTC at various concentrations for 1h. The cells were

spun down and the supernatant was analysed for PGE_2 by ELISA. Data represent means ± Standard deviation of three experiments P < 0.05 compared to the control.

4.33. Western blot analysis of *C. albicans* mediated upregulation of p38 MAP kinase in HeLa cells.

Rationale: MAP kinases mediate signal transduction cascades in response to stress. A number of these pathways are mediated via p38 MAPK [183]. The role of this pathway during *C. albicans* infection in HeLa cells was investigated.

To study the role of p38 MAP kinase pathway in HeLa cells upon *C. albicans* infection, HeLa cells were infected with *C. albicans* for 6 h and analysed by Western blotting with p38 MAP kinase specific antibody. p38 MAP kinase was found to be upregulated upon infection in HeLa cells as shown in Fig.30.

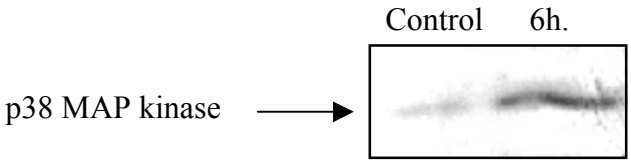


Figure 30. *C. albicans* upregulates p38 MAP kinase in Hela cells. To study the effect on p38 MAPK upon infection, HeLa cells were infected with *C. albicans* (MOI=5) for 6 h. the protein lysate was made and was analysed by Western blotting for the levels of p38 MAPK protein.

4.34. *C. albicans* does not upregulate JNK pathway in HeLa cells

Rationale: JNK signalling pathway is also involved in stress mediated response [184]. The role of JNK pathway during infection with *C. albicans* in HeLa cells was studied.

HeLa cells were infected with *C. albicans* as earlier and the protein lysate was subjected to Western blotting and probed with anti-JNK antibody. No changes in the level of JNK proteins were found. Fig. 31.

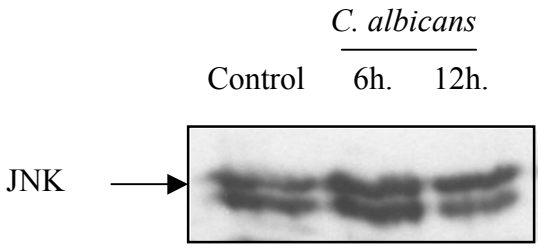


Figure 31. Upregulation of COX-2 in HeLa cells does not occur via JNK pathway. HeLa cells were incubated with *C. albicans* with multiplicity of infection of 5 the protein was analysed by Western blotting for the JNK protein.

4.35. *C. albicans* induced COX-2 upregulation is independent of PI-3 kinase pathway

Rationale: PI-3 kinase is another important stress response enzyme and in turn stimulate other pathways [185, 186, 187]. We found upregulation of PI-3 kinase during the infection of HeLa cells with *C. albicans.* Thus, it was of interest to see if PI-3 kinase was involved in *C. albicans* induced COX-2 upregulation

Significant upregulation of PI-3-kinase was observed in the infected HeLa cells within 6 h of infection. To see the role of PI-3-kinase during infection. HeLa cells were infected with *C. albicans* in presence of PI-3 kinase inhibitor, wortmannin. No alteration in COX-2 levels was observed upon treatment with wortmannin at the concentration of 10 μM (Fig. 32). It was proposed that upregulation of COX-2 upon infection was not mediated via PI-3 kinase pathway.

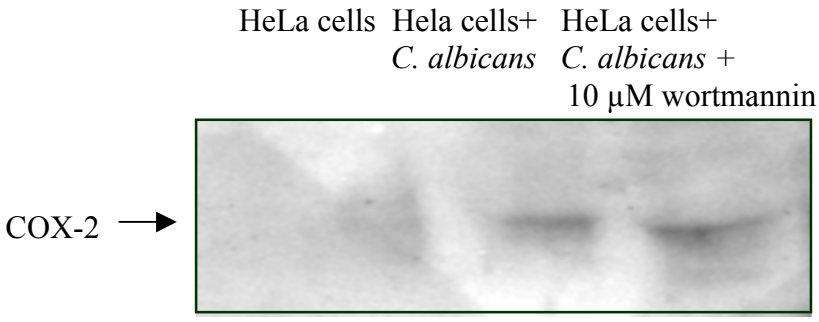
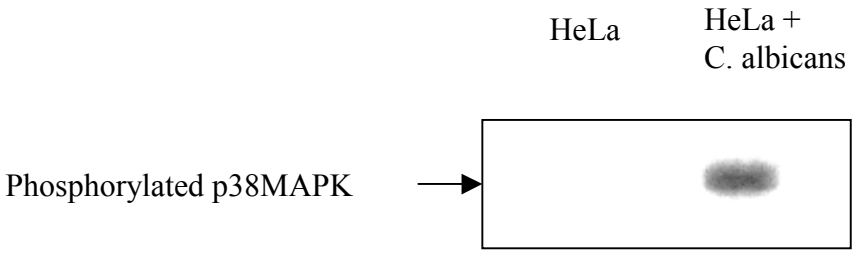


Figure 32. *C. albicans*-induced upregulation of COX-2 is not inhibited by PI-3-kinase inhibitor wortmannin. HeLa cells were preincubated with 10 μM wortmannin followed by the infection with *C. albicans* (MOI=5) for 6h. The protein lysate was analysed by Western blotting for the levels of COX-2.

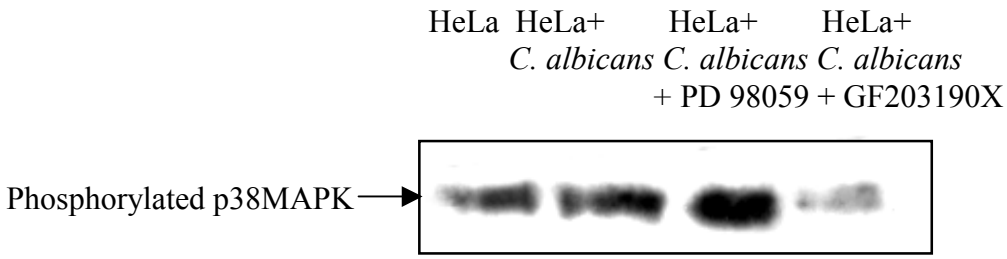
4.36. *C. albicans* induces phosphorylation of p38 MAPK via protein kinase C.

Rationale: The signal transduction cascade is a series of kinases and transcription factors activated in a sequential manner. Our aim was to establish the sequence of events between infection of HeLa cells by *C. albicans* and COX-2 activation or PGE_2 synthesis.

The protein lysate was prepared from HeLa cells infected with *C. albicans* and phosphorlyated protein was immunoprecipitated using phosphotyrosine antibody. The levels of p38 MAP kinase in the sample were determined using western analysis. We found upregulation not only in the amount of p38 MAPK but also in the amount of phosphorylated p38 MAP kinase (Fig. 33.a) in HeLa cells, which was in line with the work of Kumagai et al [188]. To determine the upstream phosphorylating agents for p38 MAP kinase, we infected HeLa cells with *C. albicans* as described following preincubation of HeLa cells with PD98059 (ERK1/2 inhibitor), and GF 203190X (PKC inhibitor) for 1 h. The protein was immunoprecipitated, using phospho-tyrosine antibody, and subjected to Western blot analysis. The inhibition of protein kinase C was associated with inhibition of phosphorylation of p38 MAP kinase. This demonstrates the involvement of Protein kinase C in the activation of p38 MAPK (Fig. 33b).



(a)



(b)

Figure 33. *C. albicans* induces phosphorylation of p38 MAPK in HeLa cells by PKC pathway. HeLa cells were subjected to infection with *C. albicans* (MOI=5) for 6 hr. The protein was subjected to immuno precipitation using phospho-tyrosine antibodies Western blotting (a). *C. albicans* infected cell pretreated with PD98059 and GF203190X were subjected to western blot analysis for the p38 MAPK protein (b).

