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Identification of the Elusive Mammalian Enzyme Phosphatidylcholine-Specific Phospholipase C

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14. ABSTRACT

The long-term **purpose** of the present proposal aims at establishing the role of the elusive mammalian protein, phosphatidycholine-specific phospholipase C (PC-PLC) in the inflammatory processes involved in progression of rheumatoid arthritis (RA).

Thus, the main **scopes** of this proposal are: 1. to identify the PC-PLC gene and protein; and 2. to test PC-PLC involvement in production of TNF alpha by monocytes.

Major findings. In the first year of funding, we have mostly worked toward the identification of PC-PLC (Aim 1). Based on results published by other groups, we proposed to compare transcriptome analysis between two PC-PLC activating conditions (treatment with LPS and oxidized LDLs) to identify candidate *PC-PLC* mRNAs. While we found that the published activation of human PC-PLC by LPS in HUVEC cells was due to Phospholipase D activity and not PC-PLC, we identified another condition (high serum) that leads to reproducible and specific activation of PC-PLC and that can be substituted to LPS in the transcriptome analysis. Moreover, we found that most of activated PC-PLC is in the cytosol with important implications for both the analysis of elevated transcripts and the possibility of chromatographic purification of PC-PLC.

15. SUBJECT TERMS
Phosphatidycholine-specific phospholipase C, lipopolisaccharide, oxidized lipoproteins, serum, rheumatoid arthritis, transcriptome sequencing, HUVECs, U937 cells

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1. INTRODUCTION:

The present proposal aims at identifying novel players that are critically involved in the progression of rheumatoid arthritis (RA). The identification of these factors may ultimately provide alternative "druggable" targets for the treatment of this debilitating disease. The specific hypothesis that is addressed by this proposal is to test whether a mammalian protein called phosphatidycholine-specific phospholipase C (PC-PLC) might represent such a novel target. Since the mammalian PC-PLC gene has not been identified yet nor the PC-PLC protein isolated the main goals of this proposal are: 1. To identify the PC-PLC gene and protein; and 2. To test PC-PLC involvement in production of TNF alpha by monocytes.

2. KEYWORDS:

Phosphatidycholine-specific phospholipase C, lipopolisaccharide, oxidized lipoproteins, serum, rheumatoid arthritis, transcriptome sequencing, HUVECs, U937 cells.

3. OVERALL PROJECT SUMMARY:

Summary of Current Objectives.

Task 1. To identify mammalian PC-PLC.

In this task, we propose to identify the gene responsible for PC-PLC activity in mammalian cells by complementing cell biology with genomic information. In order to identify mammalian PC-PLC, we proposed to take advantage of the following observations, recently reported in the literature(Zhang *et al.*, 2011, Zhang *et al.*, 2010). Certain stimuli, such as lipopolysaccharide (LPS) or oxidized LDLs (ox-LDLs), significantly increase PC-PLC protein level (tested using cross-reacting polyclonal antibodies raised against bacterial PC-PLC) and its enzymatic activity. Since the changes in both activity and protein level take place after 12-24 hours with either treatment, we hypothesize that the increase in PC-PLC is due to enhanced transcription of its gene.

In the course of the past year, we have optimized new experimental approaches both to measure PC-PLC activity *in vitro* and to stimulate PC-PLC activity in HUVECs. In fact, after extensive experimentation, we found that the published *in vitro* assay for PC-PLC activity employed to demonstrate activation of PC-PLC in response to LPS (Zhang *et al.*, 2011) was not reliable/reproducible/specific and that treatment with LPS was not effective in stimulating PC-PLC activity once a reliable PC-PLC *in vitro* assay was performed (please refer to the "Summary of Results" section for details).

Thus, we now propose to identify PC-PLC by differential expression analysis using stimulation with high serum instead of LPS. To carry out this task, we will perform the following Subtasks as indicated in the original application.

Subtask 1a: To perform whole transcriptome sequencing to identify unknown messages that are up-regulated in conditions known to increase PC-PLC protein level (such as high serum and ox-LDL treatments) (timeframe, next 1-3 months).

