

# Lipopolysaccharide Downregulates the Expression of Selected Phospholipase C Genes in Cultured Endothelial Cells

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**Abstract**—The signaling system of phosphoinositides (PI) is involved in a variety of cell and tissue functions, including membrane trafficking, ion channel activity, cell cycle, apoptosis, differentiation, and cell and tissue polarity. Recently, PI and related molecules, such as the phosphoinositide-specific phospholipases C (PI-PLCs), main players in PI signaling were supposed to be involved in inflammation. Besides the control of calcium levels, PI-PLCs contribute to the regulation of phosphatidylinositol biphosphate metabolism, crucial in cytoskeletal organization. The expression of PI-PLCs is strictly tissue specific and evidences suggest that it varies under different conditions, such as tumor progression or cell activation. In a previous study, we obtained a complete panel of expression of PI-PLC isoforms in human umbilical vein endothelial cells (HUVEC), a widely used experimental model for endothelial cells. In the present study, we analyzed the mRNA concentration of PI-PLCs in lipopolysaccharide (LPS)-treated HUVEC by using the multiliquid bioanalyzer methodology after 3, 6, 24, 48, and 72 h from LPS administration. Marked differences in the expression of most PI-PLC codifying genes were evident.

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**KEY WORDS:** HUVEC; signal transduction; inflammation; phospholipase C; gene expression.

## INTRODUCTION

When inflammation occurs, endothelial cells (EC) in the vessels are the first elements exposed to mediators circulating in the bloodstream [1]. EC react with finely tuned responses mediated by different pathways, including the phosphoinositide (PI) signal transduction system. The PI pathway contributes to a variety of cell functions, including hormone secretion, neurotransmitter signal transduction, cell growth, membrane trafficking, ion channel activity, cytoskel-

eton regulation, cell cycle control, apoptosis, embryonic development, organogenesis, and cell/tissue polarity [2–7]. A combination of compartmentalized and temporal changes in the levels of phosphorylated derivatives of PI elicits different cellular responses, including regulation of gene expression, DNA replication, and chromatin degradation [2–7].

The phosphoinositide-specific phospholipase C (PI-PLC) enzymes contribute to the regulation of the spatiotemporal balance of molecules belonging to the PI system. After activation by different stimuli, PI-PLC rapidly cleaves the polar head group of phosphatidylinositol biphosphate (PIP<sub>2</sub>), a structural component of the cell membrane. Decrease in PIP<sub>2</sub> levels affects many activities, such as cytoskeleton reorganization, cytokinesis, membrane dynamics, nuclear events, and channels activity [8–10]. Cleavage of PIP<sub>2</sub> produces two second messengers inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG) (Berridge). IP<sub>3</sub> induces the release of calcium, a crucial mediator in many cell and tissue activities, such as progression through the cell cycle, actin polymerization and subsequent cell migration

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[2–4]. DAG can be further cleaved to release arachidonic acid, which either acts as a messenger, or can activate the serine/threonine calcium dependent protein kinases C [8–12].

Thirteen mammalian PI-PLC enzymes have been identified, divided into six subfamilies on the basis of amino acid sequence, domain structure, and mechanism of recruitment:  $\beta$ (1–4),  $\gamma$ (1, 2),  $\delta$ (1, 3, 4),  $\epsilon$ (1),  $\zeta$ (1), and  $\eta$ (2) [8]. Isoforms within subfamilies share sequence similarity, common domain organization, and general regulatory mechanism [8–10].

The distribution of PI-PLC enzymes is strictly tissue specific. Probably, each isoform bears a unique function in the modulation of physiological responses [8–12]. In our previous study, the panel of PI-PLC expression was delineated in quiescent human umbilical vein EC (HUVEC), an experimental model for human vascular differentiated, mature EC [13].

Functional studies based on the measurement of PI-PLC activation products indicated the involvement in the inflammatory activation of macrophages [14]. The PI-PLC  $\gamma$  subfamily isoforms are activated in the presence of arachidonic acid that serves as a common link between receptor activation of phospholipase A2 and the PI pathway [8].

The lipopolysaccharide (LPS), a glycolipid structural component of the outer membrane of Gram-negative bacteria, is widely used as inflammatory stimulus [15–18]. In rat astrocytes, PI-PLC enzymes resulted differently expressed after LPS treatment compared to the untreated counterpart [19], suggesting that the PI-PLC pathway might be involved in the inflammatory activation.

In the present study, we treated cultured HUVEC with LPS in order to investigate whether the inflammatory stimulus might modify the expression of the genes that codify for PI-PLC isoforms.

