Chapter II

Impaired phospholipid metabolism in S. cerevisiae by NNK and NNN
1. **Abstract**

Tobacco specific nitrosamines (TSNAs) namely 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosonornicotine (NNN) are potent carcinogens present in cigarette smoke, and chronic exposure led to pulmonary cancer. However the impact on lipid metabolism remains enigmatic. NNK and NNN influence phospholipid metabolism and the mechanism is yet to be elucidated. Hence, *Saccharomyces cerevisiae* was exposed to different concentrations of NNK or NNN (0 to 400 µM) to elucidate their role in lipid metabolism. Reduced phospholipids were accompanied with increased neutral lipid content. Here we report for the first time that NNK and NNN exposure significantly increase phospholipase B (PLB), particularly plb1p activity and the preferred substrate is PC, a major phospholipid responsible for a series of metabolic functions and also a major constituent in the pulmonary surfactant. Furthermore, altered PLB activity by NNK and NNN promotes the alteration of fatty acid (FA) composition in both phospholipids and neutral lipid. It increases the long chain fatty acid (C 18 series) in phospholipids specifically phosphatidylethanolamine (PE) and PS, whereas short chain fatty acids are increased in cardiolipin (CL). NNK and NNN mediated degradation of phospholipids is associated with enhanced PLB activity and alteration of phospholipid composition is accompanied with acyl chain remodelling. Understanding the altered phospholipid metabolism produced by NNN exposure is a worthwhile pursuit because it will help us to understand the toxicity of tobacco smoke.

**Key words:** Tobacco specific nitrosamines, N-nitrosonornicotine, *S. cerevisiae* phospholipid, phospholipase B, neutral lipid, free fatty acid.
2. Introduction

Tobacco smoking is hazardous to smokers and also affects the surrounding atmosphere. Tobacco products and cigarette smoke affect the environment to a great extent, resulting in many respiratory diseases including cancer. The air pollution emitted by cigarettes is 10 times greater than diesel car exhaust (Invernizzi et al., 2004). Tobacco-specific nitrosamines (TSNAs) are toxic compounds present in cigarette smoke and tobacco products (Chen et al., 2008; Hecht and Hoffmann, 1988; Stinn et al., 2010). Specifically 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and N-nitrosonornicotine (NNN) are potent pulmonary carcinogens, reported by the International Agency for Research on Cancer as human carcinogens (IARC, 2007) and exert a wide spectrum of biological effects on the lung and lung airways, including oxidative stress, inflammation and DNA damage (Hecht and Hoffmann, 1988; Lin et al., 2010; Tyroller et al., 2005). TSNAs primarily target the lung and alter the composition of lipid-protein complex particularly surfactants. Tobacco specific nitrosamines non-specifically bind to the targeting cells based on their membrane composition (Schuster et al, 1992). The impact of the above compounds on lipids particularly phospholipid metabolism is yet to be elucidated.

Phospholipids are major components of the cellular membranes that take part in a series of metabolic events including maintenance of the cellular permeability, regulation of proteins associated with membrane and regulation of intracellular signalling (Yamashita et al., 1977; Carman and Zeimetz, 1996; Exton, 1994). Reported evidences showed that smoking is associated with reduced surfactant phospholipids in the bronchoalveolar lavage particularly phosphatidylcholine (Finley and Ladman, 1972; Scott, 2004) and the responsive compounds are yet to be elicited. Exposure to NNK and NNN increased lipid peroxidation in membrane phospholipids due to generation of reactive oxygen.
Species (ROS), and was also reported by us (Nachiappan et al., 1994). TSNAs alter the lipid composition including fatty acid changes required for membrane adaptation (Schuster et al., 1995; Vijayaraj et al., 2011a and 2011b).

The assessment of chemicals for their toxicological data normally requires a large number of animal species. An alternative approach is to use microbial system to study the effect of this compound. To achieve this, Saccharomyces cerevisiae, is exposed to NNK and NNN. S cerevisiae is an excellent and an increasingly important model system that is currently being used to explain logical and fundamental questions in eukaryotic cell biology and genetic regulation. It will play a pivotal role in advancing our understanding of phospholipid metabolism, because many aspects of metabolic processes are similar to mammalian system, and hence it is much easier to study. The obtained results could be easily compared with conventional animal models, for better understanding of the role of this compound in human pathogenesis.