Based on our results obtained in the initial funding period, two time points for each treatment (High serum and ox-LDLs) will be identified in which the activity of PC-PLC starts to increase and in which the activity has peaked, and another experiment will be conducted to collect cells at the two time points for each treatment to isolate total RNA.

RNA will be isolated and the isolated RNA will be sent to a commercial source for whole transcriptome sequences and informatic analysis, as indicated in the original aims. Eight experimental groups will be repeated in triplicate to obtain statistical fitness for a total of 24 samples. As control, PC-PLC activity will be measured in samples conducted in parallel to make sure that the activity is indeed elevated when cells are collected for RNA isolation.

For the identification of up-regulated transcripts, the high serum (20% FBS) treatment group will be first compared to the low serum (0.5% FBS) control group and the ox-LDL treatment group will be compared to the ox-LDL control group. Then, up-regulated transcripts from the 20% FBS and the ox-LDL groups will be compared to each other to find transcripts that are up-regulated in both groups. Informatic analysis of

differentially expressed transcripts will be performed by the same company that will perform transcriptome analysis.
Up-regulated transcripts will be selected based on the following features, ordered by relevance: genes of unknown function, zero or one predicted transmembrane domain and potentially carrying one or more signature domains (C1: KxxxxxxR; C2: SGH; C3:SRxxxxxHxxxD) found in lipid phosphate phosphatases (LPPs, enzymes that cleave the phosphate group off lipids).

Subtask 1b: To test the identified cDNA clones for PC-PLC activity in yeast (timeframe, next 3-6 months).

CDNA clones corresponding to up-regulated transcripts common to high serum (20% FBS) and ox-LDL treated cells will be then purchased from Applied Biosystems or other repositories and transformed in the yeast *Saccharomyces cerevisiae* using a plasmid carrying the GAL7 inducible promoter (transcriptionally induced in the presence of galactose and available to the PI) and the Flag tag. PC-PLC activity (measured *in vitro* by the fluorescent assay from Molecular Probes) will be determined in lysates prepared from yeast cultures grown for 24 hours in the presence of 2% galactose (inducing condition) or 2% glucose (non inducing condition).

Optimization of immunoprecipitation of PC-PLC from cytosol of HUVEC cells and U937 cells using the cross-reacting antibodies raised against bacterial PC-PLC (timeframe, during the time that transcriptome analysis is conducted by the company).

The identification of mammalian PC-PLC using the cross-reacting antibodies was originally considered an alternative approach in case the transcriptome analysis would fail to identify a *bona fide* PC-PLC clone. Based on the new results obtained in the past year showing that most of the mammalian PC-PLC activity in both HUVECs and U937 cells resides in the cytosol (as opposed to membrane fractions) (**Figure 2** and data not shown), this approach became very attractive and feasible. Thus, if we find immunoprecipitated PC-PLC activity from cytosolic extracts, we will then employ immuno-affinity chromatography using the cross-reacting antibodies, followed by additional refining chromatographical columns (as previously described by (Clark *et al.*, 1986, Sheikhnejad *et al.*, 1986, Wolf *et al.*, 1985) and coupled with mass spectrometry analysis to identify PC-PLC (these studies will be performed with the support of the mass spectrometry facility at Stony Brook University).

Future experimentation will include:

- To test putative PC-PLC clones in mammalian cells.

The clones that impart higher PC-PLC activity in galactose as compared to glucose will be then subcloned into a mammalian expression vector (i.e. pcDNA3.1 plasmid), transfected in Hela cells and tested for PC-PLC *in vitro* activity. The clones that confirm high PC-PLC activity as compared to the empty vector will be then down-regulated using siRNA in untransfected Hela cells (which have endogenous PC-PLC activity) to show that indeed the endogenous mammalian gene carries PC-PLC activity.

- To establish the role of PC-PLC in TNF-mediated signaling in monocytes.

Human monocytic U937 cells will be employed as experimental model were the effect of overexpression and down-regulation of the newly identified mammalian PC-PLC gene will be tested on TNF signaling.

Summary of Results.