## MATERIALS AND METHODS

### Cell Culture

HUVEC (Cambrex Corporation) were cultured following manufacturer's instructions. Endothelial Cell Growth Supplement (Endogrow, Millipore) was added to the culture medium. Confluent monolayer was obtained after 6–12 days. Cells were cultured from passage 2 to 7. Cells were stimulated by adding to the medium of culture 100 ng/ml LPS (Sigma, St. Louis, MO, USA). Cultures were stopped at the following

times: 3 and 24 h after LPS stimulation for western blot and 3, 6, 24, 48, and 72 h after LPS stimulation for RT-PCR.

### Western Blot

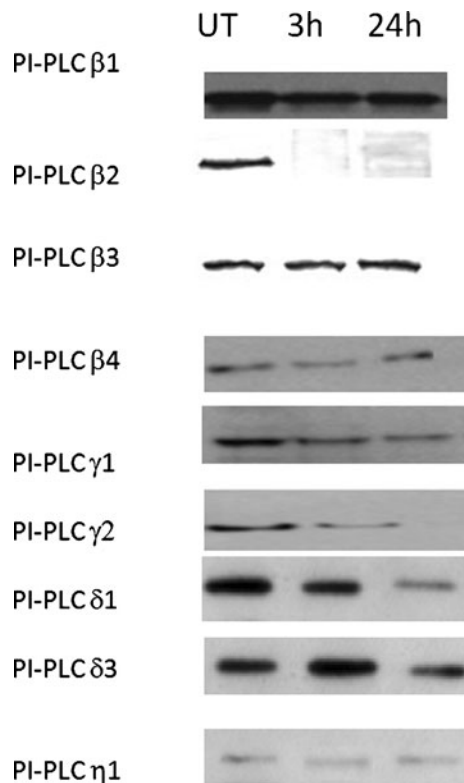
Whole-cell lysates were prepared by lysing cells in RIPA buffer (50 mM Tris pH=7.5, NP-40, 0.1 % SDS, 100 mM NaCl, 50 mM NaF, 1 mM EDTA) supplemented with a set of protease inhibitors: 10  $\mu$ g of leupeptin per milliliter, 10  $\mu$ g of aprotinin per milliliter, 1 mM sodium benzamide, and 1 mM phenylmethylsulfonyl fluoride. Proteins (50  $\mu$ g) were separated on 12 % polyacrylamide, 0.1 % SDS gel. Then, incubation with a monoclonal antibody specific for each PI-PLC isoform (Santa Cruz, CA, USA) followed. Immunoreactive bands were visualized using the enhanced chemiluminescence method (Fig. 1).

### RT-PCR and Multiquid Bioanalysis

Both treated cells and untreated negative controls, plated on six-well plates, were suspended in TRIzol reagent (Invitrogen Corporation, Carlsbad, CA, USA). Total RNA was isolated from samples following the manufacturer's instructions. The obtained RNA purity was assessed by using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA, USA). One microgram of total RNA was reverse transcribed by using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) according to manufacturer's instructions. Briefly, RT Buffer, dNTP Mix, RT Random Primers, Multiscribe Reverse Transcriptase, RNase Inhibitor and DEPC-treated distilled water were added in RNase free tubes on ice. RNA sample was added. Thermal cycler was programmed as follows: 25 °C for 10 m and 37 °C for 120 m; the reaction was stopped at 85 °C for 5 m. The final volume was 20  $\mu$ l. For PCR reactions, the following primer pairs (Bio Basic Inc., Amherst- NY, USA) were used: for PI-PLC  $\beta$ 1 (gene PLCB1; OMIM ID \*607120) forward 5'-AGCTCTCAGAACAAG CCTCCAACA-3' and reverse 5'-ATCATCGTCGTC GTCACCTTCCGT-3'; for PI-PLC  $\beta$ 2 (gene PLCB2; OMIM ID \*604114) forward 5'-AAGGTGAAGG CCTATCTGAGCCAA-3' and reverse: 5'-CTTGCAA ACTTCCCAAAGCGAGT-3'; for PI-PLC  $\beta$ 3 (gene PLCB3; OMIM ID \*600230) forward 5'-TATCTTCTT GGACCTGCTGACCGT-3' and reverse 5'-TGTGCCCT CATCTGTAGTTGGCTT-3'; for PI-PLC  $\beta$ 4 (gene PLCB4; OMIM ID \*600810) forward 5'-GCACA

