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Original article

Protective effects of polydatin in free and nanocapsulated form on changes caused by lipopolysaccharide in hippocampal organotypic cultures

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ABSTRACT

Background: Polydatin (PD) is a compound, originally isolated from the root and rhizome of the Chinese herb *Polygonum cuspidatum*. To date, various biological properties of this compound, such as analgesic, anti-pyretic or diuretic effects, have been shown. Recently, anti-oxidant and anti-inflammatory properties have been widely postulated, yet PD instability and low bioavailability limit its beneficial actions. Therefore, it has been suggested that an encapsulation process may be a promising strategy for overcoming these limitations and increasing the therapeutic efficacy of PD.
Methods: We examined the effects of PD in two forms, including free and in PD-loaded polymeric nanocapsules, on lipopolysaccharide (LPS)-induced changes in hippocampal organotypic cultures.
Results: Our results indicated that free and encapsulated PD diminished cell death processes and attenuated the secretion of pro-inflammatory cytokines induced by LPS administration. Additionally, PD in both forms strongly inhibited the production of nitric oxide and down-regulated the level of iNOS enzyme in LPS-stimulated hippocampal cultures.
Conclusion: Taken together, our study showed that PD exerts anti-inflammatory and anti-oxidant properties in LPS-treated hippocampal organotypic cultures. Furthermore, we show that the encapsulation procedure preserved the features of the free form of this compound, and therefore, the polymeric nanocapsules containing PD may be used as a novel and promising delivery system in therapeutic strategies.

Introduction

Inflammation is an important background of brain disorders including depression, schizophrenia, Parkinson's disease and Alzheimer's disease. The main purpose of the brain inflammatory processes is to remove or inactivate potentially damaging factors and restore brain homeostasis. This action is primarily mediated *via* two cell systems: glia cells in the central nervous system (CNS) and lymphocytes, monocytes, and macrophages of the haematopoietic system [1]. During prolonged neuroinflammation, the self-limitation of the inflammatory response can be disturbed. The excessive production of neurotoxic factors such as cytokines, chemokines or reactive oxygen species leads to changes in brain structure, synaptic plasticity and to subsequent neurodegeneration [2–4]. Despite many years of studies, there is still a need to search for new, more effective neuroprotective substances. Recently, one of the more interesting ideas is to concentrate research efforts on natural substances, which produce a mediation of inflammation [5].

Polyphenols constitute one of the most abundant groups of plant metabolites. Among them, resveratrol (3,4′,5-trihydroxy-trans-stilbene) has been the subject of research for many years. Unfortunately, some data show that resveratrol treatment encounters serious limitations, mainly because it is sensitive to enzymatic oxidation [6]. Thus, resveratrol derivatives, including polydatin (PD, 3,4′,5-trihydroxystilbene-3-β-d–D-glucoside) have become a subject of interest. PD was originally extracted from the

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root and rhizome of Polygonum cuspidatum Sieb. et Zucc. (Polygonaceae). It has a glucoside group bonded in position C-3 that substitutes for the hydroxyl group of resveratrol. This substitution causes conformational changes in the molecule and leads to resistance to enzymatic oxidation and active penetration through glucose carriers [7].

PD has multidirectional effects on cardiac muscle and endothelial cells as well as anti-arteriosclerosis, anti-shock, anti-tumour, and lung- and hepato- protective activity [8–13]. Interestingly, most of the above-mentioned biomedical properties of PD derive from its strong anti-oxidant and immune-regulating properties [10].

Interestingly some data postulate neuroprotective activity of PD in the brain, especially in cerebral ischaemic pathogenesis. Additionally, PD protects against learning and memory deficits and attenuated cognitive impairments as well as activates anti-oxidative enzymes in the rat model of vascular dementia through chronic cerebral hypoperfusion [8]. PD may potentiate BDNF expression in the cerebral cortex of neonatal rats [14] and effectively inhibit amyloid beta polymerization [15].

Despite the discovery of neuroprotective properties of PD in the brain, its poor solubility and low bioavailability strongly limit its beneficial actions. Therefore, PD encapsulation seems to be a promising strategy. Nanocapsules (NC) consist of a nanosized colloidal core and a polymeric shell. A convenient method for producing NC for hydrophobic drug encapsulation is layer-by-layer (LbL) deposition of polyelectrolytes on nanoemulsion droplets [8,16,17]. The polymeric shell may be modified through PEG-ylation, which prevents nanocapsules from opsonisation and prolongs the half-life of capsules or immobilization of targeting ligands [12]. Additionally, nano-carrier PEG-ylation prevents phagocytosis by microglia as well as recognition of the nanocarriers by other immune cells. Since nanocapsules exhibit similarity in their size and morphology to the naturally occurring carriers and their diameters are between 10 and 200 nm, interaction with cells in the brain is very likely [18].

To date, there have been no comparative studies concerning the application of PD in free form and PD-loaded nanoparticles as a potential protective agent against damage induced by the bacterial endotoxin (lipopolysaccharide, LPS) in hippocampal slices.

In the present study, we evaluated the effects of PD on the viability/death processes in hippocampal organotypic cultures. Additionally, we examined the potential anti-inflammatory and anti-oxidant actions of this compound in both basal and LPS-stimulated conditions. To study the putative mechanisms underlying the effects evoked by PD treatment, we focused on the intracellular signalling pathways associated with TLR4, including NF-κB signalling, which is considered to mediate the production of pro-inflammatory factors, as well as Nrf2/Keap1 signalling, as a mechanism regulating antioxidant action.

Material and methods

Nanocapsule synthesis

PD nanocarriers were synthesized by a previously described method [16–19] of direct encapsulation of nanoemulsion droplets in polyelectrolyte multilayer shells. Nanoemulsion droplets containing polydatin were formed by dispersing (through magnetic stirring) 0.1 ml of an oil phase containing chloroform, DMSO, 34 mg/ml surface-active agent dioctyl sulfosuccinate sodium salt (AOT) and 70 mg/ml polydatin into 200 ml of polycation poly-L-lysine (PLL) 0.1 mg/ml aqueous solution. Poly-L-lysine hydrobromide, (MW ~15,000 to 30,000), poly-L-glutamic acid sodium salt, (MW ~15,000 to 50,000), chloroform and sodium chloride were obtained from Sigma-Aldrich, Poland, while AOT was received from CYTEC, Poland. All materials were used as received without further purification. Subsequently, the nanoemulsion droplets containing PD were encapsulated in a polyelectrolyte multilayer shell using the layer-by-layer technique with a saturation approach [16,20,21]. A volume of 10 ml of nanoemulsion containing PD was added to the optimized volume of poly-L-glutamic acid solution under gentle mixing with a magnetic stirrer. The procedure for the adsorption of polyelectrolyte PLL and PGA was repeated until an appropriate number of polyelectrolyte layers in the shell were formed. The volumes of polyelectrolytes used for shell formation were chosen by simultaneous zeta potential measurements. The volumes were considered optimal when the zeta potential reached a constant value just after overcharging that was close to the value of the free polyelectrolytes in the solution [16]. Under those conditions, the amount of non-adsorbed polyelectrolyte was minimized because most of it was adsorbed at emulsion droplets, which induced overcharging of their surface. Those optimal volumes of polyelectrolytes for the formation of consecutive layers of the shell were as follows: 1 ml PGA, 1 ml PLL, 1.3 ml PGA, 2 ml PLL, and 2.4 ml PGA (all polyelectrolytes at a concentration of 2 mg/ml). As the result of this procedure, PD-containing nanocapsules were obtained and were positively charged with five polyelectrolyte layers $(PLL/PGA)_2PLL$ and negatively charged with six polymer layers $(PLL/PGA)_3$, which were denoted as NCPD5 and NCPD6, respectively. Chloroform was evaporated from suspensions and its final concentration measured by gas-chromatography-with-electron-capture-detector GC-ECD was ~0.04 mg/l [22]. The entire procedure of capsule preparation was performed under sterile conditions. As the reference sample, empty nanocapsules (without polydatin), NC5 and NC6, were prepared according to the same procedure.