In the first year of funding, we have worked toward the identification of PC-PLC (original Task 1). Based on results published by other groups, we proposed to identify candidate *PC-PLC* mRNAs by comparing transcriptome analysis between two PC-PLC activating conditions (treatment with LPS and oxidized LDLs).

Thus the first step in order to achieve the first task was to reproduce the published activation of PC-PLC by LPS. We followed the experimental protocol described by Zhang ((Zhang *et al.*, 2011) for both treatment of HUVEC cells and for PC-PLC activity *in vitro* (**Figure 1A**). Briefly, HUVEC cells were either serum starved (with 0.5% of Fetal Bovine Serum, FBS) or serum starved and stimulated with LPS at different concentrations (0.1 and 0.5 ug/ml). After 24 hours, cells were collected and total lysates were prepared to measure PC-PLC activity as described by Zhang (Zhang *et al.*, 2011). Unexpectedly, no activation of PC-PLC was observed

(**Figure 1A**). On the other hand, we appreciated that the PC-PLC assay as described (Zhang *et al.*, 2011) was not reliable/reproducible and specific.

The limitation of the method described by Zhang (Zhang *et al.*, 2011) for measuring *in vitro* PC-PLC activity is that it indirectly measures PC-PLC activity determining the amount of inorganic phosphate (Pi) in the aqueous phase. This will account also for non-enzymatically derived Pi, like that associated with nucleotides or peptides. In this case, a dose response with different protein concentrations (**Figure 1B**) would result in false positives as it would be interpreted as an increase of PC-PLC activity with protein while the increase of Pi is merely due to non enzymatically derived Pi. On the other hand, if the detected Pi is a result of an enzymatic activity, it should increase with the time of incubation. Indeed, as shown in **Figure 1A**, the activity of the bacterial PC-PLC positive control (bPC-PLC) doubles at 30 minutes of incubation as compared to 15 minutes. Surprisingly, the Pi from cell extracts did not increase with time (30 vs 15 minutes) suggesting that it is rather a result a non-enzymatic source of aqueous Pi.

This indicated to us that no conclusions on activation of PC-PLC by LPS could be drawn using the published assay (Zhang *et al.*, 2011).

Thus we resorted to use a fluorimetric assay kit, commercially available from Molecular Probes (EnzChek® Direct Phospholipase C Assay Kit) which also distinguishes between PC-PLC and PC-PLD activities. Using this assay we could show that the PC-PLC activity of control HUVEC cells was increasing with time (**Figure 2A**) and with protein (data not shown) indicating that the detected activity was indeed due to an enzyme. Importantly, we showed that, after separating total membranes from cytosol, most of the PC-PLC activity was in the cytosol. This is an important result since it greatly facilitates alternative approaches for the isolation of the PC-PLC protein (with affinity or traditional chromatographical purification) and it also refines the structural features of the putative PC-PLC protein (no or one transmembrane domain) that will help in the identification of the candidate PC-PLC clones from the transcriptome analysis. Also we excluded the possibility that a significant portion of PC-PLC activity was in the nuclear fraction (data not shown). Additionally, using the cross-reacting antibodies against bacterial PC-PLC we could show that a band of the expected ~60kd was immunoreactive to these antibodies in the cytosolic fraction (**Figure 2B**) suggesting that these antibodies could be a precious tool for the identification of PC-PLC.

Thus with the optimized PC-PLC activity in hand, we evaluated the effect of increasing concentrations of LPS on the activity in the cytosolic fractions (**Figure 3**). Again unexpectedly given the published report (Zhang *et al.*, 2011), no significant changes in PC-PLC activity were observed either at different concentrations of LPS (**Figure 3**) or at different times of incubation (12, 24 and 48 hours) with 0.1ug/ml LPS (data not shown). We also tested different preparation of LPS with similar effects. These unexpected results excluded LPS as one of the stimuli that could be used for the proposed comparative transcriptome analysis.

While performing these experiments though, we noticed that the basal PC-PLC activity of serum starved HUVEC cells (0.5% FBS) was significantly lower than the basal activity of HUVECs grown in 20% FBS. Thus we set up to serum starve HUVEC cells for 24 hours with 0.5% FBS and then one set of cells were stimulated with 20%FBS for 24 hours and one set was kept at 0.5% FBS. Membranes and cytosol were isolated and PC-PLC activity was measured (**Figure 4**). Indeed, a four-fold increase of PC-PLC activity was observed in serum stimulated samples. Moreover maximal stimulation was observed after 24 hours but it started at 12 hours. These results therefore support the fact that serum stimulation can be used instead of LPS for comparative transcriptome analysis.

4. KEY RESEARCH ACCOMPLISHMENTS:

- Identification of a novel experimental condition (High serum) that induces activation of PC-PLC (**Figure 4**). Besides being a novel observation in of itself, it allows us to overcome an unexpected obstacle (lack of activation following LPS stimulation) and allows us to proceed further with our proposed studies.

- Most of PC-PLC activity in basal and stimulated condition (high serum) resides in the cytosol (**Figure 2** and **Figure 4**). This is an important result since it greatly facilitates alternative approaches for the isolation of the PC-PLC protein (with affinity or traditional chromatographical purification) and it also refines the structural

features of the putative PC-PLC protein (no or one transmembrane domain) that will help in the identification of the candidate PC-PLC clones from the transcriptome analysis.

5. CONCLUSIONS:

Reumatoid arthritis is a significant medical challenge both in the military and general population. In fact, the limitation of physical activity or the acquisition of disability due to the disease together with high medical-related expenses (Gabriel *et al.*, 1997a, Gabriel *et al.*, 1997b) determines a poor health-related quality of life (Dominick *et al.*, 2004, Mili *et al.*, 2003).

Since RA cannot be cured, current treatments aim at reducing the chronic inflammation to slow down the disease and reduce the damage to cartilage, bone and ligaments. Current treatments for RA include non-steroidal anti-inflammatory drugs, steroids, disease-modifying antirheumatic drugs (such as the widely used methotrexane), immunosuppressants, and TNFα inhibitors (Saag *et al.*, 2008). One limitation with these treatments (in addition to the fact that they are not curative) is that they manifest serious negative side effects, such as heart problems, liver and kidney damage, increased susceptibility to infections and even increased risk of certain cancers, such as non-melanoma skin cancer (Amari *et al.*, 2011). Hence the need and interest in developing alternative treatments with a more targeted effect and less harmful side effects. One possible strategy would be to use agents with a narrower spectrum of action by blocking a specific target (i.e. PC-PLC) that acts in a specific cell type relevant to the progression of RA, such as monocytes.

The long-term objective of this proposal is indeed the investigation of whether PC-PLC might represent a safer alternative to current RA treatments. Thus the identification of PC-PLC through our proposed experiments will represent a potential new opportunity in the treatment against RA.

Importantly, the experimentation conducted so far has overcome obstacles ensuring that the proposed studies will proceed toward the identification of PC-PLC. In addition, they uncovered important new features of PC-PLC that can be instrumental in the pursue of alternative strategies and the refining of criteria for the planned experiments.

Once PC-PLC will be identified we will establish the role of PC-PLC in TNF-mediated signaling in monocytes.

6. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS.** Nothing to report.

7. **INVENTIONS, PATENTS AND LICENCES.** Nothing to report.

8. **REPORTABLE OUTCOMES**. Nothing to report.

9. **OTHER ACHIEVEMENTS**. Nothing to report.

10. **REFERENCES**.

