Nicotine And Salbutamol Induced Changes In Protein Kinase C Activity In Lymphocytes In COPD

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ABSTRACT

Nicotine in Tobacco smoke may activate lymphocytes by activating protein kinase C (PKC) mediated signal transduction pathway, which is not known. Therefore, role of PKC in regulation of COPD was studied in lymphocytes and the effect of nicotine, and salbutamol evaluated.

This study was conducted on Stage I, II, III of COPD patients. The results showed that PKC activity was higher in all the stages of COPD, maximum in stage III. It had significant reciprocal correlation with FEV₁% in each group. Nicotine increased total, cytosolic and membrane PKC activity in the order as stage III > II and I. Salbutamol decreased total, cytosolic and membrane PKC activity, maximum in stage II. PKC activity in stage III patients is lesser sensitive to salbutamol than in other stages of COPD. These findings demonstrate involvement of PKC mediated signal transduction pathway in regulation of COPD, which is influenced by nicotine and salbutamol.

Keywords: Signal Transduction, Lymphocytes.
INTRODUCTION:

COPD is a disease of airways characterized by airflow limitation that is not fully reversible. COPD is very prevalent, affecting about 10% of people over 45 years in developed countries and increasing especially in developing countries (1). The increasing world-wide prevalence of COPD reflects aging of the population because it is a disease of elderly people. COPD has a high morbidity and is one of the leading causes of hospitalization and loss of time from work. It has a rising mortality and is now the third most common cause of death in developed countries (2). No current treatments, including corticosteroids, reduce disease progression or mortality and have relatively little effect (~20% reduction) in preventing exacerbations, reflecting their lack of anti-inflammatory effects in this disease. There is therefore an enormous unmet need for the development of new treatments for COPD that target the underlying inflammatory process and aberrant repair mechanisms (3).

The small intrapulmonary airways are the major site of airflow limitation in both asthma and chronic obstructive pulmonary disease (4, 5). The most important feature in pathogenesis of COPD is chronic inflammation caused by accumulation of T-lymphocytes (predominantly CD8+), macrophages and neutrophils which cause the release of various inflammatory mediators such as leukotriene B4 (LTB4), interleukin (IL-8), tumor necrosis factor-α (TNF-α) etc. leading to bronchiolar constriction by acting directly on receptors on airway smooth muscle (6). Airway inflammation increased with the severity of airflow limitation, and was characterized by increased infiltrating macrophages, neutrophils, B and T lymphocytes and lymphoid follicles (7). Furthermore, there is increasing evidence that kinases are also involved in corticosteroid resistance, which is a feature of severe COPD (8).

It is usually progressive and associated with an abnormal inflammatory response of lungs to the noxious particles and gases (9). Only 15 to 20 % of smokers develop the disease and in >95% cases, smoking accounts as the main factor. A major constituent of the tobacco smoke is nicotine, which is an important pharmacological agent. Nicotine acts on lymphocytes (both T and B cells) through nicotinic receptors (10). These receptors are coupled via Gq protein to the membrane-associated enzyme phospholipase C (PLC) which is known to convert membrane phosphatidyl inositol 4,5 biphosphate (PIP2) into inositol 1,4,5 triphosphate (IP3) and 1,2 sn-diacyl glycerol (DAG). IP3 binds to specific receptors on endoplasmic reticulum, leading to intracellular release of Ca2+. This serves as active Ca2+, which binds to protein kinase C (PKC) and primes it.

The PKC family comprises a group of isoenzymes with serine/threonine protein kinase activity that is expressed ubiquitously in mammalian cells (11). The primed PKC is translocated to the membrane where it binds to DAG and becomes fully active. Activated PKC phosphorylates several proteins by catalysing covalent transfer of phosphate group from ATP to serine and threonine residues of target proteins, which induces a conformational change resulting in expression of their functional properties and ultimate biological functions (12 – 14). Mukherjee et al. addressed an elusive issue: a possible role for PKC, which is activated by phorbol esters, and Ca2+ oscillations in tuning excitation–contraction coupling in airway smooth muscle (15). Development of COPD and its severity depends upon exposure to the cigarette smoke. Smoke condensate induced expression of inflammatory mediators is inhibited by inhibitors of PKC (16). Since lymphocytes are involved in inflammatory process of lung and contain nicotinic receptors, it is speculated that activation of these cells by nicotine may involve PKC mediated pathway, which may be serving as an important mechanism operative in the airway inflammation in COPD. COPD represents an acceleration of the normal aging process in lungs and these pathways of cellular senescence are also regulated by multiple interacting kinase pathways (17). Therefore, the present study was contemplated to investigate the role of PKC in peripheral blood lymphocytes of COPD patients of different stages and evaluate the effects of nicotine on it.
MATERIALS AND METHODS

Study Design – The patients of COPD of Stage I, II and III were selected from those attending the OPD of the Clinical Research Centre of V. P. Chest Institute, as per American Thoracic Society guidelines [9]. Healthy subjects served as controls. Blood was collected followed by isolation of lymphocytes and PKC activity determined. The effect of nicotine and salbutamol was studied on PKC by incubating the cells with 100nM of the drug for 10 min, followed by centrifugation, washing of cell pellet, preparation of cell lysate, cytosol and membrane fractions, determination of total protein contents and assay of PKC activity. The optimum concentration of nicotine and incubation time was determined by performing kinetic studies on lymphocytes of healthy subjects. The kinetics for salbutamol was studied in our laboratory earlier. Statistical analysis was performed using computer based software. P<0.05 was considered significant.

Chemicals and Reagents

(i) Radioactive Chemicals: [³H] Phorbol 12, 13 dibutyrate ([³H] PDBu) (specific activity 20 Ci/mM) was purchased from MP Biomedicals Inc, Irvine, CA, USA.

(ii) Other Supplies: Bovine serum albumin (BSA), dithiothreitol (DTT), ethylene glycol bis (β-amino ethylene) tetra acet acid (EGTA), ethyl diamine tetra acet acid (EDTA), histopaque (sp. Gravity 1.077), nicotine hydrogen tartrate, phenyl methyl sulphonyl fluoride (PMSF), L-α-phosphatidyl serine (PS), salbutamol, trizma base etc. were purchased from Sigma Chemical Co., St Louis, MO, USA. Heparin (5000 I.U. /ml) was purchased from Biological Evans Ltd. (Hyderabad, India). All other chemicals used were of analytical grade and were procured from Sisco Research Laboratories Pvt. Ltd., Mumbai, India or Qualigens Fine Chemicals, Mumbai, India.

Subjects:
The study included 40 subjects. Out of these 30 were patients of COPD (10 each of stage I, II and III) and 10 healthy subjects.

i) Patients: COPD patients of either sex, age ≥ 18 years, diagnosed and classified as stage I, II and III patients as per American Thoracic Society guidelines (18) were recruited from the outpatient department of Clinical Research Center, V.P. Chest Institute, University of Delhi, Delhi. The age (mean ± S.D) of the patients of stage I was 63.60 ± 13.62 years, stage II was 55.50 ± 7.15 and stage III was 52.30 ± 11.06 years. Patients associated with history of any other disease or cancer, pregnant and lactating females, on long-term antihistamines and detected positive for parasitic infestation were excluded. None of the patients was using bronchodilator for 24 hrs before collection of blood samples.

(ii) Healthy Subjects- Healthy non-smokers of either sex, aged ≥ 18 years (23.80 ± 5.63 mean ± S.D., range 18-32 years) with no personal or family history of respiratory or systemic disease constituted the control group.

The study was approved by the Institutional Ethics Committee. Informed, written consent was obtained from each subject.

Preparation of Lymphocytes- Lymphocytes were isolated from blood by the method of Boyum (19). Venous blood, collected in heparin (30 I.U. heparin/ml of blood) under aseptic conditions, was diluted 1:1 with physiological saline (0.15M NaCl), carefully layered over histopaque (specific gravity 1.077) and centrifuged at 2000 rpm (670 x g) for 10 Min at 4°C in a refrigerated centrifuge (Plasto Craft, 4R-V/FM, India) using swing out rotor (no. 15). The interface containing lymphocytes was collected, diluted with physiological saline and centrifuged. The pellet was washed twice. Contaminating erythrocytes were removed by osmotic shock treatment by suspending the pellet in 0.02% sodium chloride followed by drop wise mixing of an equal volume of 0.16% sodium chloride after 30 seconds (20). The cell suspension was centrifuged, pellet washed twice with physiological saline and resuspended. The lymphocytes were counted in a Neubauer’s chamber and their viability determined by trypan blue exclusion which was 97.00 ± 0.52% (mean ± SEM). The cells were finally suspended in sonication buffer (25mM tris HCl, pH 7.5, 2 mM EGTA, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 250 mM sucrose) to obtain 40x10^6 cells / ml.
Preparation of Cell Lysate, Cytosol and Membrane Fractions: Lymphocyte suspensions (in ice-water bath) were sonicated using microtip (Misonix Ultrasonic Processor XL-2020) by giving five bursts of 30 sec each at an interval of one min. Cell lysate was centrifuged at 1,05,000g for 1h at 4°C in a Beckman L7-65 ultracentrifuge (Beckman Instrument Inc., CA, USA). The supernatant (cytosol) was saved. The pellet was suspended in 0.6% triton X-100 in sonication buffer and allowed to stand at 4°C for 2h. Protein contents of cell lysate, cytosol and membrane fractions were determined by the method of Lowry et al (21).

PKC Assay: PKC activity was determined by radio-ligand binding assay as described by Jaiswal et al (22). The reaction mixture consisted of 50mM tris HCl pH7.5, 1mM CaCl₂, 0.1% BSA, 20mM MgCl₂·6H₂O, 2 mM DTT, 5mg/ml PS and 1µCi/ml [³H]PDBu. The sample (25µl) containing 30-50µg protein was mixed thoroughly with 25µl of reaction mixture and incubated for 25 min at 37°C. Each test was run in triplicate. In the third tube, phorbol 12-myristate 13-acetate was added at a final concentration of 5µM. This gave the non-specific binding and served as control. The reaction was terminated by 2.5ml of 10mM tris HCl, pH7.5 and contents filtered through a 2.5cm Whatman glass fiber filter (GF/C). The tubes were washed twice, filters transferred into scintillation vials, and processed for radioactivity counting in a Beckman LS 6500 IC liquid scintillation counter (Beckman Instrument Inc., CA, USA). PKC specific PDBu binding was calculated as difference of total binding and non-specific binding. Activity was expressed as femtomoles [³H] PDBu bound per mg protein.

Effect of stimuli on protein kinase C activity in lymphocytes of healthy controls and COPD patients: Stimuli used: Nicotine and Salbutamol

Lymphocytes were prepared as mentioned before and suspended in 0.15M NaCl. Lymphocyte suspension (2.5 million cells) in saline was incubated with stimuli at 37°C for 10 minutes in metabolic shaker bath. Cells were then pelleted down by centrifugation at 6000 RPM for 2 minutes in an Eppendorf centrifuge. Three washes were given with normal saline. Resultant cell pellets were then resuspended separately in 150µl of sonication buffer, and sonicated at 0°C for 2 minutes as mentioned earlier. Cell lysate was then centrifuged at 1,05,000 X g for 1 hour at 4°C to separate the cytosol and membrane fractions. Membrane was dissolved in sonication buffer containing 0.6% Triton X-100 as described earlier. Protein estimation was done by Lowry et al method (21). PKC activity was carried out by [³H]PDBu binding assay technique in all the tubes as described earlier.

Statistical Analysis: Prism (Graph pad) software was used to analyze the results for mean ± SD (or SEM), analysis of variance (ANOVA) applying Newman-Keuls Multiple comparison test and Pearson’s correlation coefficient. P<0.05 was considered significant.

