



Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Phospholipid transfer protein (PLTP) deficiency impaired blood–brain barrier integrity by increasing cerebrovascular oxidative stress



Ting Zhou^a, Qihua He^b, Yawei Tong^a, Rui Zhan^a, Feng Xu^c, Dongsheng Fan^d, Xiangyang Guo^{d,*}, Hongbin Han^{d,*}, Shucun Qin^{e,*}, Dehua Chui^{a,d,*}

^a Neuroscience Research Institute and Department of Neurobiology, Key Laboratory for Neuroscience, Ministry of Education and Ministry of Public Health, Health Science Center, Peking University, Beijing, China

^b Center of Medical and Health Analysis, Peking University, Beijing, China

^c Department of Natural Medicines, School of Pharmaceutical Sciences, Peking University, Beijing, China

^d Peking University Third Hospital, Beijing, China

^e Key Laboratory of Atherosclerosis in Universities of Shandong, Institute of Atherosclerosis, Taishan Medical University, Taian, China

ARTICLE INFO

Article history:

Received 29 January 2014

Available online 7 February 2014

Keywords:

Phospholipid transfer protein

Blood–brain barrier

Tight junction

Oxidative stress

ABSTRACT

Phospholipid transfer protein (PLTP) regulates lipid metabolism and plays an important role in oxidative stress. PLTP is highly expressed in blood–brain barrier (BBB), but the role of PLTP in BBB integrity is not clear. In this study, BBB permeability was detected with *in vivo* multiphoton imaging and Evans blue assay. We found that PLTP deficient mice exhibited increased BBB permeability, as well as decreased expression of tight junction proteins occludin, zona occludens-1 (ZO-1) and claudin-5 in brain vessels. Cerebrovascular oxidative stress increased in PLTP deficient mice, including increased levels of reactive oxygen species (ROS) and lipid peroxidation marker 4-hydroxy-2-nonenal (HNE) and reduced superoxide dismutase (SOD) activity. Dietary supplementation of antioxidant vitamin E increased BBB integrity and tight junction proteins expression via reducing cerebrovascular oxidative stress. These findings indicated an essential role of PLTP in maintaining BBB integrity, possibly through its ability to transfer vitamin E, and modulate cerebrovascular oxidative stress.

© 2014 Elsevier Inc. All rights reserved.

1. Introduction

Phospholipid transfer protein (PLTP) is a key protein in lipid and lipoprotein metabolism, and facilitates the transfer of several amphipathic compounds including phospholipids, unesterified cholesterol, and α -tocopherol [1–3]. α -Tocopherol is the main isomer of antioxidant vitamin E, exchange of α -tocopherol between lipoproteins and cells is modulated by PLTP *in vivo* [2,3]. PLTP deficiency leads to elevated oxidative stress via reduced α -tocopherol content, increased reactive oxygen species (ROS) level and decreased superoxide dismutase (SOD) activity in peripheral arteries and brain [4–7]. Compared with the whole brain, PLTP mRNA expression level is 6.8-fold higher in cerebral vessels [8], suggests a potential role of PLTP in blood–brain barrier (BBB), but whether PLTP can affect BBB integrity is not clear.

BBB is comprised of brain microvascular endothelial cells and acts as a physical and metabolic barrier between the brain and the rest of the body [9]. The main structures responsible for the maintenance of the integrity and function of BBB are the intercellular tight junction proteins [10]. One of the main factors inducing BBB impairment is oxidative stress, which increases BBB permeability and disturbs expression of tight junction proteins zonula occludens-1 (ZO-1), occludin, and claudin-5 [11,12]. Several *in vivo* and *in vitro* studies supported a beneficial effect of vitamin E on protecting the function of BBB via its antioxidant effect [13–15]. So we raise a hypothesis that lack of vitamin E due to PLTP deficiency may increase cerebrovascular oxidative stress and impair the integrity of BBB.

This study aimed to investigate the role of PLTP in BBB integrity. BBB permeability was examined with *in vivo* multiphoton imaging and Evans blue assay, tight junction proteins occludin, ZO-1, claudin-5 expression were determined with Western blot, oxidative stress was represented by levels of ROS and 4-hydroxy-2-nonenal (HNE) and SOD activity. We found increased BBB permeability in PLTP deficient mice for the first time, as well as decreased expression of tight junction proteins occludin, ZO-1 and claudin-5 in brain vessels. Cerebrovascular oxidative stress increased in PLTP

* Corresponding authors. Address: Neuroscience Research Institute, Peking University Health Science Center, 38 Xueyuan Road, Hai Dian District, 100191 Beijing, China (D. Chui).

E-mail address: dchui@bjmu.edu.cn (D. Chui).

deficient mice, including increased levels of ROS and lipid peroxidation marker HNE and reduced SOD activity. Vitamin E supplementation attenuated cerebrovascular oxidative stress, rescued BBB impairment induced by PLTP deficiency.

2. Materials and methods

2.1. Animals

All mice were on the homogeneous C57BL/6 background. PLTP deficient mice were generated by Dr. X.C. Jiang's laboratory [16]. Mice were fed a standard chow diet or a vitamin E (Sigma, St. Louis, MO) – supplemented chow diet (800 mg/kg, 2 months) [17,18]. Mice were matched for sex and used between 8- and 9-month of age. All procedures were approved by the Animal Care Committee of Peking University Health Science Center in China.

2.2. Multiphoton *in vivo* microscopy analysis

In vivo multiphoton imaging was performed as previously described [19]. Mice were anesthetized intraperitoneally with 750 mg/kg urethane and 50 mg/kg chloralose and the cranium was firmly secured in a stereotaxic frame. A square cranial window about 2×2 mm was then opened with a high-speed drill, to gain direct access to the brain parenchyma. The tissue was kept humid constantly by subsequent additions of 200 μ l-drops of 0.9% saline. Tetramethylrhodamine (TMR) – conjugated dextran (40 kDa, 0.1 mL of 10 mg/ml, Invitrogen, Carlsbad, CA) was injected via the tail vein. At the end of the experiment mice were killed by decapitation and brains were harvested, frozen and conserved at -80°C until use.

In vivo images were acquired using a multiphoton microscope (Leica TCS SP5 MP, Chicago, IL) with 850 nm excitation and $20\times/1.0$ water immersion objective, 2 mm working distance. Once the area of interest was defined, 200 μm -thick stacks in the Z-axis (5 μm steps) were obtained with the Leica ASF software, every 5 min for a total time of 30 min.

2.3. Evans blue assay

Evans blue assay was carried out as previously described [20]. 2% Evans blue dye (4 ml/kg) was injected intravenously and allowed to circulate for 2 h. Then mice were perfused transcardially with physiological saline until no blue color was evident in effluent. The brain was removed, placed in formamide and incubated for 48 h in the dark at 60°C . The absorbance of eluted Evans blue dye in formamide solution was measured using a spectrophotometer at 650 nm, and normalized to plasma levels.

2.4. Isolation of brain capillaries

Brain capillaries were isolated using dextran gradient centrifugation as described [19]. The cortex and hippocampus were carefully dissected and the meninges were removed in ice-cold PBS containing 2% fetal bovine serum (FBS). The brain was homogenized and dextran (70 kDa, Pharmacia) was added to a concentration of 16%. The samples were then centrifuged at 6000g for 15 min. The capillary pellet located at the bottom of the tubes was collected and sequentially filtered through a 100 μm and 45 μm cell strainer. The capillaries remaining on top of the 45 μm cell strainer were collected in PBS.

2.5. Western blot analysis

Isolated brain capillaries were lysed in RIPA buffer, and Western blot analysis was carried out as previously described [21]. The protein concentration of each homogenate was determined using a

BCA kit (Pierce). Extracts (60 μg of protein) were subjected to electrophoresis, and separated proteins were transferred onto PVDF membranes, which were then immunostained with the following primary antibodies against occludin (1:500, Invitrogen, Carlsbad, CA), claudin-5 (1:500, Abcam, San Diego, CA), ZO-1 (1:500, Invitrogen, Carlsbad, CA) and β -actin (1:5000, Sigma, St. Louis, MO). The membranes were incubated with peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized with an ECL system.

2.6. Cerebrovascular oxidative stress assessment

Cerebrovascular oxidative stress was determined as described [17]. Isolated brain capillaries were incubated for 30 min in darkness in the presence of 10 mM dichlorofluorescein diacetate. Fluorescence intensity (λ_{exc} 485 nm, λ_{em} 530 nm) was read in a flexstation 3 microplate reader (molecular devices), normalized for protein concentration. SOD activity was measured using a spectrophotometric assay kit (Sigma–Aldrich) [17]. Level of HNE-protein adducts was measured by dot blot [22]. 1 μg of protein was loaded on a nitrocellulose membrane, blocking with 5% milk, and probing with an anti-HNE antibody (1:1000, alpha-diagnostics, St. Louis, MO). The membranes were incubated with peroxidase-conjugated secondary antibodies, and immunoreactive bands were visualized with an ECL system.

2.7. Statistical analysis

All data in the text and figures are expressed as mean \pm SEM of at least three independent experiments [19,21]. A one-way analysis of variance (ANOVA) followed by Dunnett's or Tukey–Kramer's post hoc tests was performed to compare groups. Mean values were considered significantly different at $*P < 0.05$ or $**P < 0.01$.

3. Results

3.1. PLTP deficiency increased BBB permeability

PLTP is highly expressed in cerebral vessels [8], we examined the impact of PLTP deficiency on BBB through multiphoton microscopy of TMR-dextran and transendothelial transport of Evans blue-labeled albumin.

Multiphoton imaging revealed an intact BBB in wild type (WT) mice, while considerable leakage of TMR-dextran into the brain parenchyma in PLTP deficient mice (Fig. 1A). Similarly, Evans blue assay revealed significantly greater leakage in PLTP deficient mice compared with WT littermates ($P < 0.01$, Fig. 1B). Both results suggested BBB integrity was impaired in PLTP deficient mice.

3.2. PLTP deficiency reduced occludin, claudin-5 and ZO-1 expression

Since the integrity of BBB is mainly mediated by the presence of tight junction, we next examined whether PLTP deficiency affects tight junction protein levels. We investigated expression of occludin, claudin-5 and ZO-1 in brain capillaries by Western blot. All the three proteins decreased significantly in PLTP deficient mice: level of occludin decreased by 56% ($P < 0.01$, Fig. 2C), claudin-5 expression decreased by 35% ($P < 0.05$, Fig. 2D), and ZO-1 expression decreased by 52% ($P < 0.01$, Fig. 2B) compared with WT, indicated that PLTP deficiency disturbed tight junction protein expression, which provided a molecular basis for BBB impairment.

3.3. PLTP deficiency increased cerebrovascular oxidative stress level

Lack of PLTP elevated oxidative stress in peripheral arteries and brain [4,17]. To confirm the impact of PLTP on cerebrovascular

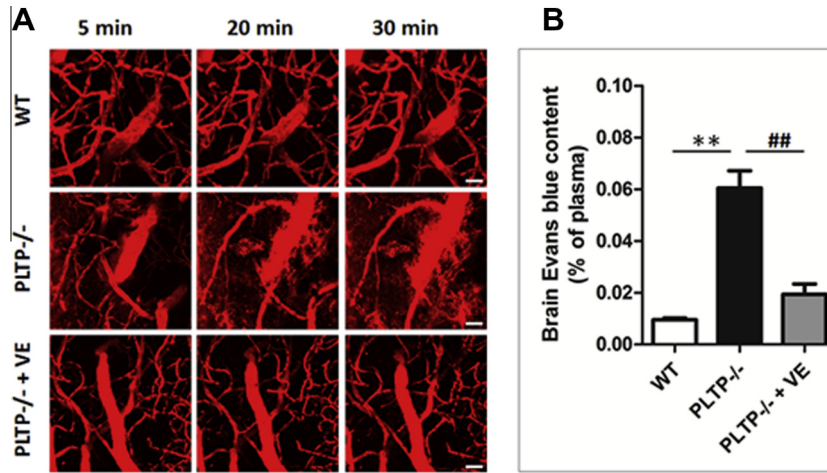


Fig. 1. Increased BBB permeability in PLTP deficient mice. (A) In vivo time-lapse multiphoton imaging of TMR-dextran (MW = 40,000; red) leakage from cortical vessels in wild type (WT), PLTP^{-/-}, and vitamin E supplemented PLTP^{-/-} mice (PLTP^{-/-} + VE). Typical profile with $n = 6$ mice per genotype. Scale bar, 20 μm . (B) In vivo assessment of Evans blue dye extravasation. Evans blue (2%, 4 ml/kg) was injected intravenously into wild type (WT), PLTP^{-/-}, and vitamin E supplemented PLTP^{-/-} mice (PLTP^{-/-} + VE) ($n = 6$) and allowed to circulate for 2 h. Brain dye content was then assessed spectrophotometrically and normalized to plasma levels. Data are means \pm SEM (** $P < 0.01$ vs. WT; ## $P < 0.01$ vs. PLTP^{-/-}). VE, vitamin E. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