4.36. *C. albicans* triggers activation of NFκB

Rationale: NFκB is an important transcription factor involved in cellular responses to cytokines, oxidative stress, and pathogen. It has also been shown to play role in cell growth, inflammation and apoptosis [189, 190, 191]. We, therefore, wanted to study involvement of NFκB in HeLa cells infected with *C. albicans.*

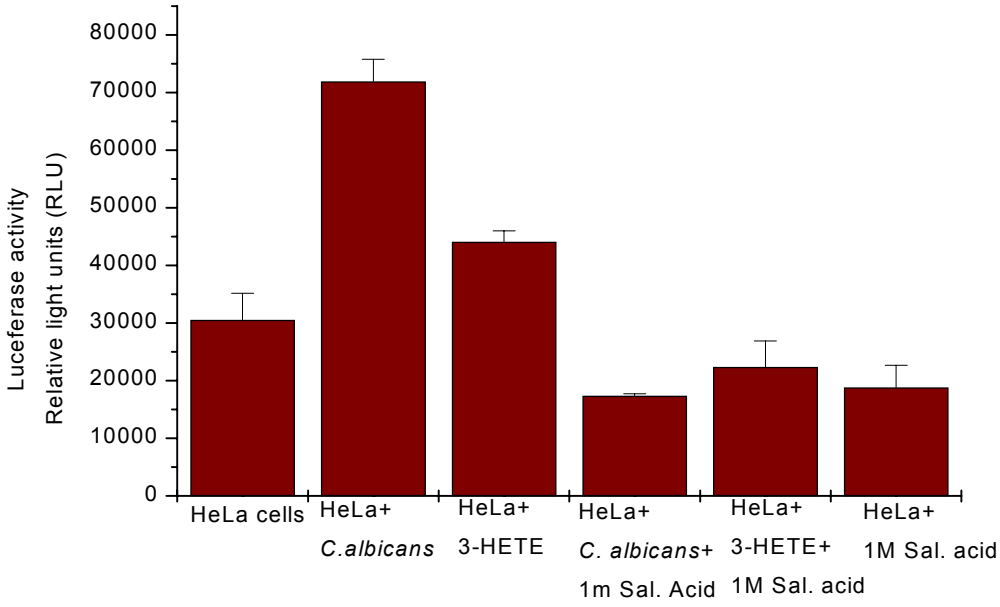


Figure 34. *C. albicans* induces upregulation of NFκB in HeLa cells. HeLa cells were transfected with pNFκB-Luc. The cells were then infected with *C. albicans* (MOI=5) for 6h and 5 µM 3-HETE, in presence and absence of 1mM salicyclic acid. Data represent means ± Standard deviation of three experiments P < 0.05 compared to the control.

In order to see whether it plays a significant role during infection with *C. albicans,* we transfected HeLa cells with pNFκB-Luc plasmid and subsequently infected them with *C. albicans* for 6 h. and measured the luciferase activity as a measure of upregulation of NFκB. It was found that NFκB was significantly upregulated upon infection with *C. albicans* (Fig.34.) this upregulation was inhibited by COX-2 inhibitor salicylic acid.

4.37. *C. albicans* induced NFκB expression is dependent upon p38 MAPK, PKC and PI-3 kinase pathways.

Rationale: A number signaling pathways have been shown to influence the expression of NFκB [191, 137]. *C. albicans* upregulated NFκB as well as MAP kinase, PKC and PI-3-

kinase pathways inHeLa cells. It was, therefore, of interest to investigate the role of these pathways in NFκB upregulation.

pNFκB-Luc transfected HeLa cells were infected with *C. albicans* in presence of p38 MAPK and PKC inhibitors and the luciferase activity was measured. It was found, that the *C. albicans* induced NFκB expression was inhibited by p38 MAPK and PKC inhibitors as shown in Fig 36. This indicates the involvement of these pathways in NFκB upregulation. We also found that PI-3 kinase pathway, which is upregulated by *C. albicans,* is involved in regulation of NFκB (Fig.35). PI-3 kinase has been reported in literature to be involved in NFκB mediated anti apoptotic function.

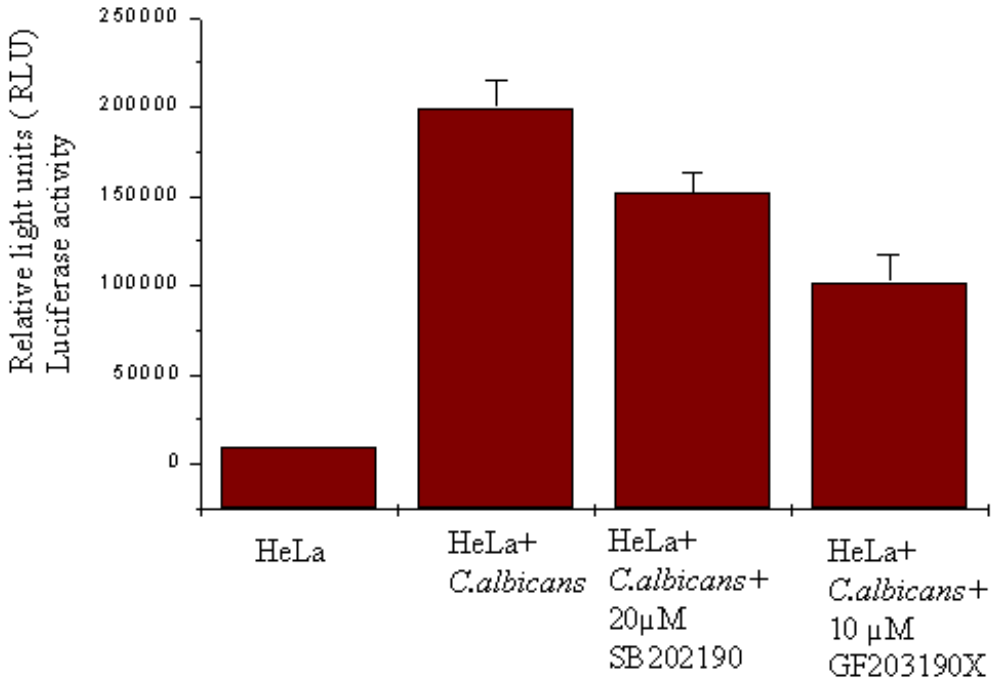


Figure 35. *Candida albicans* induced upregulation of NFκB is inhibited by SB 202190, GF 203190X. HeLa cells transfected with pNFκB-luc followed by infection with *C. albicans* (MOI=5) for 2 h. Data represent means ± Standard deviation of three experiments P < 0.05 compared to the control.

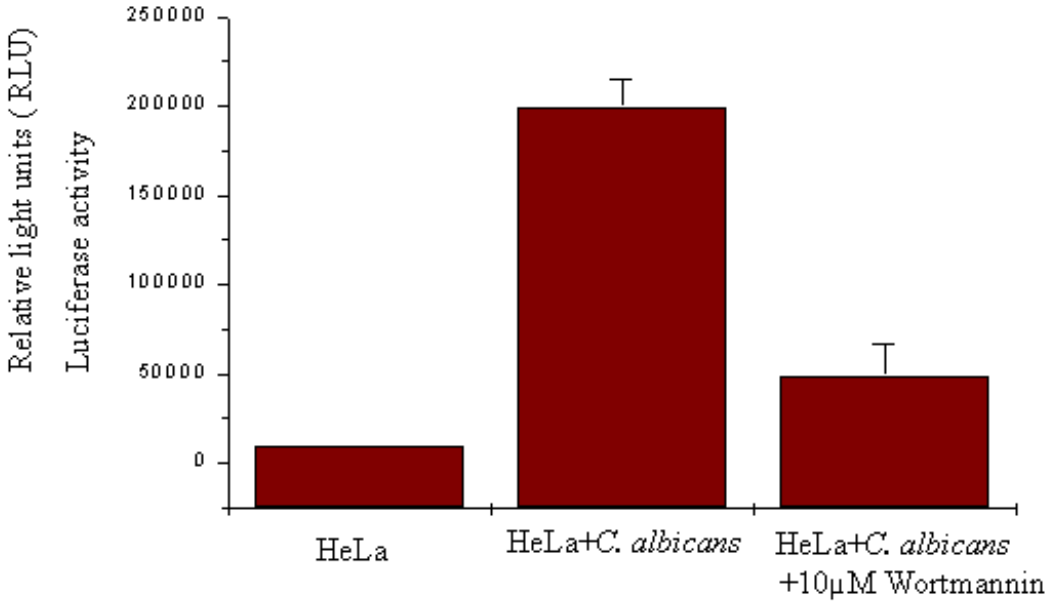


Figure 36. *Candida albicans*- induced upregulation of NFκB is inhibited by wortmannin. HeLa cells transfected with pNFκB-luc followed by infection with *C. albicans* (MOI=5) for 2 h. Data represent means ± Standard deviation of three experiments P < 0.05 compared to the control.

