Article History:
Received on 18th July, 2020
Peer Reviewed on 29th July, 2020
Revised on 11th August, 2020
Published on 27th August, 2020

Keywords:
COPD, PKC, PMA, Sphingosine

Effect of Sphingosine and PMA at Protein kinase C activity in lymphocytes of COPD patients

Anjali Vinocha, Surendra Bansal, V K Vijayan

Vallabhbhai Patel Chest Institute, University of Delhi, Delhi-07.

Background: Tobacco smoke, an important risk factor for development of COPD, contains active principle nicotine which activates lymphocytes and causes release of inflammatory mediators. It may activate these cells by activating protein kinase C (PKC) mediated signal transduction pathway, which is unknown. Therefore, in order to study the role of PKC in regulation of COPD in lymphocytes effect of Sphingosine and PMA was evaluated.

Methods: The study was conducted on forty subjects; ten each of Stage I, II, III of COPD patients and healthy subjects. Peripheral blood lymphocytes were prepared followed by preparation of cell lysate, cytosol and membrane fractions, assay of PKC activity, evaluation of effect of Sphingosine and PMA.

Result: The results showed that PKC activity was higher in all the stages of COPD, maximum in stage III, as compared to healthy subjects. It had significant reciprocal correlation with FEV₁% in each group. PMA showed increased total, cytosolic and membrane PKC activity. However, the distribution of PKC activity in both the fractions remained unchanged. Sphingosine decreased the PKC activity, with the maximum decreased observed in stage III.

Conclusion: We conclude that PKC activity increases in lymphocytes of COPD patients and is proportional to the degree of airway obstruction in each stage. These findings clearly demonstrate involvement of PKC mediated signal transduction pathway in regulation of COPD, which is influenced by PMA and Sphingosine.
INTRODUCTION:

Chronic obstructive pulmonary disease (COPD) is an airway disease which causes irreversible airway limitation. It is one of the most common cause of public health concern in people more than 40 years age. Currently, it is 4th leading cause of death and predicted to be in 7th number on the world disease burden list in 2030.(1)

Although the pathogenesis of COPD is not fully understood but the role of chronic inflammation which is caused by accumulation of T-lymphocytes (mostly CD8+), neutrophils and macrophages leading to various inflammatory mediators like interleukin (IL-8), leukotriene B4 (LTB4), tumor necrosis factor-α (TNF-α) cannot be denied(2). The progressive nature of COPD is associated with abnormal inflammatory response of lungs to various noxious particles and gases, most common being tobacco smoke. Nicotine present in tobacco smoke, acts on lymphocytes (both B and T lymphocytes) through nicotinic receptors (3). These receptors once coupled via Gq protein to the membrane-associated enzyme phospholipase C (PLC) convert membrane phosphatidyl inositol 4,5 biphosphate (PIP2) into inositol 1,4,5 triphosphate (IP3) and 1,2 sn-diacyl glycerol (DAG). This IP3 binds to specific receptors present on endoplasmic reticulum, leading to intracellular release of Ca2+ and this Ca2+ binds to protein kinase C (PKC) and hence primed. This primed PKC is translocated to the membrane and binds to DAG leading to its activation.(4)(5)(6). Hence PKC is one of the key regulatory enzymes in signal transduction pathway.

Hence, development of COPD and its severity depends upon exposure to the cigarette smoke condensate induced expression of inflammatory mediators which is inhibited by inhibitors of PKC (7). Therefore, the present study was contemplated to investigate the role of PKC in peripheral blood lymphocytes of COPD patients of different stages. To understand the mechanism of regulation of PKC and to confirm the changes observed in PKC activity, the effect of agonist and antagonist of PKC (viz. PMA and sphingosine) was studied. This will also be required for further identification and classification of receptor subtypes and dissecting their roles in normal Body function and in diseased state.

Material and Methods:

Study Design – The study was done on COPD patients attending OPD of Clinical Research Centre of V. P. Chest Institute. The patients of COPD were classified as Stage I, II and III per American Thoracic Society guidelines(8). Healthy subjects selected as controls. This study was approved by the Institutional Ethics Committee. Informed, written consent was obtained from each subject. Blood was collected for the isolation of lymphocytes and PKC activity was determined. To study the effect of PMA and Sphingosine on PKC, cells were incubated with 100nM of the drug for 10 min, then centrifugated, washing of cell pellet, preparation of cell lysate, membrane fractions and cytosol, lastly determination of total protein contents and assay of PKC activity.