GCACACAAAGGAATGGTCA-3' and reverse 5'-CGCAT TTCCTTGCTTTCCCTGTCA-3'; for PI-PLC  $\gamma$ 1 (gene PLCG1; OMIM ID \*172420) forward 5'-TCTACCTGGA GGACCTGTGAA-3' and reverse 5'-CCAGAAAGAGAG CGTGTAGTCG-3'; for PI-PLC  $\gamma$ 2 (gene PLCG2; OMIM ID \*600220) forward 5'-AGTACATGCAGATGAATCA CGC-3' and reverse 5'-ACCTGAATCCTGATTTGA CTGC-3'; for PI-PLC  $\delta$ 1 (gene PLCD1; OMIM ID \*602142) forward 5'-CTGAGCGTGTGGTTCCAGC-3' and reverse 5'-CAGGCCCTCGGACTGGT-3'; for PI-PLC  $\delta$ 3 (gene PLCD3; OMIM ID \*608795) forward 5'-CCAGAACCCTCTCAGTATCCA-3' and reverse 5'-GCCATTGTTGAGCAGTAGTCAG-3'; for PI-PLC  $\delta$ 4 (gene PLCD4; OMIM ID \*605939) forward 5'-AGACACGTCCCAGTCTGGAACC-3' and reverse 5'-CTGCTTCCTCTTCTCATATTC-3'; for PI-PLC  $\epsilon$  (gene PLCE; OMIM ID \*608414) forward 5'-GGGGCCACG GTCATCCAC-3' and reverse 5'-GGGCCTTCATACCG TCCATCCTC-3'; for PI-PLC  $\eta$ 1 (gene PLCH1; OMIM ID \*612835) forward 5'-CTTTGGTTCGGTTCCTGTGTGG-3' and reverse 5'-GGATGCTTCTGTACAGTCCCTCC-3'; for PI-PLC  $\eta$ 2 (gene PLCH2; OMIM ID \*612836) forward 5'-

GAAACTGGCCTCCAAACACTGCCCGCCG-3' and reverse 5'-GTCTTGTGGAGATGCACGTGCCCT TGC-3'. The specificity of the primers was verified by searching in National Center for Biotechnology Information database possible homology to cDNAs of unrelated proteins. Each PCR tube contained the following reagents: 0.2  $\mu$ M of both sense and antisense primers, 1–3  $\mu$ l (about 1  $\mu$ g) template cDNA, 0.2 mM dNTP mix, 2.5 U REDTaq Genomic DNA polymerase (Sigma-Aldrich) and 1 $\times$  reaction buffer. MgCl<sub>2</sub> was added at variable (empirical determination by setting the experiment) final concentration. The final volume was 50  $\mu$ l. The amplification was started with an initial denaturation step at 94 °C for 2 min and was followed by 35 cycles consisting of denaturation (30 s) at 94 °C, annealing (30 s) at the appropriate temperature for each primer pairs and extension (1 min) at 72 °C. The PCR products were analyzed by 1.5 % TAE ethidium bromide stained agarose gel electrophoresis (Agarose Gel Unit, BIO-Rad Laboratories Inc., UK). PC-assisted CCD camera UVB lamp (Vilber Lourmaret, France) was used for gel documentation. Gel electrophoresis of the amplification products revealed single DNA bands with nucleotide lengths as expected for all primer pairs. To exclude possible DNA contamination during the RT-PCR, RNA samples were amplified by PCR without reverse transcription. No band was observed, suggesting that there was no DNA contamination in the RNA preparation procedure (data not shown). The PCR products were analyzed and quantified with the Agilent 2100 bioanalyzer using the DNA 1000 LabChip kit (Agilent Technologies, Germany). All the experiments and concentration dosages were repeated at least three times.



**Fig. 1.** Western blot results: PI-PLC isoforms in untreated (UT) HUVEC, and after 3 and 24 h from LPS treatment.

## RESULTS

### Western Blot

Accordingly to previous literature data [20], the treatment of cells with LPS induced reduction in the levels of PI-PLC  $\beta$ 1 and PI-PLC  $\beta$ 2 proteins. Accordingly to previous literature data, the treatment of cells with LPS did not significantly modify the levels of PI-PLC  $\beta$ 3, PI-LPC  $\beta$ 4, PI-PLC  $\gamma$ 1, and PI-PLC  $\delta$ 1. No further data were available regarding the other isoforms. In the present analysis, the levels of PI-PLC  $\gamma$ 2 and PI-PLC  $\delta$ 3 proteins were reduced. The levels of PI-PLC  $\eta$ 1 were not significantly modified (Fig. 1).