Reported evidences mainly focused on the effect of TSNAs on carcinogenicity and there is paucity or lack of report of their effect in phospholipid metabolism and membrane remodelling. The chosen compounds NNK and NNN interact directly with the membrane components, hence we aimed to investigate the impact of NNK and NNN on phospholipid metabolism during acute exposure. Further, in S. cerevisiae PLBs are encoded by plb1, plb2, and plb3 and are associated with the plasma membrane, cell wall and periplasmic space respectively. plb1 and plb2 preferred to hydrolyse PC, PE, PS and PI whereas Plb3 hydrolyses only PI and PS (Merkel et al., 2005). To identify the role of TSNAs on these enzyme activities, we performed phospholipase assay with all three mutants and wild type cells.
3. Materials

Wild-type *S. cerevisiae* strain BY4741 [MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0] was procured from Open Biosystems (AL, USA) and its isogenic plb1Δ, plb2Δ and plb3Δ were gifted by CIMAP, India. [³²P]orthophosphate (5000 Ci/mmol) was obtained from Bhabha Atomic Research Centre (Mumbai, India) and [1-¹⁴C]acetate (1000 Ci/mmol) was obtained from PerkinElmer Life Sciences (USA). Yeast medium, lipid and fatty acid standards were obtained from Sigma (India). Silica gel 60F254 TLC plates were from Merck (India). All chemicals and solvents were purchased from Sigma unless specifically mentioned.

NNK and NNN were obtained from Sigma (India). We performed purity assessment by liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) for both compounds and it was about 99% pure (Figure A). Stock solution was prepared in dimethyl sulphoxide (DMSO) and stored at -20ºC till use. Maximum DMSO in growth media was 0.05% (v/v), a concentration that had no detectable effect on yeast growth (data not shown).

*Figure A. Purity assessment of NNK and NNN by LC/ESI-MS/MS*
3.1  Growth conditions and sensitivity assay

Yeast cells were grown in YPD (1% yeast extract, 2% peptone, and 2% dextrose) medium (pH 7.0) with aeration at 30 °C. The cells were grown in 5 ml of YPD medium for 12 h and harvested, transferred to 25 ml of YPD media with indicated concentrations of NNK, NNN (0 to 400 µM). Cells were grown at 30 °C at 180 rpm and cell growth was studied by measuring the $A_{600}$ of the cultures at frequent time intervals. The sensitivity of the yeast cells to both NNK and NNN was observed on solid medium supplemented by spotting experiments. For this the cells were serially diluted and spotted on YPD plates containing 2% agar with or without NNK/NNN and incubated for 48 h at 30ºC. The serial dilutions used were $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ and $10^{-5}$.

3.2  Labelling of yeast lipids.

Phospholipid alterations were analysed by $[^{32}\text{P}]$orthophosphate labelling (Ghosh et al., 2008) and neutral lipids were analysed by $[1-{^1}\text{C}]$acetate labelling (Rajakumari and Daum. 2010). Briefly, the cells were grown at 30 °C in YPD medium with or without different concentrations of NNK, NNN (0 to 400 µM). To this either 100 µCi of $[^{32}\text{P}]$orthophosphate (5000 Ci/mmol) or 0.5 µCi of $[1-{^1}\text{C}]$acetate (1000 Ci/mmol) was added. Cells were grown for 12 h and equal number of cells were taken and harvested by centrifugation. Unused radio label were removed by washing with 2% phosphoric acid were added and, subjected to lipid extraction.

3.3  Lipid extraction and Identification.

Total lipids from yeast cells were extracted by the method of Bligh and Dyer (1959). Briefly, to the cell pellet 400 µl of methanol and 200 µl of chloroform were added and vortexed. To this, 400 µl of acidified water (2% orthophosphoric acid) was added and vigorously vortexed. Chloroform extracts containing total
lipids were subjected to two-dimensional thin layer chromatography (TLC) with Silica Gel. In TLC plates phospholipid separation was done by using the following solvents, for first dimension chloroform/methanol/ammonia (65:25:5, v/v) and for the second dimension chloroform/methanol/acetone/acetic acid/water (50:10:20:15:5, v/v). Neutral lipids were separated by using petroleum ether: diethyl ether: acetic acid (70:30:1, v/v) as the solvent system. Individual lipids were located by comparing the $R_f$ values of the unknown with the $R_f$ values of the standard. The radioactive spots were developed by autoradiography for 12 h. The spots were scraped from the TLC plate, and the incorporation was counted in liquid scintillation counter (PerkinElmer Life Sciences, USA).

