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Expression of Phosphoinositide-Specific Phospholipase C Isoenzymes in Cultured Astrocytes Activated After Stimulation With Lipopolysaccharide

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ABSTRACT

Signal transduction pathways, involved in cell cycle and activities, depend on various components including lipid signalling molecules, such as phosphoinositides and related enzymes. Many evidences support the hypothesis that inositol lipid cycle is involved in astrocytes activation during neurodegeneration. Previous studies investigated the pattern of expression of phosphoinositide-specific phospholipase C (PI-PLC) family isoforms in astrocytes, individuating in cultured neonatal rat astrocytes, supposed to be quiescent cells, the absence of some isoforms, accordingly to their well known tissue specificity. The same study was conducted in cultured rat astrocytoma C6 cells and designed a different pattern of expression of PI-PLCs in the neoplastic counterpart, accordingly to literature suggesting a PI signalling involvement in tumour progression. It is not clear the role of PI-PLC isoforms in inflammation; recent data demonstrate they are involved in cytokines production, with special regard to IL-6. PI-PLCs expression in LPS treated neonatal rat astrocytes performed by using RT-PCR, observed at 3, 6, 18 and 24 h intervals, expressed: PI-PLC beta1, beta4 and gamma1 in all intervals analysed; PI-PLC delta1 at 6, 18 and 24 h; PI-PLC delta3 at 6 h after treatment. PI-PLC beta3, delta4 and epsilon, present in untreated astrocytes, were not detected after LPS treatment. Immunocytochemical analysis, performed to visualize the sub-cellular distribution of the expressed isoforms, demonstrated different patterns of localisation at different times of exposure. These observations suggest that PI-PLCs expression and distribution may play a role in ongoing inflammation process of CNS. J. Cell. Biochem. 109: 1006–1012, 2010.

KEY WORDS: PHOSPHOLIPASE C; SIGNAL TRANSDUCTION; ASTROCYTES; INFLAMMATION; LIPOPOLYSACCHARIDE; RT-PCR; FLUORESCENCE IMMUNOCYTOCHEMISTRY

Phosphoinositides (PIs) are involved in many key functions such as cell signaling, intracellular membrane trafficking [Cocco et al., 1996; Manzoli et al., 1996; Irvine, 2002], and nuclear signaling based on lipid hydrolysis [Cocco et al., 2001, 2003]. In the inositol lipid pathway, phosphatidyl inositol 4,5-bisphosphate (PIP2) is hydrolyzed by enzymes of the PI-specific phospholipase C (PI-PLC) family in response to a wide panel of stimuli, including growth factors, hormones, and neurotransmitters, that act on specific receptors localized at the plasma membrane. The hydrolysis of PIP2 generates two second messengers: 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) [Cocco et al., 1996; Hisatsune et al., 2005]. The former activates DAG-dependent protein kinase C (PKC) isoforms or it is converted to phosphatidic acid, which also plays signaling functions; the latter liberates calcium from intracellular stores. The PI-PLC family includes numerous enzymes, codified by different genes; these enzymes differ for structure and tissue distribution. The most important are 10 mammalian isoforms, classified into four sub-families. Four isoforms belong to PI-PLC beta sub-family, two to PI-PLC gamma, three to PI-PLC delta [Irino et al., 2004], and one to PI-PLC epsilon [Wang and Reiser, 2003]. The inositol lipid cycle seems to be involved in glial activation during neurodegenerative processes and in rat astrocyte inflammatory

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activation via increase of IL-6 level [Vitale et al., 2004]. A panel of glial PI-PLC isoforms was determined in a previous study [Lo Vasco et al., 2007], performed on neonatal (which are supposed to be quiescent cells) rat astrocytes. Performing mRNA expression analyses by using reverse transcriptase polymerase chain reaction (RT-PCR), we individuated the presence of PI-PLC isoforms beta4, gamma1, delta1, delta3, delta4, and epsilon. Immunocytochemical analyses revealed that the isoforms have different patterns of subcellular localization, being some of them (with special regard to PI-PLC beta3, which was absent in the cytoplasm) localized in the nucleus and others localized mainly in the cytoplasm (PI-PLC gamma1 and epsilon, which are weakly expressed in the nucleus) [Lo Vasco et al., 2007].