RESULTS & DISCUSSION

Results: PKC Activity and its Sub Cellular Distribution:

PKC activity increased with severity of the disease, maximum being in stage III (Figure 1A). The increase was statistically significant in stage II (P<0.05) and III (P<0.001) of COPD as compared to the healthy group. PKC activity increased in cytosol and membrane fractions of lymphocytes of COPD patients (Figure 1A). The increase in cytosolic PKC activity of COPD patients was not significant as compared to healthy subjects. However, in membrane fraction it increased significantly in stage II and III (P<0.01) in comparison to healthy subjects and in stage III than stage I (P<0.05) of COPD patients. The distribution of PKC activity (mean ± SEM) showed that in cytosol in healthy group, it was 60.17 ± 0.91%, while in COPD patients, it was 58.92 ± 1.90% in stage I, 55.51 ± 1.94% in stage II and 54.76 ± 1.91% in stage III and the remaining was present in membrane (Figure 1B).

The correlation coefficient (r) analysis between total PKC activity in lymphocytes of COPD patients and FEV1% of predicted showed a significant reciprocal correlation of -0.95 (P<0.0001), -0.89 (P<0.0005) and -0.84 (P<0.002) in stages I, II and III respectively (Figure 2A,B,C), suggesting that increase in airway obstruction in COPD is associated with increase in PKC activity.
**Figure 1.** Total PKC activity in lymphocytes of control group and of COPD patients of various stages. A) Total PKC activity and its distribution in cytosol and membrane fractions B) Percent distribution (% of total) of PKC activity in cytosol and membrane fractions. PKC activity expressed as femtomoles $[^3]H$]PDBu bound per mg protein. The data represent mean ± SEM in each group (n=10). $P < 0.05$, $** < 0.01$, $*** < 0.001$. **Experimental conditions:** Lymphocytes were prepared from the peripheral blood of healthy subjects and patients of COPD of various stages. The lysate was prepared in sonication buffer and centrifuged in an ultracentrifuge to obtain the cytosol and membrane fractions. Protein contents were determined by the method of Lowry et al. (1951). The PKC activity was assayed by radio ligand binding method. The details are given in materials and method section.

**Figure 2.** Correlation between total PKC activity in lymphocytes and FEV1 (% of predicted) of COPD patients: A) Stage I, B) Stage II, C) Stage III. PKC activity expressed as femtomoles $[^3]H$]PDBu bound per mg protein.
Figure 3. Effect of nicotine on the PKC activity of peripheral blood lymphocytes of healthy subjects (n=5) at varying A) Concentration, B) time. PKC activity expressed as femtomoles $[^3]$H]PDBu bound per mg protein (mean + SEM). 

**Experimental conditions**: Lymphocytes were incubated with varying concentrations of nicotine at 37°C in a metabolic shaker at 100 oscillations per minute. After incubation, the lymphocytes were quickly centrifuged at 0°C, washed twice with physiological saline and finally suspended in sonication buffer. The cell lysate was prepared and PKC activity assayed. The details are given in the materials and method section.

![Figure 3](image)

Figure 4. Effect of nicotine and of salbutamol on PKC activity in lymphocytes of healthy subjects and COPD patients of various stages. A) total, B) cytosolic and C) membrane fractions. PKC activity was expressed as femtomoles $[^3]$H]PDBu bound per mg protein. The data represent mean + SEM (n=10) in each group. P *<0.05, **<0.01, ***0.001.

**Experimental conditions**: Lymphocytes were prepared from the peripheral blood of healthy subjects and patients of COPD of various stages and incubated at 37°C with 100 nM concentration of the drug (nicotine or salbutamol) for 10 minutes. The cell lysates were prepared in sonication buffer and centrifuged in an ultracentrifuge.

![Figure 4](image)
Correlation between PKC Activity and Airway Obstruction

A significant correlation (r) between age of the patients and PKC activity did not exist in stage I (-0.073), stage II (-0.349) and stage III (-0.0287). Similarly, no significant correlation existed between the years of smoking and PKC activity in the patients of stage I (-0.383), stage II (-0.581) and stage III (-0.237).

These findings suggest that age and duration of smoking are not the confounding factors for increase in PKC activity in COPD patients.