4.38. Upregulation of COX-2 by *C. albicans* occurs via NFκB

Rationale: NFκB is an important transcriptional factor involved in inflammation. It has been observed that oxidative stress mediated by free radicals cause upregulation of NFκB activity and the pathways dependent on it [192, 193]. Previous reports have also demonstrated involvement of NFκB during upregulation of COX-2 [194]. Furthermore, our results indicates that the MAP kinase pathways influence COX-2 activity via NFκB. Hence, we investigated the role of NFκB in COX-2 upregulation using IKK dominant negative vector. To mediate its activity, NFκB has to separate from a complex with IκKB and translocate to the nucleus. This process is dependent upon the phosphorylation of IκKB. The IKK dominant negative protein has mutations which prevent its phosphorylation, thereby, preventing the release of NFκB.

To study whether the upregulation of COX-2 occurred via NFκB, HeLa cells were co-transfected with COX-2 promoter and IKK dominant negative plasmid followed by incubation for expression of the plasmid for 18h. The cells were then infected with *C. albicans* with multiplicity of infection 5 and incubated for 6h. and the luciferase activity were determined as expression of COX-2. It was found that *Candida albicans* induced

COX-2 expression was partially blocked when NFκB was suppressed by the dominant negative vector as shown in Fig. 37.

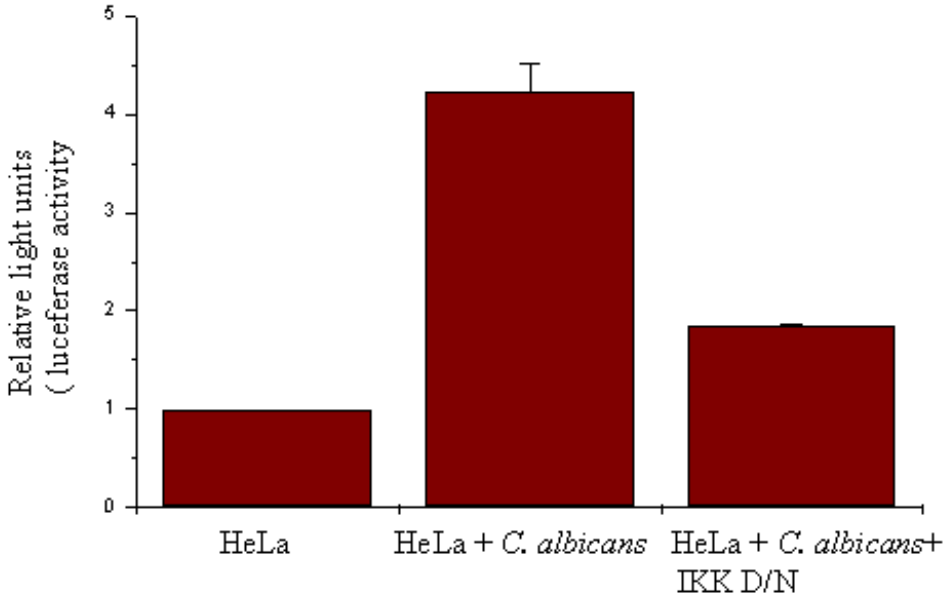


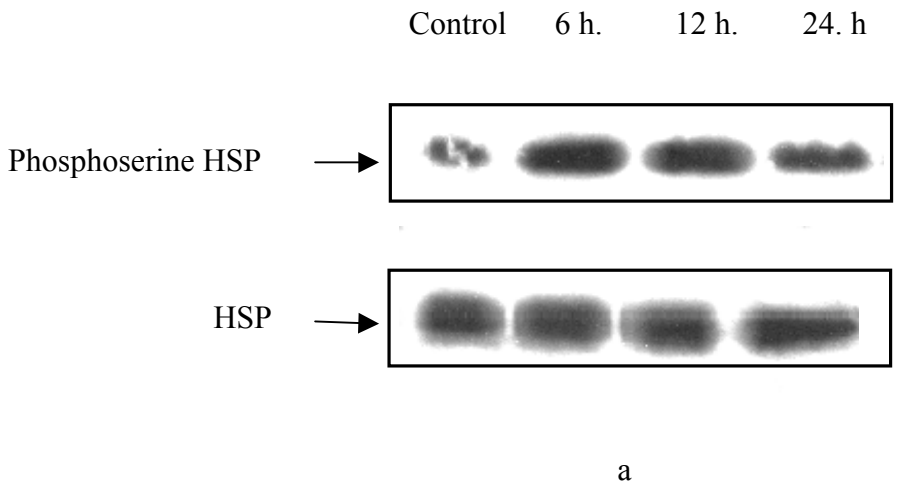
Figure 37. Involvement of NFκB in the upregulation of *C. albicans* induced COX-2. HeLa cells were co-transfected with COX-2 promoter and IKK dominant negative plasmid (I:6), and was incubated for 18 hr for the expression. Cells were then infected with *C. albicans* (MOI=5) for 2 h and analysed for luciferase activity using scintillation counter.

4.39. *C. albicans* induces phosphorylation of HSP27

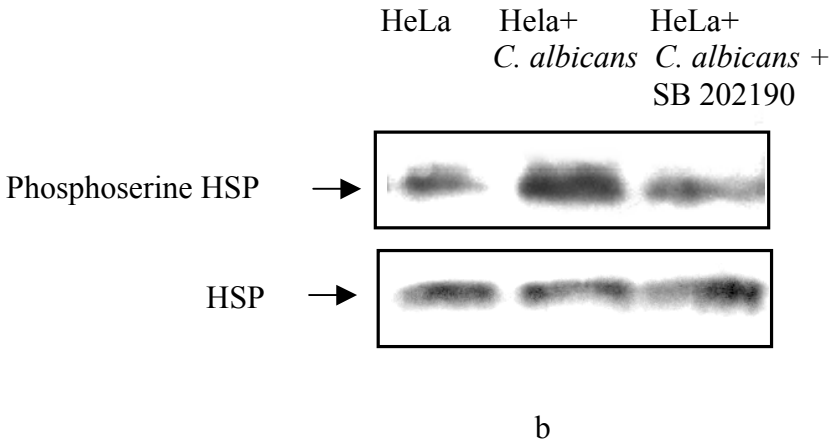
Rationale: Heat shock proteins are expressed under stress conditions. These proteins have chaperone like properties and act as inhibitors of actin polymerisation. HSP27 is one such protein which has been shown as inhibitor of actin polymerisation. This process is dependent upon its phosphorylation [195]. Hence, it was of interest to investigate whether *C. albicans* infected HeLa cells bring about phosphorylation of HSP27.

To see the effect of *C. albicans* on HSP levels, we infected HeLa cells with *C. albicans* as described in material and methods. The protein was then analysed by Western blot using anti-HSP27 antibody. No changes in amount of HSP27 protein levels were found. However, on immunoprecipitation of the phosphorylated protein, an upregulation in the amount of phosphorylation level of HSP 27 (Fig. 38a) was observed. To find the role of HSP 27 pathway in *C. albicans* induced cell signalling, we infected the HeLa cells in

presence of p38 MAP kinase inhibitor SB202190. Inhibition of p38 MAPK also inhibited phosphorylation of HSP 27 protein level as shown in Fig. 38b.



a



b

Figure 38. *C. albicans* induces phosphorylation of HSP27. HeLa cells were infected with *C. albicans* with multiplicity of infection 5 for different time periods. The protein was subjected to immunoprecipitation using phosphoserine antibody, followed by Western blotting using anti-HSP27 antibody. (a) Cells were preincubated with p38 MAP kinase inhibitor SB202190 for 1h. prior to infection for 6h (b).

4.40. *C. albicans* triggers p38 MAPK induced cytoskeletal changes in HeLa cells

Rationale: Since, microtuble depolymerization drugs have been shown to stimulate COX-2 protein [196], and we found induction and involvement of p38 MAP kinase during infection of HeLa cells with *C.albicans.* It was of interest to see whether cytoskeletal changes occurred upon infection and the role of p38 MAPK.

