ผลของ piperine ต่อการบาดเจ็บและการเปลี่ยนแปลงด้านออกซิเดชั่นซึ่งชักนำด้วย lipopolysaccharide ใน glial cell เพาะเลี้ยงจากสมองหนูขาว

นางสาวปิยนุช วงศ์อนันต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF PIPERINE ON LIPOPOLYSACCHARIDE-INDUCED INJURIES AND OXIDATIVE CHANGES IN RAT BRAIN GLIAL CELL CULTURES

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ปิยนุช วงศ์อนันต์ : ผลของ piperine ต่อการบาดเจ็บและการเปลี่ยนแปลงด้านออกซิเดชั่นซึ่งชักนำด้วย lipopolysaccharide ใน glial cell เพาะเลี้ยงจากสมองหนูขาว. (EFFECTS OF PIPERINE ON LIPOPOLYSACCHARIDE-INDUCED INJURIES AND OXIDATIVE CHANGES IN RAT BRAIN GLIAL CELL CULTURES) อ. ที่ปรึกษา: ผศ. ดร. สุรชัย อัญเชิญ, 84 หน้า. ISBN 974-17-3019-5

Oxidative stress มีส่วนเกี่ยวข้องกับโรคที่เกิดเนื่องจากความเสื่อมของเซลล์ประสาท ในการทดลองนี้จึงมุ่งศึกษาผลของ piperine ต่อการบาดเจ็บและการเปลี่ยนแปลงด้านออกซิเดชั่นซึ่งชักนำให้เกิดขึ้นด้วย lipopolysaccharide (LPS) ใน glial cells เพาะเลี้ยง จากการทดลองพบว่า เมื่อ incubate ด้วย piperine ที่ความเข้มข้นต่ำๆ (1-10 μM) เป็นเวลา 12 และ 24 ชั่วโมง สามารถกระตุ้น mitochondrial metabolic activity ของ glial cells โดยไม่มีผลต่อการอยู่รอดของเซลล์ ในทางตรงกันข้ามเมื่อ incubate ด้วย piperine ที่ความเข้มข้นสูงขึ้น (25-100 μM) เป็นเวลา 6, 12 และ 24 ชั่วโมงพบว่า mitochondrial metabolic activity และ การอยู่รอดของ glial cells มีค่าลดลง ในขณะที่การ incubate glial cells เพาะเลี้ยงด้วย LPS ความเข้มข้น 0.01-100 μg/ml เป็นเวลา 96 ชั่วโมงพบว่า mitochondrial metabolic activity มีค่าลดลงประมาณ 20-30% โดยไม่มีผลต่อการอยู่รอดของเซลล์ การ incubate ด้วย piperine ความเข้มข้น 1-10 μM หรือ trolox ความเข้มข้น 100 μM เป็นเวลา 24 ชั่วโมง ก่อนการ incubate ด้วย LPS ความเข้มข้น 1 μg/ml เป็นเวลา 96 ชั่วโมง และการ incubate ด้วย piperine ความเข้มข้น 5 และ 7.5 μM หรือ trolox ความเข้มข้น 100 μM เป็นเวลา 24 ชั่วโมง หลังการ incubate ด้วย LPS ความเข้มข้น 1 μg/ml เป็นเวลา 96 ชั่วโมง สามารถกระตุ้น mitochondrial metabolic activity ที่ลดลงเนื่องจากผลของ LPS ได้อย่างมีนัยสำคัญทางสถิติ โดยไม่ส่งผลต่อการอยู่รอดของ glial cells แต่เมื่อ incubate ด้วย piperine ความเข้มข้น 1-10 μM หรือ trolox ความเข้มข้น 100 μM ร่วมกับ LPS ความเข้มข้น 1 μg/ml เป็นเวลา 96 ชั่วโมง จะไม่มีผลต่อ mitochondrial metabolic activity และการอยู่รอดของเซลล์เมื่อเปรียบเทียบกับกลุ่มที่ incubate ด้วย LPS เพียงอย่างเดียว ขณะที่การ incubate glial cells เพาะเลี้ยงด้วย LPS ความเข้มข้น 1 μg/ml เป็นเวลา 24 ชั่วโมงไม่มีผลเปลี่ยนแปลง mitochondrial metabolic activity และ การอยู่รอดของเซลล์ อย่างไรก็ตาม mitochondrial metabolic activity มีค่าเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติเมื่อ incubate LPS ความเข้มข้น 1 μg/ml ร่วมกับ piperine ความเข้มข้น 10 μM หรือ trolox ความเข้มข้น 100 μM เป็นเวลา 24 ชั่วโมง เมื่อ incubate glial cells เพาะเลี้ยงด้วย piperine ความเข้มข้น 5 μM หรือ trolox ความเข้มข้น 100 μM เป็นเวลา 24 ชั่วโมง สามารถเพิ่มปริมาณ glutathione ที่ลดลง ในขณะที่ไม่มีผลเปลี่ยนแปลงปริมาณ nitrite ที่เพิ่มขึ้นจากการ incubate ด้วย LPS ความเข้มข้น 1 μg/ml เป็นเวลา 96 ชั่วโมง จากการทดลองแสดงให้เห็นว่า piperine ที่ความเข้มข้นต่ำๆ สามารถกระตุ้น metabolic activity และ function ของ glial cells ในกระบวนตอบสนองต่อการอักเสบของสมอง

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สาขาวิชา เภสัชวิทยา ลายมือชื่ออาจารย์ที่ปรึกษา.....