3.4 Fatty acid analysis

The cells were grown in YPD medium with 50 µM concentration of NNK, NNN alone or together. Both neutral lipids and phospholipids were extracted from the cells and fatty acids were analyzed by Gas chromatography-mass spectrometry (GC/MS). Lipids were separated by TLC and individual lipids were extracted from the silica gel with chloroform/methanol (2:1 v/v), and subjected to methanolysis using BF3/methanol for conversion to methyl esters (Morrison and smith. 1964). Fatty acid methyl esters were separated by GC/MS and quantification was referred to heptadecanoic methyl ester (C17), as an internal standard.

3.5 Preparation of phospholipid substrates

Radioactively labelled phospholipids were prepared biosynthetically by incubating yeast cells with $[^{32}\text{P}]$ orthophosphate for 12 h, followed by separation by 2D-TLC and extraction (Wagner and Paltauf. 1994). Purity was typically >95%, as checked by TLC. Phospholipid micelles were prepared by combining the extracted lipids that are dissolved in chloroform/methanol (2:1, v/v). Solvent was
evaporated under vaccum and dispersed in ethanol followed by sonication (Gheriani-Gruszka et al, 1988).

3.6 Phospholipase assays

Phospholipase activity was assayed using ethanol-solubilized $[^{32}\text{P}]$ labelled phospholipids. The concentration of PC was 2 mM and the specific radioactivity was 5000 Ci/mmol (Fisher et al., 1992). The buffer (200 µl) contained 50 mM Tris-HCl pH 8.0 / 1 mM-EGTA / 10 mM-CaCl$_2$. The reaction was initiated by addition of enzyme source (50µg) from corresponding yeast lysate (wild type and plbΔ strains treated with or without NNK/NNN) and incubated at 37°C for 60 min. The assay was also performed in the presence or absence of calcium. The reactions were stopped by adding chloroform: methanol: 2% phosphoric acid (1:2:1, v/v) and lipids were extracted, separated by TLC and quantified. Protein was determined by the method of Bradford (1976).

4. Results

4.1 Effect of TSNAs on the incorporation of $[^{32}\text{P}]$orthophosphate into phospholipids.

Phospholipid alteration was studied by measuring the $[^{32}\text{P}]$orthophosphate labelling in both control and compound treated cells. Yeast cells were independently treated with different concentrations of both NNK and NNN (0 – 400 µM) and it was depicted in Figure1A and B respectively. The incorporation of $[^{32}\text{P}]$orthophosphate into phospholipids decreased significantly when compared with control and the observed reductions were in a concentration dependent manner. Significant reduction was observed at 400 µM in both NNK (~63%) and NNN (~57%) as compared to control, however 50 µM of NNK (52%) and NNN (40%) showed profound reduction in both cases when compared to other concentrations. The 400 µM of NNK and NNN were further enhanced only ~11%
and 17% greater than 50 µM of TNSAs respectively. Hence 50 µM was chosen for further studies.
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Figure 1. The *S. cerevisiae* cells were grown in YPD medium containing 100 µCi of [32P]orthophosphate in the presence or absence of different concentrations of NNK (A) or NNN (B) and incorporation of [32P]orthophosphate into yeast phospholipids were measured as described in materials and methods. (C) Autoradiography of yeast phospholipid in the presence of NNK. The cells were grown in the presence or absence of 50 µM of either NNK or NNN alone or together. Equal OD’s of cells were taken in each group, lipids were extracted and resolved on two-dimensional silica TLC (1, LPL; 2, PI; 3, PS; 4, PG; 5, PC; 6, PE; 7, CL; 8, PA; O, origin). (D) Amount of [32P]orthophosphate incorporated into various phospholipids with/without 50µM of NNK. Values are the mean of three separate experiments. Data are means ± S.D, † p < 0.05, *p<0.01 and *p<0.001 versus control.

Figure 1C, showed the autoradiography of 50 µM TSNAs on individual phospholipids and its quantitative data was depicted in Figure 1D. Depletion in PC (~59%), ~56% in PS followed by PE (~53%) in NNK exposed cells. In NNN treated cells significant (p< 0.001) reduction was in PS (63%), PC (42%) and PE (36%). Interestingly, the synergestic reduction was observed highly in PG (72%), PE (68%) and PC (67%) whereas both PS and CL showed ~61% reduction. Reduction of phospholipid was accompanied with simultaneous increase in lysophospholipids (Figure 1B). Our [32P]orthophosphate labelling studies suggest that TSNAs decrease the phospholipid content associated with lysophospholipid generation and the maximum reduction was seen in NNK+NNN treated cells.

