Toxicol. Res.
Vol. 28, No. 1, pp. 5-9 (2012)
Open Access http://dx.doi.org/10.5487/TR.2012.28.1.005
pISSN: 1976-8257 eISSN: 2234-2753

Official Journal of Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Synthetic Prion Peptide 106-126 Resulted in an Increase Matrix Metalloproteinases and Inflammatory Cytokines from Rat Astrocytes and Microglial Cells

Kibbeum Song, Ji-Young Na, Myung-Hoon Oh, Sokho Kim, Young-Ha Kim, Byung-Yong Park, Gi-Wook Shin, Bumseok Kim, Myungjo You and Jungkee Kwon

Bio-Safety Research Institute, College of Veterinary Medicine, Chonbuk National University, Jeonju 561-156, Korea

(Received February 8, 2012; Revised March 12, 2012; Accepted March 21, 2012)

It has been shown that the accumulation of prion in the cytoplasm can result in neurodegenerative disorders. Synthetic prion peptide 106-126 (PrP) is a glycoprotein that is expressed predominantly by neurons and other cells, including glial cells. Prion-induced chronic neurodegeneration has a substantial inflammatory component, and an increase in the levels of matrix metalloproteinases (MMPs) may play an important role in neurodegenerative development and progression. However, the expression of MMPs in PrP induced rat astrocytes and microglia has not yet been compared. Thus, in this study, we examined the fluorescence intensity of CD11b positive microglia and Glial Fibrillary Acidic Protein (GFAP) positive astrocytes and found that the fluorescent intensity was increased following incubation with PrP at 24 hours in a dose-dependent manner. We also observed an increase in interleukin-1 beta (IL-1β) and tumor necrosis factor alpha (TNF-α) protein expression, which are initial inflammatory cytokines, in both PrP induced astrocytes and microglia. Furthermore, an increase MMP-1, 3 and 11 expressions in PrP induced astrocytes and microglia was observed by real time PCR. Our results demonstrated PrP induced activation of astrocytes and microglia respectively, which resulted in an increase in inflammatory cytokines and MMPs expression. These results provide the insight into the different sensitivities of glial cells to PrP.

***Key words*:** Prion peptide, Astrocytes, Microglia, Matrix metalloproteinase

INSTRUCTION

Prion infection of the central nervous system causes a progressive and ultimately lethal degeneration of neuronal tissue; however, the underlying pathomechanisms of this degeneration are still not well understood (Rezaie and Lantos, 2001). When activated in the course of neuroinflammatory responses, glial cells morphology changes from a ramified to an amoeboid shape. When activated glial cells produce proinflammatory molecules, including cytokines and chemokines, levels of these molecules are thought to be correlated with the pathogenesis of various neurodegenerative diseases (Meda *et al*., 2001). In prion diseases, the reactive gliosis is most likely a consequence of the accumulation of disease-associated isoforms of the prion. In agreement with this hypothesis, microglia cells are activated by the aggregated, amyloid-like, form of a Prion peptide 106-126 (PrP) *in vitro*. Supernatants from PrP treated microglia cultures activate astrocytes and are neurotoxic (Forloni *et al*., 1993). Thus, cytokines and chemokines released by PrP-activated glial cells may contribute indirectly to the pathogenesis of prion infection by acting as mediators of gliosis and/or directly via cytotoxicity to neurons (Williams *et al*., 1997).

The matrix metalloproteinases (MMPs) are a large family of zinc-dependent endopeptidase enzymes and responsible for remodeling the extracellular matrix (ECM) in the CNS and cleaving proteins present in myelin (Proost *et al*., 1993) and tight junction complexes (Gurney *et al*., 2006). Together with the tissue inhibitors of metalloproteinases (TIMPs), the MMPs form a dynamic system that maintains the homeostatic balance of ECM breakdown and regeneration (Crocker *et al*., 2006). In many disease states, this balance is lost, and excessive MMP proteolytic activity is thought to contribute to the pathogenesis of a number of CNS diseases, including cerebral ischemia, Parkinson's disease, Alzheimer's disease, and traumatic injury (Crocker *et*

Correspondence to: Jungkee Kwon, Department of Laboratory Animal Medicine, College of Veterinary Medicine, Chonbuk National University, 664-14, Jeonju 561-156, Korea
E-mail: jkwon@jbnu.ac.kr

al., 2004). Especially, MMP-1, MMP-3 (Choi *et al*., 2008; Haorah *et al*., 2007) and MMP-11 (Crocker *et al*., 2008) are implicated in CNS inflammation. Previous studies showed the MMP expressions profile in purified cell cultures of astrocytes and microglia. However, it has been never shown that MMPs expression have systematically compared in glial cells treated with PrP, although the evidence for MMP involvement in chronic neurodegeneration has been accumulated. The objective of the present study, therefore, was to identify the comparative expression of the inflammatory cytokines and MMP gene expressions in glial cells and to better understand the difference in sensitivity of glial cells to PrP.