### RT-PCR

The following median concentrations were obtained for untreated cells (measured at 24 and 48 h): PLCB1 (6.5 ng/ml), PLCB2 (6.98 ng/ml), PLCB3 (5.35 ng/ml), PLCB4 (1.67 ng/ml), PLCG1 (9.07 ng/ml), PLCG2 (0.87 ng/ml), PLCD1 (6.19 ng/ml), PLCD3 (7.88 ng/ml), PLCD4 (0), PLCE (0), PLCH1 (3.21 ng/ml), PLCH2 (0), and PLCZ (0). After stimulation with LPS, the following median concentrations were obtained: 3-h treatment PLCB1 (3.8 ng/ml), PLCB2 (2.12 ng/ml), PLCB3 (3.81 ng/ml), PLCB4 (0), PLCG1 (5.41 ng/ml), PLCG2 (0 ng/ml), PLCD1 (1 ng/ml), PLCD3 (3.32 ng/ml), PLCD4 (0), PLCE (0), PLCH1 (0), PLCH2 (0), PLCZ (0); 6-h treatment PLCB1 (4.36 ng/ml), PLCB2 (0), PLCB3 (3.59 ng/ml), PLCB4 (0), PLCG1 (5.84 ng/ml), PLCG2 (0), PLCD1 (3.75 ng/ml), PLCD3 (2.41 ng/ml), PLCD4 (0), PLCE (0), PLCH1 (0), PLCH2 (0), PLCZ (0); 24-h treatment PLCB1 (6.06 ng/ml), PLCB2 (0), PLCB3 (3.77 ng/ml), PLCB4 (0), PLCG1 (4.36 ng/ml), PLCG2 (0), PLCD1 (1.88 ng/ml), PLCD3 (0), PLCD4 (0), PLCE (0), PLCH1 (0), PLCH2 (0), PLCZ (0); 48-h treatment PLCB1 (7.33 ng/ml), PLCB2 (0), PLCB3 (4.61 ng/ml), PLCB4 (0.64 ng/ml), PLCG1 (1.78 ng/ml), PLCG2 (0), PLCD1 (3.4 ng/ml), PLCD3 (4.11 ng/ml), PLCD4 (0), PLCE (0), PLCH1 (0), PLCH2 (0), PLCZ (0); 72-h treatment PLCB2 (0), PLCG1 (0.64 ng/ml), PLCG2 (0), PLCD3 (7.55 ng/ml) (Table 1).

Statistical analysis of the results did not show significant differences in three dosages obtained for each isoform. Results in different experiments were comparable. The standard deviation of the compared three dosages was not statistically significant for each isoform.

### DISCUSSION

The endothelium acts dynamically, producing and modifying vasoactive molecules [20]. The morphology, molecular expression, and function of EC are influenced by the surrounding environment, leading the endothelium to adapt [21–23].

EC are exposed to mediators acting during the inflammation cascade [1]. Then, cells react by activating different signal transduction pathways. Efforts addressed to identify and investigate the signal transduction pathways might contribute to highlight the cascade of molecular events occurring in EC during inflammation [18, 24].

Evidences suggested that LPS induces activation of selected PI-PLC isoforms [19]. In our previous study, we delineated in HUVEC the expression panel of PI-PLC enzymes. In quiescent HUVEC, the following isoforms were detected: PI-PLC  $\beta$ 2, PI-PLC  $\beta$ 3, and PI-PLC  $\beta$ 4; the isoforms of  $\gamma$  subfamily (PI-PLC  $\gamma$ 1, and PI-PLC  $\gamma$ 2); the isoforms of  $\delta$  subfamily (PI-PLC  $\delta$ 1, PI-PLC  $\delta$ 3, and PI-PLC  $\delta$ 4); and the isoforms of  $\eta$  subfamily (PI-PLC  $\eta$ 1 and PI-PLC  $\eta$ 2). The remaining PI-PLC  $\beta$ 1, PI-PLC  $\epsilon$ , and PI-PLC  $\zeta$  resulted in unexpressed isoforms [13]. In order to verify whether in HUVEC LPS might act upon gene expression of PI-PLC enzymes, we analyzed the mRNA transcript of all isoforms after LPS stimulation.

In the present study, the isoforms PI-PLC  $\beta$ 1, PI-PLC  $\delta$ 4, and PI-PLC  $\eta$ 2 were differently expressed in untreated HUVEC with respect to our previous study [13]. PI-PLC  $\beta$ 1 was unexpressed in the previous study, while in the present experiments we found it to be expressed [13]. By contrast, PI-PLC  $\delta$ 4 and PI-PLC  $\eta$ 2 resulted expressed in quiescent HUVEC in the previous study [13]. In the present experiments, they are not expressed in untreated HUVEC as well as in LPS treated counterpart. At the moment, no explanation is possible for these different findings. However, the primary HUVEC lines we processed in our two studies were obtained from different batches. Literature data showed a number of genetic differences in HUVEC primary lines [25]. That might partially explain this controversial finding.

According to previous literature data [20], the treatment of cells with LPS induced reduction in the levels of PI-PLC  $\beta$ 1 and PI-PLC  $\beta$ 2 proteins. Accordingly to previous literature data, the treatment of cells with LPS did not significantly modify the levels of PI-PLC  $\beta$ 3, PI-LPC  $\beta$ 4, PI-PLC  $\gamma$ 1, and PI-PLC  $\delta$ 1. No further data were available regarding the other isoforms. In the present analysis, the levels of PI-PLC  $\gamma$ 2 and PI-PLC  $\delta$ 3 proteins were reduced. The levels of PI-PLC  $\eta$ 1 were not significantly modified.

In the present experiments, PLCB1 transcript decreases about 50 % after 3 h from LPS treatment, increases after 6 h, and reaches the initial levels after 24 h. Therefore, the expression of PI-PLC  $\beta$ 1 is rapidly and briefly modified by LPS. In rat astrocytes, Pi-Plc  $\beta$ 1, unexpressed in untreated cells, was expressed after LPS treatment [19]. PI-PLC  $\beta$ 1 seemed to be downregulated in endometriosis, both in eutopic and ectopic endometrium, compared to the endometrium of unaffected women [26]. Endometriosis is a multifactorial disease,

**Table 1.** RT-PCR Results, Multiquid Analyzer Dosages of PLC genes

	B1	B2	B3	B4	D1	D3	G1	G2	H1	GAPDH
NT	6.5	6.98	5.35	1.67	6.19	7.88	9.07	0.87	3.21	2.9
LPS										
3 h	3.8	2.12	3.81	0	1	3.32	5.41	0	0	5.3
6 h	4.36	0	3.59	0	3.75	2.41	5.84	0	0	5.7
24 h	6.06	0	3.77	0	1.88	0	4.36	0	0	6.1
48 h	7.33	0	4.61	0.63	3.4	4.11	1.78	0	0	6.7
72 h	nq	0	nq	nq	nq	7.55	0.64	nq	0	4.1

NT untreated concentrations, nq not quantified. Concentrations are intended in nanograms per milliliter

with a strong inflammatory component. Therefore, although the present results remain to be confirmed, our data might suggest that under inflammatory stimulus the transcription of the PLCB1 gene is modified with respect to untreated counterpart. However, the regulation of PLCB1 is controversial. Further studies are required in order to investigate the regulation of PI-PLC  $\beta$ 1 expression during the inflammation cascade.

In the present experiments, the transcription of PLCB2 gene decreases about 40 % after 3 h from LPS treatment. The observation was prolonged to 72 h, and no transcript was detected during the whole period. Unfortunately, it was not possible to further prolong the observation for more than 72 h, as HUVEC primary lines die after eight to nine passages [25]. By the present results, LPS treatment seems to efficiently silence the expression of PI-PLC  $\beta$ 2 in HUVEC. PI-PLC  $\beta$ 2 isoform is strictly expressed in hematopoietic cell lines [27, 28]. In fact, in our previous studies, PI-PLC  $\beta$ 2 was not expressed in quiescent cell lines as well as in the pathological counterpart [13, 19, 26, 29]. PI-PLC  $\beta$ 2 is known to be involved in the pathogenesis of some autoimmune diseases [30]. Moreover, interleukin-8, a pro-inflammatory chemokine, induces activation of G-protein which results in the activation of PI-PLC  $\beta$ 2 [31]. However, further studies are required in order to investigate the role of PI-PLC  $\beta$ 2 and the relationship with the elements of the immune system [31].

In the present experiments, the transcription of PLCB3 gene is uniformly reduced about 40 % after LPS treatment since 3–72 h. Therefore, the effect of LPS treatment seems to stably reduce the expression of PLCB3 in HUVEC. PI-PLC  $\beta$ 3 findings are controversial compared to our previous studies. We described downregulation during astrocyte activation after LPS stimulation [19]. By contrast, we found upregulation of PI-PLC  $\beta$ 3 expression in the ectopic endometrium [27].

Further studies are required in order to investigate the differences in PLCB3 expression. In fact, the different regulation of PLCB3 transcription might only partially be explained with the different features of the analyzed cytotypes (rat astrocytes *versus* human endometrium and EC).

In the present experiments, after LPS treatment, PLCB4 transcript decreases since 6–24 h. PI-PLC  $\beta$ 4 isoform is highly expressed in the nervous system, and is involved in the nociceptive pathway in mouse [32]. In our previous studies, PI-PLC  $\beta$ 4 was expressed both in untreated and in LPS-treated rat astrocytes [19]. Unfortunately, we had not quantified the transcript concentrations. Therefore, it is not possible to evaluate whether in rat astrocytes Plcb3 as well as genes codifying for other isoforms were downregulated.

In the present experiments, the expression of PI-PLC  $\delta$ 1 is reduced about 80 % after LPS treatment, then stably increases about 50 % since 6 h. Therefore, the downregulating effect of LPS acts rapidly upon PLCD1 transcription and continues, although less evident, for a prolonged period. These observations accord to our previous studies. In rat astrocytes, LPS stimulation acted rapidly and briefly downregulating the expression of Plcd1. Moreover, PI-PLC  $\delta$ 1 seemed to be downregulated in the ectopic endometrium [26]. Interestingly, evidences suggested a critical role for PI-PLC  $\delta$ 1 in the progression the cell cycle from G(1)-to-S-phase [33]. Further literature data indicated that lack of Plcd1 in knock-out (KO) mice induces skin inflammation [33]. Further studies are required in order to investigate the role played by PI-PLC  $\delta$ 1 during inflammation.