4.2 Effect of TSNAs on the incorporation of [14C]acetate into neutral lipids.

Declined phospholipid synthesis suggested that there might be a rapid metabolic switchover from phospholipid synthesis to neutral lipid leading to its
Impaired phospholipid metabolism in *S. cerevisiae* by NNK and NNN accumulation. Hence, [14C]acetate labelling studies was performed to assess the impact of TSNAs on neutral lipid metabolism and was showed in Figure 2.

**Figure 2** Increased accumulation of neutral lipid in the presence of NNK and NNN. (A) Autoradiography of yeast neutral lipid in the presence of 50µM of NNK/NNN. The cells were grown in YPD medium containing 0.5 µCi of [14C]acetate for 12 h in the presence or absence NNK/NNN. Equal OD's of cells were taken in each group, neutral lipids were extracted. (B) Amount of [14C]acetate incorporated into various neutral lipids. The values are presented as counts/min per A_{600} of cells after 12 h of labelling. Values are the mean of three separate experiments. Data are means ± S.D. † *p* <0.05, *# p* <0.01 and *#* *p* <0.001 versus control.

TSNAs significantly increase the neutral lipid synthesis in compound treated cell as compared to control. Accumulation of TAG was significantly higher in NNK+NNN treated cells, it was ~2.8 fold whereas ~2.1 fold with NNN and ~1.8 fold in NNK exposed cells. Similar synergism was observed with all other neutral lipids. On the contrary in NNK+NNN exposed cells DAG was increased ~ 1.3 fold only whereas it was ~ 1.8 fold in NNK and ~3.0 fold in NNN treated cells. In addition, sterol and sterol esters also increased significantly in TSNAs treated cells. The overall labelling studies clearly showed significant (*p*< 0.001) reduction.
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in phospholipids accompanied with significant increase (p< 0.001) in neutral lipids. Interestingly we also observed increased free fatty acid (FFA) in NNN+NNK (~2.1 fold) and it was ~1.9 fold in either NNN or NNK treated cells.

**4.3. Involvement of phospholipases on phospholipid degradation.**

To provide evidence for TSNAs induced hydrolysis of phospholipid, we analyzed phospholipase activity using [32P]orthophosphate labelled phospholipid as substrate and TSNAs-treated or untreated yeast lysate as an enzyme source. Figure 3A depicts the effect of the TSNAs on hydrolysis of various phospholipids by phospholipase. On exposure of TSNAs to yeast cells, the phospholipase activity was enhanced for PC degradation followed by PE, PS and PG. The hydrolysis of PC was substantially higher with 50 µM NNN (~1.6 fold) whereas in NNK it was ~1.3 fold, followed by ~1.2 fold in NNK+NNN treated cells.
Impaired phospholipid metabolism in *S. cerevisiae* by NNK and NNN

**Figure 3** NNK and NNN enhances the phospholipase B activity. The wild type cells were grown for 12 h in the absence or presence of NNK/NNN (50µM) and its lysates were used as an enzyme source. The obtained cell lysate was incubated with [32P]orthophosphate labelled phospholipid at 37°C for 60 min and activity was measured. (A) Effect of NNK/NNN (50 µM) on hydrolysis of various phospholipids. The rate of PC hydrolysis was measured in wild type and plbΔ strains (plb1Δ, plb2Δ and plb3Δ) exposed to various concentration of NNK (B) or NNN (C). (D) The rate of PC hydrolysis was measured in wild type and plbΔ strains (plb1Δ, plb2Δ and plb3Δ) exposed to 5050 µM of NNK/NNN. The influence of metal ion on hydrolysis of PC was measured in wild type and plbΔ strains with or without Ca2+ (E). *p<0.001 versus wild type). The values are presented as three separate experiments. Data are means ± S.D, *p<0.01 and **p<0.001 versus control.

To assess the effect of TSNAs on hydrolysis of phospholipid with different phospholipase B, we used wild type and three plbΔ strains (plb1Δ, plb2Δ and plb3Δ). Yeast cells were independently treated with different concentrations of both NNK and NNN (0 – 400 µM) and its lysate was used as an enzyme source throughout. Concentration-dependent hydrolysis of phospholipids was observed in wild type as well as in mutant strains exposed to NNK (Figure 3B) and NNN (Figure 3C). The activity was greatly enhanced by TSNAs, particularly NNN in wild type, whereas mutant cells showed diminished activity (plb1Δ< plb2Δ< plb3Δ <wild type). Figure 3D depicted the impact of 50 µM TSNAs on phospholipase activity. plb1Δ cells showed ~16% diminished activity in NNN treated cells whereas ~10% and ~7% in the presence of NNK and NNK+NNN respectively, as compared to wild type. Both plb2Δ and plb3Δ responded almost similar to wild type in presence of TSNAs. These results suggest that enhanced phospholipase activity may be due to plb1 stimulated by TSNAs exposure.
### Table 1A- Phospholipid fatty acid composition

<table>
<thead>
<tr>
<th>Fatty acid (%)</th>
<th>C10:0</th>
<th>C12:0</th>
<th>C14:0</th>
<th>C16:0</th>
<th>C16:1</th>
<th>C18:0</th>
<th>C18:1</th>
<th>C18:2</th>
<th>C16/C18</th>
<th>UFA/SFA</th>
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<td><strong>Phosphatidylinositol</strong></td>
<td></td>
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<td></td>
<td></td>
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</tr>
<tr>
<td>Control</td>
<td>5.61 ± 0.51</td>
<td>11.90 ± 0.53</td>
<td>41.24 ± 1.53</td>
<td>78.3 ± 1.42</td>
<td>19.41 ± 1.51</td>
<td>12.21 ± 0.32</td>
<td>1.77 ± 0.73</td>
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<td>15.27</td>
<td>0.10</td>
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<tr>
<td>NNK</td>
<td>5.24 ± 0.91*</td>
<td>12.0 ± 1.35</td>
<td>47.6 ± 2.32*</td>
<td>6.71 ± 2.21*</td>
<td>17.8 ± 1.43*</td>
<td>9.12 ± 1.32*</td>
<td>1.46 ± 0.81*</td>
<td>2.67</td>
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<tr>
<td>NNN</td>
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<td>5.74 ± 0.42*</td>
<td>49.03 ± 2.30*</td>
<td>3.43 ± 1.55*</td>
<td>27.82 ± 2.43*</td>
<td>9.52 ± 0.84*</td>
<td>3.62 ± 0.92*</td>
<td>1.76</td>
<td>0.19</td>
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<tr>
<td>NNK+NNN</td>
<td>10.99 ± 0.52*</td>
<td>6.47 ± 0.65*</td>
<td>38.74 ± 2.32#</td>
<td>2.37 ± 0.83*</td>
<td>12.48 ± 1.95*</td>
<td>28.87 ± 2.42*</td>
<td>7.20 ± 1.65*</td>
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<td><strong>Phosphatidic acid</strong></td>
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<tr>
<td>Control</td>
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<td>21.04 ± 0.42</td>
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<td>NNK</td>
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<td>15.6 ± 0.86*</td>
<td>14.1 ± 0.71*</td>
<td>24.3 ± 1.42*</td>
<td>8.82 ± 1.76*</td>
<td>14.1 ± 0.73*</td>
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<td>NNN</td>
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<td>21.01 ± 1.32</td>
<td>9.84 ± 1.24*</td>
<td>15.00 ± 0.57*</td>
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<tr>
<td>NNK+NNN</td>
<td>5.64 ± 0.43*</td>
<td>17.50 ± 1.56*</td>
<td>15.52 ± 0.61‡</td>
<td>21.04 ± 0.66</td>
<td>9.13 ± 1.43*</td>
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<td>12.71 ± 0.82*</td>
<td>3.31 ± 0.61‡</td>
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VN’s Lab, BDU
**Table 1B - Neutral lipid fatty acid composition**

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<th>C16:0</th>
<th>C16:1</th>
<th>C18:0</th>
<th>C18:1</th>
<th>C18:2</th>
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</tr>
<tr>
<td>Control</td>
<td></td>
<td>10.11 ± 0.62</td>
<td>14.08 ± 0.94</td>
<td>31.74 ± 2.07</td>
<td>4.85 ± 1.18</td>
<td>23.41 ± 3.22</td>
<td>11.42 ± 0.92</td>
<td>4.37 ± 2.07</td>
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</tr>
<tr>
<td>NNN</td>
<td>0.02 ± 0.009</td>
<td>7.13 ± 1.01*</td>
<td>8.45 ± 1.73*</td>
<td>30.53 ± 2.96</td>
<td>10.84 ± 1.42*</td>
<td>26.26 ± 5.43*</td>
<td>7.78 ± 1.32*</td>
<td>8.96 ± 2.01*</td>
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<td>0.33</td>
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<tr>
<td>NNN+NNK</td>
<td>11.7 ± 0.42</td>
<td>13.86 ± 0.51</td>
<td>42.17 ± 1.98</td>
<td>3.48 ± 1.22</td>
<td>14.01 ± 0.34</td>
<td>11.54 ± 1.12</td>
<td>3.18 ± 0.75</td>
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<td>Triglyceride</td>
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<tr>
<td>Control</td>
<td>0.11 ± 0.008</td>
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<td>NNN</td>
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<td>15.57 ± 3.22</td>
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<tr>
<td>NNN+NNK</td>
<td>0.07 ± 0.02</td>
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</table>

**Table 1. Effect of NNK and NNN on alterations of fatty acid composition in yeast phospholipid.** Lipids were extracted from both control and NNK/NNN treated cells and subjected to methylation with BF3 - methanol. Methylated fatty acids were analysed by GC/MS. The values are presented as three separate experiments. Data are means ± S.D, † p <0.05, # p<0.01 and *p <0.001 versus control. UFA, unsaturated fatty acids; SFA, saturated fatty acids.
It is worth assessing the effect of Ca\(^{2+}\) on TSNAs mediated phospholipid hydrolysis. We performed the enzyme assay in the presence of Ca\(^{2+}\) in wild type and all three plb\(\Delta\) strains that were exposed to compounds. In the presence of TSNAs, phospholipase activity was greatly enhanced (~30-55\%) by the addition of 10 mM Ca\(^{2+}\) in wild type and plb\(\Delta\) strains as compared with their respective controls (Figure 3E). However, plb1\(\Delta\) showed lesser activation as compared to others stains. In addition wild type cells, we observed increased enzyme activity (~15-25\%) in TSNAs-treated cell in the absence of Ca\(^{2+}\) also (Figure 3E). Hence, our results suggest that TSNAs decrease phospholipid content through PLB activity and also through other phospholipases that are involved thereby leading to an increase in LPL.

4.4 Involvement of TSNAs in phospholipid molecular species alteration

Table 1 shows the changes in FA composition in both phospholipids and neutral lipids of TSNAs treated cells. Cells exposed to TSNAs showed an overall decrease in C16 series of fatty acids (C16:0 and C16:1) and a concomitant increase in C18 series fatty acids (C18:0 and C18:1) in phospholipids, particularly in PE and PS with NNN treatment. In PE monounsaturated FA (C18:1) was increased significantly in NNN treated cells where as NNK+NNN increases C18:1 and C18:2 fatty acids in PS with significant reduction of C16 series fatty acids. Among the phospholipids, medium chain FA content (C12:0 and C14:0) was increased in CL of NNK and NNN treated cells (Table 1A). It was reverse in neutral lipid fatty acid composition. In sterol and TAG, C16 series of fatty acids were increased whereas significant reduction in C18 series of fatty acids of TSNAs treated cells was observed when compared to control (Table 1B).
4.5 Cell viability assessment

Figure 4 Growth and viability: Effect of NNK and NNN on the growth and viability of cells were monitored at 30°C. (A) Effect of 50 µM of NNK/NNN cell growth was studied by measuring the $A_{600}$ of the cultures at frequent time intervals. (B) The cells ($A_{600} = 1.0$) were serially diluted and spotted on to solid YPD plates with or without NNK/NNN and incubated for 48 h at 30°C.

Finally the cell viability was assessed with 50 µM of TSNAs by growth curve (Figure 4A) and spotting (Figure 4B) experiments. We observed ~ 15 - 20% growth reduction with compounds, it was significantly higher in NNK+NNN treated cells.

5. Discussion

Tobacco-specific nitrosamines are a group of carcinogens present in tobacco and tobacco smoke. Group I carcinogens, NNK and NNN have notable attention because of their carcinogenic effect and abundance in the regular tobacco products. Reduction of phospholipid content with TSNAs exposure was fully corroborated, using S. cerevisiae as a model system for investigating membrane lipid homeostasis. In order to understand the impact of TSNAs on lipid metabolism, S. cerevisiae cells were treated over a wide range of NNK and NNN concentration. The alterations in total phospholipid levels were determined in
presence of NNK or NNN and a concentration dependent reduction in total phospholipid content (Figure 1A and 1B) was observed. Reported evidences showed that NNK and NNN exposure alters the lipid composition of the membrane during adaptation (Schuster et al., 1995; Sauk and Norris, 1988).