MATERIALS AND METHODS

CELL CULTURE

Astrocyte cultures were prepared from mixed primary glial cultures obtained from neonatal rat cortex as described previously [Levi et al., 1993], in accordance with national guidelines for care and use of laboratory animals. Glial cells were seeded into 75-cm^2 flasks at initial density of $2–3 \times 10^6$ cells per flask, maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Invitrogen, Carlsbad, CA) and cultured to confluence (14 days) in a 5% CO_2 incubator at 37°C with media changes every 2–3 days. Astrocyte cultures were characterized for purity as previously described [Amruthesh et al., 1993] and consisted of more than 95% glial fibrillary acidic protein-positive cells (data not shown). Upon confluence, cells were lifted from the flask surface using 0.25% trypsin/0.02% EDTA. Next the cells were washed, plated in six-well plates for molecular biology studies or plated on coverslips (15 mm) for immunocytochemistry studies (all the reagents were obtained from Invitrogen). The astrocytes were then stimulated by adding to the medium of culture 100 ng/ml LPS (Sigma, St. Louis, MO). For molecular biology analysis, cells, both treated and untreated negative controls, plated on six-well plates, were suspended in TRIzol reagent (Invitrogen) at 3, 6, 18, and 24 h after LPS addiction to the medium of culture. For immunocytochemical analysis, cells, both treated and untreated negative controls, plated on coverslips, were grown until they underwent fixation at 3, 6, 18, and 24 h after LPS addiction to the culture medium.

RT-PCR

Total RNA was isolated from samples by using TRIzol reagent (Invitrogen) following the manufacturer's instructions. The obtained RNA was quantitated by the UV absorption ratio 260:280 nm by using a UV/visible spectrophotometer (SmartSpec 3000, Bio-Rad Laboratories, Hercules, CA); 1 μg of total RNA was reverse transcribed by using SuperScript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. Briefly, total RNA, oligo(dT), dNTP mix, and DEPC-treated distilled water were heated for 5 min at 65°C and then buffer (250 mM Tris, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) containing DTT and recombinant RNase inhibitor was added to the mixture. All reagents were obtained from Invitrogen. After incubation for 2 min at 42°C, SuperScript III reverse transcriptase (200 U) was added and incubated for 50 min. Reaction was stopped by heating for 15 min at 70°C. The final volume was 20 μl. For PCR reactions, the following primer pairs (M-Medical, Florence, Italy) were used: for PI-PLC beta1 reverse: 5′-CTG AGC TCA CCC ATG ACA GA-3′, forward: 5′-TGC ATA CGT GTC TGG GAC AT-3′; for PI-PLC beta2 reverse: 5′-GAC ACA CTC GCT TTG GGA AG-3′, forward: 5′-GGG CTC AGC TGC ATT TTA AG-3′; for PI-PLC beta3 reverse: 5′-GGA GGA GGA GGA TCA TAG GC-3′, forward: 5′-CCC AGA AAG AGT TGG AGC TG-3′; for PI-PLC beta4 reverse: 5′-GGA TGC TCT ATC CGA TCC AA-3′, forward: 5′-GCT CCT TTG CGT GTT TCT TC-3′; for PI-PLC delta1 reverse: 5′-TCA TCC TGT CCC TGG AGA AC-3′, forward: 5′-TCA GAC ACG TCA GTG GCT TC-3′; for PI-PLC delta3 reverse: 5′-GGG TTG GAA CAG CAG ATT GT-3′, forward: 5′-AGT GGG TAC ACT CGG GTC AG-3′; for PI-PLC delta4 reverse: 5′-CCT CTG GGA ACA AGA GCA AG-3′, forward: 5′-GCT TCA TGG GAT TGA AGG AA-3′; for PI-PLC epsilon reverse: 5′-CGG ATC AAA GGA GGA ATG AA-3′, forward: 5′-GCC TCT GGT TGT CAG AAA GC-3′; for PI-PLC gamma1 reverse: 5′-TAC AGC AGG AAG GCC AGA CT-3′, forward: 5′-CTC TCT GGG CCT TGT AGT CG-3′; for PI-PLC gamma2 reverse: 5′-GGT TCT GAT GGG CTG TGT TT-3′, forward: 5′-GGC AAG ATG GTC TTC AGC TC-3′. The specificity of the primers was verified by searching in NCBI database possible homology to cDNAs of unrelated proteins. Each PCR tube contained the following reagents: 0.2 μM of both sense and antisense primers, 3–5 μl template cDNA, 0.2 mM dNTP mix (Invitrogen), 2.5 U Platinum Taq DNA polymerase (Invitrogen), and 1× reaction buffer (Invitrogen). $MgCl_2$ was added at variable (empirical determination by setting the experiment) final concentration. The final volume was 50 μl. 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% TBE agarose gel electrophoresis (Submarine Agarose Gel Unit, Hoefer, San Francisco, CA). Gels were previously stained with ethidium bromide (50 ng/100 ml). A PC-assisted CCD camera (GelDoc 2000 System/Quantity One Software; Bio-Rad) was used for gel documentation and quantification. Optical densities were normalized to the mRNA content of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a typical reference constitutive transcript. 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).