Statistical analysis was performed using computer-based software.

Chemicals and Reagents: [3H] Phorbol 12, 13 dibutyrate ([3H] PDBu; specific activity 20 Ci/mM) was purchased from MP Biomedicals Inc, Irvine, CA, USA. Bovine serum albumin (BSA), ethylene glycol bis (β-amino ethylene) tetra acetic acid (EGTA), dithiothreitol (DTT), ethyl diamine tetra acetic acid (EDTA), phenyl methyl sulphonyl fluoride (PMSF), histopaque (sp. Gravity 1.077), L-α-phosphatidyl serine (PS), PMA, Sphingosine and 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). Rest other chemicals used were of analytical grade and were procured from Qualigens Fine Chemicals, Mumbai, India or Sisco Research Laboratories Pvt. Ltd.

Test and Control groups: This study included 40 subjects. Out of these 30 were COPD patients (10 each of stage I, II and III) and 10 healthy subjects. COPD patients of either sex, age ≥ 18 years, diagnosed and classified as stage I, II and II patients. Patients with history of any other disease or cancer, lactating and pregnant females, on long-term antihistamines and diagnosed with parasitic infestation were excluded.
from the study population. None of the patients was using bronchodilator for 24 hrs before collection of blood samples. Healthy non-smokers individuals of either sex, aged ≥ 18 years with no personal or family history of any respiratory or systemic disease was enrolled as control group.

**Lymphocyte Preparation**: Isolation of lymphocytes from blood was done as per the method of Boyum taking venous blood, heparin (30 I.U. heparin/ml of blood), physiological saline (0.15M NaCl) and histopaque (specific gravity 1.077) (9). 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 (10). 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.

**Incubation of Lymphocytes with PMA and Sphingosine**: Lymphocyte suspensions (50 μl, 2.0x10^6 cells) were pipetted in separate tubes, mixed with PMA and Sphingosine solution (stock solutions: PMA 100 mM, Sphingosine, 100 mM; diluted with sonication buffer at the time of experiment) and final volume made upto 200 μl. The suspensions were incubated at 37°C, centrifuged, cell pellet washed thrice and suspended in 150 μl sonication buffer.

**Cell Lysate, Membrane Fractions and Cytosol Preparation**: Lymphocyte suspensions (in ice-water bath) was sonicated and resulting cell lysate was centrifuged in a Beckman L7-65 ultracentrifuge (Beckman Instrument Inc., CA, USA)at 1,05,000g for 1h at 4°C. The cytosol (supernatant) was stored. The pellet left behind was suspended in 0.6% triton X-100 in sonication buffer at 4°C for 2h. Protein contents of cytosol, membrane fractions and cell lysate were determined as per method of Lowry et al (11).

**PKC Activity Assay**: To determine the PKC activity by radio-ligand binding assay we had used method as per Jaiswal et al using 50mM tris HCl pH7.5, 1mM CaCl_2, 0.1% BSA, 20mM MgCl_2, 6H2O, 2 mM DTT, 5mg/ml PS and 1μCi/ml [3H]-PDBu(12). The difference of total binding and non-specific binding served as PKC specific PDBu binding. This 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**: We had studied the effect of Phorbol 12-Myristate 13-acetate (PMA) and Sphingosine. For this, lymphocytes were prepared and suspended in 0.15M NaCl as described earlier. Lymphocyte suspension containing 2.5 million cells was incubated with stimuli at 37°C for 10 minutes in metabolic shaker bath. After these cells were centrifuged at 6000 RPM for 2 minutes in an Eppendorf centrifuge and pellets were formed. Three washing was done with normal saline, resultant cell pellets were resuspended separately in 150μl of sonication buffer, and sonicated at 0°C for 2 minutes. Cell lysate was then separated from the cytosol and membrane fractions by centrifugation at 1,05,000 X g for 1 hour at 4°C to separate. Resulting membrane was dissolved in sonication buffer containing 0.6% Triton X-100 and protein estimation was done as per Lowry et al method(11). Lastly, PKC activity was assessed by [3H]PDBu binding assay technique in all the tubes as described earlier.