During the last two decades, carcinogenicity of TSNA compounds and its associated problems has been the subject of intensive investigation and tremendous progress has been made. However understanding membrane remodelling, associated with carcinogenicity, particularly phospholipid alteration, is yet to be studied. Pathophysiological mechanisms of TSNA depend on membrane organisation/composition. One of the first steps of carcinogenesis by TSNA is by inhalation of tobacco smoke and the generation of reactive oxygen species within the cells (Alavanja, 2002). We have reported that increased membrane damage occurs through reactive oxygen species (ROS) during NNN exposure (Nachiappan et al., 1994). Interaction of NNN with targeted cells mainly depends on lipid and fatty acid composition (Schuster et al., 1992). A moderate reduction in phospholipid content and alteration in fatty acid pattern was also observed in presence of NNN by us and others (Vijayaraj 2011a and 2011b; Schuster et al., 1986 and 1990; Sauk and Norris, 1988). Reported evidence suggests that TSNA may contribute to the alteration in the structure and function of membrane lipids and also to its remodelling enzyme activity. Lands proposed that a deacylation-reacylation cycle was involved in the remodelling system. In the lands cycle phospholipid cleavage occurs by phospholipases to produce lysophospholipid. ATP-driven activation of another FFA by acyl-CoA synthetase, and transfer of the fatty acid from the acyl-CoA to the lysophospholipid by acyl-CoA:lysophospholipid acyltransferases (Lands and Crawford, 1976) occurs.
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Interactions of TSNAs with targeted cells mainly depend on lipid and fatty acid composition (Schuster et al., 1992). NNK and NNN are potent pulmonary carcinogens in TSNAs. Phospholipids are important components required to maintain the normal pulmonary function. Decreased secretion of PC (~50%) was observed in rat alveolar type II cells by cigarette smoke exposure (Wirtz and Schmidt, 1996; Giammona et al., 1971). Clinical reports showed decreased surfactant phospholipids in the lavage fluid of smokers (Hohlfeld et al., 1997; Finley and Ladman, 1972). In our studies we monitored the quantitative and qualitative content of both phospholipids and neutral lipids in *S. cerevisiae* that were treated with NNK and NNN. Cells exposed to TSNAs showed approximately 40% (Figure 1D) decrease in the overall phospholipid content and was observed using $[^{32}\text{P}]$orthophosphate labelling and significant reduction was noted in PC, PS and PE. The changes in phospholipid composition will alter the membrane composition of the cells. According to De Kruijff (2006), the cells adjust their membrane properties to environmental conditions by changing the ratio of PC/PE. Decreased PC content results in the loose packing, as found in disordered membranes (Juresic et al., 2009). In addition, altered acyl chain composition was also observed by us during PC depletion (Vijayaraj et al 2011a; 2011b). PC and PE have a different influence on the conformation of transmembrane proteins and consequently on the membrane organization and function (Boumann et al., 2006; Kruijff, 2006; Storey et al., 2001). PS is a key intermediate in the synthesis of PC and PE in yeast (Birner et al., 2003; Carman and Zeimetz, 1996) and we observed a substantial reduction in PS. Schuster et al (1995) report strengthened our results and the formation of LPL (24%) in our study is higher.

Each phospholipid has a vital role in the normal function of the cells and its alteration leads to membrane dysfunction (Ghosh et al., 2008). Regulation of phospholipid and neutral lipid synthesis are interrelated (Carman and
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Phosphatidic acid (PA) is a vital phospholipid that acts as a biosynthetic precursor for the formation of both phospholipids and neutral lipids (Rajakumari et al., 2008; Carman and Henry, 2007; Athenstaedt and Daum, 1999). In TSNAs treated cells there was increased neutral lipids and PA may be channelized for neutral lipid synthesis. In our study, depletion of phospholipids was observed with $[^{32}\text{P}]}$orthophosphate labelling and simultaneously the $[1-^{14}\text{C}]$acetate labelling depicted increased neutral lipid synthesis when exposed to TSNAs. Here we have shown that there was a substantial increase in DAG, TAG, FFA and sterol levels with NNN treated cells (Figure 2). Our data are in accordance with the observation that, defective phospholipid metabolism leads to accumulation of neutral lipids (Malanovic et al., 2008). Increased TAG in presence of NNN might be due to increased activity of either DAG-acyltransferase (Cases et al., 1998) or phospholipid: diacylglycerol acyltransferase (PDAT) (Dahlqvist et al., 2000). There are several phospholipases namely phospholipases B, C, and D (Merkel et al., 2005; Flick and Thorner, 1993; Mayr et al., 1996). Phospholipase C produces DAG (Flick and Thorner, 1993) whereas phospholipase D produces PA (Mayr et al., 1996; Rima et al., 2009). In addition, phospholipid depletion may be due to dephosphorylation of PA by PA-phosphatase to produce DAG, which is subsequently converted to TAG (Gaspar et al., 2008).