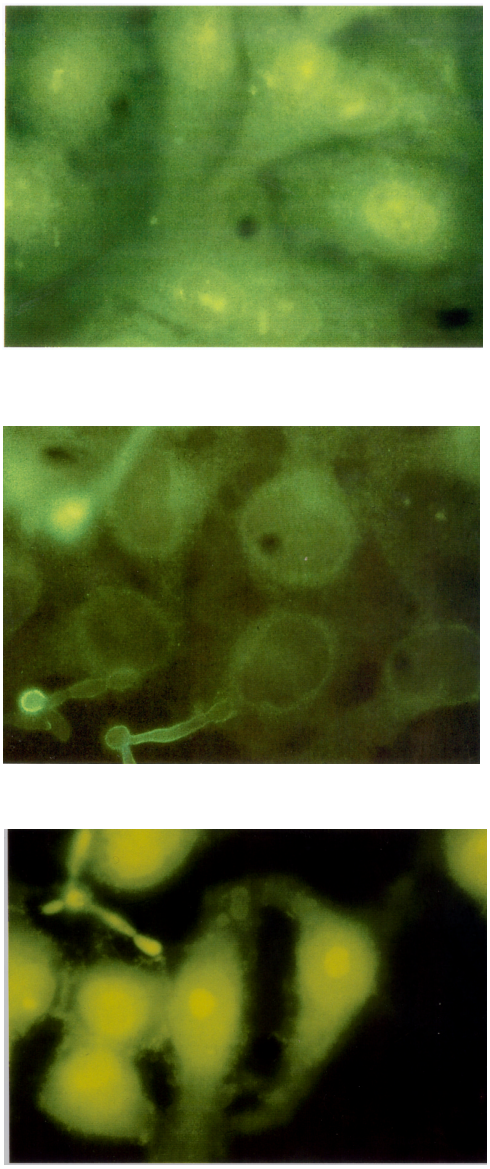


Figure 39. *C. albicans* triggers p38 MAPK induced cytoskeletal changes in HeLa cells. HeLa cells were grown on coverslips in DMEM containing 10% FCS. Coverslips were infected with *C. albicans* (b, c) for 6 h. in presence (c) or absence (a) of 20µM SB 202190. Coverslips after incubation were washed with PBS, fixed using 4% paraformaldehyde for 15 min. The coverslips were washed and incubated with anti-actin antibody for 30 min in a humid chamber followed by secondary anti rabbit-FITC conjugate antibody. The coverslips were visualised under immunofluorescent microscope.

To study the effect of *C. albicans* on the cytoskeleton, HeLa cells were grown on coverslips and infected with *C. albicans* for 6 h followed by immunostaining with anti-

actin antibody. To study whether these changes in cytoskeleton are mediated via p38 MAPK, we incubated cells with SB202190 during infection followed by immuno-staining using anti-actin antibody. *C. albicans* was found to induce cytoskeletal changes in HeLa cells as visualised by rearrangement of actin fibers from the periphery of cell (b), which was inhibited by SB202190 (c) to a large extent. These results are in line with earlier reportes [197, 198], which suggested that the phosphorylation of HSP27 modulates the function of a protein at the level of actin cytoskeleton. *In vivo* phosphorylation of HSP27 have been shown to be an important element in the modulation of f-actin dynamics upon stimulation when the cell is under stress [199, 200, 201].

4.41. *C. albicans* induces apoptosis in HeLa cells.

Rationale: In host pathogen interaction either host cells either phagocytise the pathogen and destroy it or they undergo programmed cell death, apoptosis [202, 203]. It was therefore of interest to study *C. albicans*-HeLa cell interaction and fate of host cells upon infection.

a. C. albicans induced DNA fragmentation in HeLa cells.

To study whether *C. albicans* induces apoptosis, DNA fragmentation assay was performed. HeLa cells were incubated with *C. albicans*. Within 4h. of incubation *C. albicans* caused DNA laddering in HeLa cells, demonstrating DNA damage, the final step of apoptosis as shown in (Fig. 40).

a b c

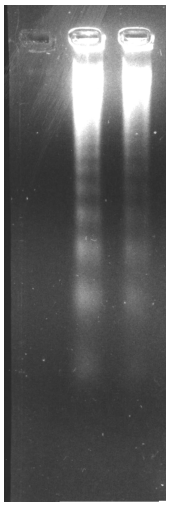
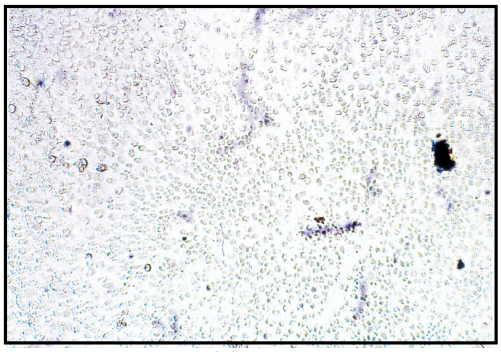
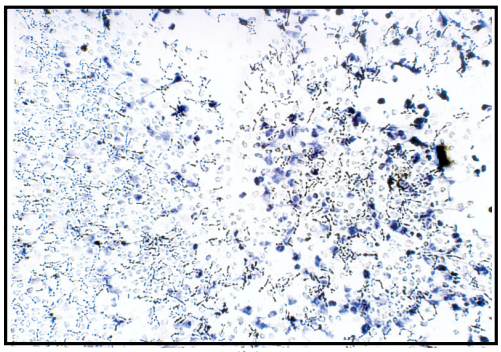


Figure 40. *C. albicans* induces DNA fragmentation in HeLa cells. HeLa cells were grown. DNA was extracted with phenol-chloroform-isoamyl alcohol (50:49:1) and was separated by electrophoresis in 3% agarose gel. DNA from $1.5x10^7$ untreated HeLa cells (Lane a), Cells incubated with 1µM/ml of staurosporin (lane b) and cells infected with *C. albicans* with multiplicity of infection = 5 (lane c).

a. *C. albicans* induces apoptosis as determined by TUNEL assay



(a)



(b)

Figure. 41. TUNEL assay. HeLa cells were grown on coverslips and were infected with *C. albicans* for 6h with a MOI of 5 in DMEM containing 1% FCS. Cells were fixed and permeabilized using 0.5% saponin. Coverslips were washed once and were incubated for 2 minutes in 100 mM cacodylate, pH 6.8, 0.1 mM DTTg 150 U/ml TdT (terminal deoxynuclueotide transferase) and 0.5 µM dUTP-Biotin in a humidified chamber at 37°C for 30 min. Coverslips were washed, blocked with 2% BSA. The coverslips were then incubated with streptavidin-HRP conjugate in blocking buffer, followed staining with TrueBlue for 10 minutes at RT, washed and observed under microscope.

C. albicans was found to induce apoptosis in HeLa cells. We found That upon infection within 6h. Nicks in DNA induced by *C. albicans* could be clearly seen by TUNEL assay as shown in Fig. 41. DNA laddering, which is a mark of final DNA damage occurring during apoptosis as shown in Fig 40. The experiment clearly demonstrate that HeLa cells as a protective measure upon infection undergo apoptosis.

4.42. *C. albicans* induces apoptosis via caspase-3 activity.

Rationale: Experiments were performed to elucidate the mechanism of *C. albicans* induced apoptosis. Caspase-3 is a proteolytic enzyme, involved in degradation of proteins during apoptosis [204]. Involvement of caspase-3 on *C. albicans* induced apoptosis was investigated.

To study whether *C. albicans* induces apoptosis in HeLa cells by the activation of caspase-3. As shown in Fig. 42, *C. albicans* stimulated production of caspase-3 in HeLa cells within 6 h of infection with *C. albicans*. At 12h. the activity was maximum which then decreased after 24h.

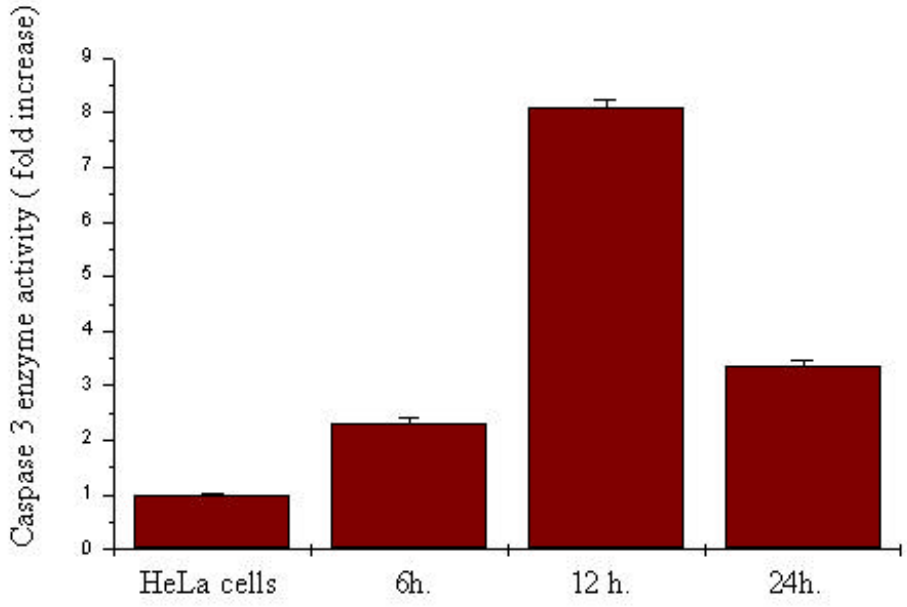


Figure 42. *C. albicans* induces caspase-3-mediated apoptosis. HeLa cells ($5x10^5$) were grown in six well plates and were infected with *C. albicans* (MOI=5) for 6 h. The cell were lysed using caspase lysis buffer and were incubated with caspase3 substrate – AcDEVD-pNA. and the amount of caspase 3 degraded was measured by spectrophotometer at 405 nm. The figure represent the fold change of Caspase-3 enzyme compared to the control HeLa cells.