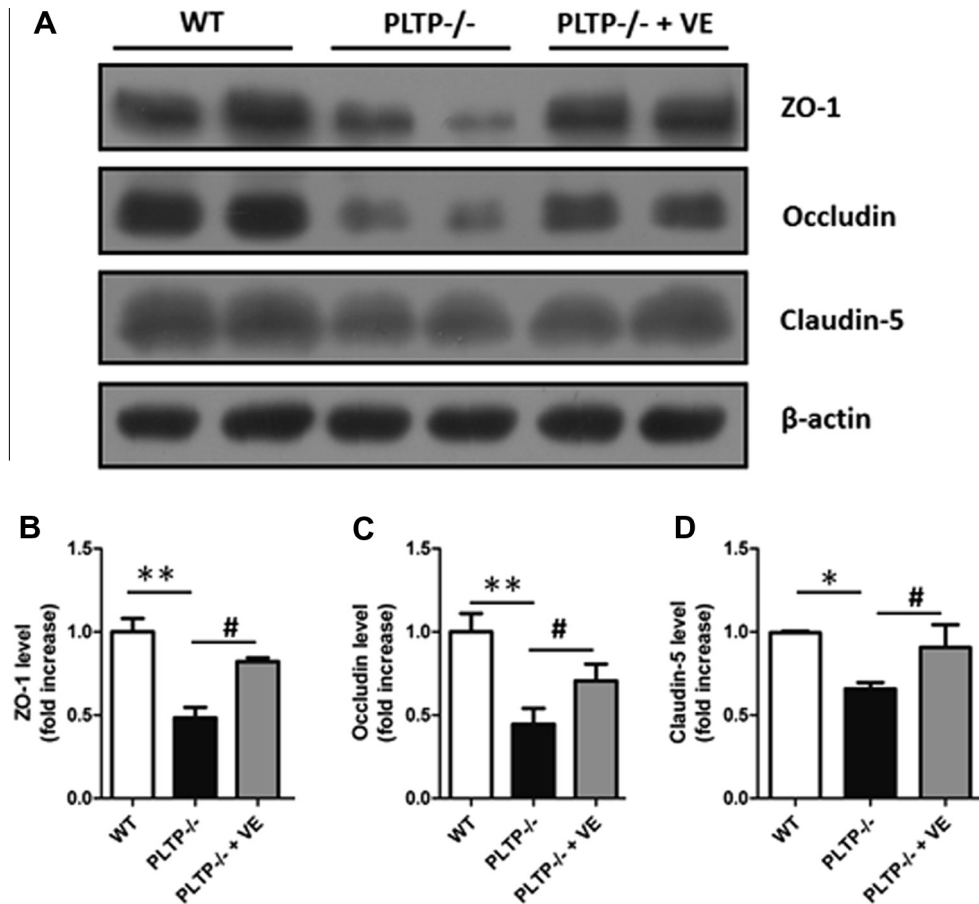


Fig. 2. Decreased expression of tight junction proteins in brain vessels of PLTP deficient mice. (A) Representative Western blots of the expression of ZO-1, occludin, and claudin-5 in brain vessels of wild type (WT), PLTP^{-/-}, and vitamin E supplemented PLTP^{-/-} mice (PLTP^{-/-} + VE) ($n = 6$). β -Actin was used as a loading control. Quantitative analysis of the expression levels of ZO-1 (B), occludin (C), and claudin-5 (D). Data are means \pm SEM (* $P < 0.05$, ** $P < 0.01$ vs. WT; # $P < 0.05$ vs. PLTP^{-/-}). VE, vitamin E.

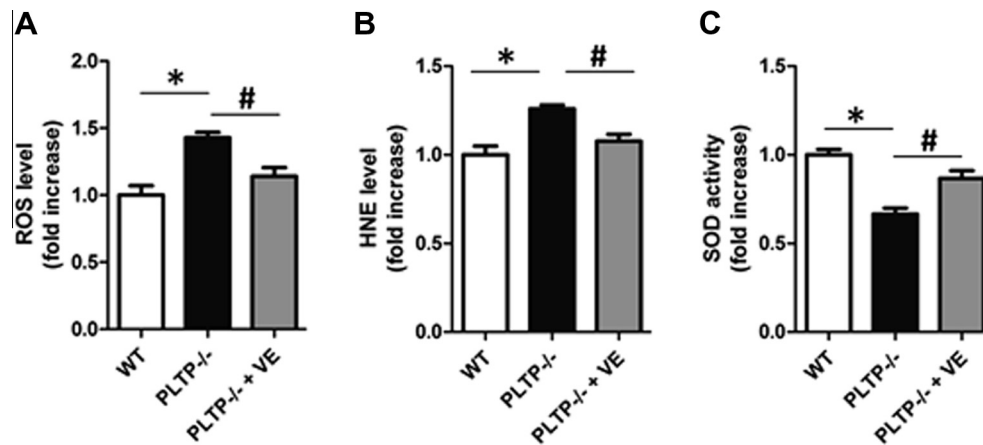


Fig. 3. Increased ROS and HNE levels and decreased SOD activity in PLTP deficient mice. ROS level (A), HNE level (B) and SOD activity (C) in brain vessels of wild type (WT), PLTP^{-/-}, and vitamin E supplemented PLTP^{-/-} mice (PLTP^{-/-} + VE) ($n = 6$). ROS level was measured with dichlorofluorescein diacetate assay, SOD activity was detected using a spectrophotometric assay kit, HNE level was determined with dot blot. Data are means \pm SEM (* $P < 0.05$ vs. WT; # $P < 0.05$ vs. PLTP^{-/-}). VE, vitamin E.

oxidative stress, we demonstrated that in PLTP deficient mice, ROS level increased by 43% compared with WT mice ($P < 0.05$, Fig. 3A), lipid peroxidation marker HNE increased by 26% ($P < 0.05$, Fig. 3B), while SOD activity decreased by 34% compared with WT littermates ($P < 0.05$, Fig. 3C). Our results suggested that increased BBB permeability in PLTP deficient mice may be due to elevated oxidative stress at BBB.