The transcription of PLCD3 decreases more than 50 % after 3 h from LPS treatment of HUVEC. It further decreases after 6 h (60 %) and no mRNA was detected after 24 h. The expression begins after 48 h from LPS treatment. In addition to its role in the cell cycle control

and osmotic response, recent evidences indicate that PI-PLC  $\delta 1$  might act as an antioncogene [33, 34]. PI-PLC  $\delta$  isozymes, the most primitive and evolutionary conserved, are known to be the most sensitive to calcium. Our previous studies described the variation of PI-PLC  $\delta 1$  and PI-PLC  $\delta 3$  expression during activation after LPS stimulation and in endometriosis [26]. Both isoforms seem to be downregulated in a time-dependent manner under inflammatory conditions [13, 16]. That suggested that both PI-PLC  $\delta 1$  and PI-PLC  $\delta 3$  might be involved in the fine tuning and regulation of the inflammation cascade. However, their role, timing of action, and possible interplay during the complex inflammation mechanism remains to be elucidated.

In the present experiments, the transcription levels of PLCG1 progressively decrease. The transcript is reduced about 45–50 % since 3 h, about 75 % at 48 h and 95 % at 72 h. Therefore, LPS acts rapidly and progressively. Moreover, LPS effects last until 72 h of observations. In our previous study, Pi-Plc  $\gamma 1$  was expressed both in untreated and in LPS-treated astrocytes. However, results are not comparable to the present data. In fact, in our previous study, we did not quantify the transcript. Literature data demonstrated that PI-PLC  $\gamma 1$  is essential for T cell development, activation and tolerance. In KO mice, deficiency of *Plcg1* impairs the functionality of regulatory T cells, causing inflammatory/autoimmune symptoms [35].

In the present experiments, no transcript for PLCG2, low in quiescent HUVEC, was detected until 48 h after LPS treatment. PI-PLC  $\gamma 2$  transcript was detected exclusively after 72 h, although reduced about 25 % compared to untreated levels. PI-PLC  $\gamma 2$  isoform is tissue restricted to the hematopoietic lineage [8, 36]. In our previous study, PI-PLC  $\gamma 2$  was unexpressed in untreated and LPS-treated astrocytes, and in endometrium, according to literature data. PI-PLC  $\gamma 2$  was also identified as a promising target in inflammatory arthritis. In fact, recent studies suggested that PI-PLC  $\gamma 2$  isoform might represent a critical regulator of the cellular and molecular mechanisms occurring in bone and immune cells during autoimmune inflammation [37].

In the present experiments, PI-PLC  $\eta 1$ , expressed in quiescent HUVEC, resulted unexpressed after LPS treatment, and no transcript was detected after 72 h from stimulation. LPS seems to act rapidly and definitely upon PLCH1 transcription. Unfortunately, no literature data are available about the possible role of PI-PLC  $\eta$  subfamily in the inflammation cascade. The remaining isoforms, PI-PLC  $\delta 4$ ,  $\epsilon$ , and  $\eta 2$  remained unexpressed both in untreated controls and LPS treated HUVEC.

Resuming, our data suggest that some PI-PLC isoforms might be involved in the metabolic pathways activated by LPS-induced inflammatory stimulation of HUVEC. For some isoforms, namely PI-PLC  $\beta 2$  and  $\gamma 2$ , our results accord to literature data suggesting the involvement of PI-PLC in the regulation of the immune system. The present data partially contrast with previous results obtained in quiescent HUVEC, remarkably with regard to PI-PLC  $\beta 1$ ,  $\delta 4$ , and  $\eta 2$ . In rat astrocytes, LPS seems to upregulate PI-PLC  $\beta 1$  expression [19]. In the present experiment, LPS seems to downregulate the transcription of *PLCB1* gene in HUVEC. The differences we observed might be due to the peculiar role played by PI-PLC  $\beta 1$  in the nervous system [32]. The remaining findings partially or completely accord to literature data.

The present results suggest that the treatment with LPS affects the gene expression of PI-PLC enzymes, and thus contribute to regulate the production of the enzymes. LPS seems to downregulate the overall expression of most PI-PLC isoforms. That is an interesting long-term effect of LPS, otherwise known to induce the activation of the corresponding enzymes, probably followed by depletion of the intracellular stores. Although all to demonstrate, in HUVEC the effects of LPS upon PLC genes expression might be the attempt to self-limit the inflammatory reaction or might contribute to the regulation of the cell cycle. In this perspective, the role of the PI-PLC enzymes remains to be highlighted.

To understand the complex network that regulates the inflammatory process and the ability to manipulate the EC might offer a powerful tool of considerable practical and clinical importance, providing useful insights for potential therapeutic strategies.