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Astrocytes grown on coverslips were fixed in MetOH/acetic acid (3:1). After fixation, cells were washed several times in phosphate buffer (PBS) and first saturation was performed with 1% BSA in PBS; then incubation with primary antibodies was performed, following the manufacturer's indications with regard to the dilutions, lasting overnight at +4°C. The used primary antibodies (purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were: mouse monoclonal anti-PI-PLC beta1, rabbit polyclonal anti-PI-PLC beta3,

TABLE I. RT-PCR Results

Untreated astrocytes	PI-PLC isoforms	LPS-treated astrocytes 3 h	6 h	18 h	24 h
−	Beta 1	+	+	+	+
−	Beta 2	−	−	−	−
+	Beta 3	−	−	−	−
+	Beta 4	+	+	+	+
+	Gamma1	+	+	+	+
−	Gamma2	−	−	−	−
+	Delta1	−	+	+	+
+	Delta3	−	+	−	−
+	Delta4	−	−	−	−
+	Epsilon	−	−	−	−

RT-PCR expression of PI-PLC isoforms in untreated neonatal rat astrocytes (left column) and in LPS-treated neonatal rat astrocytes at different times after treatment (right columns).

rabbit polyclonal anti-PI-PLC beta4, mouse monoclonal anti-PI-PLC gamma1, mouse polyclonal anti-PI-PLC delta1, goat polyclonal anti-PI-PLC delta3, goat polyclonal anti-PI-PLC delta4, goat polyclonal anti-PI-PLC epsilon. After several washes with PBS, a second incubation was performed with the secondary fluorochrome-conjugated antibody 60 min at room temperature in humidified dark chamber. The secondary antibodies were purchased from: Sigma (for Cy3-conjugated anti-mouse and anti-rabbit); Santa Cruz Biotechnology, Inc. (for Texas Red-conjugated anti-goat); negative controls were performed by omitting the primary antibody (for the experiments testing PI-PLC beta3, beta4, delta1, delta3, and delta4) or by using normal goat IgG (for the experiments testing PI-PLC beta1, gamma1, and epsilon) (data not shown). After several washes performed with PBS in dark chamber, the coverslips were counter-stained with DAPI, mounted on slides, and observed with a fluorescence microscope (Nikon Eclipse 800) equipped with ACT image analysis and acquisition program.

RESULTS

RT-PCR

PI-PLC beta1, PI-PLC beta4, and PI-PLC gamma1 were expressed at 3, 6, 18, and 24 h after LPS treatment. PI-PLC delta1 was not expressed at 3 h, while it was present at 6, 18, and 24 h after LPS treatment. PI-PLC delta3 was expressed only at 6 h after LPS treatment and was absent at 3, 18, and 24 h. PI-PLC beta2, PI-PLC beta3, PI-PLC gamma2, PI-PLC delta4, and PI-PLC epsilon were not expressed at any of the analyzed time of exposure to LPS treatment (Table I) (Fig. 1). Briefly at 3 h were present PI-PLC beta1, PI-PLC beta4, and PI-PLC gamma1; at 6 h were present PI-PLC beta1, PI-PLC beta4, and PI-PLC gamma1 and appear PI-PLC delta1 and PI-PLC delta3; at 18 h were still present PI-PLC beta1, PI-PLC beta4, PI-PLC gamma1, and PI-PLC delta1 while PI-PLC delta3 was no more expressed; at 24 h were present PI-PLC beta1, PI-PLC beta4, PI-PLC gamma1, and PI-PLC delta1 (Table I) (Fig. 1).