3.4. PLTP deficiency induced cerebrovascular damage is reversed by vitamin E supplementation

PLTP mediates exchange of vitamin E from blood to endothelial cells, PLTP deficiency leads to vitamin E depletion, thus increases oxidative stress [4–6,17]. To demonstrate whether impaired BBB integrity in PLTP deficient mice is due to elevated oxidative stress, we fed PLTP deficient mice with vitamin E supplemented diet for 2 months [17,18], and examined effect of vitamin E supplementation on BBB permeability, tight junction expression and levels of oxidative stress markers. We first found that vitamin E supplementation significantly restored BBB integrity in both multiphoton microscopy of TMR-dextran (Fig. 1A) and Evans blue assay (Fig. 1B), as well as increased tight junction occludin, claudin-5 and ZO-1 (Fig. 2), probably through decreasing ROS and HNE, while increasing SOD activity (Fig. 3), indicated that impaired BBB integrity caused by elevated oxidative stress due to lack of vitamin E in PLTP deficient mice can be rescued by chronic vitamin E supplementation.

4. Discussion

The current study was undertaken to investigate the role of PLTP in BBB integrity in vivo. We first found that PLTP deficient mice displayed leakage of both endogenous and exogenous macromolecules from blood to brain parenchyma, indicating that BBB integrity was impaired. Tight junction proteins have an indispensable role in maintaining BBB integrity [23], our results further showed that expression of the main tight junction proteins occludin, claudin-5 and ZO-1 decreased in brain vessels of PLTP deficient mice. Disruption of tight junction protein has been widely found in inflammation, ischemia and metabolism diseases, while oxidative stress is recognized as a hallmark of these neurological disorders [24,25]. PLTP deficient mice showed increased oxidative stress and decreased SOD activity in brain [17]. We also demonstrated that levels of ROS and lipid peroxidation marker HNE increased,

while SOD activity decreased in cerebral vessels of PLTP deficient mice, indicated that PLTP deficiency increased cerebrovascular oxidative stress, led to disturbed tight junction proteins expression and impaired BBB integrity.

How did PLTP deficiency induce increased oxidative stress and BBB impairment? PLTP is highly expressed cerebrovascular endothelial cells and involved in HDL biogenesis and remodeling at the BBB [8]. PLTP mediates transfer of many amphipathic compounds including α -tocopherol, phospholipids and unesterified cholesterol [1–3]. In vivo study has found that PLTP deficiency decreased level of α -tocopherol in mouse brain, but not contents of cholesterol, phosphatidylcholine, sphingomyelin or $n-3/n-6$ fatty acids ratio [17]. α -Tocopherol is the main isomer of antioxidant vitamin E. Lack of vitamin E in periphery vessels and brain due to PLTP deficiency increases levels of ROS, cholesterol oxides, and decreases SOD activity [4–6,17], which leads to impairment of endothelial function in periphery aorta [3,4] and increases vulnerability to toxic effect induced by $A\beta_{25-35}$ [17]. It has been reported that chronic dietary supplementation of vitamin E can prevent $A\beta_{25-35}$ – induced memory deficits, reduced cerebral oxidative stress and toxicity in PLTP deficient mice [17]. In this study, our results also showed that vitamin E supplementation decreased oxidative stress in brain vessels, and restored tight junction proteins expression and BBB integrity, indicated that PLTP maintained BBB integrity by mediating transfer of vitamin E.

In summary, we demonstrated for the first time that PLTP deficiency increased BBB permeability and decreased tight junction expression by elevating cerebrovascular oxidative stress, provided a potential role of PLTP in maintaining BBB integrity.

Acknowledgments

This work was supported by the National High Technology Research and Development Program of China (Nos. 2012CB911000, 2012CB911004) and the National Natural Science Foundation of China (NSFC; Grants No. 81171015).

References

- [1] L. Lagrost, C. Desrumaux, D. Masson, V. Deckert, P. Gambert, Structure and function of the plasma phospholipid transfer protein, *Curr. Opin. Lipidol.* 9 (1998) 203–209.
- [2] G.M. Kostner, K. Oettl, M. Jauhainen, C. Ehnholm, H. Esterbauer, H. Dieplinger, Human plasma phospholipid transfer protein accelerates exchange/transfer of alpha-tocopherol between lipoproteins and cells, *Biochem. J.* 305 (Pt 2) (1995) 659–667.

- [3] C. Desrumaux, V. Deckert, A. Athias, D. Masson, G. Lizard, V. Palleau, P. Gamber, L. Lagrost, Plasma phospholipid transfer protein prevents vascular endothelium dysfunction by delivering alpha-tocopherol to endothelial cells, *FASEB J.* 13 (1999) 883–892.
- [4] C. Desrumaux, V. Deckert, S. Lemaire-Ewing, C. Mossiat, A. Athias, D. Vandroux, L. Dumont, S. Monier, J.P. Pais de Barros, A. Klein, E. De Maistre, D. Blache, A. Beley, C. Marie, P. Garnier, L. Lagrost, Plasma phospholipid transfer protein deficiency in mice is associated with a reduced thrombotic response to acute intravascular oxidative stress, *Arterioscler. Thromb. Vasc. Biol.* 30 (2010) 2452–2457.
- [5] X.C. Jiang, A.R. Tall, S. Qin, M. Lin, M. Schneider, F. Lalanne, V. Deckert, C. Desrumaux, A. Athias, J.L. Witztum, L. Lagrost, Phospholipid transfer protein deficiency protects circulating lipoproteins from oxidation due to the enhanced accumulation of vitamin E, *J. Biol. Chem.* 277 (2002) 31850–31856.
- [6] C. Desrumaux, P.Y. Risold, H. Schroeder, V. Deckert, D. Masson, A. Athias, H. Laplanche, N. Le Guern, D. Blache, X.C. Jiang, A.R. Tall, D. Desor, L. Lagrost, Phospholipid transfer protein (PLTP) deficiency reduces brain vitamin E content and increases anxiety in mice, *FASEB J.* 19 (2005) 296–297.
- [7] J. Pillgram-Larsen, T.E. Ruud, J.O. Stadaas, A.O. Aasen, Hemodynamic consequences of multitherapy pretreatment in experimental endotoxemia, *Prog. Clin. Biol. Res.* 236A (1987) 227–234.
- [8] A.P. Chirackal Manavalan, A. Kober, J. Metso, I. Lang, T. Becker, K. Hasslitzler, M. Zandl, E. Fanaee-Danesh, J.B. Pippal, V. Sachdev, D. Kratky, J. Stefulj, M. Jauhiainen, U. Panzenboeck, Phospholipid transfer protein is expressed in cerebrovascular endothelial cells and involved in HDL biogenesis and remodeling at the blood–brain barrier, *J. Biol. Chem.* (2013) [Epub ahead of print].
- [9] B.V. Zlokovic, The blood–brain barrier in health and chronic neurodegenerative disorders, *Neuron* 57 (2008) 178–201.
- [10] N.J. Abbott, A.A. Patabendige, D.E. Dolman, S.R. Yusof, D.J. Begley, Structure and function of the blood–brain barrier, *Neurobiol. Dis.* 37 (2010) 13–25.
- [11] C.M. Zehendner, L. Librizzi, J. Hedrich, N.M. Bauer, E.A. Angamo, M. de Curtis, H.J. Luhmann, Moderate hypoxia followed by reoxygenation results in blood–brain barrier breakdown via oxidative stress-dependent tight-junction protein disruption, *PLoS ONE* 8 (2013) e82823.
- [12] J.J. Lochhead, G. McCaffrey, C.E. Quigley, J. Finch, K.M. DeMarco, N. Nametz, T.P. Davis, Oxidative stress increases blood–brain barrier permeability and induces alterations in occludin during hypoxia-reoxygenation, *J. Cereb. Blood Flow Metab.* 30 (2010) 1625–1636.
- [13] M. Hossain, P. Mazzone, W. Tierney, L. Cucullo, In vitro assessment of tobacco smoke toxicity at the BBB: do antioxidant supplements have a protective role?, *BMC Neurosci* 12 (2011) 92.
- [14] B. Oztas, S. Akgul, F.B. Seker, Gender difference in the influence of antioxidants on the blood–brain barrier permeability during pentylentetrazol-induced seizures in hyperthermic rat pups, *Biol. Trace Elem. Res.* 118 (2007) 77–83.
- [15] M. Kaya, V. Cimen, R. Kalayci, M. Kucuk, C. Gurses, N. Arican, I. Elmas, Catalase and alpha-tocopherol attenuate blood–brain barrier breakdown in pentylentetrazole-induced epileptic seizures in acute hyperglycaemic rats, *Pharmacol. Res.* 45 (2002) 129–133.
- [16] X.C. Jiang, C. Bruce, J. Mar, M. Lin, Y. Ji, O.L. Francone, A.R. Tall, Targeted mutation of plasma phospholipid transfer protein gene markedly reduces high-density lipoprotein levels, *J. Clin. Invest.* 103 (1999) 907–914.
- [17] C. Desrumaux, A. Pisoni, J. Meunier, V. Deckert, A. Athias, V. Perrier, V. Villard, L. Lagrost, J.M. Verdier, T. Maurice, Increased amyloid-beta peptide-induced memory deficits in phospholipid transfer protein (PLTP) gene knockout mice, *Neuropsychopharmacology* 38 (2013) 817–825.
- [18] N. Ogier, A. Klein, V. Deckert, A. Athias, G. Bessedé, N. Le Guern, L. Lagrost, C. Desrumaux, Cholesterol accumulation is increased in macrophages of phospholipid transfer protein-deficient mice. normalization by dietary alpha-tocopherol supplementation, *Arterioscler. Thromb. Vasc. Biol.* 27 (2007) 2407–2412.
- [19] R.D. Bell, E.A. Winkler, I. Singh, A.P. Sagare, R. Deane, Z. Wu, D.M. Holtzman, C. Betsholtz, A. Armulik, J. Sallstrom, B.C. Berk, B.V. Zlokovic, Apolipoprotein E controls cerebrovascular integrity via cyclophilin A, *Nature* 485 (2012) 512–516.
- [20] S.L. Park, D.H. Lee, S.E. Yoo, Y.S. Jung, The effect of Na⁽⁺⁾/H⁽⁺⁾ exchanger-1 inhibition by sabiporide on blood–brain barrier dysfunction after ischemia/hypoxia in vivo and in vitro, *Brain Res.* 1366 (2010) 189–196.
- [21] M. Sun, T. Zhou, L. Zhou, Q. Chen, Y. Yu, H. Yang, K. Zhong, X. Zhang, F. Xu, S. Cai, A. Yu, H. Zhang, R. Xiao, D. Xiao, D. Chui, Formononetin protects neurons against hypoxia-induced cytotoxicity through upregulation of ADAM10 and sAbetaPPalpha, *J. Alzheimer's Dis.* 28 (2012) 795–808.
- [22] M.A. Erickson, K. Hansen, W.A. Banks, Inflammation-induced dysfunction of the low-density lipoprotein receptor-related protein-1 at the blood–brain barrier: protection by the antioxidant *N*-acetylcysteine, *Brain Behav. Immun.* 26 (2012) 1085–1094.
- [23] A.M. Enciu, M. Gherghiceanu, B.O. Popescu, Triggers and effectors of oxidative stress at blood–brain barrier level: relevance for brain ageing and neurodegeneration, *Oxidative Med. Cell. Longevity* 2013 (2013) 297512.
- [24] T.A. Clark, H.P. Lee, R.K. Rolston, X. Zhu, M.W. Marlett, R.J. Castellani, A. Nunomura, G. Casadesus, M.A. Smith, H.G. Lee, G. Perry, Oxidative stress and its implications for future treatments and management of Alzheimer disease, *Int. J. Biomed. Sci.* 6 (2010) 225–227.
- [25] J. Vina, A. Lloret, E. Giraldo, M.C. Badia, M.D. Alonso, Antioxidant pathways in Alzheimer's disease: possibilities of intervention, *Curr. Pharm. Des.* 17 (2011) 3861–3864.