However, although HUVEC represent a widely used, useful experimental model for human macrovascular EC, limitations in the present study remain in that they cannot fully represent the metabolic properties and interactions of the EC distributed in the living organism. Further studies are required to analyze the role and the crosstalk among the PI-PLC isoforms and the reciprocal regulation in order to tune the cell responses.

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## REFERENCES

1. Gonzalez, M.A., and A.P. Selwyn. 2003. Endothelial function, inflammation, and prognosis in cardiovascular disease. *Am J Med* 115(Suppl 8A): 99S–106S.
2. Berridge, M.J. 1981. Phosphatidylinositol hydrolysis: a multifunctional transducing mechanism. *Mol Cell Endocrinol* 24(2): 115–140.
3. Berridge, M.J., and R.F. Irvine. 1989. Inositol phosphates and cell signalling. *Nature* 341(6239): 197–205.
4. Berridge, M.J. 2009. Inositol trisphosphate and calcium signalling mechanisms. *Biochim Biophys Acta* 1793(6): 933–940.
5. Comer, F.I., and C.A. Parent. 2007. Phosphoinositides specify polarity during epithelial organ development. *Cell* 128(2): 239–240.
6. Croke, C.E., A. Pozzi, and G.F. Carpenter. 2009. PLC-gamma1 regulates fibronectin assembly and cell aggregation. *Exp Cell Res* 315(13): 2207–2214.
7. Mi, L.Y., D.S. Ettenson, and E.R. Edelman. 2008. Phospholipase C-delta extends intercellular signalling range and responses to injury-released growth factors in non-excitable cells. *Cell Prolif* 41(4): 671–690.
8. Suh, P.G., J. Park, L. Manzoli, L. Cocco, J.C. Peak, M. Katan, K. Fukami, T. Kataoka, S. Yuk, and S.H. Ryu. 2008. Multiple roles of phosphoinositide-specific phospholipase C isozymes. *BMB Reports* 41: 415–434.
9. Bunney, T.D., and M. Katan. 2011. PLC regulation: emerging pictures for molecular mechanisms. *Trends Biochem Sci* 36(2): 88–96.
10. Katan, M. 2005. New insights into the families of PLC enzymes: looking back and going forward. *Biochem J* 391(Pt 3): e7–e9.
11. Nakamura, Y., and K. Fukami. 2009. Roles of phospholipase C isozymes in organogenesis and embryonic development. *Physiology* 24: 332–341.
12. Hisatsune, C., K. Nakamura, Y. Kuroda, T. Nakamura, and K. Mikoshiba. 2005. Amplification of Ca<sup>2+</sup> signaling by diacylglycerol-mediated inositol 1,4,5-trisphosphate production. *J Biol Chem* 280(12): 11723–11730.
13. Lo Vasco, V.R., L. Pacini, T. Di Raimo, D. D’Arcangelo, and R. Businaro. 2011. Expression of phosphoinositide-specific phospholipase C isoforms in HUVEC. *J Clin Pathol* 64(10): 911–915.
14. Lo, C.J., H.G. Cryer, and R.V. Maier. 1996. Prostaglandin E2 production by endotoxin-stimulated alveolar macrophages is regulated by phospholipase C pathways. *J Trauma*. 40(4): 557–62. discussion 563.
15. Tang, X., E.M. Edwards, B.B. Holmes, J.R. Falck, and W.B. Campbell. 2006. Role of phospholipase C and diacylglyceride lipase pathway in arachidonic acid release and acetylcholine-induced vascular relaxation in rabbit aorta. *Am J Physiol Heart Circ Physiol* 290(1): H37–H45.
16. Loppnow, H., and P. Libby. 1989. Comparative analysis of cytokine induction in human vascular endothelial and smooth muscle cells. *Lymphokine Res* 8(3): 293–299.
17. Reyes, C.L., and G. Chang. 2005. Structure of the ABC transporter MsbA in complex with ADP.vanadate and lipopolysaccharide. *Science*. 308(5724): 1028–31. Retraction in: Chang G, Roth CB, Reyes CL, Pornillos O, Chen YJ, Chen AP. *Science*. 2006. 22;314(5807):1875.
18. Zhang, X.M., A. Morikawa, K. Takahashi, G.Z. Jiang, Y. Kato, T. Sugiyama, M. Kawai, M. Fukada, and T. Yokochi. 1994. Localization of apoptosis (programmed cell death) in mice by administration of lipopolysaccharide. *Microbiol Immunol* 38(8): 669–671.