MATERIALS AND METHODS

Cell cultures and immunocytochemistry. Primary glial mixed cultures were obtained from the cerebral cortex of 1-day-old SD rats (Daehan Bio-link, Chungju, Korea) as previously described (Shim *et al*., 2011). Briefly, newborn rats were euthanized by decapitation according to the guidelines presented by the IACUC of Chonbuk National University (CBU 2010-0056). Brain tissue was then mechanically dissociated in culture medium (MEM containing 10% FBS, HEPES, 1% antibiotics), and cells were seeded onto culture flasks (75T-flask, Nunc, Netherland). Upon reaching confluency (14 days), flasks were shaken and hit strongly 10~20 times by hand. Cells that remained attached were removed by incubation with trypsin and seeded on culture dishes or poly-L-lysine-coated coverslips in culture medium (DMEM containing 10% FBS, 1% antibiotics). The purified astrocytes cultures were then used for experiments. The floating cells were also utilized to establish primary microglia cultures as described previously (Shim *et al*., 2011). Briefly, microglia-containg supernatants were plated using medium (DMEM containing 10% FBS, 1% antibiotics). Medium was replaced 1 hour after plating to further select the microglia cell population. Astrocytes and microglia were pre-plated for 24 h and then treated with 10, 30 and 50 μM PrP 106-126 (Peptron, Daejon, Korea).

Microglial cells were seeded on poly-L-lysine-coated coverslips for staining. For immunofluorescence measurements, cultures were use on precoated coverslide in non-coated dishes or plates. All incubations and washes were performed at room temperature. Cells were fixed with 100% methanol, washed three times with PBS, permeabilized with 0.1% Triton X-100/PBS for 10min and then washed three times with PBS. Fixed cells were incubated for 30 min with 1% BSA in PBS. Cells were incubated overnight with diluted primary monoclonal antibodies, GFAP (1 : 1,000 dilution, Chemicon, Temecula, CA) and CD11b (1 : 200 dilution, Chemicon, Temecula, CA, USA) for double staining. After rinsing in PBS, these cells were incubated for 2 h with diluted secondary antibodies (goat anti-mouse, rabbit Alexa Fluor 596, goat anti-rabbit Alexa Fluor 488) and washed with PBS. As a negative control, the primary antibodies were not added. Images were obtained by fluorescence microscopy on a scope A1 (Carlzeiss, Germany).

Western blotting. Total cell extracts were harvested in RIPA buffer and then centrifuged at 15,000 rpm for 15 min at 4°C. Quantification of total protein was performed using the BCA protein assay reagent (Bio-Rad Laboratories, Hercules, CA). Proteins were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane. After blocking in 5% skim milk in PBS with 0.1% Tween-20 (PBS-T), the membranes were incubated overnight at 4°C with specific primary antibodies for IL-1β, TNF-α and β-actin (Cell Signaling, Danvers, USA) that had been diluted by 1 : 1,000 in 1% skim milk in PBS-T. After washing, the blots were incubated at room temperature for 1 h with peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Millipore, USA) that had been diluted by 1 : 10,000 in PBS-T. Signals were detected using a supersignal west dura extended duration substrate (Thermo, CA, USA), according to the manufacturer's instructions. Densitometric analysis was conducted directly from the blotted membrane using a Chemi Imager analyzer system (Alpha Innotech, San Leandro, CA, USA).

Real-time PCR. Total cellular RNA prepared from the cells was precipitated with Ribo EX (Geneall, Daejeon, Korea) and dissolved in DEPC-treated distilled water. Total RNA (2 μg) was treated with RNase-free DNase (Invitrogen) and the first strand cDNA was generated with oligo's primers provided in Maxime RT PreMix (Intron, Seongnam, Korea) using the first-strand cDNA synthesis kit according the manufacturer's instruction. Specific primers for each gene were designed using Primer Express soft-

Table 1. Oligonucleotide sequences of the primers used in RT-PCR

Gene	Sequences	
	Forward	Reverse
MMP-1	GCCATTACTCACAACAATCCTC	ACACAATATCACCTTCCTCCTC
MMP-3	TTGATGATGATGAACGATGGAC	GTGGAGGACTTGTAGACTGG
MMP-11	TATGACGAGACTTGGACTATTGG	CTGAGCACCTTGGAAGAACC
GAPDH	TGAACGGGAAGCTCACTGG	TCCACCACCCTGTTGCTGTA

ware (Applied Biosystems, Singapore) as shown in Table 1. The real-time PCR reaction mixture consisted of 10 ng reverse-transcribed total RNA, 167 nM forward and reverse primers, and 2 × PCR master mixture in a final volume of 20 μ*l*. The PCR was carried out in 48 well plates using the ABI Step one plus Sequence Detection System (Applied Biosystems, Singapore). All experiments were performed in triplicate.

RESULTS

The majority (95%) of astrocytes and microglia in this culture system expressed astrocytes and microglia specific marker, GFAP and CD11b, respectively (Fig. 1). Fig. 1 showed the activation of astrocytes and microglia treated by PrP. The fluorescence intensity of astrocytes and microglia treated by PrP was increased as compared to control. The relationship between glial activation and neurodegeneration is still unclear, although several cytokines and inflammatory mediators produced from activated glial cells may initiate or exacerbate the progression of neuropathology. As shown in Fig. 2, protein expression of IL-1β and TNF-α was increased in astrocytes and microglia treated with PrP. The PrP dose-dependent increase in these cytokines was

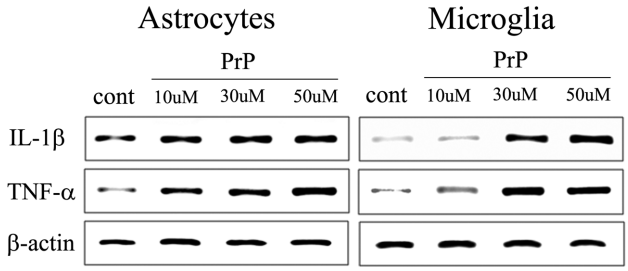

Fig. 2. Protein expression of IL-1β and TNF-α in PrP treated astrocytes and microglia. Astrocytes and microglia were treated with various concentrations of PrP for 24 hours (10, 30 and 50 μM). Western blotting analysis was conducted directly from the blotted membrane by using a Chemi Imager analyzer system.

different between the two glial cells, where microglial cells were more sensitive to PrP treatment than astrocytes. Fig. 3 showed that microglia treated with PrP have more sensitive with higher gene expression than astrocytes. MMP-1 and MMP-11 gene expressions in microglia showed significantly increase in a PrP dose-dependent manner, whereas MMP-3 gene expression showed no change in microglia. There was no significant difference in MMP-3 and MMP-11 gene expressions in astrocytes except for MMP-1 gene

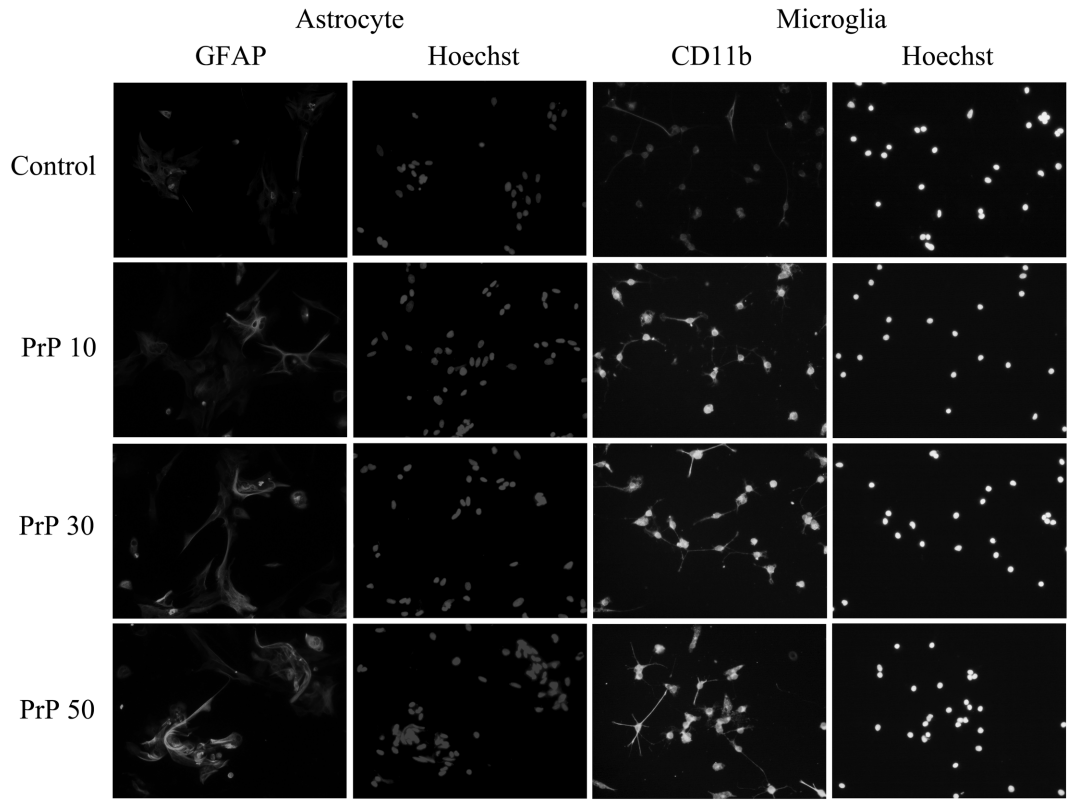

Fig. 1. Activation of astrocytes and microglia induced by PrP. Astrocytes and microglia were treated with various concentrations of PrP for 24 hours (10, 30 and 50 μM). Immunocytochemistry staining for GFAP (red), CD11b (green) and Hoechst (blue). Fluorescence intensity was observed using a florescence microscope. Original magnification, 200 ×.

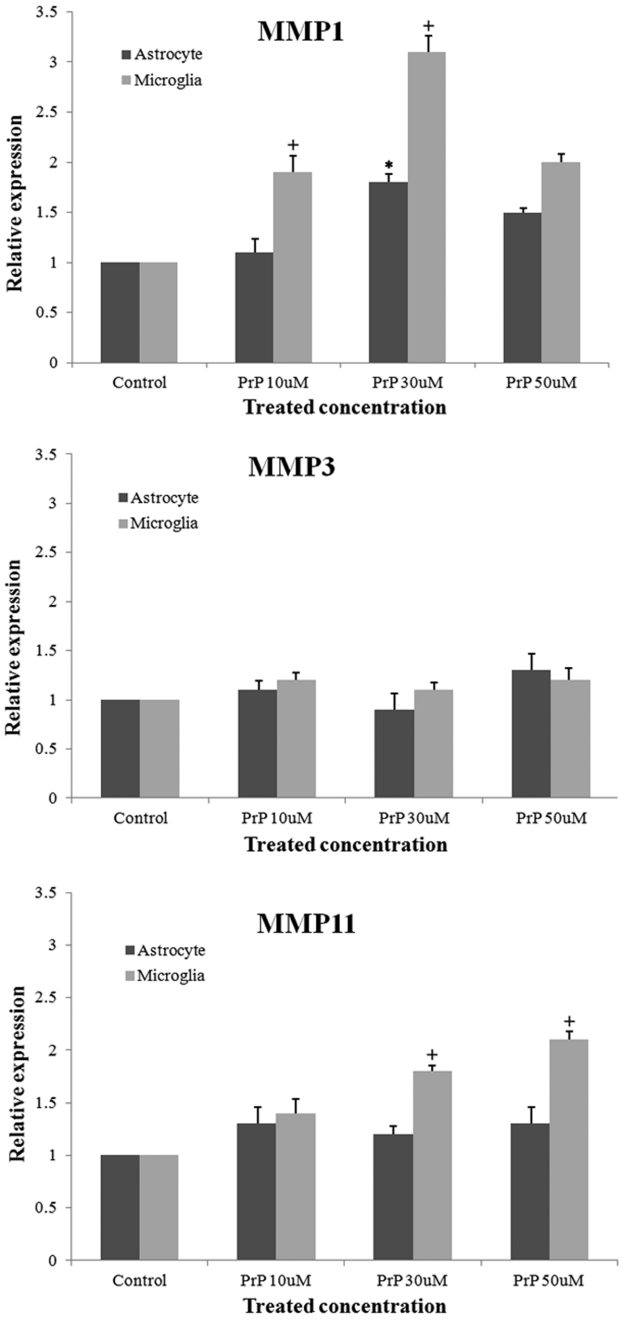

Fig. 3. Real time RT-PCR analysis of MMPs gene expression. Real time RT-PCR analysis of MMPs genes was performed as described in the materials and methods. MMP-1 and MMP-11 gene expressions showed significantly increase in a PrP dose dependant manner in microglia. MMP-3 and MMP-11 gene expressions showed no changed in astrocytes. Each value is expressed as the mean ± SEM of triplicate. Significance by *t*-test: *$p < 0.05$ vs astrocytes control, +$p < 0.05$ vs microglia control.

expression. These results indicated that astrocytes and microglia treated with PrP have different inflammatory cytokines responses and expressions of MMPs genes after PrP treatment.

DISCUSSION

The presence of activated microglia adjacent to PrP deposits is a common feature in the infected brains of humans and animals (Guiroy *et al*., 1994). Moreover, *in vivo* accumulation of activated microglia is concomitant with PrP deposition, which precedes neuronal death and clinical signs of prion disease (Betmouni *et al*., 1996). Using PrP 106-126 as an *in vitro* model, previous reports have shown that the presence of activated microglia is required for neurotoxicity. Furthermore, PrP 106-126 has been shown to exhibit *in vitro* chemotactic properties for human monocytes through direct interactions with cell surface receptors (Le *et al*., 2001). However, little is known about the nature of the molecular signals and cell types responsible for astrocytes and microglia in the vicinity of PrP aggregates (Kim *et al*., 2005). MMPs have been implicated in a variety of neuroinflammatory diseases, including EAE (Pagenstecher *et al*., 1998), multiple sclerosis, Parkinson's disease, cerebral ischemia and nerve regeneration (Larsen *et al*., 2003). In many instances, activation of MMPs is associated with tissue injury and cell death. For example, MMP-3 has been implicated in the neuroinflammatory cascade as a secreted factor released from apoptotic cells. This study showed that active MMP-3 increased expression of cytokines that resulted in the activation of microglia, accounting for the localized activation of microglia around apoptotic neurons in the CNS (Kim *et al*., 2005). Elevated MMP-3 levels have been reported in several autoimmune and inflammatory CNS disease states (Hafiz *et al*., 2000).

To the best of our knowledge, this is the first study to demonstrate a complementary response of astrocytes and microglia to prion infection leading to MMPs gene expression *in vitro*. We hypothesize that activation of astrocytes and microglia in the vicinity of PrP aggregates and their subsequent activation of MMPs gene expression after infection and long before the onset of the disease may contribute to both neuronal damage and pathogenesis. We also demonstrated that TNF-α and IL-1β were up-regulated in astrocytes and microglia after PrP treatment, which lead to MMPs gene expression. In this study, we compared the MMPs gene expression profile between astrocytes and microglia after PrP treatment. Although we did not identify the detailed pathway or mechanism of PrP induced MMPs gene expression, our experiments suggest the existence of functional interactions that might induce transduction signal pathways. MMPs genes are widely expressed in the adult CNS, and their expression is regulated in response to stressful and pathological conditions. They have been involved in the manifestation of various brain disorders such as Alzheimer's disease. Further investigations will be needed to better understand how PrP aggregates influence the MMPs profile expression and signal pathways in both astrocytes

and microglia.

REFERENCES

Betmouni, S., Perry, V.H. and Gordon, J.L. (1996). Evidence for an early inflammatory response in the central nervous system of mice with scrapie. *Neuroscience*, **74**, 1-5.

Choi, D.H., Kim, E.M., Son, H.J., Joh, T.H., Kim, Y.S., Kim, D., Flint Beal, M. and Hwang, O. (2008). A novel intracellular role of matrix metalloproteinase-3 during apoptosis of dopaminergic cells. *J. Neurochem*., **106**, 405-415.

Crocker, S.J, Frausto, R.F., Whitton, J.L. and Milner, R. (2008). A novel method to establish microglia-free astrocyte cultures: Comparison of matrix metalloproteinase expression profiles in pure cultures of astrocytes and microglia. *Glia*., **56**, 1187-1198.

Crocker, S.J., Milner, R., Pham-Mitchell, N. and Campbell, I.L. (2006). Cell and agonist-specific regulation of genes for matrix metalloproteinases and their tissue inhibitors by primary glial cells. *J. Neurochem*., **98**, 812-823.

Crocker, S.J., Pagenstecher, A. and Campbell, I.L. (2004). The TIMPs tango with MMPs and more in the central nervous system. *J. Neurosci. Res*., **75**, 1-11.

Forloni, G., Angeretti, N., Chiesa, R., Monzani, E., Salmona, M., Bugiani, O. and Tagliavini, F. (1993). Neurotoxicity of a prion protein fragment. *Nature*, **362**, 543-546.

Guiroy, D.C., Wakayama, I., Liberski, P.P. and Gajdusek, D.C. (1994). Relationship of microglia and scrapie amyloid-immunoreactive plaques in kuru, Creutzfeldt-Jakob disease and Gerstmann-Straussler syndrome. *Acta. Neuropathol.*, **87**, 526-530.

Gurney, K.J., Estrada, E.Y. and Rosenberg, G.A. (2006). Blood-brain barrier disruption by stromelysin-1 facilitates neutrophil infiltration in neuroinflammation. *Neurobiol. Dis.*, **23**, 87-96.

Haorah, J., Ramirez, S.H., Schall, K., Smith, D., Pandya, R. and Persidsky, Y. (2007). Oxidative stress activates protein tyrosine kinase and matrix metalloproteinases leading to blood-brain barrier dysfunction. *J. Neurochem*., **101**, 566-576

Hafiz, F.B. and Brown, D.R. (2000). A model for the mechanism of astrogliosis in prion disease. *Mol. Cell Neurosci.*, **16**, 221-232.

Kim, Y.S., Kim, S.S., Cho, J.J., Choi, D.H., Hwang, O., Shin, D.H., Chun, H.S., Beal, M.F. and Joh, T.H. (2005). Matrix metalloproteinase-3: a novel signaling proteinase from apoptotic neuronal cells that activates microglia. *J. Neurosci*., **25**, 3701-3711.

Larsen, P.H., Wells, J.E., Stallcup, W.B., Opdenakker, G. and Yong, V.W. (2003). Matrix metalloproteinase-9 facilitates remyelination in part by processing the inhibitory NG2 proteoglycan. *J. Neurosci*., **23**, 11127-11135.

Le, Y., Yazawa, H., Gong, W., Yu, Z., Ferrans, V.J., Murphy, P.M. and Wang, J.M. (2001). The neurotoxic prion peptide fragment PrP(106-126) is a chemotactic agonist for theGprotein-coupled receptor formyl peptide receptor-like 1. *J. Immunol*., **166**, 1448-1451.

Meda, L., Baron, P. and Scarlato, G. (2001). Glial activation in Alzheimer's disease: the role of Abeta and its associated proteins. *Neurobiol. Aging*, **22**, 885-893.

Pagenstecher, A., Stalder, A.K., Kincaid, C.L., Shapiro, S.D. and Campbell, I.L. (1998). Differential expression of matrix metalloproteinase and tissue inhibitor of matrix metalloproteinase genes in the mouse central nervous system in normal and inflammatory states. *Am. J. Pathol*., **152**, 729-741.

Proost, P., Van Damme, J. and Opdenakker, G. (1993). Leukocyte gelatinase B cleavage releases encephalitogens form human myelin basic protein. *Biochem. Biophys. Res. Comm.*, **192**, 1175-1181.

Rezaie, P. and Lantos, P.L. (2001). Microglia and the pathogenesis of spongiform encephalopathies. *Brain Res. Rev.*, **35**, 55-72.

Shim, S., Kim, S., Choi, D.S., Kwon, Y.B. and Kwon, J. (2011). Anti-inflammatory effects of [6]-shogaol: potential roles of HDAC inhibition and HSP70 induction. *Food Chem. Toxicol*., **49**, 2734-2740.

Williams, A., Van Dam, A.M., Ritchie, D., Eikelenboom, P. and Fraser, H. (1997). Immunocytochemical appearance of cytokines, prostaglandin E2 and lipocortin-1 in the CNS during the incubation period of murine scrapie correlates with progressive PrP accumulations. *Brain Res*., **754**, 171-180.