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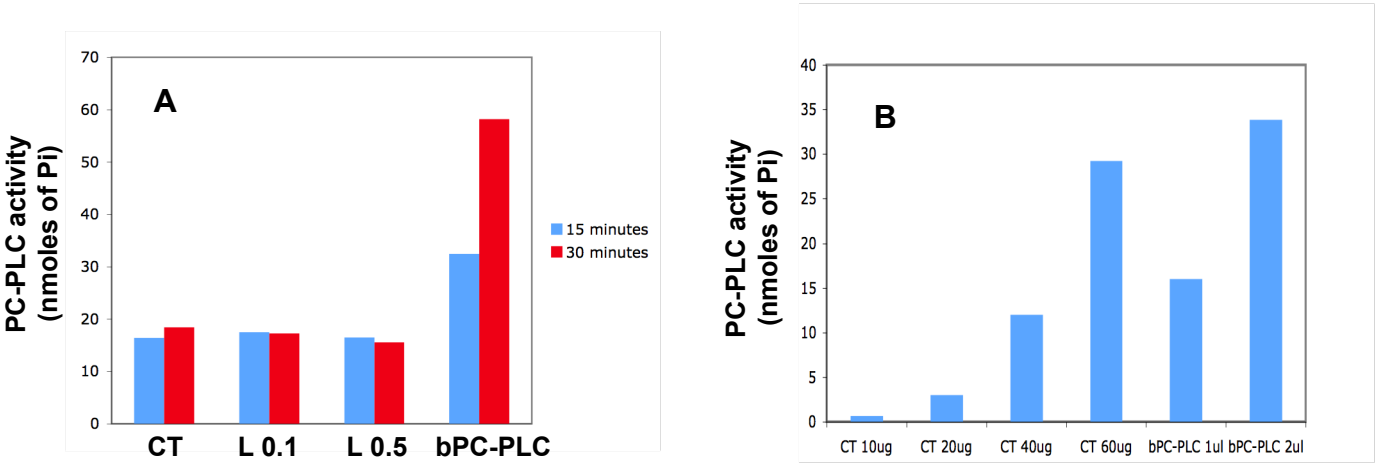

Figure 1. Absence of true enzymatic PC-PLC activity in total lysate of HUVECs as measured according to . A. HUVECs were treated with 0.1 and 0.5 ug/ml of LPS for 24 hours. Total lysates (40ug from each condition) were prepared to measure PC-PLC activity *in vitro* after 15 and 30 minutes of incubation at 37˚C. Purified bacterial PC-PLC (bPC-PLC) was included as positive control. **B.** Different amounts of lysate from control HUVEC cells (CT) were prepared to measure PC-PLC activity *in vitro* after 15 minutes iof ncubation at 37˚C. Results representative of 2 independent experiments.
CT= control cells; L0.1=cells treated with 0.1 ug/ml LPS; L0.5=cells treated with 0.1 ug/ml LPS.

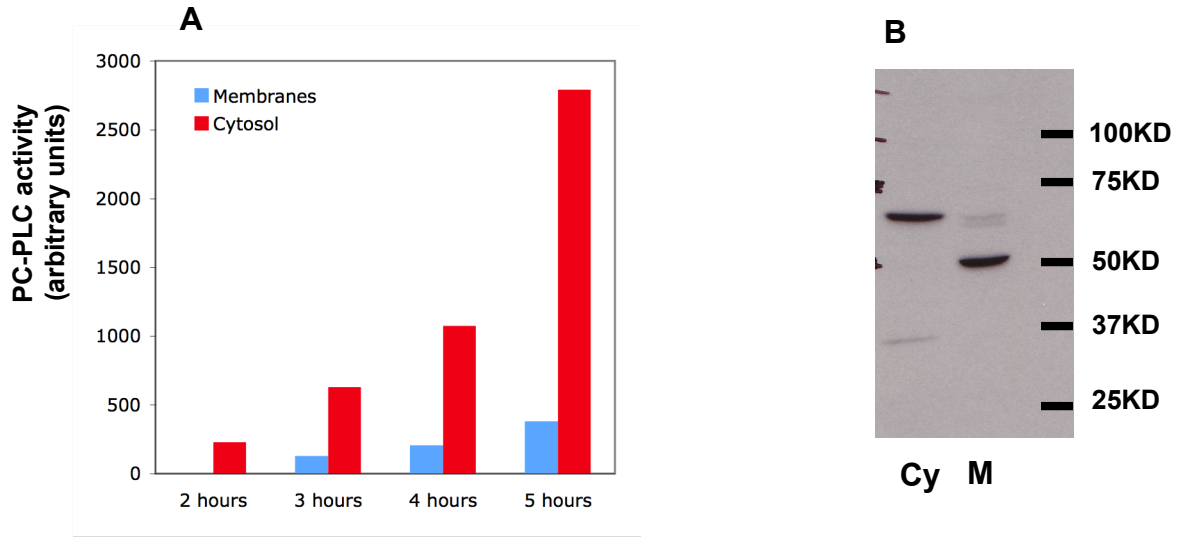

Figure 2. Optimization of an alternative method to measure PC-PLC activity in vitro showing maximum PC-PLC activity in cytosol. A. Total lysates from control HUVECs were fractionated to separate total membranes and cytosol. Equal fractions of membranes and cytosol (corresponding to 50ug of membranes) were compared for PC-PLC activity in vitro at different times of incubation. **B**, **Western blottin**g. Equivalent fractions of membranes (M) and cytosol (Cy) from control cells (20% FBS) were probed for PC-PLC levels using anti PC-PLC antibodies raised against bacterial PC-PLC. Results are representative of at least 3 independent experiments.

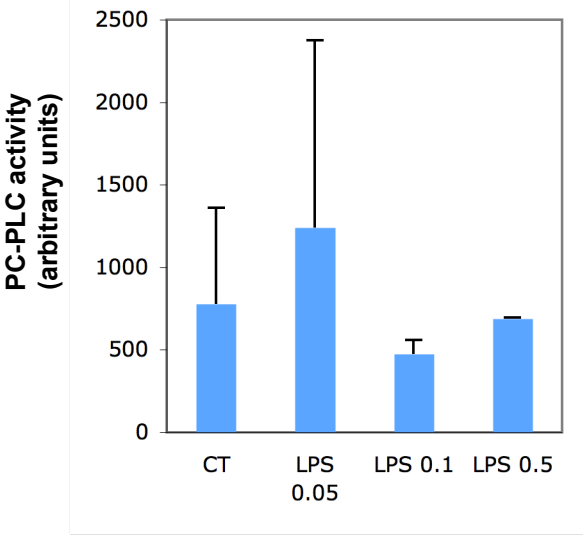

Figure 3. LPS did not activate PC-PLC in HUVECs. Serum starved HUVECs were treated with 0.05, 0.1 and 0.5 ug/ml of LPS for 24 hours. Cytosols (50ug from each condition) were prepared to measure PC-PLC activity *in vitro* and incubated for 4 hours at 37˚C.

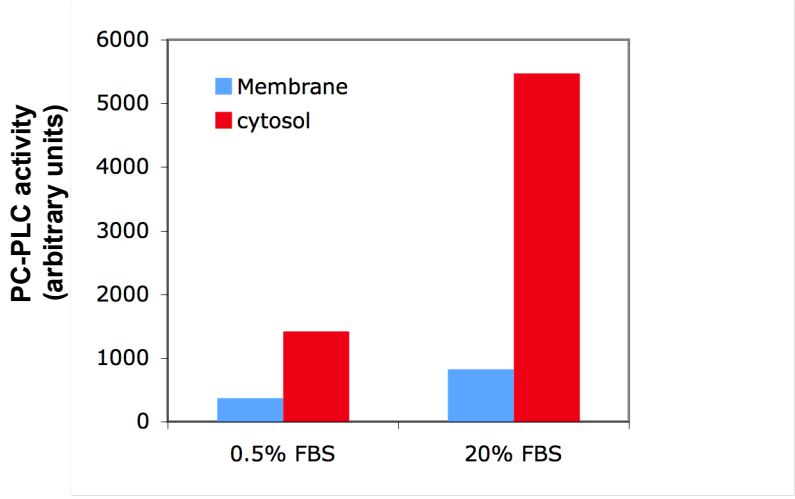

Figure 4. Serum activates PC-PLC in HUVECs. HUVEC cells were either serum starved (0.5% FBS) or serum stimulated (20% FBS) for 24 hours. Membranes and cytosol were prepared and PC-PLC activity was measured in equivalent fractions (corresponding to 50ug of membranes) for 4 hours at 37˚C. Results representative of two independent experiments.