19. Lo Vasco, V.R., C. Fabrizi, L. Fumagalli, and L. Cocco. 2010. Expression of phosphoinositide specific phospholipase C isoenzymes in cultured astrocytes activated after stimulation with lipopolysaccharide. *J Cell Biochem* 109(5): 1006–1012.
20. Grinberg, S., G. Hasko, D. Wu, and S.J. Leibovich. 2009. Suppression of PLCbeta2 by endotoxin plays a role in the adenosine A(2A) receptor-mediated switch of macrophages from an inflammatory to an angiogenic phenotype. *Am J Pathol* 175(6): 2439–2453.
21. Gloe, T., H.Y. Sohn, G.A. Meininger, and U. Pohl. 2002. Shear stress-induced release of basic fibroblast growth factor from endothelial cells is mediated by matrix interaction via integrin alpha(v)beta3. *J Biol Chem* 277(26): 23453–23458.
22. Christ, M., and M. Wehling. 1998. Cardiovascular steroid actions: swift swallows or sluggish snails? *Cardiovasc Res* 40(1): 34–44.
23. Grienddling, K.K., and W.R. Alexander. 1996. Endothelial control of the cardiovascular system: recent advances. *FASEB J* 10: 283–292.
24. Cuschieri, J., J. Billigren, and R.V. Maier. 2006. Endotoxin tolerance attenuates LPS-induced TLR4 mobilization to lipid rafts: a condition reversed by PKC activation. *J Leukoc Biol* 80(6): 1289–1297.
25. Unterluggauer, H., E. Hütter, R. Voglauer, J. Grillari, M. Vöth, J. Bereiter-Hahn, P. Jansen-Dürr, and M. Jendrach. 2007. Identification of cultivation-independent markers of human endothelial cell senescence *in vitro*. *Biogerontology* 8(4): 383–397.
26. Lo Vasco, V.R., M. Leopizzi, C. Chiappetta, R. Businaro, P. Polonia, C. Della Rocca, and P. Litta. 2012. Expression of phosphoinositide-specific phospholipase C enzymes in normal endometrium and in endometriosis. *Fertil Steril* 98(2): 410–414.
27. Gratacap, M.P., B. Payrastre, C. Viala, G. Mauco, M. Plantavid, and H. Chap. 1998. Phosphatidylinositol 3,4,5-trisphosphate-dependent stimulation of phospholipase C-gamma2 is an early key event in FcgammaRIIA-mediated activation of human platelets. *J Biol Chem* 273(38): 24314–24321.
28. Miao, J.Y., K. Kaji, H. Hayashi, and S. Araki. 1997. Inhibitors of phospholipase promote apoptosis of human endothelial cells. *J Biochem* 121(3): 612–618.
29. Lo Vasco, V.R., C. Fabrizi, M. Artico, L. Cocco, A.M. Billi, L. Fumagalli, and F.A. Manzoli. 2007. Expression of phosphoinositide-specific phospholipase C isoenzymes in cultured astrocytes. *J Cell Biochem* 100(4): 952–959.
30. Jakus, Z., E. Simon, D. Frommhold, M. Sperandio, and A. Mócsai. 2009. Critical role of phospholipase C gamma2 in integrin and Fc receptor-mediated neutrophil functions and the effector phase of autoimmune arthritis. *J Exp Med* 206(3): 577–593.
31. Atta-ur-Rahman, Harvey K., and R.A. Siddiqui. 1999. Interleukin-8: an autocrine inflammatory mediator. *Curr Pharm Des* 5(4): 241–253.
32. Lo Vasco, V.R. 2012. The phosphoinositide pathway and the signal transduction network in neural development. *Neuroscience Bulletin* 28(6): 789–800.
33. Kaproth-Joslin, K.A., X. Li, S.E. Reks, and G.G. Kelley. 2008. Phospholipase C delta 1 regulates cell proliferation and cell-cycle progression from G1- to S-phase by control of cyclin E-CDK2 activity. *Biochem J* 415(3): 439–448.
34. Ichinohe, M., Y. Nakamura, K. Sai, M. Nakahara, H. Yamaguchi, and K. Fukami. 2007. Lack of phospholipase C-delta1 induces skin inflammation. *Biochem Biophys Res Commun* 356(4): 912–918.
35. Fu, G., Y. Chen, M. Yu, A. Podd, J. Schuman, Y. He, L. Di, M. Yassai, D. Haribhai, P.E. North, J. Gorski, C.B. Williams, D. Wang, and R. Wen. 2010. Phospholipase C{gamma}1 is essential for T cell development, activation, and tolerance. *J Exp Med* 207(2): 309–318.
36. Kurosaki, T., A. Maeda, M. Ishiai, A. Hashimoto, K. Inabe, and M. Takata. 2000. Regulation of the phospholipase C-gamma2 pathway in B cells. *Immunol Rev* 176: 19–29.
37. Faccio, R., and V. Cremasco. 2010. PLCgamma2: where bone and immune cells find their common ground. *Ann N Y Acad Sci* 1192: 124–130.