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PI-PLC beta1 was detected both in the nucleus and the cytoplasm at 3 h, while at 6, 18, and 24 h it was present only in the cytoplasm. PI-PLC beta4 was detected only in the cytoplasm at 3 and 6 h, being absent in the nucleus, while it was detected both in the nucleus and cytoplasm at 18 and 24 h. PI-PLC gamma1 was absent in the nucleus at 3 h, it was detected both in the nucleus and the cytoplasm at 6 and 18 h, being once again absent in the nucleus at 24 h, when it was detected in the cytoplasm. PI-PLC delta1 was detected both in the nucleus and the cytoplasm at 6, 18, and 24 h. PI-PLC delta3 was detected in the cytoplasm at 6 h, being absent for the rest of the observation period (Table II) (Fig. 2). Briefly, after LPS treatment, in the nucleus were present: at 3 h PI-PLC beta1; at 6 h PI-PLC gamma1 and delta1; at 18 h PI-PLC beta4, gamma1, and delta1; at 24 h PI-PLC beta4 and delta1. In the cytoplasm were present: at 3 h PI-PLC beta1, beta4, and gamma1; at 6 h PI-PLC beta1, beta4, delta1, and delta3; at 18 and 24 h PI-PLC beta1, beta4, gamma1, and delta1 (Table II) (Fig. 2).

DISCUSSION

Transduction signals involved in processes such as differentiation, proliferation, and apoptosis induce changes in inositol metabolism and, vice versa, inositol metabolism is involved in the cascade of sequential events composing these complex processes [Noh et al., 1995; Martelli et al., 2002; Cocco et al., 2003]. A combination of compartmentalized and temporal changes in PIP2 or phosphatidyl inositol 3,4,5 trisphosphate (PIP3) levels might be detected by PI-binding proteins to elicit different cellular responses, including regulation of gene expression, DNA replication, or chromatin degradation. Enzymes interfering with PI metabolism such as the PI-PLCs, divided into four sub-families comprising various isoforms, are involved in myogenic [Faenza et al., 2004] and adipogenic differentiation [O'Carroll et al., 2009], neoplastic proliferation [Lo Vasco et al., 2004; Cocco et al., 2009], and apoptosis [Ledeen and

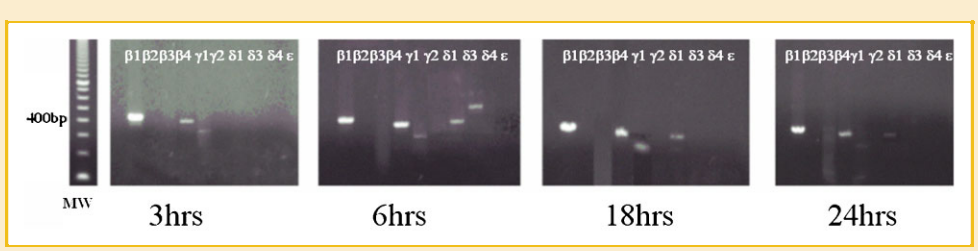

Fig. 1. Results of gel electrophoresis of RT-PCR products in LPS-treated neonatal rat astrocytes.

TABLE II. Immunocytochemistry Results in LPS-Treated Neonatal Rat Astrocytes

	3 h		6 h		18 h		24 h	
PI-PLC isoforms	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm
Beta 1	+	+	−	+	−	+	−	+
Beta 4	−	+	−	+	+	+	+	+
Gamma1	−	+	+	+	+	+	−	+
Delta1	−	−	+	+	+	+	+	+
Delta3	−	−	−	+	−	−	−	−

Wu, 2004]. Moreover, also the sub-cellular distribution of PI-PLCs seems to contribute to the regulation of these events [Choi et al., 1989; McBride et al., 1991; Bertagnolo et al., 1995; Pawelczyk and Matecki, 1998]. Among the numerous signal transduction pathways involved in the cascade of events characterizing the inflammation process, special attention is paid to the production of interleukin 6 (IL-6), especially operating in the actions induced by endothelin peptides (ETs) [Rubanyi and Polokoff, 1994]. In astrocytes the ET-G protein mediated PI-PLC activation leads to PI hydrolysis and rapid formation of IP3 and DAG accumulation [MacCumber et al., 1990]. The activation of the latter leads to PKC activation. IL-6 is considered not only a pro-inflammatory cytokine, but also a modulating factor displaying also anti-inflammatory properties [Benveniste, 1998]. Increases in IL-6 levels are PI-PLC dependent but do not always require calcium entry, even if it probably an agonist of the selective endothelin receptor sub-type B (ET_B), named IRL1620, mobilizes intracellular calcium content from the cytoplasm stores [Morga et al., 2000]. It has also been analyzed [Miyata et al., 2003] the role of PI-PLC beta4 in mouse inflammatory pain, showing that the nociception in formalin test second phase, resulting from tissue inflammation, is attenuated in PI-PLC beta4 knockout mice; these data effectively demonstrate that PI-PLC beta4 may be involved in the nociceptive response, more than in the inflammatory cascade. PI-PLC gamma2, a strictly tissue-specific isoform, expressed in hematopoietic cell lines, is suspected to be [Yu et al., 2005] a key regulator in autoimmune and/or inflammatory diseases mediated by B cells and non-B, non-T hematopoietic cells, but it is not involved [Moreno-Garcìa et al., 2005] in the regulation of CD38 signaling that leads to B lymphocyte activation. Another study negates the involvement of PI-PLC gamma2 gene in the pathogenesis of Wegener granulomatosis [Jagiello et al., 2005], a human disease of unknown etiology characterized by granulomata of the respiratory system and systemic necrotizing vasculitis. PI-PLC gamma2 seems also to be involved in the activation (secretion and aggregation) of human platelets [Gratacap et al., 1998].

In agreement with their role in the maintenance of brain parenchyma homeostasis, astrocytes are able to react to inflammatory stimuli. Various cytokines and substances like bacterial LPS induce astrocytes to adopt a reactive phenotype. In the presence of LPS, astrocytes synthesize both IL-6 and TNFα, probably via a PI-PLC-dependent pathway. Both these events can be inhibited by LaCl3 and by a PI-PLC agonist, U73122. In the presence of LPS, the addition of these inhibitors shows that the induced cytokine production is PI-PLC dependent and requires calcium entry [Morga et al., 2000]. IRL1620 enhances the production of IL-6 and TNFα induced by LPS, inducing a significant increase of their measurable

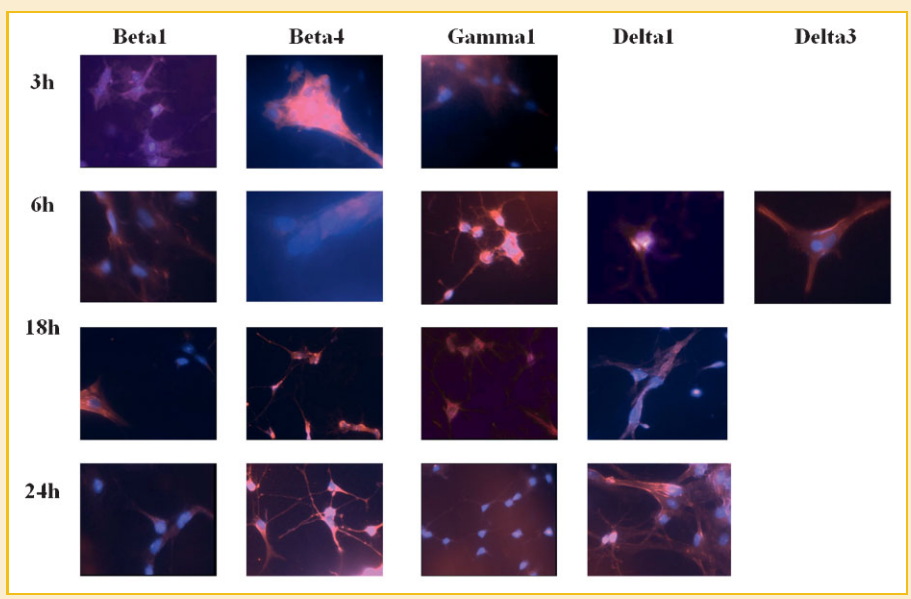

Fig. 2. Microscopy immunofluorescence imaging after immunocytochemistry analysis in LPS-treated neonatal rat astrocytes (40× magnification).

levels. Furthermore, IRL1620 also enhances the LPS-induced NO production [Morga et al., 2000]. Also the latter effect requires PI-PLC activation [Wang and Reiser, 2003]. In the presence of LPS, IRL1620 increases nitric oxide synthase (NOS) type II synthesis. The IRL1620 dependent rise of nitric oxide (NO) production induced by LPS can be inhibited by both U73122 and LaCl3, demonstrating that this mechanism uses a PI-PLC-dependent activation pathway, depending on calcium entry [Morga et al., 2000; Wang and Reiser, 2003].