Effect of Nicotine

The optimum concentration of nicotine and time of incubation was derived from our kinetic studies on lymphocytes of healthy subjects by incubating them with varying concentrations and time intervals (Figure 3A, B). The lymphocytes of COPD patients were incubated with 100nM nicotine for 10 minutes. The results showed that nicotine significantly increased PKC activity in COPD patients of stage I and II (P<0.01), and III (P<0.001) as compared to the increase in healthy group III (Figure 4A). It caused an increase in PKC activity (mean ± SEM) by 9.48 ± 0.48% in healthy group, 28.9 ± 4.88% in stage I, 30.17 ± 3.01% in stage II and 41.42 ± 5.47% in stage III. Thus, nicotine increased PKC activity in the order of stage III>stage II> stage I as compared to the healthy subjects.

The increase in PKC activity by nicotine was associated with its increase in cytosol and membrane fractions (Figure 4B, C). In cytosol nicotine increased the activity (mean ± SEM) by 6.01 ± 1.85% in healthy group, 20.56 ± 10.79% in stage I, 25.64 ± 4.26% in stage II and 44.87 ± 8.05% in stage III, which was significant in stage III (P<0.01) in comparison to the healthy controls. Similarly, in membrane fraction, it increased the activity (mean ± SEM) by 15.51 ± 3.26% in healthy group, 50.66 ± 18.99% in stage I, 36.49 ± 3.38% in stage II and 51.73 ± 11.17% in stage III. The increase was significant in stage I and II (P<0.05).

Effect of Salbutamol

Studies from our lab have shown that salbutamol, a β₂ agonist that is widely used as a drug for the treatment of COPD, causes a decrease in PKC activity in lymphocytes of healthy subjects and the maximum decrease was at 100nM in a time dependent manner upto 12 min.

In the present study, incubation of lymphocytes with 100nM salbutamol for 10 min decreased the PKC activity in COPD patients of various stages and healthy subjects (Figure 4A). The decrease was maximum in stage II. It caused a decrease in total PKC activity (mean ± SEM) by 23.26 ± 2.91% in healthy group, 30.86 ± 3.40% in stage I, 32.71 ± 3.29% in stage II and 29.76 ± 5.15% in stage III. These results suggest that the total activity of PKC of lymphocytes was decreased by salbutamol in the milieu.

The decrease in PKC activity was due to its decrease in cytosol and membrane fractions (Fig 4B, C). In cytosol salbutamol decreased the activity (mean ± SEM) by 27.86 ± 4.87% in healthy group, 33.42 ± 6.22% in stage I, 33.42 ± 4.21% (mean ± SEM) in stage II and 33.82 ± 7.88% in stage III. Similarly in membrane it decreased the PKC activity (mean ± SEM) by 15.87 ± 1.69% in healthy group, 24.54 ± 4.9% in stage I, 31.13 ± 3.64% in stage II and 22.16 ± 7.40% in stage III. This shows that salbutamol decreased the PKC activity in cytosol and membrane of lymphocytes of COPD patients and healthy controls. It was maximum in the membranes of the Stage II patients.

DISCUSSION:

PKC is one of the key regulatory enzymes in signal transduction pathway. Protein kinases are selective for Ser/Thr or Tyr residues in their target proteins and recognize a consensus sequence around these residues rather than all of the residues on a particular target protein (23). Our results show enhanced total, cytosolic and membrane PKC activity in lymphocytes of COPD patients of all the stages as compared to the healthy subjects. The increased PKC activity in membrane fraction suggests increased translocation of PKC from cytosol to membrane. A 5% translocation of PKC from cytosol to membrane is adequate for biological activity of lymphocytes (24). In the present study, the translocation of PKC to the membrane was higher than 5%, suggesting persistent physiological activation of the cells in COPD patients. Further, we
observed a significant reciprocal correlation between PKC activity in lymphocytes and FEV1% in various stages of COPD patients suggesting that increase in airway obstruction is associated with increase in PKC activity in each stage of the disease.