**Statistical Analysis**: We had used Prism (Graph pad) software to analyse results for mean ± SD (or SEM), analysis of variance (ANOVA) applying Newman-Keuls Multiple comparison test and Pearson’s correlation coefficient.

**RESULT**: In this study, age (mean ± S.D) of the patients of stage I was found to be 63.60 ± 13.62 years, stage II and stage III was 55.50 ± 7.15 and 52.30 ± 11.06 years respectively. As published earlier by Vinocha et al., PKC activity was increased with severity of the disease, maximum seen in stage III. As shown in that study, increase in the activity of PKC was statistically significant in stage II (P<0.05) and III (P<0.001) of COPD when compared with the healthy subjects. The increase in PKC activity in cytosol of COPD patients...
(study subjects) was not significant as compared to healthy controls. Although, in membrane fraction PKC activity increased significantly in stage II and III (P<0.01) as compared to healthy subjects and also in stage III than stage I (P<0.05) of COPD patients. In the same study, distribution of PKC activity (mean ± SEM) in cytosol in healthy group, was found to be 60.17 ± 0.91%, while in COPD patients (test group), same was 58.92 ± 1.90% in stage I, 55.51 ± 1.94% and 54.76 ± 1.91% in stage II and stage III respectively and the remaining was present in membrane(13).

**Figure 1a.** Effect of PMA on total PKC activity of lymphocytes of healthy subjects and COPD patients of various stages. PKC activity was expressed as femtomoles [3H]PDBu bound per mg protein. The data represent mean ± SEM of 10 subjects in each group.

![Figure 1a](image1a.png)

**Figure 1b.** Effect of PMA on distribution of PKC activity in cytosol and membrane fractions of lymphocytes of healthy subjects and COPD patients of various stages. PKC activity was expressed as femtomoles [3H]PDBu bound per mg protein. The data represent mean ± SEM of 10 subjects in each group.

![Figure 1b](image1b.png)
Figure 1c. Effect of PMA on percent distribution (% of total) of PKC activity in cytosol and membrane fractions of lymphocytes of healthy subjects and COPD patients of various stages. The data represent mean ± SEM.

Figure 2a. Effect of Sphingosine on total PKC activity of lymphocytes of healthy subjects and COPD patients of various stages. PKC activity was expressed as femtomoles [3H]PDBu bound per mg protein. The data represent mean ± SEM of 10 subjects in each group.

Figure 2b. Effect of Sphingosine on distribution of PKC activity in cytosol and membrane fractions of lymphocytes of healthy subjects and COPD patients of various stages. PKC activity was expressed as femtomoles [3H] PDBu bound per mg protein. The data represent mean ± SEM of 10 subjects in each group.
Effect of PMA
Lymphocytes were incubated with PMA at 100 nM concentration for 10 minutes. PMA caused an increase in total PKC activity by 23.32 ± 3.30% in control group, 31.94 ± 0.73% in stage I, 38.69 ± 2.81% in stage II and 42.16 ± 4.53 in stage III. It was observed that the increase in the total PKC activity was statistically significant between controls and stage I, control and stage II and control and stage III.
PMA caused an increase in cytosol PKC activity by 15.73 ± 2.27% in control group, 35.88 ± 1.51% in stage I, 46.93 ± 1.71% in stage II and 68.31 ± 5.24% in stage III. PMA caused an increase in membrane PKC activity by 34.74 ± 8.93% in control group, 27.64 ± 3.03% in stage I, 30.82 ± 5.92% in stage II and 23.27 ± 3.83% in stage III. As shown in figure 1 (1a, 1b and 1c) that in cytosol, the increase was statistically significant between control and stage I, control and stage II, control and stage III and stage I and III. However, in membrane the PKC activity increased statistically between control and stage II and control and stage III.

Experimental conditions: Lymphocytes were prepared from the peripheral blood of healthy subjects and patients of COPD of various stages and incubated with PMA at 100 nM concentration for 10 minutes. The cell 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.