Nanocapsule size measurements

The nanocapsule size distribution (hydrodynamic diameter) was determined by Dynamic Light Scattering (DLS) using the Zetasizer Nano Series from Malvern Instruments. Each value was obtained as an average from at least three runs with at least 10 measurements. All measurements were performed in 0.015 M NaCl at 25 °C.

Nanocapsule Zeta potential and stability measurements

The zeta potential of the nanocapsules was determined with a Laser Doppler Electrophoresis (LDA) method using the Zetasizer Nano Series from Malvern Instruments. The obtained values were an average from at least three runs (with at least 20 measurements). All measurements were performed at 25 °C in 0.015 M NaCl. Additionally, the stability of nanocapsules was evaluated by the periodic measurements of their hydrodynamic diameter.

Nanocapsule concentration measurements

The concentration of nanocapsules was determined at 25 °C using Nanoparticle Tracking Analysis (NanoSight NS500, Malvern Instruments).

Nanocapsule visualization

Nanocapsules were visualized by Scanning Electron Microscopy (Cryo-SEM) using a JEOL JSM 7600 F equipped with cold stage for

cryogenic measurements. Samples were prepared by high-pressure freezing according to a previously described protocol [23]

Hippocampal organotypic culture (OHCs)

Hippocampal organotypic cultures were prepared from 6- to 7-day-old Sprague-Dawley rats [18]. After decapitation, brains were removed to an ice-cold working buffer (96% HBSS, 3.5% glucose, 0.5% penicillin/streptomycin). Hippocampi were cut into 400-μm slices using a McIlwain tissue chopper. In the next step of the experiment, the slices were put on Millicell-CM membranes (Millipore, USA) in 6-well plates with 1 ml of medium (20% HBSS; 25% horse serum; 50% DMEM; 5 mg/ml glucose; 1% amphotericin B; 0.4% penicillin-streptomycin; 2% B-27 supplement; pH = 7.4). Cultures were grown in a medium containing 25% horse-serum, which was then gradually (from DIV 4^{th} until 7^{th}) changed to a serum-free medium (45% HBSS; 50% DMEM F-12; pH 7.4; 5 mg/ml glucose; 1% penicillin-streptomycin; amphotericin B; 2% N-2 supplements and 2% B-27). Hippocampal cultures were maintained for 7 days in an incubator (37 °C) with an adjustable CO_2 flow (5%) before the treatment.

Treatment of hippocampal organotypic cultures

Hippocampal slices were pre-treated for 1 h with free PD (5–50 μM; Sigma-Aldrich, Poland), empty nanocapsules (NC5, NC6; 2×10^{11}-2.5×10^{10}), as well as PD-loaded nanocapsules (NCPD5, NCPD6; 2×10^{11}-5×10^{10}). The concentration of polydatin-loaded nanocapsules was 2×10^{11}, 1×10^{11}, 5×10^{10} particles/ml, which corresponds to the polydatin concentration of 15.8 μg/ml, 7.9 μg/ml and 3.95 μg/ml respectively. Next, lipopolysaccharide (LPS, *Escherichia coli* O111:B4; potency 500000 EU/mg; 1 μg/ml, Sigma-Aldrich) was added to the cultures for 24 h or 48 h. Control slices were treated with PBS buffer-vehicle.

Lactate dehydrogenase activity (LDH)

The cell death was quantified by the measure of the level of lactate dehydrogenase released into the culture medium 24 h or 48 h after stimulation. Culture supernatants were incubated with the reagent mixture according to the supplier's instructions (Cytotoxicity Detection Kit, Roche Diagnostic GmbH, Germany). The intensity of the red colour formed in the assay is proportional to LDH activity. The data were normalized to the activity of LDH released from control slices (100%) and are expressed as a percentage of the control ± SEM (standard error of the mean).

Metabolic activity (MTT)

The metabolic activity of hippocampal slices was determined by the MTT (3-[4,5-dimethylthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, Germany) test as previously described [18]. At 24 h or 48 h after treatment, MTT at a concentration of 0.15 mg/ml was added to each well and incubated for 1 h at 37 °C. Next, the culture medium was discarded, and a mixture of 0.1 M HCl and isopropanol was added to dissolve the formazan dye. Using a multi-well spectrophotometer (Multiscan, Thermo Labsystem, Finland), the absorbance value was measured at 570 nm. The data were normalized to the absorbance in the control slices (100%) and are expressed as a percentage of the control ± SEM.

Nitrite accumulation assay

Nitrite (indicator of the production of nitric oxide) secreted in the culture medium was measured by a Griess reaction. After 24 h or 48 h of treatment, 50 μl of supernatant was mixed with equal volumes of Griess reagents (0.1% N-1-naphthylethylenediamine dihydrochloride and 1% sulphanilamide in 5% phosphoric acid) and then incubated for 10 min at room temperature. Absorbance was measured at 540 nm in a microplate reader (Multiscan, Thermo Labsystem, Finland). The data were normalized to nitrite level in control slices (100%) and are expressed as a percentage of the control ± SEM.

Preparation of whole cell extracts and fractions

The slices were washed with ice-cold PBS and lysed in RIPA buffer with protease and phosphatase inhibitors. Next samples were centrifugated (4 °C, 20 min, 10,000 g) and prepared for western blot analysis.

Proteins from nuclear and cytoplasmatic fractions were extracted using NE-PER®Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific, China) according to the manufacturer's instructions.

Western blotting

The lysates (equal amounts of protein) of whole or different fractions were mixed with 4x Laemmli sample buffer (Bio-Rad, USA). Proteins were separated by SDS-PAGE (4%–20% gel) under constant voltage (200 V) and were transferred electrophoretically to PVDF membranes (Trans-Blot Turbo; Bio-Rad, USA) which were washed twice with TBS, pH = 7.5, blocked in 5% non-fat milk for 1 h at room temperature and incubated overnight at 4 °C with the primary antibodies: anti-iNOS (sc-650), anti-NRF2 (sc-722), anti-p65 (sc-372), anti-phospho-p65 (sc-33039), anti-TLR4 (sc-16240) (Santa-Cruz Biotechnology, Inc., USA), anti-Keap-1 (#4678, Cell Signaling). The membranes were washed 4 times with TBS containing 0.1% Tween-20 (TBST) and then incubated with horseradish peroxidase-linked secondary antibodies, including goat anti-rabbit IgG HRP (PI-1000, Vector Laboratories, UK) or horse anti-mouse IgG HRP (PI-2000, Vector Laboratories, UK), for 1–2 hrs. Afterwards, the membranes were washed 4 times with TBST, and the immunoblots were visualized with the Pierce® ECL Western blotting Substrate (Thermo Fisher, Pierce Biotechnology, USA). Next, membranes were washed 2 times for 5 min each in TBS, stripped using stripping buffer (100 ml of Tris-HCl (pH = 6.7), 2% SDS and 700 μl of 2-mercaptoethanol all from Sigma-Aldrich, USA), and washed 2 times for 5 min each in TBS, blocked and reprobed with antibodies against β-actin (sc-130656, Santa-Cruz Biotechnology, Inc., USA) and anti-H3 (06-755, Merck, Millipore, USA) as internal loading controls in the SignalBoost Immunoreaction Enhancer Kit. In the case when we cut the membrane and determine two different proteins on it, one β-actin band corresponds to two proteins (NRF-2 and Keap-1). The relative levels of immunoreactivity were quantified densitometrically using Fujifilm Multi Gauge software (Fuji Film, Tokyo, Japan).

Enzyme-linked immunosorbent assay (ELISA)

Hippocampal slices were pre-treated with free PD or NCPD6 for 1 h and then stimulated with LPS (1 μg/ml). The medium of slices was collected for TNF-α (tumour necrosis factor alpha), IL-1β (interleukin beta) and IL-4 (interleukin-4) measurement after 48 h. The protein levels of TNF-α, IL-1β, (both R&D Systems, USA) and IL-4 (USCN Life Science Inc., China) in the culture medium were measured using commercially available enzyme-linked immunosorbent assay kits according to the manufacturer's instructions. The detection limits were as follows: TNF-α, 5 pg/ml; IL-1β, 5 pg/ml; IL-4, 6.3 pg/ml. Inter-assay precision levels were as

follows: TNF-α: <8.8%; IL-1β <4.4%; IL-4 < 12%; intra-assay precision: TNF-α: <2.1%; IL-1β: <3.9%; IL-4: <10%.

Statistical analysis

Statistical analysis was performed using Statistica 10.0 Software (Statsoft, Tulusa, USA). Homogenates of slices from the same culture were combined for biochemical experiments. All data were obtained from independent experiments. All data are presented as percentage of the control ± SEM.

All groups were compared by one- or two-way analysis of variance (ANOVA), followed by Duncan's *post hoc* tests for multiple pair-wise comparisons, as appropriate. p Values less than 0.05 were considered to be statistically significant.

Results

Effects of free PD on cell viability/death tests in LPS-stimulated hippocampal organotypic cultures (OHC)

PD alone at 5–25 μM did not evoke any significant changes in LDH activity after 24 or 48 h. Only the dose of 50 μM after 24 h caused increased cell death (LDH) in control slices ($F_{(4,166)} = 2.63$; $p < 0.05$) (Fig. 1A). Therefore, this dose was not used for further experiments. On the other hand, in LPS-stimulated OHC, PD at doses of 5 and 10 μM ($F_{(4,166)} = 3.26$; $p < 0.05$) after 24 h, and at a dose of 5 and 10μM ($F_{(3,130)} = 6.74$; $p < 0.05$) after 48 h, significantly reduced lactate dehydrogenase release (Fig. 1A). In the MTT test either in basal or in LPS conditions, PD in all tested doses did not evoke any changes (Fig. 1B).

Synthesis and characterization of PD nanocapsules

Nanoemulsion containing polydatin stabilized by a surfactant/polyelectrolyte (AOT/PLL) interfacial complex with an 80-nm droplet size was prepared by the procedure described in the previous section. The zeta potential of PD-containing nanoemulsion droplets was high enough (+ 68 mV) to provide sufficient electric stabilization against their aggregation. On such prepared nanoemulsion droplets, polyelectrolyte shells were constructed in a layer-by-layer approach with the saturation technique (Fig. 2A, B, C). The adsorption of consecutive polyelectrolyte layers resulted in changes in the zeta potential values arranged in a typical saw-like pattern. The absolute values of zeta potentials of formed nanocapsules were higher than 30 mV, which provided sufficient stability against aggregation or agglomeration. The average size of ~120 nm of five-(NCPD5) and six-(NCPD6) layer nanocapsules was measured by the DLS. Additionally, the size of nanocapsules visualized by the cryo-SEM was in good agreement with values obtained by the DLS. Some aggregates observed in the micrograph can be the result of the sample preparation procedure. Because PD is practically not soluble in water, 100% efficiency of encapsulation was assumed. Thus, the final PD concentration in a stock solution of six-layer nanocapsules was 79 mg/l, while the particle concentration determined by NTA was ~2×10^{12} nanocapsules per ml. Stability studies of nanocapsules indicated no significant changes in size and zeta potential during the period of 60 days.

Cytotoxic effects of NC5 and NC6 in hippocampal organotypic cultures

As shown in Table 1, NC5 were toxic at all used dilutions: (2×10^{11} to 2.5×10^{10} particles) in LDH ($F_{(4,63)} = 26.85$; $p < 0.05$) as well as MTT ($F_{(4,63)} = 74.95$; $p < 0.05$) tests after 24 h. On the other hand NC5 alone did not affect nitrites production. Based on the obtained results we did not use NC5 nanocapsules in next set of experiments.

In contrast, NC6 in concentrations ranging from 2×10^{11} to 2.5×10^{10} particles did not change the lactate dehydrogenase release, metabolic activity (MTT test) and nitric oxide production in hippocampal organotypic slices after 24 h or 48 h of incubation (Table 1). We chose only negatively charged six-layer nanocapsules for polydatin encapsulation for further experiments.

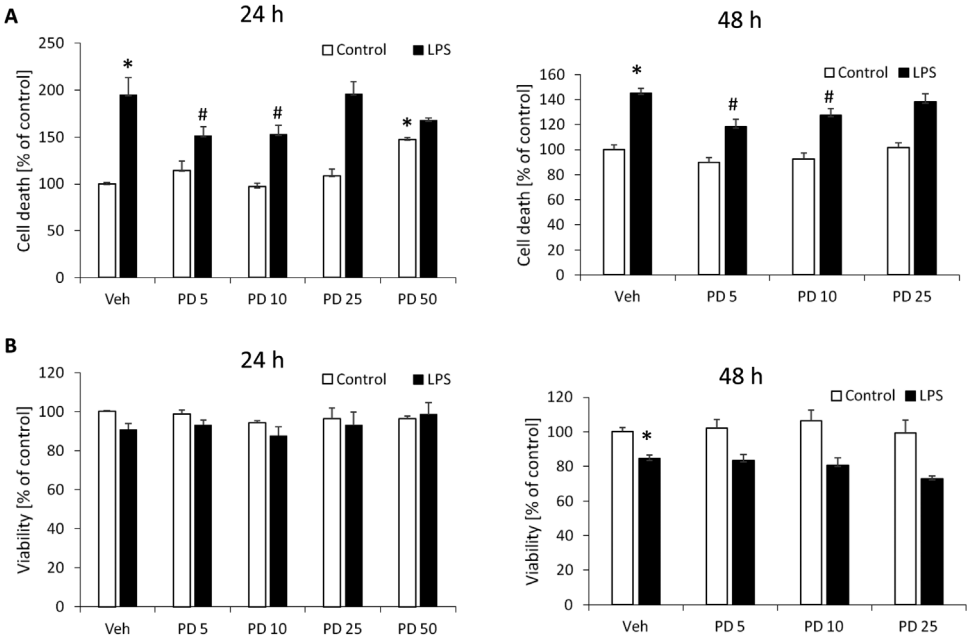

Fig. 1. The effect of free PD and lipopolysaccharide (LPS) on cell death/viability parameters: (A) LDH release and (B) MTT reduction in hippocampal organotypic slices. Hippocampal slices were pre-treated for 1 h with various concentrations (5–50 μM) of free (non-encapsulated) polydatin and next stimulated for 24 h and/or 48 h with lipopolysaccharide (LPS; 1 μg/ml). Control slices were treated with appropriate vehicle. Results are shown as the percent of control ± SEM, $^{*}p < 0.05$ *vs.* control Veh, $^{\#}p < 0.05$ *vs.* control LPS (n = 4–5 in each group).