Nicotine, a major active principle of tobacco smoke, activates inflammatory cells which results in airway inflammation. It acts on lymphocytes through nicotinic receptors (10). Receptor occupancy may lead to activation of PKC activity (25). In the present investigation we studied the effect of nicotine on PKC activity in lymphocytes of healthy subjects and various stages of COPD patients in vitro. In healthy subjects, nicotine at 100 nM induced maximum increase in PKC activity, a concentration which is comparable to its concentration in plasma of smokers (140nM to 215nM) (26), reflecting its physiological relevance. At this concentration in COPD patients, it caused an increase in PKC activity by >29% in all the stages, maximum being in stage III (44.9%) while in healthy subjects it was only 9.5%. The increase in cytosolic PKC activity was >20.6% in all the stages of COPD, maximum being in stage III (44.9%) and only 6% in healthy group. Similarly, it caused >36% increase in PKC activity in membrane fraction in all the stages. The maximum increase was in stage III (51.7%) while in healthy subjects the increase was only by 15.5%. These findings suggest that nicotine activates PKC in lymphocytes. The activation is more pronounced in COPD patients and is maximum in stage III. Since the increase in COPD patients is much higher than healthy subjects, it suggests that the cells of COPD patients are primed to nicotine. The translocation of enzyme from cytosol to membrane was also maximum in stage III, suggesting further activation of the cells already primed to nicotine. A study by Sushma M et al also stated that there is no significant difference in the levels of PKC in peripheral blood lymphocytes between healthy subject group and healthy smokers group without COPD (27).

The magnitude of activation of PKC depends upon activation of the signaling system operating via second messengers (28). Nicotine suppresses T-cell by depletion of IP3 sensitive intracellular Ca2+ stores (29). In our study, the increase in PKC activity may be either due to Ca2+ independent novel and atypical subclass isoenzymes of (30,31) or there may be influx of Ca2+ from extracellular milieu through Ca2+ channels in the membrane, which may transiently increase the intracellular calcium.

Salbutamol, a β2 agonist, is widely used as drug for treatment of COPD. The present study reveals that in lymphocytes it decreased the total PKC activity in stage I, II and III. The fall was >29% in all the stages and the maximum decrease was in stage II patients (32.71%). Similarly, the PKC activity decreased in cytosol (>33%) and membrane fractions (>22%) in all the three stages. The decrease in cytosol was almost equal in all the stages, but in membranes, maximum fall (>31%) was observed in stage II patients and minimum in stage III patients. It is evident that the effect of salbutamol was less pronounced in stage III than stage I and stage II, suggesting that the PKC activity in stage III patients is lesser sensitive to salbutamol than other lesser severe stages of COPD or healthy subjects. This contention further gets support from translocation of PKC from cytosol to membrane which also had the similar pattern. Thus one of the modes of action of salbutamol in COPD patients may be through inhibition of PKC activity by the drug.

It is thus evident that, the activity of PKC significantly increases in all the 3 stages. This increase has a significant reciprocal correlation with FEV1 (%) of predicted, suggesting that with the increase in the airway obstruction in COPD, there is an increase in the PKC activity or vice-versa. The lymphocytes in the COPD patients seem to be primed for activation, since exposure to the nicotine caused an increased expression of PKC activity, suggesting increased potential of these cells in response to the external factors like nicotine. The role of PKC in COPD is supported by our studies on the effect of drug salbutamol, which decreased the activity if the enzyme in lymphocytes of COPD patients. However, the effect was not so pronounced in stage III as compared to controls and stages I and II, suggesting that the PKC activity in stage III patients does not remain as sensitive to the action of salbutamol as in other lesser severe stages of COPD or healthy controls. This may be speculated to be one of the reasons for poor response of salbutamol in COPD patients of stage III.
CONCLUSIONS:

Thus, it can be concluded that PKC mediated signal transduction pathway plays a significant role in the development and perpetuation of manifestation of the symptoms of COPD, which is triggered by nicotine of tobacco smoke and inhibited by salbutamol treatment. This makes PKC as a potential candidate for studies on its isoenzymes to explore them as a target for the development of new therapeutics (32 – 34). Thus, we provided the first evidence that PKCζ is a potential target for the treatment of COPD by selective small molecules. Pharmacological inhibition of protein kinase C (PKC)ζ downregulates the expression of cytokines involved in the pathogenesis of chronic obstructive pulmonary disease (COPD).

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