Effect of Sphingosine
The lymphocytes were incubated with sphingosine at 100 nM concentration for 10 minutes. Sphingosine caused a decrease in total PKC activity by 30.66 ± 0.94% in control group, 32.89 ± 4.84% in stage I, 34.24 ± 5.37% in stage II and 40.19 ± 2.69 in stage III. These results indicate that the total activity of PKC of lymphocytes decreased by incubating them with sphingosine.

Sphingosine caused a decrease in total PKC activity in cytosol by 34.32 ± 1.60 in control group, 24.74 ± 5.80 in stage I, 29.94 ± 7.45% in stage II and 38.78 ± 2.69% in stage III. Sphingosine caused a decrease in membrane PKC activity by 25.04 ± 1.69 in control group, 42.71 ± 3.65% in stage I, 38.18 ± 4.25 in stage II and 41.62 ± 3.45% in stage III. As shown in figure 2 (2a and 2b) that sphingosine decreased the PKC activity in lymphocytes, both in cytosol and membrane fraction. Although the decrease in the membrane PKC activity was found to be statistically significant, it did not reveal any significant difference among the various groups of healthy subjects and COPD patients.

Experimental conditions: Lymphocytes were prepared from the peripheral blood of healthy subjects and patients of COPD of various stages and incubated with sphingosine at 100 nM concentration for 10 minutes. 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.

DISCUSSION:
COPD is characterized by a chronic inflammatory process predominant in the small airways and lung parenchyma. It is primarily a disease of the adults (Sharma & Graham, 2005). The present study included 30 patients of COPD, whose age ranged between 32 to 85 years. Out of these 25 were males and 5 were females showing predominance of the males, which is similar to the findings of other workers, showing a median ratio of 1.56:1(M: F) (Jindal et al. 2006)(14). A recent study has shown that cigarette smoke induces PKC activity in endothelial cells (Kalra et al.1994)(15). Noonan et al. (1987) reported that PKC is involved in activation of T-lymphocytes following which there is production of interleukin-2 (IL-2)(16). In our study the activity of PKC was increased in both cytosol and membrane fractions, and its distribution in cytosol and membrane remained almost unchanged. The PKC activity was mainly present in cytosol. PKC is known to be present in tissues and organs mainly in cytosol as inactive form (Nishizuka, 1984)(17). Its activity is linearly related to its translocation from cytosol to membrane in a Ca²⁺ dependent fashion, where it binds to an acidic phospholipid and diacylglycerol (Kaibuchi et al. 1983; Hirasawa & Nishizuka, 1985)(18). This may be the
reason for its increased activity in membrane fraction of lymphocytes of COPD patients.

We have studied the effect of PMA (Known agonist of PKC) was studied on PKC activity in lymphocytes from healthy volunteers and COPD patients. PMA, one of the tumor promoting agents, is a known agonist of PKC which activates it by binding to its regulatory domain, followed by release of the active site, which becomes available to the substrate. It activates PKC by mimicking the action of its physiological activator, DAG. The activation due to phorbol ester is stable and sustained while that induced by DAG is short-lived (Leach & Blumberg, 1985; Blumberg, 1988; Nishizuka, 1992). It binds to PKC at regulatory domain in a manner similar to DAG (Nishizuka, 1992). PMA and DAG bind with PKC in a stochiometric ratio of 1:1 (Newton, 1997). The binding of PMA to PKC in a molar ratio has been exploited to determine activity of PKC in a ligand binding assay.

In the present study, PKC activity assayed in lymphocytes of healthy subjects and COPD patients increased on treatment with PMA. It caused an increase in total PKC activity of 23.3% in control, 31.9% in Stage I, 38.7% in Stage II and 42.2% in Stage III as compared to total PKC activity in respective groups without PMA. The increase in cytosolic PKC activity was by 15.7% in control group, 35.9% in Stage I, 46.9% in Stage II and 68.3% in Stage III as compared to cytosolic PKC activity in respective groups without PMA. The increase in PKC activity in membrane fraction by 34.7% in controls, 27.6% in Stage I, 30.8% in Stage II and 17% in Stage III as compared to membrane PKC activity without PMA in respective groups. The distribution in cytosol and membrane remained almost unchanged. Thus, our findings get support from the available literature that PMA increases the PKC activity, which in turn increases the phosphorylation of its substrate proteins (Steinberg et al., 2008)(19).