4.43. Inhibition of PI-3 kinase by wortmannin increases the *C. albicans* induced caspase-3 activity in HeLa cells

Rationale: PI-3 kinase was found to be upregulated by *C. albicans* and is known to be involved in apoptosis, we investigated its role in HeLa cell-*C. albicans* interaction.

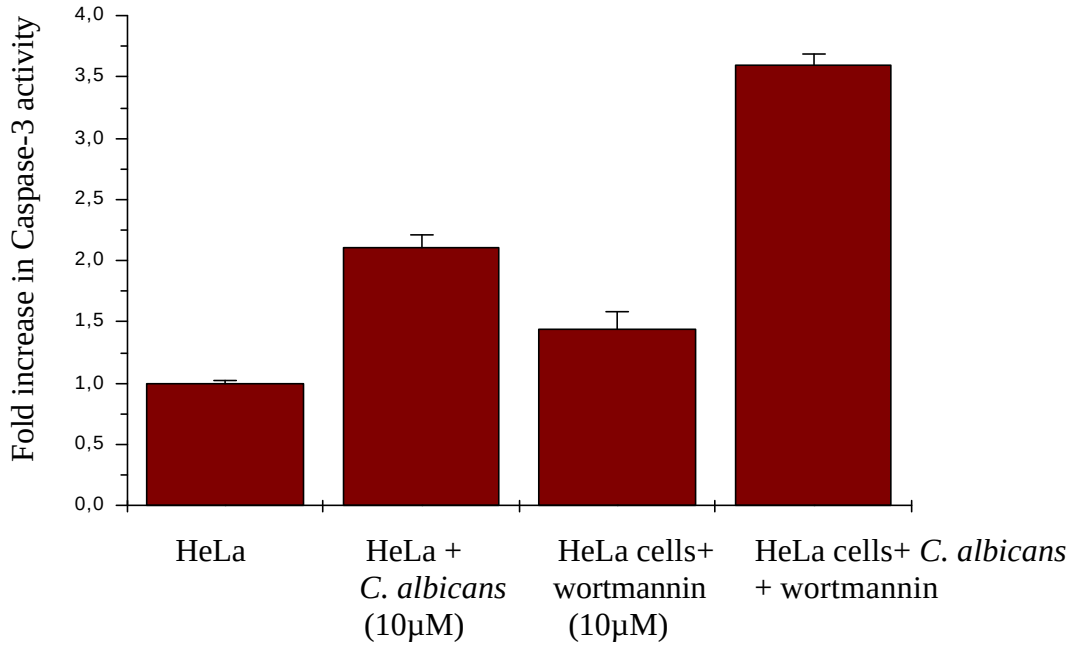


Figure 43. Inhibition of PI-3-kinase upregulates *C. albicans* induced apoptosis. HeLa cells ($5x10^5$), grown in six well plates, were infected with *C. albicans* (MOI=5) for 6 h. The cells were lysed using caspase lysis buffer and were incubated with caspase3 substrate – AcDEVD-pNA. The activity was measured by spectrophotometer at 405 nm. The figure represents fold increase in caspase activity.

HeLa cells were infected with *C. albicans* for 6h (MOI of 5) in DMEM containing 1% FCS with a preincubation of 10µM wortmannin for 1h. The caspase-3 enzyme activity was found to be increased by the inhibition of PI-3-kinase with wortmannin. As shown in Fig. 43, enhancement was more then that observed with *C. albicans* alone indicating anti-apoptotic activity of PI-3- Kinase in *C. albicans*-HeLa cell interaction.

4.44. Inhibition of NF-kB by dominant negative of IKK reduces *C. albicans* induced upregulation of caspase-3

Rationale: NFκB is an important molecule in the process of apoptosis. Since we find upregulation of NFκB during infection of HeLa cells with *C. albicans*, we studied its role in the process of apoptosis.

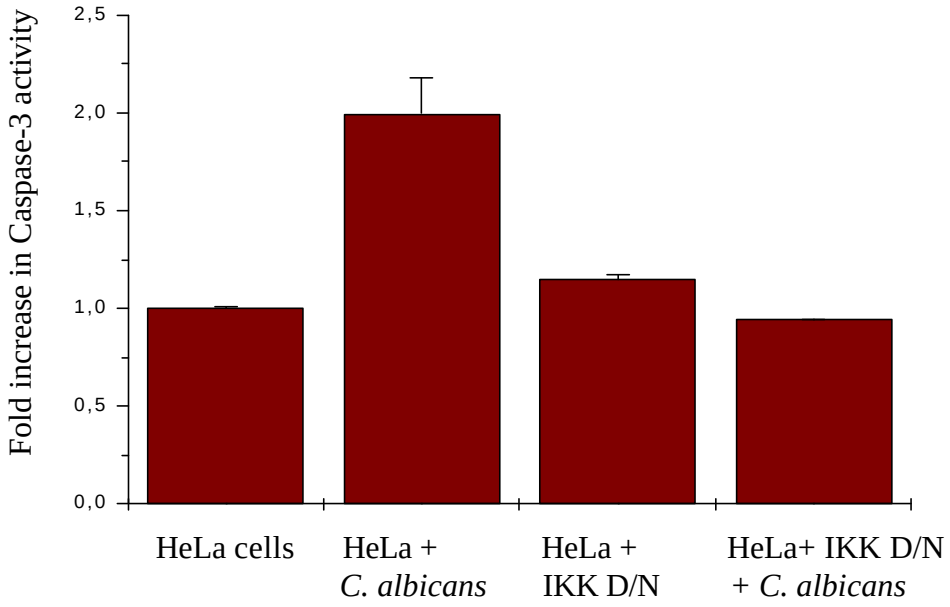


Figure 44. Inhibition of NF-kB by dominant negative of IKK reduces *C. albicans*-induced upregulation of caspase-3. HeLa cells were transfected with IKK dominant negative, the cells were kept for 24 h for expression followed by infection with *C. albicans* (MOI=5) for 2 h. The cells were lysed and assayed for for caspase activity.

Prior to the infection, HeLa cells were transfected with Inhibitor kappa kinase dominant negative (IKK D/N) plasmid. 24 hr later the cells were infected with *C. albicans* with a MOI of 5 in DMEM containing 1% FCS. Caspase-3 activity was assayed, by monitoring the degradation of caspase-3 substrate – AcDEVDpNA, in HeLa cell lysate. As shown in Fig. 43, inhibition of NFκB, was coupled with decreased caspase-3 activity, demonstrating the prominent role of NFκB in apoptosis.

4.45. *C. albicans* induces cleavage of p65 in HeLa cells

Rationale: NFκB is generally attributed with an anti-apoptotic role, though there are reports of its pro-apoptotic activity [205, 206]. Since, in our system NFκB shows a proapoptotic activity, it was of interest to elucidate this pathway. Truncated p65 molecule has been shown to reduce anti-apoptotic activity of NFκB and thus induce apoptosis in the cell. We wanted to study whether *C. albicans* induces apoptosis by this mechanism.

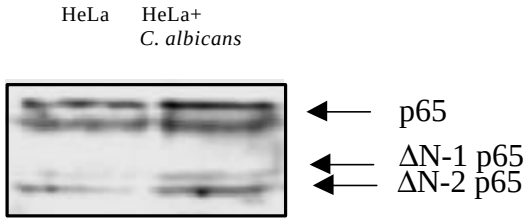


Figure 45. *C. albicans* induces cleavage of p65 in HeLa cells. HeLa cells were infected with *C. albicans* (MOI=5). The nuclear protein was resolved on 12% SDS-Polyacralymide gel electrophoresis, transferred on to nitrocellulose membrane and was subjected to Western analysis with primary antibody against NF-kB p65 for 1 h. The blots were visualised by chemiluminiscence after incubation with secondary antibody.

We found that upon infection of HeLa cells with *C. albicans,* there occurs a cleavage of p65. Since the primary antibody was against the amino terminus of p65, the data indicate to the cleavage of the carboxy terminus of p65 as decsribed by Levkau, et al. [132]. We found that this cleavage of p65 was involved in mediating the apoptosis process. The carboxy terminal cleavage of p65 and its role in apoptosis was confirmed in our system.

4.46. *C. albicans* induces nuclear translocation of cRel and p50

Rationale: Rel C and p50 have been shown to be the proapoptotic subunits of NFκB [206]. The nuclear translocation of these subunits was investigated as an additional causative factor in our system.

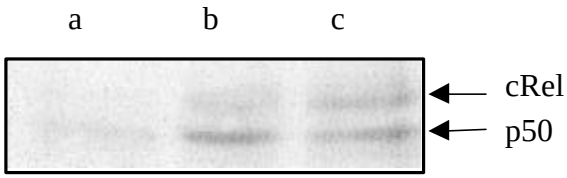


Fig. 46. *C. albicans* induces nuclear translocation of cRel and p50. Hela cells were in fected with *C. albicans* (a, b) in absence (b) or presence of 10μM wortmannin(c). The nuclear extract were prepared and were analyed for cReL and p50 using specific antibodies by Western blot.

We find that both Rel C and p50 get translocated into the nucleus upon infection of HeLa cells with *C. albicans*. Both subunits have been shown to be substrates for caspase-3 and as inducers of the pro-apoptotic function to NFκB [206].

5. DISCUSSION

C. albicans and arachidonic acid metabolism

Infection with *C. albicans* induces activation of defence mechanism of the host. As a defence mechanism, the host cells produce arachidonic acid (AA) which further gets converted into eicosanoids that mediate inflammation. In the present study, interaction between *Candida albicans* as pathogen and HeLa cells was investigated. The mechanism of infection process by *C. albicans* was further elucidated. *C. albicans*, apart from the simple sugars, is able to utilise various other complex substrates like ethanol, acetate and butyrate as the carbon source for energy generation. Aim of this study emanates from previous studies that AA can be converted by fungi to a growth factor 3-hydroxy eicosotetraenoic acid (3-HETE), although these fungi do not contain C 20:4 fatty acids (AA). 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. It was found that the AA is an appropriate substrate for the human fungal pathogen *Candida albicans*. Although, this fungus does not contain AA of its own, it is capable of utilising it as a good substrate for the energy supply. This became evident from the observations that the fungus grew on AA as a sole carbon source. *C. albicans* possesses machinery for both mitochondrial and peroxisomal β-oxidation [63,165]. Normally, β-oxidation of poly-unsaturated fatty acid with even double bond occurs in peroxisomes by enzymes MFE-2 . In our case incomplete β-oxidation takes place in mitochondria, causing 3R-hydroxylation of arachidonic acid, leading to formation of 3-HETE [107]. It is found that presence of 5Z,8Z-diene system in fatty acids is a prerequisite for 3R-hydroxylation [100]. Acetyl Co-A which is formed during the metabolism of AA, enters the citric acid cycle for energy generation. We find that a significant amounts of labelled CO_2 is formed from [1-^{14}C]-arachidonic acid by this mechanism, which comes from the decarboxylation reaction of TCA cycle. Fatty acids have been reported to induce the isocitrate lyase, a key enzyme of the glyoxalate shunt of the citrate cycle [66, 233]. The glyoxylate shunt directs acetyl-CoA derived from fatty acid metabolism to *de novo* synthesis of biomolecules from fatty acid. Acetyl-CoA forms succinate bypassing the decarboxylation reaction of TCA cycle. Oxaloacetate formed from glyoxylate bypass is used up for *de novo* synthesis of proteins and carbohydrates. Using this machinery AA was found to be used as source of energy as well as substrate for the

formation of building blocks for carbohydrate biosyntheses. As high as two-thirds of the radioactivity from arachidonic acid metabolism was found in the carbohydrates of *C. albicans*.

Hydrogen atoms derived during the metabolism of arachidonic acid, enter the electron transport chain through a series of carriers including cytochromes, iron-sulfur proteins and flavin linked proteins, bringing about oxidative phosphorylation and ultimately to molecular oxygen. Antimycin A inhibits the flow of transport of electron from cytochrome b and c_1, thus inhibiting respiration. Unlike human cells, *C. albicans* is reported to possess alternative terminal oxidase [207]. The gene of this enzyme in the alternative oxidase pathway has been recently identified [168]. This pathway differs from the main respiratory pathway in that the oxygen uptake is accompanied by heat production instead of ATP formation. Alternative pathway branches off from the main respiratory chain at the site of cytochrome b, and thus is insensitive to antimycin A or cyanide [234]. AA was found to have a salicylhydroxamic acid-like action in the inhibition of alternative oxidase of the mitochondrial electron transfer system pathway. This demonstrats that arachidonic acid metabolites which have biological importance in the life cycle of *C. albicans* and in pathogenicity in humans are formed with the supression of alternative mode of energy generation. In higher plants, the expression of alternative oxidase has been reported to be stimulated both in post-germinative development of cotyledons [208]. It is also involved in infection-mediated necrosis [209]. It is tempting to speculate that in *C. albicans* the alternative oxidase is involved in the alteration of morphogenesis. Thus, its selective inhibition by arachidonic acid could play a role in the infection process.

The observation that *C. albicans* grows on arachidonic acid is in line with the facts that certain fungi of the genus Candida are capable of growing readily on both hydrocarbons of variable chain length [66, 210] and oleic acid [211]. Hydrocarbons are first oxidised to the corresponding monocarboxylic acid and then in some species to the α, ω-dicarboxylic acid. The further degradation proceeds via activation by an acyl CoA synthetase, peroxisomal β-oxidation and citrate/glyoxalate cycle. Fatty acids have been reported to induce the isocitrate lyase, a key enzyme of the glyoxalate shunt of the citrate cycle which is involved in the effective conversion of arachidonic acid to carbohydrates [214]. Thus, *C. albicans* is able to survive in the glucose deficient environment using glyoxylate shunt upon internalisation by host cells [212].

Upon feeding *C. albicans* with AA, a novel 3-hydroxyl eicosanoid was found to be formed. This compound was first detected by its immunoreactivity towards an antibody specific to 3-OH group. It was then identified as by GS-MS analysis of the lipid extract as 3,18-dihydroxy-5,8,11,14-eicosatetraenoic acid. Its formation was strongly suppressed by salicylate that also inhibits the formation of 3(R)-HETE in *Dipodascopsis uninucleata* suggesting the occurrence of a common biosynthetic pathway to 3(R)-hydroxy-oxylipins in fungi.

In a recent study (Ciccoli, R et al. submitted for publication), we found the formation of 3,18-DiHETE in *D. uninucleata* through mitochondrial-dependent and independent pathways. In *D uninucleata*, 3R-HETE was formed exclusively from AA. 3-hydroxylation occurs in mitochondria by enoyl-hydratase. The R-configurationof 3(R)-OH fatty acids prevent their β-oxidation at the level of 3-OH fatty acid, 3(S) configuration is a suitable substrate for β-oxidation. In *C. albicans* 3-hydroxylation is followed by 18-hydroxylation. Although these organisms do not have arachidonic acid, they are able to metabolise endogenous fatty acids with the formation of bioactive oxylipins like LA derived 3-HTDE.

Even though the fungus was grown in the absence of arachidonic acid, using a group-specific antibody against 3(R)-hydroxy fatty acids, the presence of such compounds could be detected *in situ* in the hyphae of *C. albicans*. Again, the immunofluorescence was strongly suppressed, when the fungus was grown in the presence of acetylsalicylic acid or salicylate. Since *C. albicans* does not contain arachidonic acid, the endogenous 3(R)-hydroxy-oxylipin(s), which are important in the life cycle of this fungus, must be different from 3,18-DiHETE. It may be speculated that the endogenous immunoreactive compounds are metabolites of linoleic acid, which is one of the major fatty acids in the lipids of *C. albicans* [55, 58], Moreover, a linoleic acid-derived 3(R)-hydroxy-oxylipin was identified as 3-HTDE [100] in *Dipodascopsis uninucleata*. However, if exogenous AA is available to *Candida albicans*, 3,18-DiHETE is formed as an additional metabolite upon interaction with mammalian cells. It remains to be established whether the formation of 3,18-DiHETE affects the pattern of endogenous 3(R)-hydroxy-oxylipins and whether the various oxylipins reveal different biological activities. The possible biological role of the oxylipins produced by Candida species is unknown. In a number of other fungi oxylipins of

different structures are proposed to play a regulatory role in the reproductive cycle of the respective fungi.

Aspirin was found to suppress the growth of *C. albicans* and the formation of hyphal and other multicellular structures due to inhibition of the biosynthesis of 3(R)-hydroxyoxylipins by the pathogen. Oxylipins are found to have many biological effects in fungi. In *D. uninucleata*, 3-HETE was found to be involved in the sexual phase [109]. Although *C. albicans* does not exhibit classical sexual cycle of fungi with distinct sexual forms, it possess distinct differences in the morphological forms in its life cycle. It was found that 3-hydroxy oxylipins is found selectively in hyphal stage of *C. albicans* and thus it seems to have an important role in the morphogenesis. The mode of action of azole fungicides is via inhibition of cytochrome p450 and ergosterol biosynthesis [51] hence inhibits 18-hydroxylation mechanism. Aspirin, which is a β-oxidation inhibitor was found to inhibit the 3-hydroxylation mechanism. For this reason combination of clotrimazole and aspirin to inhibit 18-hydroxylation and 3-hydroxylation of 3,18-DiHETE was found to be more effective. The widespread use of azole antifungal drugs has led to the development of drug resistance in *C. albicans*, creating a major problem in the treatment of yeast infections. 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 types of morphogenetic forms are apparently necessary for colonisation and invasion of 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. We found that the aspirin, which inhibited formation of 3-hydroxyoxylipins, also inhibited formation of filamentous forms as well as the growth of 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. Previous studies in fibroblast cells demonstrated that the inhibition of beta- oxidation occurs due to structural similarities between aspirin and 3-hydroxyacyl-CoA and 3-ketoacyl-CoA, which are substrates for 3-hydroxyacyl-CoA-dehydrogenase [170].

The first step of infection includes adhesion of pathogen to host. *C. albicans* which adheres rapidly is found to be more virulent, and is able to proceed further to establish the infection process. The ability of *Candida albicans* to respond to diverse environments capacity for multiplication inside the host and resistance to environmental influences, is critical for its success as a pathogen. We find that aspirin is able to decrease the adhesion of *C. albicans* to HeLa cells. This ability along with the ability to inhibit candidal forms and 3hydroxyoxylipins, demonstrate its potentiality for the chemotherapy against *C. albicans* infection.

Host Pathogen Interaction

Host factors affecting the pathogenicity of *Candida albicans*

C. albicans is able to release arachidonic acid from HeLa cells upon infection which is subsequently converted into eicosanoids, such as prostaglandin E_2. COX-1 is the constitutive form expressed at basal level in most of the tissues while COX-2 is induced by a wide range of mitogens, tumor promoters and cytokines [79, 80]. The primary observation has been the ability of *Candida albicans* to upregulate the production of COX-2 enzyme in HeLa cells and the production of PGE_2. Cyclooxygenase enzymes convert arachidonic acid to PGG_2, which is further converted to PGH_2 by the peroxidase activity of prostaglandin H synthase. The upregulation of COX-2 was clearly evident within 6 hours of infection. This corresponds to the concurrent increase in the levels of PGE_2. Selective neutralisation of PGE_2 production has been used to block pain and inflammation. Cyclooxygenase inhibitors are the most widely used class of painkillers and anti-inflammatory agents. In *Candida albicans*, PGE_2 enhances the germ-tube formation. Germ-tube formation has been characterised as a sign of pathogenicity of *C. albicans* [22]. This activity of PGE_2 seems to inadvertently promote the infectivity of *C. albicans* by inducing germ-tube formation which is necessary for penetration into the tissue. The data demonstrates that *C. albicans* selectively induces COX-2 gene and protein synthesis but not COX-1.

We also observed that 3-HETE, at a concentration as low as 5 µM is able to upregulate COX-2 in HeLa cells, indicating that it is an important compound responsible for *C.*

albicans-induced upregulation of COX-2. It has been also observed that 3-OH oxylipins are associated with the hyhyphal forms in *Candida albicans* [170]. It has been observed that the host cells, especially monocytes and HeLa cells, engulf *Candida albicans* upon infection (Rosseau S et al and present study). This internalisation process results in the production of a novel arachidonic acid metabolite 3,18-DiHETE, which is believed to be responsible for the process of inflammation.

Signal transduction cascade in COX-2 upregulation

Induction of COX-2 expression and prostaglandin formation has previously been demonstrated to be associated with activation of the MAPK signaling pathway in various cell systems. For example, Xie *et al.* provided evidence for the role of JNK and c-Jun in mediating v-Src-induced COX-2 gene expression [214]. p38 MAP kinase has been implicated in interleukin-1-stimulated COX-2-dependent prostaglandin production in fibroblasts, and human umbilical vein endothelial cells [177, 215]. Other signaling elements of the prostaglandin pathway have also been linked with activation of MAPKs. For example, both ERK ½ and p38MAP kinase mediate biosynthetic activation of cytosolic phospholipase A_2 [211]. PKC and p38 MAP kinase are primarily serine/threonine kinases and their activation is itself dependent upon the phosphorylation by other kinases. ERK1/2 is another kinase shown to be involved the COX-2 upregulation.

Investigation of MAPK signaling pathways for the *C. albicans* induced activation of COX-2 showed the stimulation of p38 MAPK pathway without involvement of ERK1/2 and JNK pathways. Moreover, it was found that p38 MAPK was rapidly phosphorylated by PKC, as GF203190X, a PKC inhibitor, abrogated p38MAPK protein and COX-2 activation. Previous studies have shown the involvement of PKC and p38 MAPK in the production of COX-2 [88].

However, inhibition of this kinase by PD98059 did not modulate the expression of COX-2, or the amount of PGE_2 as seen in other systems. These results suggest key roles for PKC as well as for p38 MAP kinase cascades in the biosynthesis of PGE_2, likely by upregulating the induction of cyclo-oxygenase-2 in *C. albicans* infected HeLa cells. PI-3 kinase was also activated by *Candida albicans* infection, although inhibition by wortmannin failed to

suppress the COX-2 upregulation. This observation suggests a different role for PI-3-kinase upon *C. albicans* mediated infection of HeLa cells.

Activation of NF-κB and MAP kinases as signalling events play a pivotal role in cytokine, LPS, or stress induced gene expression. One of the important DNA binding proteins in many stress inducible responses is NF-κB. Proteins of the NF-κB family are important transcription factors retained in the cytoplasm through heterodimerization with one of several types of IκB proteins. Stress inducing factors trigger the phosphorylation and degradation of IκB, resulting in the release of NF-κB to enter the nucleus, where it activates gene transcription [191, 217]. Following stimulation with agonists, IκB is phosphorylated at the serine residue by inhibitory kappa β kinase (IKK) complex, and subsequently degraded. NFκB then translocates to the nucleus, where it docks to DNA at binding sites within promoter region of target genes [218]. In addition to regulating cellular responses to cytokines, oxidative stress and pathogens, NFκB plays role in inflammation [217], apoptosis [219] and tumor cell proliferation. The involvement of this pathway was investigated in *C. albicans* infected HeLa cells. We also investigated the role of the other members of the COX-2 cascade on the NF-κB activation. Remarkably high levels of NF-κB promoter binding activity are detected in HeLa cells during infection with *C. albicans* in HeLa cells. Sodium salicylate, an inhibitor of COX-2 activity, abolilshed the NFκB activity. The inhibition was even beyond the basal level of NFκB activity. Seemingly, the effect on cell cycle is specific to cell type [221].