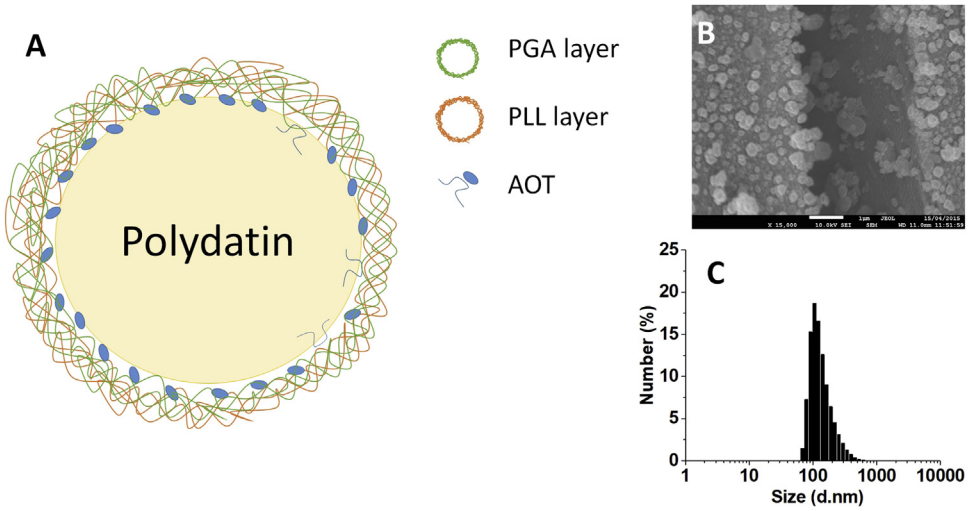

Fig. 2. Scheme of nanocapsules NC5 and NC6 with PD (A), cryo-SEM image of NC6 nanocapsules (B) and the size distribution of NC6 nanocapsules (C). Concentration of nanocapsules 10^{12}/ml; concentration of PD - 0,079 g/l; The concentration of PD-loaded nanocapsules was 2×10^{11}, 1×10^{11}, 5×10^{10} particles/ml, which corresponds to the PD concentration of 15.8 μg/ml, 7.9 μg/ml and 3.95 μg/ml respectively. AOT - dioctyl sulfosuccinate sodium salt, PLL – poly-L-lysine; PGA – poly-L- glutamic acid.

Table 1
The effect of empty NC5 and NC6 nanocapsules on cell death/viability as well as nitrites release in hippocampal organotypic cultures.

		LDH	MTT	Nitrites
NC 5	Veh	100 ± 2	100 ± 2	100 ± 1
24 h	2×10^{11}	170 ± 9 *	56 ± 2 *	93 ± 3
	1×10^{11}	159 ± 6 *	70 ± 1 *	96 ± 2
	5×10^{10}	158 ± 4 *	75 ± 2 *	97 ± 2
	2.5×10^{10}	160 ± 3 *	87 ± 2 *	95 ± 1
NC 6	Veh	100 ± 3	100 ± 4	100 ± 1
24 h	2×10^{11}	96 ± 3	104 ± 5	99 ± 1
	1×10^{11}	90 ± 4	107 ± 4	98 ± 1
	5×10^{10}	93 ± 4	113 ± 3	106 ± 4
	2.5×10^{10}	91 ± 4	111 ± 6	101 ± 2
NC 6	Veh	100 ± 4	100 ± 3	100 ± 1
48 h	2×10^{11}	102 ± 4	104 ± 2	97 ± 2
	1×10^{11}	100 ± 5	103 ± 2	98 ± 2
	5×10^{10}	99 ± 8	101 ± 2	97 ± 2
	2.5×10^{10}	99 ± 11	107 ± 4	105 ± 2

Hippocampal slices were treated for 24 h and/or 48 h with various concentrations of empty NC5 or NC6 nanocapsules. Results are shown as the percent of control ± SEM. * $p < 0.05$ *vs.* control. (n = 4–5 in each group).

Effects of nanocapsules loaded with PD (NCPD6) on the cell viability/death in LPS-stimulated hippocampal organotypic cultures

We examined the effects of different concentrations of NCPD6 on cell viability and death parameters in hippocampal slices for both basal as well as LPS-stimulated conditions. As shown in Fig. 3A NCPD6 at the basal condition had no effect on LDH release, while NCPD6 diminished LPS-induced cell death processes in the LDH test. The protective effect of NCPD6 was observed for both 1×10^{11} and 2×10^{11} doses after 48 h ($F_{(3,111)} = 3.25$; $p < 0.05$) of treatment. On the other side NCPD6 alone or in LPS-stimulated slices, did not affect cell viability (MTT test) in all tested dilutions after 24- and 48-h treatments (Fig. 3B).

Impact of free PD and NCPD6 on cytokine production in hippocampal organotypic cultures stimulated by LPS

We evaluated the effects of free PD on the production of IL-1β, TNF-α and IL-4 in LPS-stimulated OHC. The protein levels were determined after 48 h of bacterial endotoxin treatment. In control (unstimulated) slices, PD did not affect the release of any of the tested cytokines (Table 2A). As expected, stimulation of OHC with LPS led to significant enhancement of the levels of IL-1β ($F_{(1,29)} = 1210.41$; $p < 0.05$), TNF-α ($F_{(1,39)} = 1474.33$ $p < 0.05$), and IL-4 ($F_{(1,18)} = 59.28$; $p < 0.05$). Interestingly, pre-treatment with PD (5 and 10 μM) suppressed the LPS-evoked overproduction of pro-inflammatory TNF-α ($F_{(2,39)} = 8.43$; $p < 0.05$), and at a dose of 5 μM, it inhibited also the secretion of IL-1β ($F_{(2,29)} = 2.08$; $p < 0.05$). PD had no effect on increases induced by LPS as well as secretion of the anti-inflammatory cytokine IL-4 (Table 1A). In the next set of our experiment we measured the impact of NCPD6 on the IL-1β, TNF-α and IL-4 production after 48 h of LPS administration. In control (unstimulated) slices, NCPD6 had no effect on the production of any of the tested cytokines. On the other hand, pre-treatment with NCPD6 at $1x10^{11}$ decreased LPS-evoked overproduction of TNF-α ($F_{(1,18)} = 59.28$; $p < 0.05$) (Table 2B).

Effects of free PD and NCPD6 on nitrites production and iNOS levels in LPS-stimulated hippocampal organotypic cultures

In hippocampal slices, PD pre-treatment (5 μM and 10 μM) decreased the LPS-evoked nitrite accumulation only after 48 h ($F_{(3,132)} = 15.97$; $p < 0.05$) (Fig. 4A). Next we determined the protein levels of iNOS by western blotting method. Consistent with the down-regulation of nitrite level, PD significantly attenuated LPS-induced iNOS levels after 48 h ($F_{(2,63)} = 7.04$; $p < 0.05$) (Fig. 4B). Moreover in hippocampal organotypic cultures, NCPD6 pre-treatment (2×10^{11} and 1×10^{11}) effectively decreased LPS-stimulated nitrite production ($F_{(3,124)} = 8.02$; $p < 0.05$) also only after 48 h of incubation (Fig. 5A). Consistent with the inhibition of nitrite production, NCPD6 significantly down-regulated LPS-induced iNOS levels after 48 h ($F_{(2,62)} = 4.83$; $p < 0.05$) (Fig. 5B).

Impact of free PD and NCPD6 on the TLR4/NF-κB and Keap1/Nrf2 pathways in LPS-stimulated hippocampal organotypic cultures

Several studies have showed that the TLR4-related NF-κB pathway plays an important role in modulating the synthesis of pro-inflammatory factors such as cytokines and NO in LPS-stimulated cells. As shown in Fig. 6A, we did not observe any effects of LPS or PD on TLR4 expression after 48 h of stimulation. Additionally, 48 h after stimulation with bacterial endotoxin, there was no activation of the NF-κB factor (Fig. 6A). Similar to free PD,

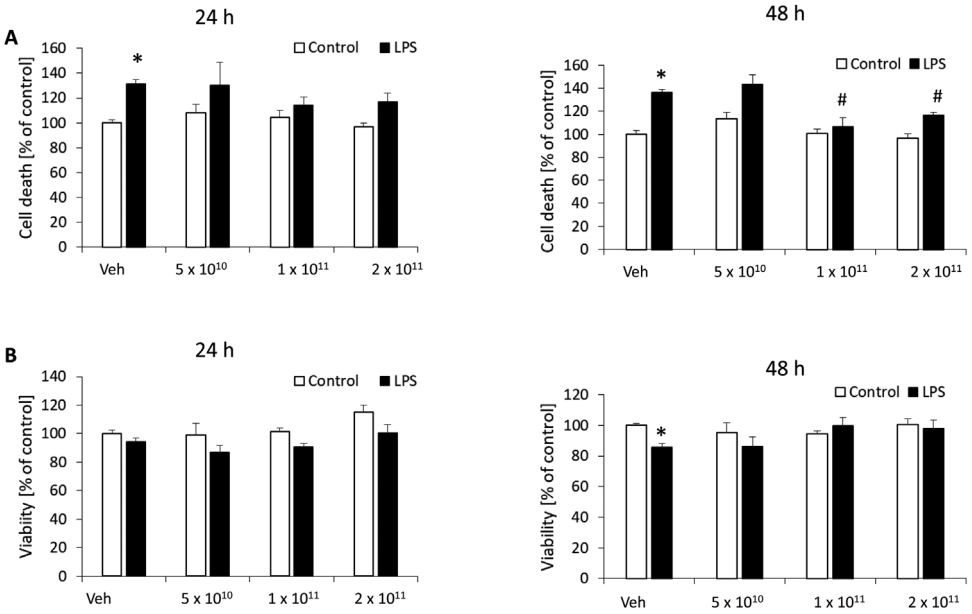

Fig. 3. The effect of nanocapsules loaded with polydatin (NCPD6) and lipopolysaccharide (LPS) on cell death/viability parameters: (A) LDH release and (B) MTT reduction in hippocampal organotypic slices. Hippocampal slices were pre-treated for 1 h with various concentrations (5×10^{10}–2×10^{11}) of encapsulated polydatin and next stimulated for 24 h and/or 48 h with lipopolysaccharide (LPS; 1 μg/ml). Results are shown as the percent of control ± SEM, $^{*}p < 0.05$ *vs.* control Veh, $^{\#}p < 0.05$ *vs.* control LPS (n = 4–5 in each group).

Table 2
The effect of free polydatin (PD) and nanocapsules loaded with polydatin (NCPD6) as well as lipopolysaccharide (LPS) on the production of pro- and anti- inflammatory cytokines: IL-1β, TNF-α and IL-4 in hippocampal organotypic cultures.

A

	Control + Veh	LPS + Veh	Control + PD 5	LPS + PD 5	Control + PD 10	LPS + PD 10
IL-1β	100.00 ± 1.96	**866.92 ± 25.75***	104.31 ± 1.84	769.23 ± 17.64#	103.39 ± 3.62	826.37 ± 29.77
TNF-α	100.00 ± 4.20	**3438.65 ± 139.69***	107.79 ± 3.13	2754.57 ± 262.75#	103.29 ± 4.54	2921.25 ± 185.29#
IL-4	100.00 ± 6.88	**229.12 ± 40.74***	91.48 ± 3.68	203.85 ± 8.01	87.36 ± 4.07	180.22 ± 9.19

B

	Control + Veh	LPS + Veh	Control + 2×10^{11}	LPS + 2×10^{11}	Control + 1×10^{11}	LPS + 1×10^{11}
IL-1β	100.00 ± 1.52	**765.13 ± 30.69***	103.82 ± 3.01	752.39 ± 20.55	104.46 ± 2.49	760.69 ± 12.53
TNF-α	100.00 ± 3.89	**3154.46 ± 174.28***	104.48 ± 5.79	3046.43 ± 9.45	102.25 ± 2.97	2719.64 ± 198.56#
IL-4	100.00 ± 2.89	**227.16 ± 18.22***	100.89 ± 5.53	228.36 ± 10.24	99.4 ± 7.09	211.34 ± 19.56

Hippocampal slices were pre-treated for 1 h with various concentrations of PD (5 and 10 μM) or NCPD6 (2×10^{11} and 1×10^{11}) and next stimulated 48 h with LPS (1 μg/ml). Results are shown as the percent of control ± SEM. $^{*}p < 0.05$ *vs.* control Veh, $^{\#}p < 0.05$ *vs.* control LPS (n = 4-5 in each group).

NCPD6 loaded with PD did not evoke any changes in the activation of TLR4 as well as NF-κB factor levels (Fig. 6B).

The Nrf2/Keap1 signalling pathway is considered to mediate the antioxidant activity of many compounds. Therefore, we tested the effects of PD on the level of Keap1 and Nrf2 factors. Bacterial endotoxin at a concentration of 1 μg/ml did not change Keap1 protein level after 48 h (Fig. 7a A). Additionally, NCPD6 nanocapsules were not able to change the protein level of Keap1 (Fig. 7a B).

However, stimulation with LPS strongly decreased the total levels of Nrf2 ($F_{(1,16)} = 12.20$; $p < 0.05$) as well as cytoplasmatic ($F_{(1,18)} = 9.18$; $p < 0.05$) (Fig. 7b). Furthermore, free PD in all tested doses, at baseline as well as stimulated by LPS conditions, did not evoke any changes in expression of Nrf2 total, cytoplasmatic and nuclear protein levels (Fig. 7b A). Moreover nanocapsules NCPD6 were not able to change the protein levels of Nrf2 factor (total, nuclear and cytoplasmatic fraction) (Fig. 7b B).

Discussion

The present studies clearly demonstrate that PD in both forms, free as well as encapsulated in polyelectrolyte shell nanocapsules, possesses beneficial properties working against LPS-induced changes in hippocampal organotypic cultures. We observed that this compound diminishes the cell death processes and exerts anti-oxidative potential on the nitrites release as well as iNOS expression. Moreover, PD established anti-inflammatory activity expressed as suppression of the pro-inflammatory TNF-α and IL-1β cytokine production.

In our study, we used a hippocampal organotypic culture model because the usefulness of this technique in basic research has increased in recent years. The main advantage of this model is the ability to replicate many aspects of the *in vivo* context. The organotypic cultures preserve the interactions of cells in the brain, including neurons, astrocytes and microglia [24].

The first findings of our study indicate that regardless of the form, PD with high potency decreases cell death processes evoked by LPS in organotypic cultures, which were estimated as lactate dehydrogenase release. On the other hand, PD had no effect on cell viability in the MTT test. These discrepancies observed in our study may come from the fact that both tests measure different aspects of the vital status of hippocampal slices.

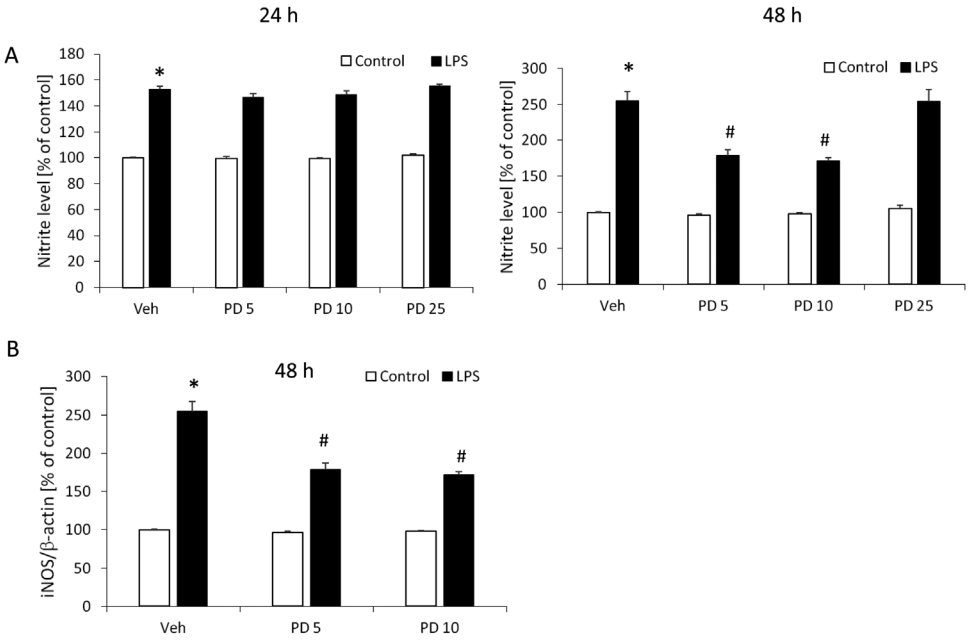

Fig. 4. The effect of free polydatin (PD) and lipopolysaccharide (LPS) on: (A) nitrite level and on (B) iNOS level in hippocampal organotypic slices Hippocampal slices were pre-treated for 1 h with various concentrations (5–50 μM) of free (non-encapsulated) PD and next stimulated 24 h and/or 48 h with lipopolysaccharide (LPS; 1 μg/ml). Results are shown as the percent of control ± SEM. Western blot results (iNOS) are normalized to β-actin. $^{*}p < 0.05$ *vs.* control Veh, $^{\#}p < 0.05$ *vs.* control LPS (n = 4–5 in each group).

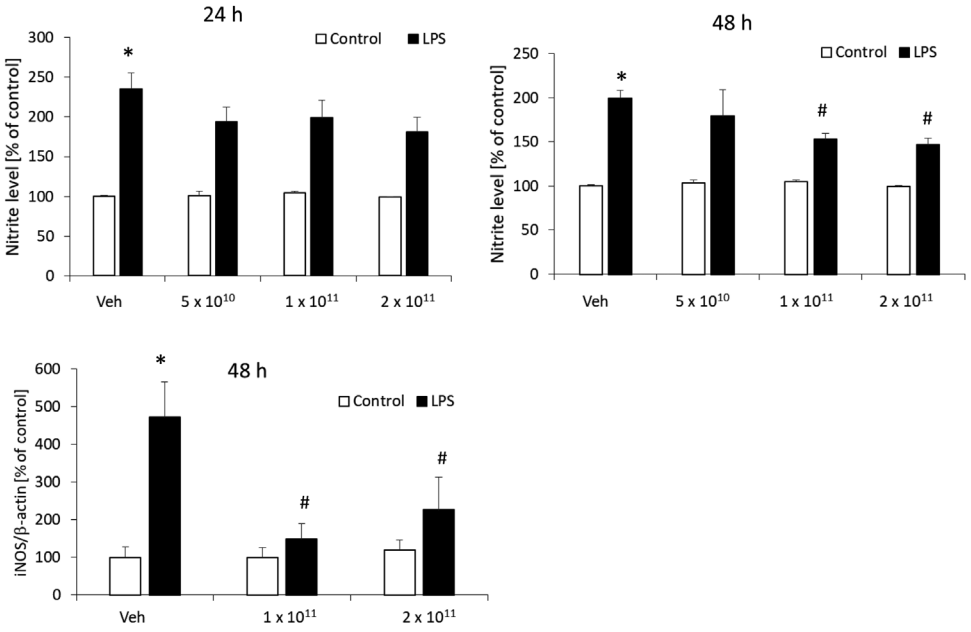

Fig. 5. The effect of nanocapsules loaded with polydatin (NCPD6) and lipopolysaccharide (LPS) on: (A) nitrite level and on (B) iNOS level in hippocampal organotypic slices. Hippocampal slices were pre-treated for 1 h with various concentrations (5×10^{10}–2×10^{11}) of encapsulated polydatin and next stimulated 24 h and/or 48 h with lipopolysaccharide (LPS; 1 μg/ml). Results are shown as the percent of control ± SEM. Western blot results (iNOS) are normalized to β-actin. $^{*}p < 0.05$ *vs.* control Veh, $^{\#}p < 0.05$ *vs.* control LPS (n = 4–5 in each group).

The protective properties of PD on cell death/viability have been observed in other experimental models. However, our study is the first to show that encapsulation of PD in a polyelectrolyte shell by the LbL approach maintains the protective ability of PD. Xu and collaborators reported that PD reduced the level of LDH release in pheochromocytoma cells [7]. Additionally, several studies reported that PD potentiated the viability parameters in the MTT test. The protective effects of PD in this assay were not observed in the present study after LPS treatment but were observed in an H_2O_2- or ethanol-induced injury model [25,26].

Using the bacterial endotoxin LPS stimulation model in our study provided a broader perspective because LPS administration not only affected cell death/viability parameters, but primarily induced inflammatory activation. Although LPS does not closely reflect the neuroinflammation observed in pathological conditions, according to the published data, this model may be successfully used for evaluation of whether the compounds under study exhibit anti-inflammatory or anti-oxidant activity [13].

A growing amount of evidence indicates the antioxidant effects of PD. In our observation, we demonstrated that in organotypic slices,

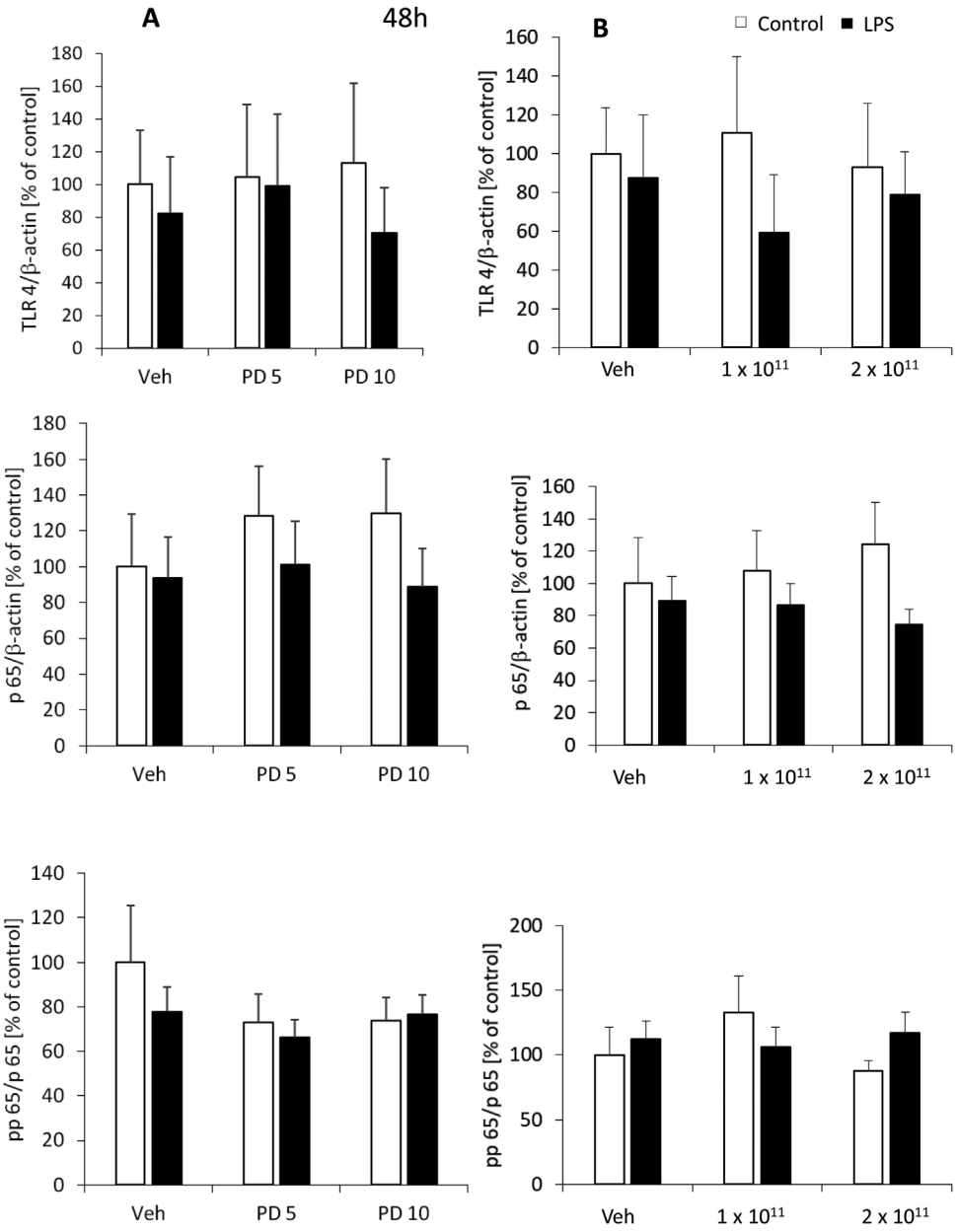

Fig. 6. The effect of free polydatin (A) and nanocapsules loaded with polydatin (NCPD6) (B) as well as lipopolysaccharide (LPS) on TLR4 level and phosphorylation of p65 (NF-κB) in hippocampal organotypic cultures. Hippocampal slices were pre-treated for 1 h with various concentrations of free (non-encapsulated) polydatin or nanocapsules loaded with polydatin (NCPD6) and next stimulated 48 h with lipopolysaccharide (LPS; 1 μg/ml). Results are normalized to β-actin and shown as the percent of control ± SEM, $^{*}p < 0.05$ *vs.* control Veh, $^{\#}p < 0.05$ *vs.* control LPS (n = 4–5 in each group).

PD in both forms, reduces the production of nitric oxide. Additionally, PD strongly diminished the enhancement of the level of iNOS enzyme after LPS treatment. Lou et al. reported that in RAW 264.7 cells stimulated by bacterial endotoxin, PD effectively inhibited NO as well as PGE2 production by reduction of iNOS and COX-2 expression [27]. Furthermore, in the MCAO model of ischaemia, injection of PD at doses of 25 or 50 mg/kg protects against brain damage and up-regulates the expression of glioma-associated oncogene homolog1, patched-1 and SOD1. Additionally, the highest dose of PD (50 mg/kg) up-regulated the expression of claudin-5 to sustain the integrity of the blood-brain barrier, which was disrupted by cerebral ischaemia. [28]. The beneficial property of PD may also be reflected by an increase in SOD, GSH-Px and CAT activities, as recently demonstrated in CCl4–induced liver injury in mice [29].

Apart from the anti-oxidant action, PD displays anti-inflammatory potential. We demonstrated that PD in free form reduces the production of two main pro-inflammatory cytokines: IL-1β and TNF-α. These results agree with numerous studies that have reported that free PD modulates the secretion of cytokines but mostly on the periphery. Among them, diminished production of IL-17 in activated human peripheral blood mononuclear cells after PD treatment was observed [30]. Additionally, PD blocked the expression of IL-1β, IL-6 and TNF-α and alleviates inflammatory damage in mice with ulcerative colitis [31]. Free PD also modulated IL-6, IL-8 and TNF-α gene expression in a heat-stressed HaCaT cell line [32].

Despite the abundance of data, there is a lack of studies concerning the anti-inflammatory action of PD loaded in nanoparticles. In the present study, we demonstrated that nanoparticles

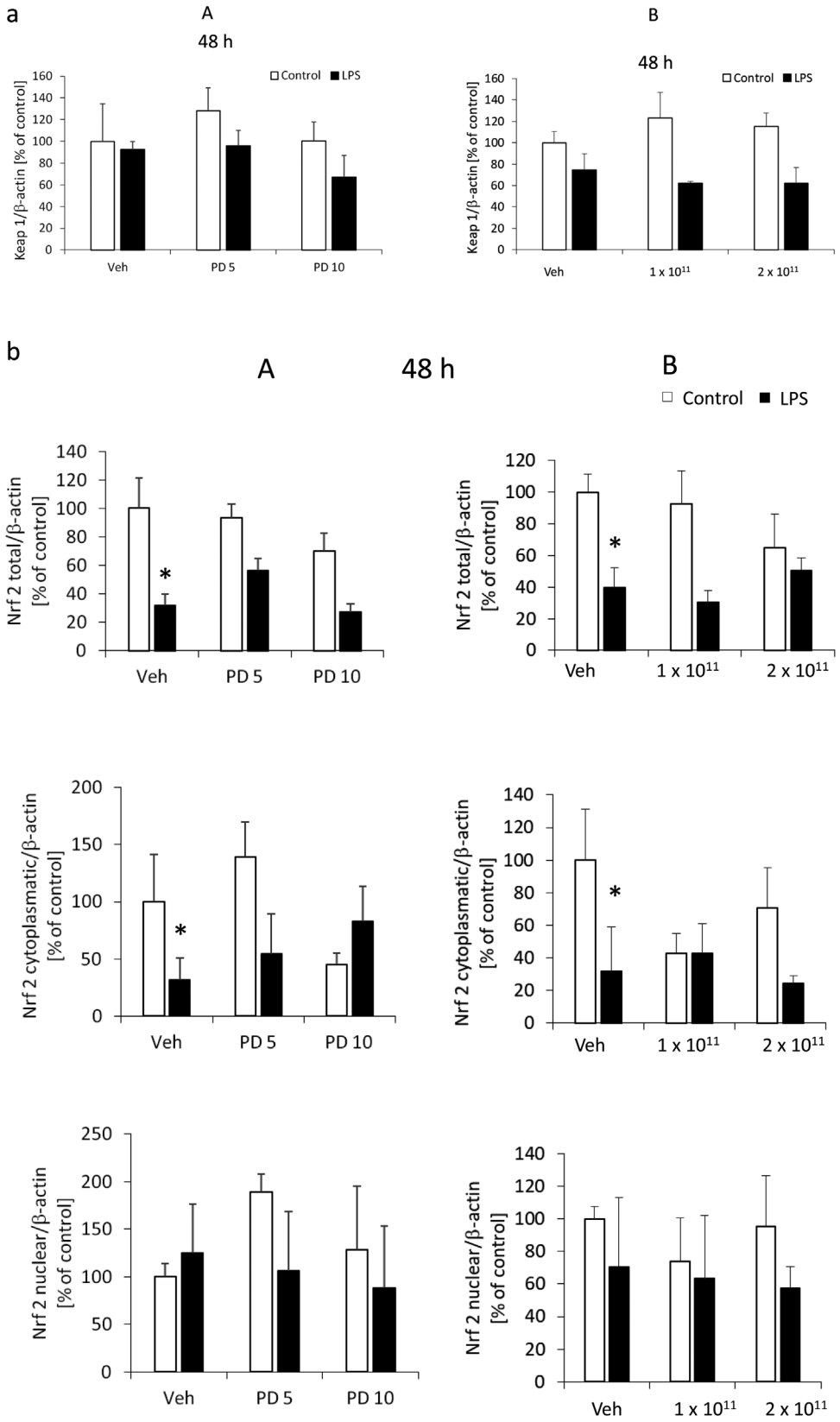

Fig. 7. (a) The effect of free polydatin (A) and nanocapsules loaded with polydatin (NCPD6) (B) as well as lipopolysaccharide (LPS) on the Keap1 level in hippocampal organotypic cultures. Hippocampal slices were pre-treated for 1 h with various concentrations of free (non-encapsulated) polydatin or nanocapsules loaded with polydatin (NCPD6) and next stimulated 48 h with lipopolysaccharide (LPS; 1 μg/ml). Results are normalized to β-actin and shown as the percent of control ± SEM, $^{*}p < 0.05$ *vs.* control Veh, $^{\#}p < 0.05$ *vs.* control LPS (n = 4–5 in each group). (b) The effect of free polydatin (A) and nanocapsules loaded with polydatin (NCPD6) (B) as well as lipopolysaccharide (LPS) on total Nrf2, cytoplasmatic Nrf2 and nuclear Nrf2 fractions. Hippocampal slices were pre-treated for 1 h with various concentrations of free (non-encapsulated) polydatin or nanocapsules loaded with polydatin (NCPD6) and next stimulated 48 h with lipopolysaccharide (LPS; 1 μg/ml). Results are normalized to β-actin and shown as the percent of control ± SEM, $^{*}p < 0.05$ *vs.* control Veh, $^{\#}p < 0.05$ *vs.* control LPS (n = 4–5 in each group).

loaded with PD diminished the response evoked by LPS of release only of TNF-α. Therefore, based on our finding, it can be postulated that the encapsulation procedure changed the release of PD and attenuated its action on the inflammatory status caused by LPS, but this suggestion requires further study.

It is well accepted that the release of potentially harmful factors such as pro-inflammatory cytokines or nitric oxide is linked to activation of certain transcription factors. After bacterial endotoxin treatment, two factors, NF-κB and Nrf2/Keap1, are activated [33,34]. Moreover recent data suggested strong cross–talk between them. NF-κB is composed of p50/p65 and IkBα. Upon activation, IκBα is phosphorylated and degraded, which allows for phosphorylation of the p65 subunit and subsequent translocation of the dimer p50/p65 into the nucleus. Importantly, after LPS activation, the stimulation of NF-κB is attributed to TLR4 [35]. In fact, previous data, indicated that treatment with bacterial endotoxin increases the expression of TLR4 after 1 h or 24 h of LPS stimulation [36]. Interestingly, some studies demonstrated that free PD also affects the NF-κB/TLR4 pathway. In RAW 264.7 cells stimulated with LPS, PD significantly ameliorated the activation of NF-κB [27]. Furthermore, PD down-regulated the high expression of TLR4, MyD88, and NF-κB caused by LPS simulation in human bronchial epithelial BEAS-2B cells [37]. Additionally, free PD seems to be able to protect the brain from the damage caused by pMCAO through down-regulation of NF-κB expression [28].

In the present study, after a longer incubation, we did not observe the impact of LPS as well as free and encapsulated PD on the TLR4 level. Moreover LPS–evoked stimulation showed only a trend to increase the p65/NF-κB factor expression in hippocampal cultures, while free PD slightly affected these changes. Analysis of our present results can lead to the suggestion that 48 h of incubation with LPS is too long to still maintain the TLR4 or NF-κB up-regulation. These inaccuracies limit our research but are due to the fact that the beneficial properties of encapsulated PD were observed in the present study until 48 h. Recently data pointed that the NF-κB factor interferes with Nrf2 signalling, which comprises one of the major cellular defence mechanisms against oxidative stress and xenobiotic damage. Studies have provided convincing evidence to support the hypothesis that Nrf2 has an antagonistic effect on the NF-κB pathway. [23,38,39].

Interestingly, we demonstrated for the first time, that 48 h of stimulation of organotypic hippocampal cultures with LPS diminished the total protein level of Nrf2 factor as well as the estimated cytoplasmatic fractions. On the other hand, PD in both free and encapsulated forms did not significantly modulate the Nrf2 factor level. Furthermore, we did not observe the impact of either LPS or PD (in both forms) on Keap1 protein expression. In contrast, some immune stimulation or stress modifies Keap1 thiol groups and alters its conformation to lead to the release of Nrf2. *De novo* synthesized Nrf2, not marked for degradation, translocate to the nucleus and binds to the antioxidant response elements (AREs) of target genes and enhances their expression [23]. Therefore we can speculate that bacterial endotoxin *via* reduction of the activity of Nrf2 factor in hippocampal slices, causes a diminished expression of antioxidant enzymes and consequently leads to damage in the hippocampal cells.

Taken together, the present findings showed that PD not only in free but also in encapsulated form exhibits protective effects in LPS-stimulated hippocampal organotypic cultures, which was confirmed by the ability of PD to reduce cell death and lower pro-inflammatory factor levels, including cytokines release, nitric oxide production and iNOS level. Although the action of PD on the NF-κB and Nrf2/Keap1 signalling pathway requires further study, nonetheless based on the obtained results, we can strongly postulate that encapsulation of some natural substances may be considered an attractive strategy for the attenuation of toxicity and inflammatory processes in the brain. Additionally, an applied novel delivery system based on the LbL encapsulation of hydrophobic compounds can be a new very promising therapeutic strategy.

Conflict of interest

None.

Acknowledgements

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