To understand and confirm the role of PKC in COPD, the effect of antagonist of PKC, viz sphingosine was studied on lymphocytes of healthy subjects and COPD patients. Sphingosine, a known inhibitor of PKC, inhibits PKC at low concentration and also inhibits translocation of the enzyme from cytosol to membrane (Hannun & Bell, 1989)(20). It acts by binding at the regulatory site of PKC (DAG binding site) and inhibits DAG competitively and Ca$^{2+}$ binding non competitively (Das et al. 2014)(21). It can be easily delivered into the cell due to its amphipilic nature and is assumed to get partitioned in membrane. In the present study, we found that the total PKC activity in the lymphocytes of the healthy subjects and COPD patients decreased after incubation with sphingosine. Inhibition of PKC was observed both in cytosolic and membrane fractions, which may be due to binding of sphingosine at the regulatory domain of the enzyme, thereby inhibiting it and its translocation to the membrane. It caused a decrease in the total PKC activity by 30.66% in controls, 32.89% in Stage I, 34.24% in Stage II and 40.19% in Stage III as compared to total PKC activity in their respective groups without sphingosine. The decrease in cytosolic PKC activity was by 34.3% in control, 24.74% in Stage I, 29.94% in Stage II and 38.78% in Stage III as compared to cytosolic PKC activity in their respective groups without sphingosine. It caused a decrease in PKC activity in membrane fraction by 25.04% in controls, 42.71% in Stage I, 38.18% in Stage II and 41.62% in Stage III as compared to membrane PKC activity without sphingosine in their respective groups. This shows that PKC activity in lymphocytes in healthy and COPD patients is regulated by sphingosine by its action on regulatory domain. Since the activity is not fully inhibited the regulation by PKC may be considered to be partial. From these findings, it is evident that the effect of agonist and antagonist (viz. PMA and sphingosine) shows that the enzyme activity is regulated by the action of these drugs in COPD, by their action on regulatory domain partially.

CONCLUSION:
Thus, it can be concluded that the PKC activity is increased in the lymphocytes in COPD patients, which is associated with the physiological activation of the cells. The increase in PKC activity is proportional to the degree of airway obstruction. For further characterization of PKC activity in lymphocytes from healthy subjects and COPD patients, the effect of its known agonist (PMA) and its known antagonist
(Sphingosine) was studied. PMA (known agonist of PKC) activated PKC activity in lymphocytes of healthy subjects and COPD patients of various stages. It increased the total PKC activity and the increase was directly proportional to the increase in the severity of airflow obstruction, maximum being in stage III. It increased the PKC activity both in cytosol and membrane fractions; however, distribution of PKC activity in both the fractions remained unchanged. PMA activates PKC by binding at its regulatory domain.

Sphingosine is a known antagonist of PKC. It inhibited PKC activity in lymphocytes in healthy subjects and COPD patients. It decreased the total PKC activity, the maximum decrease was in stage III. It is known to inhibit PKC and its translocation from cytosol to membrane. It acts by binding at the regulatory site of PKC (DAG binding site) and inhibits DAG competitively and Ca$^{2+}$ binding non-competitively. It decreased the PKC activity both in cytosol and membrane fractions.

The present study clearly demonstrates that PKC activity is significantly increased in lymphocytes of patients of COPD at various stages. The increase in activity shows a reciprocal relationship with FEV1 (%) of predicted, suggesting that the increase in airway obstruction, is associated with increase in PKC activity and involvement of PKC mediated signal transduction pathway. PMA activated PKC activity in lymphocytes of healthy subjects and COPD patients of various stages and Sphingosine inhibited PKC activity in lymphocytes of healthy subjects and COPD patients. Though there was substantial inhibition of PKC by sphingosine, it was not complete, therefore the possibility of the regulation of the enzyme at its catalytic domain, at least in part, also needs further investigation. The literature also mentions the role of different isoenzymes of PKC and differential activation of isoenzymes of PKC, which may be another area of investigation. These findings clearly demonstrate the role of PKC mediated signal transduction pathway in the development and perpetuation of manifestation of the symptoms of COPD.

ACKNOWLEDGEMENT:
The authors would like to acknowledge Dr. V.K. Vijayan for providing us with all the required samples for the study.

REFERENCE:


