PHOSPHOLIPIDS BLOCK NUCLEAR FACTOR-KAPPA B AND TAU PHOSPHORYLATION AND INHIBIT AMYLOID-BETA SECRETION IN HUMAN NEUROBLASTOMA CELLS

N. R. PANDEY, K. SULTAN, E. TWOMEY AND
D. L. SPARKS*

Lipoprotein and Atherosclerosis Research Group, University of Ottawa Heart Institute, 40 Ruskin Street, Ottawa, ON, K1Y 4W7, Canada

Abstract—Inflammation and oxidative stress have been shown to play a critical role in the pathophysiology that leads to neurodegeneration. Omega-6 phospholipids, e.g. dilinoleoylphosphatidylcholine (DLPC), have been shown to have anti-inflammatory properties and therefore experiments were undertaken to determine whether DLPC can prevent inflammatory neurodegenerative events in the model neuronal cell line, SH-SY5Y. Tumor necrosis factor (TNF-α) and H2O2 activate mitogen-activated protein kinase (MAPK) in SH-SY5Y cells within 5 min and this activation is completely blocked by DLPC (12 µM). DLPC blocks IκBα phosphorylation in the SH-SY5Y cells and prevents the phosphorylation and activation of nuclear factor-kappa B (NF-κB). The phospholipid inhibits induction of MAPK and NF-κB in similar fashion to the MEK1/2-inhibitor, U0126 (10 µM), DLPC completely abolishes TNF-α, H2O2 and lipopolysaccharide (LPS)-induced neuronal tau phosphorylation. Cellular amyloid precursor protein levels are reduced by DLPC and LPS-induced amyloid-β expression and secretion in SH-SY5Y cells are completely blocked by DLPC. Taken together, these data suggest that DLPC can act through MAPK to block neuronal inflammatory cascades and prevent potential pathological consequences in the neuronal metabolism of amyloid and tau proteins. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: phosphatidylcholine, nuclear factor-kappa B (NF-κB), tumor-necrosis factor-alpha (TNF-α), MAPK, amyloid-β, tau phosphorylation.

Neurodegeneration in Alzheimer’s disease (AD) is associated with the accumulation of extracellular plaques comprised of small peptides, called amyloid-β (Aβ), and intracellular neurofibrillary tangles, consisting of aggregates of hyperphosphorylated tau protein (Selkoe, 2001). Evidence suggests that inflammatory events are involved in the pathogenesis of AD. Pro-inflammatory molecules are present at sites of Aβ plaques and anti-inflammatory drugs slow the progression of the disease (McGeer et al., 1996; Kalaria, 1999; Akiyama et al., 2000).

*Corresponding author. Tel: +1-613-761-4822; fax: +1-613-761-5102. E-mail address: dsparks@ottawaheart.ca (D. L. Sparks).

Abstract—Inflammation and oxidative stress have been shown to play a critical role in the pathophysiology that leads to neurodegeneration. Omega-6 phospholipids, e.g. dilinoleoylphosphatidylcholine (DLPC), have been shown to have anti-inflammatory properties and therefore experiments were undertaken to determine whether DLPC can prevent inflammatory neurodegenerative events in the model neuronal cell line, SH-SY5Y. Tumor necrosis factor (TNF-α) and H2O2 activate mitogen-activated protein kinase (MAPK) in SH-SY5Y cells within 5 min and this activation is completely blocked by DLPC (12 µM). DLPC blocks IκBα phosphorylation in the SH-SY5Y cells and prevents the phosphorylation and activation of nuclear factor-kappa B (NF-κB). The phospholipid inhibits induction of MAPK and NF-κB in similar fashion to the MEK1/2-inhibitor, U0126 (10 µM), DLPC completely abolishes TNF-α, H2O2 and lipopolysaccharide (LPS)-induced neuronal tau phosphorylation. Cellular amyloid precursor protein levels are reduced by DLPC and LPS-induced amyloid-β expression and secretion in SH-SY5Y cells are completely blocked by DLPC. Taken together, these data suggest that DLPC can act through MAPK to block neuronal inflammatory cascades and prevent potential pathological consequences in the neuronal metabolism of amyloid and tau proteins. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

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Abbreviations: Aβ, amyloid-β; AD, Alzheimer’s disease; APP, amyloid precursor protein; DLPC, dilinoleoylphosphatidylcholine; DMSO, dimethyl sulfoxide; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; PL, phospholipids; PPARα, peroxisome proliferator-activated receptor-α; TNF-α, tumor necrosis factor;
dependent signaling in neuroblastoma cells, which results in reduced Aβ secretion and tau hyperphosphorylation.

EXPERIMENTAL PROCEDURES

Chemicals

DLPC was obtained from Avanti Polar Lipids Inc., Alabaster, AL, USA. The MEK1/2 inhibitor, U0126, and its inactive isoform, U0124, were purchased from Calbiochem (La Jolla, CA, USA). Lipopolysaccharide (LPS) and hydrogen peroxide were from Sigma Chemical Co. Anti-human hyper-phosphorylated tau (paired helical filament-PHF) clone-AT8 antibody (Cat # MN1020) doubly phosphorylated at Ser202/Thr205 was procured from Thermo Scientific (Rockford, IL, USA). Total tau-B11E8 antibody (Cat # sc58855) and donkey anti-goat IgG-HRP (cat# sc-2020) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Amyloid-β antibody (Cat # ab 17905–50) was from Abcam and amyloid precursor protein (APP-N terminus; Cat # A8967) antibody was from Sigma Aldrich, Inc. (St. Louis, MO, USA). Phospho-NF-κB p65 (Cat # 3036), NF-κB p65 (Cat # 3034), phospho-IκBα (Cat # 9246), IκBα (Cat # 9242), phospho-ERK1/2 (Cat # 9101), ERK1/2 (Cat # 9102), total anti-β-actin (Cat # 4967) and anti-rabbit IgG-HRP (Cat # 7074) were obtained from Cell Signaling Technology, (Danvers, MA, USA). Affinity purified peroxidase linked goat anti-mouse antibody (Cat# 4741806) was purchased from Kirkengaard and Perry Laboratories (Garthersburg, MD, USA). Unless otherwise stated, drugs and inhibitors were of analytical grade and were solubilized in dimethyl sulfoxide (DMSO). All chemical inhibitors were used at reported IC50 concentrations to block various signaling pathways.

Cells and cell culture

Human neuroblastoma (SH-SY5Y) cells were obtained from American Type Cell Culture (ATCC Number CRL-2266; Manassas, VA, USA). SH-SY5Y cells were cultured and maintained in F12: DMEM (1:1, v/v) media, supplemented with 10% FBS and 1% penicillin/streptomycin. Passages 3–10 were used and pre-confluent cells were subjected to stimulation with drugs for indicated times under serum-starved conditions.

Preparation of phospholipid vesicles

Phospholipid vesicles were prepared by sonication as previously described (Pandey et al., 2008). Briefly, pure DLPC in chloroform was dried down under N2 gas and 1 ml of DMSO added and then vortexed for 1 min. The mixture was sonicated in a Branson 5200 waterbath sonicator for 3×10 min pulses with brief vortexing between pulses.

Western blot analysis

After incubation with drugs for the indicated times and doses, cells were washed twice with ice cold PBS on ice. Cells were lysed in NP40 lysis buffer (Biosource, Camarillo, CA, USA) (50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 50 mM Na3VO4, 1% NP-40 and 0.02% Na3VO4) supplemented with 1 mM PMSF and 1× protease inhibitor cocktail (Sigma, Saint Louis, MO, USA) (AEBSF, aprotinin, bestatin hydrochloride, E-64, EDTA and leupeptin hemisulfate salt). Equal amounts of cell protein were separated by SDS-PAGE and analyzed by Western blot using specific antibodies to TNF-α, Aβ, APP, phospho-ERK1/2, ERK1/2, phospho-tau, tau, phospho-IκBα, IκBα, phospho-NF-κB, NF-κB and β-actin. Band intensity was analyzed with the Alpha-Innotech FluorChem™ HD Imager (Alpha Innotech Corp., San Leandro, CA, USA).

Amyloid-β quantification

Aβ immuno-quantification was performed in conditioned media after incubation with the inhibitors/agonists and/or phospholipids for the indicated times and doses. Briefly, the conditioned media was removed and briefly centrifuged to remove any cells. Equal amounts (30 μl) of a 1:1 dilution of conditioned media in Laemmlli’s sample buffer containing mercaptoethanol were separated by 12% SDS-PAGE. The proteins were transferred to a PVDF mem-

Fig. 1. DLPC blocks ERK1/2 phosphorylation. Pre-confluent SH-SY5Y cells were pre-incubated with 12 μM DLPC for 30 min and then incubated with TNF-α (10 ng/ml) for the indicated times. Phospho and total ERK1/2 expression were analyzed by Western blotting. Histograms representing densitometry analysis of phospho-ERK1/2 (p-ERK1/2) and the values are presented relative to total ERK1/2 control and are expressed as mean±SEM of at least three independent experiments. * P<0.05 versus TNF-α alone.
brane and probed using a specific antibody for Aβ and an IgG linked HRP secondary antibody in 5% Milk/TBST. Blots were developed using the West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL, USA) on the Fluorochem Alphalager. Band intensities were analyzed using the Spot-densitometer application of the AlphaEaseFC software and values were corrected with total cell protein. The cells were washed twice with ice-cold PBS on ice and then lysed with NP40 lysis buffer (Biosource, Camarillo, CA, USA). Total protein concentration was determined using the BCA protein assay (Pierce, Rockford, IL, USA).

Fig. 2. UO126 inhibits TNF-α-induced ERK and NF-kB phosphorylation. SH-SY5Y cells were pre-incubated with the MEK1/2-inhibitor U0126 (10 μM) or its inactive analog U0124 (10 μM) for 30 min and then incubated with TNF-α (10 ng/ml) for 5 min. Phospho and total ERK1/2, IκBα and NF-κB expression were analyzed by Western blotting. Western blot images are representative of at least three independent experiments.

Fig. 3. DLPC inhibits TNF-α-induced IκBα phosphorylation and degradation. SH-SY5Y cells were pre-incubated with DLPC (12 μM) for 30 min and then incubated with TNF-α (10 ng/ml) for the indicated times. Phospho and total IκBα expression were analyzed by Western blotting. Histograms representing densitometry ratio analysis of phospho-IκBα (p-IκBα) and IκBα and the values are presented relative to total β-actin control and are expressed as mean±SEM of at least three independent experiments. * P<0.001 versus TNF-α alone.
Statistical analysis

Values are shown as Mean±SEM for at least three independent experiments and *P<0.05 is considered significant. Differences between mean values were evaluated by one-way analysis of variance by a pairwise multiple comparison using the Student–Newman–Keuls post-hoc test (SigmaStat; Systat Software, Inc., San Jose, CA, USA).

RESULTS

DLPC blocks ERK phosphorylation in SH-SY5Y cells

Experiments were undertaken to determine if DLPC can block a TNF-α activation of MAPK in neuroblastoma cells. SH-SY5Y cells were treated with DLPC (12 μM) and/or TNF-α (10 ng/ml) and ERK 1/2 phosphorylation was quantified by immunoblotting. DLPC was shown to completely block ERK1/2 phosphorylation for 30 min after treatment with TNF-α (Fig. 1). DLPC was also shown to block a hydrogen peroxide induction of MAPK in SH-SY5Y cells (Supplemental Fig. 1).

DLPC blocks NF-κB activation

The effect of a selective MEK1/2-inhibitor, U0126, on a TNF-α induction of MAPK and nuclear factor-kappa B (NF-κB) was evaluated. Fig. 2 shows that pretreatment of SH-SY5Y cells with U0126 (10 μM) for 30 min blocked the TNF-α-induced (10 ng/ml for 5 min) phosphorylation of ERK1/2 and reduced the phosphorylation of both IκBα and NF-κB by ~50%. While treatment with the inactive analog, U0124, had a slight effect on ERK1/2 phosphorylation, the compound had no effect on IκBα and NF-κB phosphorylation status. DLPC treatment of SH-SY5Y cells completely blocked TNF-α-induced IκBα phosphorylation (Fig. 3, left panel) and slightly reduced IκBα degradation (Fig. 3, right panel). DLPC also inhibited TNF-α-induced NF-κB phosphorylation. TNF-α induced a three-fold increase in NF-κB phosphorylation in SH-SY5Y cells 5 min after treatment (Fig. 4). DLPC blocked the increase in neuronal NF-κB phosphorylation by ~50% over 5–30 min post treatment with TNF-α.

DLPC blocks tau phosphorylation

The effect of pro-inflammatory and oxidative stress agonists on tau phosphorylation were also evaluated in SH-SY5Y cells. Pretreatment of the cells with U0126 completely blocked TNF-α-induced phosphorylation of tau (AT8) at 5 min (Fig. 5). TNF-α and LPS (100 ng/ml) both increased tau phosphorylation and DLPC blocked the effect of both agonists and gave rise to tau phosphorylation levels that were lower than control values by 30 min (Figs. 6 and 7). Similar results were observed with a hydrogen peroxide induction of tau phosphorylation (Supplemental Fig. 2).

DLPC blocks LPS-induced amyloid-β secretion in SH-SY5Y cells

Fig. 8 shows that DLPC decreased the basal expression of APP by ~25% in unstimulated neuroblastoma cells. Treatment of SH-SY5Y cells with LPS for 24 h significantly...
increased Aβ expression and secretion (Fig. 9). DLPC decreased the basal secretion of Aβ from SH-SY5Y cells by 65% and completely blocked the LPS-induced increase in Aβ secretion (Fig. 9).

Fig. 5. U0126 blocks TNF-α-induced tau phosphorylation. SH-SY5Y cells were pre-incubated with U0126 (10 μM) or U0124 for 30 min and then incubated with TNF-α (10 ng/ml) for 5 min. Phospho and total tau expression were analyzed by Western blotting. Histograms representing densitometry ratio analysis of phospho-tau (p-tau) and total tau are presented relative to control values and are expressed as mean±SEM of at least three independent experiments. * P<0.001 versus TNF-α alone.

Fig. 6. TNF-α-induced tau phosphorylation is blocked by DLPC. SH-SY5Y cells were pre-incubated incubated with DLPC (12 μM) for 30 min and incubated with TNF-α (10 ng/ml) for indicated times. Phospho and total tau expression were analyzed by Western blotting. Histograms representing densitometry ratio analysis of phospho-tau (p-tau) and total tau are presented relative to control values and are expressed as mean±SEM of at least three independent experiments. * P<0.001 versus TNF-α alone.
DISCUSSION

Phospholipids (PL) are important components of the human body and constituents of the circulating plasma lipoproteins. PL has been suggested to have therapeutic value in treating inflammatory and neurodegenerative diseases (Little et al., 1985; Wilson et al., 1998; Lieber, 2000; Higgins and Flicker, 2003). PL have been shown to have anti-inflammatory effects in both the liver and intestinal track (Lieber, 2005; Treede et al., 2007). PL protect against alcoholic liver injury (Lieber, 2000, 2005; Cao et al., 2002b) and have also been shown to be effective at alleviating gastrointestinal inflammation caused by ulcerative colitis (Stremmelm et al., 2005). Other studies have shown therapeutic value of PL for both Alzheimer’s Disease (AD) and other neurological diseases (Little et al., 1985; Higgins and Flicker, 2003). PL have shown the potential to improve memory and cognitive function in rodents (Chung et al., 1995; Sakai et al., 1996; Suzuki et al., 2001) and PL have also been used for treating senile dementia and other neurodegenerative disorders in humans (Funfgeld et al., 1989; Amaducci et al., 1991; Engel et al., 1992; Crook et al., 1992; Schreiber et al., 2000).

Our research has shown that the acyl chain composition of PL directly impact cellular signaling and transcriptional processes (Pandey et al., 2008; Pandey and Sparks, 2008). Soy PL are enriched in the omega-6 fatty acid, linoleic acid, an 18 carbon acyl chain with two unsaturations (18:2). Omega-6 PL act through PPARα pathways to stimulate hepatic HDL/apoA-I secretion and raise plasma HDL levels (Burgess et al., 2005; Pandey et al., 2008; Pandey and Sparks, 2008). Decreased plasma HDL and apoA-I levels are highly correlated with the severity of AD (Merched et al., 2000; Fan et al., 2001). Niacin also acts through PPARα and has been shown to have both HDL raising therapeutic value as well as anti-inflammatory properties (Kuvin et al., 2006; Thoenes et al., 2007). Dietary niacin may also directly protect against AD and age related cognitive decline (Morris et al., 2004).

Inflammation is causal to neurodegeneration in AD and neuronal response to inflammatory stimuli is governed by both MAPK and NF-κB pathways. Studies have shown that MAPK plays a role in the activation of NF-κB and induction of TNF-α production (Jang and Surh, 2005; Lecureur et al., 2005; Wu and Cederbaum, 2008). DLPC acts through MAPK to prevent activation of NF-κB. Dose titration studies showed that >12 μM DLPC significantly increases PPARα expression. 12 μM DLPC is also able to completely block a peroxide and TNF-α activation of MAPK in SH-SY5Y cells by preventing phosphorylation of ERK 1/2 (Fig. 1). This result is similar to that observed with other ERK phosphorylation inhibitors. UO126 is a MEK 1/2 specific inhibitor that blocks ERK phosphorylation and inhibits AP-1, a potent transcriptional regulator of immune response genes (Duncia et al., 1998). Inhibition of MAPK activation with UO126 has been previously shown to block...
Aβ induction of NF-κB, COX-2 expression and PGE(2) production (Jang and Surh, 2005).

Omega-6 PL act to block NF-κB activation in SH-SY5Y cells by preventing the phosphorylation of IkBα and NF-κB (Figs. 3 and 4). DLPC completely blocks a TNF-α-induced phosphorylation of IkBα (Fig. 3). A reduction in IkBα phosphorylation would be expected to prevent NF-κB phosphorylation and Fig. 4 confirms this view. Other experiments in this laboratory have shown that DLPC can block an LPS and TNF-α induction of NF-κB in HepG2 and Caco-2 cell lines (Pandey et al., unpublished observations). Studies in other laboratories have shown that DLPC is anti-apoptotic (Mak et al., 2003) and decreases induction of TNF-α secretion and NF-κB activation in Kupffer cells of ethanol-fed rats (Cao et al., 2002a,b). DLPC therefore has significant NF-κB inhibitory activity in different tissues.

One of the earliest AD mechanistic hypothesis involves the inflammatory-induced accumulation of hyperphosphorylated tau proteins in the neuron (Goedert et al., 1991a,b). Tau hyperphosphorylation in neuronal cells is initiated by MAPK activation (Guise et al., 2001). The protein is phosphorylated by both the stress kinase p38 and glycogen synthase kinase 3 (GSK3) (Gomez-Ramos et al., 2004) and GSK3 is activated by Aβ and p38 by oxidative stress. Oxidative stress, LPS and TNF-α all increase tau phosphorylation in SH-SY5Y cells and DLPC completely prevents the hyperphosphorylation (Figs. 6 and 7). Tau fibrillary tangles are believed to promote microtubule disintegration and destroy the neuron’s transport system and therefore DLPC would be expected to prevent neuronal malfunction and cell death.

AD is also thought to be a protein misfolding disease and results in the accumulation of abnormally folded amyloid protein fragments in the brains of AD patients (Kayed et al., 2003; Shankar et al., 2008; Irvine et al., 2008). Aβ [1–42] is the more pathogenic fragment that is associated with disease states. To determine if omega-6 PL impact the amyloid metabolism in SH-SY5Y cells, basal APP and Aβ levels were probed in cells treated with DPLC. PL treated cells showed a significant reduction in basal APP levels (Fig. 8) and a 65% reduction in basal Aβ secretion (Fig. 9). Aβ secretion has been linked to an activation of MAPK and NF-κB (Furukawa et al., 1996; Combs et al., 2001a,b) and therefore it would be expected that an inhibition of these pathways would reduce the production and secretion of Aβ. NF-κB inhibitors are effective at blocking the formation of Aβ in cell culture systems (Paris et al., 2007) and inhibition of MAPK and NF-κB activation in vivo reduce the level of Aβ peptides and NF-κB in the brains of the transgenic mouse model of AD (TG2576) (Sung et al., 2004). Thus, it is possible that the DLPC-induced inhibition of Aβ
secretion may be a through a blockage of MAPK and NF-κB activation. DLPC-induced inhibition of MAPK and NF-κB may directly involve the modulation in the β- or γ-secretase activities. Studies have shown that inhibition of MAPK and NF-κB pathways inhibit β- and γ-secretase activities and reduce Aβ production in both LPS and Aβ-treated mice (Lee et al., 2008, 2009a,b). Therapeutic strategies that regulate secretase activity and reduce Aβ secretion would therefore be expected to have value in the treatment of AD (Walsh et al., 2005; Yin et al., 2007; Lee et al., 2009b).

PL enriched in unsaturated fatty acids are crucial to the normal neurological function of the brain. Neurodegeneration has been shown to be associated with abnormal phospholipid metabolism in the brain (Farooqui et al., 1992, 2004; Bazan, 2005). Brain tissue from AD patients has been shown to have alterations in brain membrane composition and metabolism (Soderberg et al., 1991; Nitsch et al., 1992; Wells et al., 1995; Pettegrew et al., 2001). Most notably, AD brains are deficient in unsaturated phospholipids and enriched with saturated (Soderberg et al., 1991). Erythrocyte membrane phospholipid composition has been shown to correlate to brain phospholipid composition (Connor et al., 1990) and may be a useful marker for neurological disease (Fenton et al., 2000; Sumiyoshi et al., 2008). Since erythrocyte phospholipid composition is impacted by dietary habits, the therapeutic administration of omega-6 PL, such as DLPC, would be expected to increase the concentration of these lipids in both the plasma and brain and promote neuronal anti-inflammatory events, by directly inhibiting NF-κB.

REFERENCES


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Fig. 9. LPS-induced amyloid-β secretion is blocked by DLPC. SH-SY5Y cells were pretreated with DLPC (12 μM) for 30 min and then incubated LPS (100 ng/ml) for 24 h. Conditioned media from each experiment were collected and subjected to Western blot analysis for amyloid-β (1–42). Sample loading volumes were adjusted relative to total cell protein. Histograms representing densitometry analysis of amyloid-β are expressed relative to control values, as mean±SEM of at least three independent experiments. * P<0.001 versus control and ** P<0.001 versus LPS alone.


Supplementary data