C. albicans induced activation of NF-κB was inhibited upon inhibition of p38 MAPK with SB202190, PKC with GF203190X and PI-3 kinase with wortmannin. PI-3 kinase pathway was found to be involved in the upregulation of NF-κB activity. Inhibitor-mediated repression of COX-2 by IKK dominant negative demonstrated only partially inhibition of COX-2 upregulation and showed some residual promoter activity. This indicated that factors other than NFκB may be involved in COX-2 promoter activation. Possible candidate could be epidermal growth factor receptor (EGFR), an inducer of COX-2 [222], and Vascular endothelial growth factor (VEGF), a potent angiogenic factor [223]. Increased synthesis of PGE_2 may also stimulate IL-8 gene, another angiogenic factor reported from human colonic epethelial cells [224]. Thus, many pathways may simultaneously exist for the upregulation of COX-2. We find the involvement of free

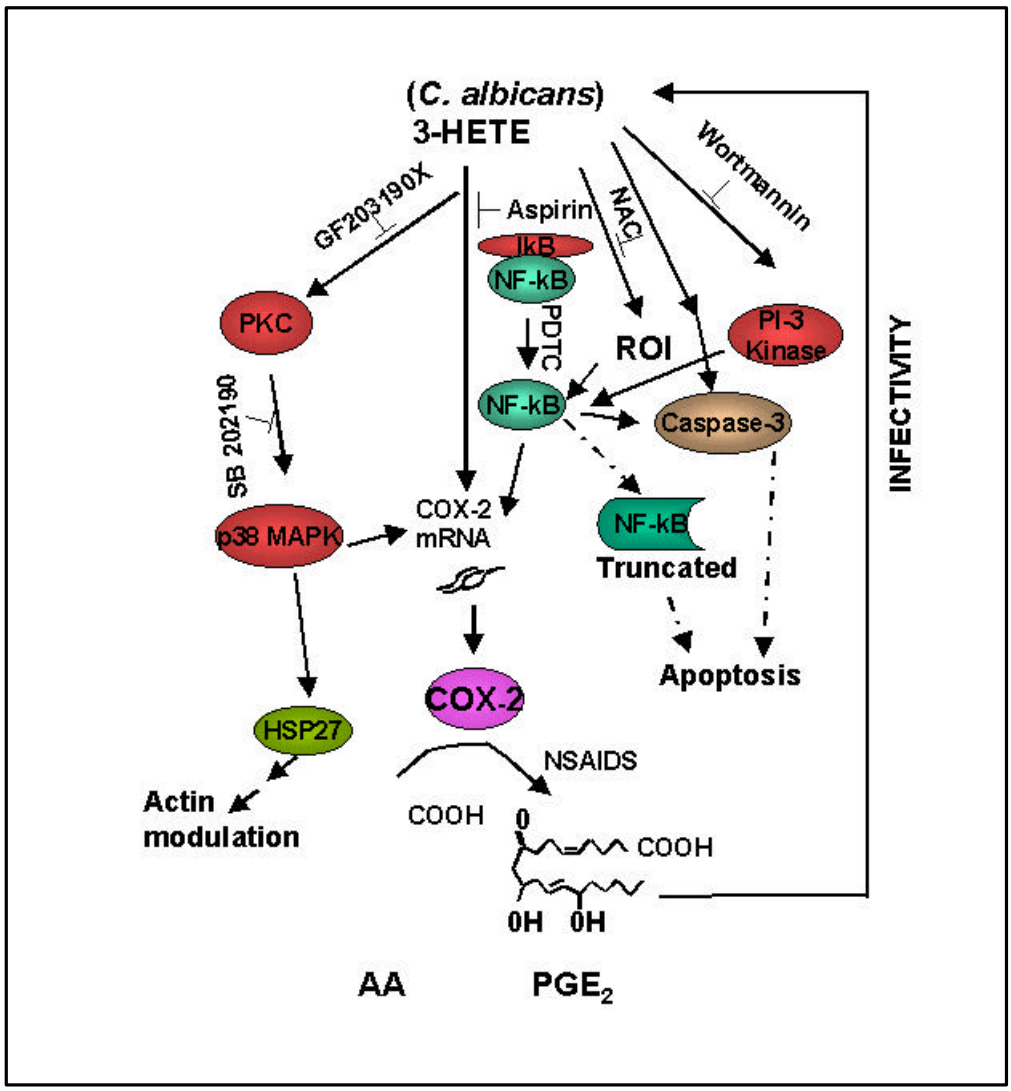
radicals in COX-2 and PGE_2 upregulation during the infection of HeLa cells with *C. albicans,* which demonstrate involvement of reactive oxygen species and NFκB. Aspirin is an inhibitor of cyclooxygenase and is able to inhibit NFκB [171]. Thus, it can act via dual pathways in combating *C. albicans* induced infection. Specific inhibition by free radical scavenger PDTC, and NAC leads to specific inhibition of COX-2 promoter activity and PGE_2 synthesis, providing a functional link between *C. albicans*-stimulated production of Reactive Oxygen Intermediates (ROIs) and COX-2 activation.

HeLa cells and smooth muscle cells are important sources of xanthine oxidase [225,226] which might generate reactive oxygen species (ROI). ROIs in turn cause cellular inflammatory response by activation of intracellular stress kinases and subsequent tissue injury [227]. We find that NAC, a precursor of glutathione, and PDTC, a free radical scavenger, strongly decreased the COX-2 activity. These findings are consistent with the earlier reports in which oxidative stress has been assigned a key role in the activation of COX-2 [181]. Oxidative stress has been linked with intracellular MAP kinase signaling pathways and that are associated with COX-2 gene expression. Using various inhibitors of MAPK signalling pathway, as well as transient transfection with IKK dominant negative plasmid, we could demonstrate that *C. albicans* influences COX-2 pathway via NFκB.

PGE_2 produced by *C. albicans*-induced HeLa cells induces activation of PI-3 kinase which is either through G protein coupled cytoplasmic PGE_2 receptors or through PPAR γ receptors [228]. The presence of PPAR γ receptors as well as COX-2 on nuclear envelop pinpoint the feasibility that prostaglandins, analogous to 15-deoxy-PGJ_2 may exert their cell protective role through these receptors [232]. 3-HETE which is a β-oxidation product of arachidonic acid produced by *C. albicans,* was found to be an excellent ligand for PPARγ, which increased PPAR gamma promoter activity in transient transfected cells. In similar experiments, albeit transfected with PPAR gamma dominant negative plasmid, 3-HETE failed to enhance the promoter activity (unpublished results). Therefore, it can not be ruled out that the action of *C. albicans* on HeLa cells is mediated by 3-HETE or its derivative 3,18-DiHETE produced by *C. albicans*

Extensive programmed cell death (apoptosis) was observed after 24 h in Hela cells infected with *C. albicans* as shown by genomic DNA laddering and TUNEL assay. After 6 h. postinfection, upregulation of caspase-3 activity was observed. Caspase-3 is an effector

caspase which is involved in the cleavage of cellular proteins during apoptosis. Further more, using DNA microarray technology, we showed that *C. albicans* upregulated PI-3 kinase in HeLa cells. PI-3 kinase is a prominent mediator of anti-apoptotic functions via AKT and p65 NFκB. Its inhibition by wortmannin increased the caspase-3 activity in infected cells. IκB dominant negative transfected cells showed no caspase-3 activity upon infection. This result was surprising as p65 NFκB, whose release is prevented by the dominant negative plasmid, is an anti-apoptotic molecule. This paradoxical reaction was clarified by the observation of truncated forms of p65 NFκB in *C. albicans* infected HeLa cells. Carboxy terminal truncation of p65 NFκB by caspase-3 renders the molecule inactive, thereby preventing its anti-apoptotic function.



Scheme Representing signaling mechanism by *C. albicans* and 3-HETE in HeLa cells.

The pro-apoptotic subunits of NFκB, p50 and c-REL, were found to be nuclear translocated in *C. albicans* infected cells. Wortmannin mediated inhibition of PI-3-kinase strongly inhibited the activation of NF-κB leading to speculation on multiple roles of NFκB dependent pathways involved in cell signaling.

p38 MAP kinase is not only involved in the upregulation of COX-2, but it is also involved in the induction of cytoskeletal changes in HeLa cells [201]. Previous studies have shown [197, 198, 199] the involvement of Heat Shock Protein 27 (HSP27) and p38 MAP kinase in the modulation of actin cytoskeleton. Heat Shock Proteins, a ubiquitous family of highly conserved proteins, function to stabilize cellular proteins during a variety of conditions such as heat shock, infection, and inflammation. Phosphorylation of HSP27, primarily chaperones, by p38 MAP kinase has been demonstrated as an important element in the modulation of f-actin dynamics in cellular stress conditions. *C. albicans* induces serine phosphorylation of HSP27 in HeLa cells. The changes in actin modulation are inhibited by p38 MAP kinase inhibitor, indicating another function of p38 MAP kinase, other than the upregulation of COX-2. Whether this modification in the shape of the cell has an influence on the progression of *C. albicans* infection has to be further investigated.

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Rupal Deva, Roberto Ciccoli, Lodewyk Kock, Tankerd Schewe and Santosh Nigam Metabolism of arachidonic acid and formation of oxylipins in fungi. Poster (no. 18) presented at International symposium on non mammalian eicosanoids and bioactive lipids, May, 2000, Berlin Germany.

Santosh Nigam Rupal Deva, Roberto Ciccoli, Inka Leo-Rossberg, Dandre P. Smith, Lodewyk Kock, Tankard Schewe. Aspirin-sensitive oxylipins in *Candida albicans*. Implications for treatment of vulvovaginal candidiasis. Poster (no. 16) presented at International symposium on non mammalian eicosanoids and bioactive lipids, May, 2000, Berlin Germany.

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