In our previous studies, performed in cultured neonatal rat astrocytes [Lo Vasco et al., 2007], PI-PLC beta3, beta4, gamma1, delta1, delta3, delta4, and epsilon were expressed, while PI-PLC beta1, beta2, and gamma2 were not found. In the present study, we observed that, after LPS treatment, some isoforms (PI-PLC beta3, delta4, and epsilon) that were expressed in untreated astrocytes, disappear; that one isoform (PI-PLC beta1), absent in untreated cells, appears after LPS stimulation and, finally, that other isoforms (PI-PLC delta1 and delta3) were differently expressed at different stages of stimulation. These observations altogether suggest that PI-PLCs may play a role in the modulation (up- or downregulation) of the activation response of astrocytes.

In fact, by RT-PCR analysis (Fig. 1), an interesting pattern of expression was observed during a 24-h observation period after having treated cultured neonatal rat astrocytes with LPS (Table I).

PI-PLC beta1, unexpressed in untreated astrocytes, after LPS treatment was expressed at every one of the intervals of time we had chosen to perform the expression analyses (3, 6, 18, and 24 h), meaning probably that it was expressed during the whole time of the 24 h observation period.

PI-PLC beta2 was unexpressed in LPS-treated astrocytes, just like observed in the untreated counterpart, according to data literature demonstrating its tissue specificity restricted to hematopoietic lineage.

PI-PLC beta3, expressed in untreated astrocytes, was unexpressed at any of the observation intervals after LPS treatment.

PI-PLC beta4 and PI-PLC gamma1 were expressed both in untreated and in LPS-treated astrocytes during the whole observation period.

PI-PLC gamma2 was unexpressed in LPS-treated astrocytes, just like observed in the untreated counterpart, according to data literature demonstrating its tissue specificity restricted to hematopoietic lineage.

PI-PLC delta1, expressed in untreated astrocytes, was unexpressed at 3 h, but it was expressed at 6, 18, and 24 h after LPS treatment, meaning probably that it is expressed since 6–24 h.

PI-PLC delta3, expressed in untreated astrocytes, was expressed only at 6 h after LPS treatment.

Both PI-PLC delta4 and PI-PLC epsilon, expressed in untreated astrocytes, were unexpressed at any of the observation intervals after LPS treatment (Table I) (Fig. 1).

Moreover, immunocytochemical analysis (Fig. 2) showed a different sub-cellular localization of the expressed PI-PLC isoforms at different times of exposure to LPS during the 24 h observation period (Table II).

In fact, in the present experiment, PI-PLC beta1 was detected in the nucleus only at 3 h, while it was detected in the cytoplasm at 3, 6, 18, and 24 h. As PI-PLC beta1 was undetected in untreated astrocytes (both in our previous studies and in the negative controls of the present experiment), the unexpected appearing of this isoform might suggest its involvement in the activation of the astrocytes, being initially (3 h) detected in the nucleus and later in the cytoplasm (at 6, 18, and 24 h). The presence of PI-PLC in the nucleus, but mainly in the cytoplasm of LPS stimulated astrocytes, represents an interesting observation, because this isoform is considered mainly, if not exclusively, nuclear [Cocco et al., 2001], even if our previous report indicated that PI-PLC beta1 was detected in the cytoplasm, but not in the nucleus, of the neoplastic counterpart of rat astrocytes, represented by rat astrocytoma C6 cells [Lo Vasco et al., 2007].

PI-PLC beta3, detected in the nucleus of untreated astrocytes (both in our previous studies and in negative controls of the present experiment), in the present experiment was not detected, according to RT-PCR results, in any sub-cellular compartment (data not shown).