Among the deacylating phospholipases, PLB catalyzed reactions are having vital role in the regulation of lipid synthesis, with potential implications for lipid pathologies (Hurley and McCormick, 2008). The activation of this enzyme results in the generation of lysophospholipids and FFAs that can directly affect other cellular processes (Schweizer, 2004). In this present study, we observed a significant decrease in the phospholipid content and a simultaneous increase in LPL (Figure 1D) and FFA (Figure 2B). Reported evidences suggest that cigarette smoke reduces the phospholipid content (Scott, 2004) by activated phospholipase...
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A2 (Oulton et al., 1991). Reduction of phospholipids may be due to increased degradation and this was confirmed by enzymatic hydrolysis of phospholipids by phospholipase assay. We found that the enhanced phospholipase activity in the presence of TSNAs, and the hydrolysis of PC was significantly higher when compared to other phospholipids (Figure 3A). Current study suggested that the reduction of phospholipids is mainly the result of NNN mediated phospholipase activity and PC is a preferred substrate. Apart from PC, marked hydrolysis was also observed in PS, PE, and PG in the presence of TSNAs (Figure 3A).

To identify the role TSNAs on these enzyme activities, we performed phospholipase assay with all three mutants and wild type cells. Our results suggest that TSNAs significantly enhance the plb1 activity particularly with NNN as compared to plb2 and plb3 (Figure 3D). PC hydrolysis was markedly decreased when compared with wild-type cells. Plb1 appears to be primarily responsible for the degradation of mainly PC and to some extent PI (Lee et al., 1994; Henry et al., 1998) and we also observed similar results. Interestingly, over expression of Plb2 does not result in degradation of PC or PI, while Plb3 hydrolysed PI, but not PC (Merkel et al., 2005). In addition, increased plb1 activity leads to a significant increase in TAG and a concomitant decrease in cellular phospholipids (Merkel et al., 2005), suggesting that fatty acids used for TAG synthesis are derived partly from PLB catalysed phospholipid degradation.

Phospholipases are activated either at low Ca\(^{2+}\) concentrations or at high concentrations. To check whether Ca\(^{2+}\) is required for TSNAs mediated phospholipid breakdown *in vitro*, we tested phospholipase assay with or without Ca\(^{2+}\) in wild type cells treated with or without NNK, NNN. The phospholipase activity was greatly enhanced by the addition of Ca\(^{2+}\) in the presence of NNN followed by NNK. In addition, we also observed increased enzyme activity in
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TSNAs-treated cell even in the absence of Ca$^{2+}$. It may be due to PC degrading enzyme(s) other than Ca$^{2+}$ dependent phospholipase that are activated under certain conditions. This reveals the existence of other PC degrading enzymes other than plb1 that are activated (Market et al., 2005) Reported evidence showed that PLB from yeast can be either Ca$^{2+}$ or other cations dependent or independent. (Merkel et al., 2005; Fyrst et al., 1999; De Kroon. 2007). Results suggest that TSNAs decrease the phospholipid content by enhancing PLB activity and is associated with an increase in LPL and FFA content.

Decreased C16 series fatty acids (C16:0 and C16:1) and increased C18 series fatty acids (C18:1) were observed in phospholipids with NNN treated cells. The changes were more predominant in PE the most abundant phospholipid during PC depletion. Ferreira et al., (2004) observed excess saturated FAs are stored mainly in phospholipids and not in TAG and steryl esters. The acyl chain composition of CL is important for its function and we investigated the CL profile in the presence of TSNAs. Fatty acid length was decreased, specifically with increase in C12:0 and C14:0 and a decrease in C16 series. The increase in myristic acid might have a vital role in protein modification, an essential step in signaling reactions such as the Ras pathway, thus fatty acid modification alters cellular proliferation and function (Tehlivets et al., 2007). Cell viability was assessed with 50 µM of TSNAs and ~20% reduction in growth was depicted. Possibly, the low PC levels interfere with the diauxic shift; leading to increased cell death (Boumann et al., 2006).

To summarize, we demonstrated the changes in lipids by TSNAs in *S. cerevisiae*. Our results suggest the decreased phospholipid content may be due to enhanced PLB activity associated with increased generation of lysophospholipids and FFAs. We also observed accumulation of neutral lipids and substantial
changes in fatty acid composition. PLB/PLA₂ is a key enzyme involved in the pathology of many clinically distinct lipid dysfunctions (Hurley and McCormick, 2008). Based on these results, we conducted the experiments in animal model to further understand the impact of TSNAs on phospholipid metabolism and it will be discussed in the following chapters.