PI-PLC beta4, detected both in the nucleus and the cytoplasm of untreated astrocytes (both in our previous studies and in negative controls of the present experiment), in LPS-treated astrocytes was detected in the nucleus at 18 and 24 h and in the cytoplasm during the whole observation period. Being PI-PLC beta4 expressed in untreated astrocytes and also in astrocytoma C6 cells [Lo Vasco et al., 2007], it is not possible to formulate any consideration regarding its involvement in the activation of astrocytes.

PI-PLC gamma1, detected in the nucleus and in the cytoplasm of untreated astrocytes (both in our previous studies and in negative controls of the present experiment), in LPS-treated astrocytes was detected in the nucleus at 6 and 18 h, while it was detected in the cytoplasm during the whole period. Being PI-PLC gamma1 expressed in untreated astrocytes and also in the cytoplasm of astrocytoma C6 cells [Lo Vasco et al., 2007], it is not possible to formulate any consideration regarding its involvement in the activation of astrocytes. PI-PLC beta4 and PI-PLC gamma1 are expressed at every interval of time we analyzed and seem to be constitutively present, excepting for their sub-cellular distribution, that slightly varies during the 24 h observation time and requires further investigations for both of these isoforms.

PI-PLC delta1, detected in untreated astrocytes (both in our previous studies and in negative controls of the present experiment), in LPS-treated astrocytes was undetected at 3 h, but it was detected both in the nucleus and the cytoplasm at 6, 18, and 24 h.

PI-PLC delta3, detected in the nucleus and the cytoplasm of untreated astrocytes (both in our previous studies and in negative controls of the present experiment), in LPS-treated astrocytes was detected in the cytoplasm at 6 h after LPS treatment.

PI-PLC delta4 and epsilon, both detected in the nucleus and the cytoplasm of untreated astrocytes (both in our previous studies and in negative controls of the present experiment), in LPS-treated astrocytes were not detected, according to RT-PCR results, in any sub-cellular compartment (data not shown).

Resuming, after cultured neonatal rat astrocytes stimulation with LPS, at a very early stage (3 h) astrocytes expressed PI-PLC beta1 (localized in the nucleus), beta4 and gamma1 (localized in the cytoplasm). At 6 h after stimulation astrocytes expressed PI-PLC

beta1, beta4 (localized in the cytoplasm), gamma1, delta1 (localized both in the nucleus and the cytoplasm), and delta3 (localized in the cytoplasm). At 18 h after stimulation astrocytes expressed PI-PLC beta1 (localized in the cytoplasm), beta4, gamma1, and delta1 (both localized in the nucleus and the cytoplasm). At 24 h after stimulation astrocytes expressed PI-PLC beta1, delta1, gamma1 (localized in the cytoplasm) and beta4 (localized both in the nucleus and the cytoplasm) (Table II) (Fig. 2). Remarkably, during the period ranging about 6 h after having stimulated the astrocytes with LPS, the five expressed isoforms (PI-PLC beta1, beta4, gamma1, delta1, and delta3) are contemporarily expressed and present in the cytoplasm of the cells, while two of them (PI-PLC gamma1 and delta1) are present also within the nucleus (Table II) (Fig. 2).

These observations suggest that the PI-PLC isoforms may play a role in the fine tuning in ongoing central nervous system inflammation mimicked by LPS stimulation through a contribute due to the temporal modulation of their expression and sub-cellular distribution [Morga et al., 2000]. In cells undergone to inflammation stimuli the presence of PI-PLC beta1, unexpressed in non-stimulated astrocytes, the sporadic (limited at the period about 6 h after LPS treatment) expression of PI-PLC delta3, expressed in non-stimulated astrocytes, and the absence of PI-PLC beta3, delta4, and epsilon, expressed in non-stimulated astrocytes, suggest that the inflammatory stimuli might have induced changes in PI pathway including the expression of PI-PLC beta1, the brief time lasting expression of PI-PLC delta3 and the suppression of the expression of PI-PLC beta4, delta4, and epsilon. These data suggest that such isoforms might be involved in the cascade of events leading to astrocyte activation induced by inflammatory stimuli. Further analyses will be necessary to identify the involved cytokines, individuate, and correlate the steps following the PI-PLCs involvement in the inflammation cascade and clarify the role played by every one of the isoforms apparently involved.

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