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PIYANUCH WONGANAN: EFFECTS OF PIPERINE ON LIPOPOLYSACCHARIDE-INDUCED INJURIES AND OXIDATIVE CHANGES IN RAT BRAIN GLIAL CELL CULURES. THESIS ADVISOR: ASSISTANT. PROF. DR. SURACHAI UNCHERN, 84 pp. ISBN 974-17-3019-5

Oxidative stress plays a role in the aging process and is one of the pathogenic causes in a variety of neurodegenerative disorders. In this study, effects of piperine on lipopolysaccharide (LPS)-induced injuries and oxidative changes in cultured glial cells from rat brains were investigated. Treatment of cultured glial cells with piperine displayed bimodal effects. At low concentrations (1-10 μM), it significantly increased mitochondrial metabolic activity after 12 and 24 hr of incubation with no apparent effects on cell survival. In contrast, mitochondrial activity and the number of surviving glial cells were markedly decreased at higher concentrations of piperine (25-100 μM) after 6, 12 and 24 hr of incubation. After an exposure to 0.01-100 μg/ml of LPS for 96 hr, mitochondrial metabolic activity of glial cells was decreased by approximately 20-30% without significant effects on cell viability. Treatment with 1-10 μM of piperine or 100 μM of trolox for 24 hr before exposure to 1 μg/ml of LPS for 96 hr and treatment with 5 and 7.5 μM of piperine or 100 μM of trolox for 24 hr after exposure to 1 μg/ml of LPS for 96 hr significantly boosted up mitochondrial activity of LPS-exposed glial cells. Under the same condition, the number of cell survived was unaffected. Treatment with 7.5 and 10 μM of piperine during exposure to 1 μg/ml of LPS for 96 hr did not affect mitochondrial metabolic activity and the number of surviving glial cells when compared with LPS-treated group. While incubation with 1 μg/ml of LPS for 24 hr had no effect on mitochondrial metabolic activity and cell viability. However, mitochondrial activity was significantly increased after treatment with 10 μM of piperine or 100 μM of trolox during exposure to 1 μg/ml of LPS for 24 hr. Post-incubation with 5 μM of piperine or 100 μM of trolox reversed LPS-induced glutathione diminution but did not alter LPS-induced nitrite accumulation in cultured glial cells. These results suggest that piperine, especially at low concentrations, might have stimulatory effect on glial cell metabolic activity and facilitate glial cell functions in brain inflammatory responses.

Inter-Department of Pharmacology Student's signature…….…….
Field of study Pharmacology Advisor's signature……..……
Academic 2002

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LIST OF ABBREVIATIONS

%	=	percent
°C	=	degree Celcius
μg	=	microgram
γGluCys	=	gamma-glutamyl-cysteine
γGT	=	gamma-glutamyl transpeptidase
μl	=	microliter
6-OHDA	=	6-hydroxydopamine
Aβ	=	β-amyloid protein
AD	=	Alzheimer's disease
AIDS	=	acquired immunodeficiency syndrome
ALS	=	amyotrophic lateral sclerosis
AP	=	alkaline phosphatase
BDNF	=	brain-derived neurotrophic factor
bFGF	=	basic fibroblast growth factor
BSO	=	buthionine sulfoximine
CaMKs	=	calmodulin regulated protein kinases
$CC l_4$	=	carbon tetrachloride
cm	=	centimeter
CNS	=	central nervous system
DMEM	=	Dulbecco's modified Eagle's medium
DMSO	=	dimethyl sulfoxide
DNase I	=	deoxyribonuclease I
DPBS	=	Dulbecco's phosphate buffered saline
DPPH	=	1,1-diphenyl-2-picrylhydrazyl
DS	=	Down's syndrome

EC_{50}	=	Median effective concentration
EDTA	=	ethylenediaminetetraacetic acid
et al.	=	et alii (and other)
FBS	=	fetal bovine serum
g	=	gram
GCM	=	glial conditioned medium
GDNF	=	glial cell-derived neurotrophic factor
GPT	=	glutamate pyruvate transaminase
GPx	=	glutathione peroxidase
GRx	=	glutathione reductase
GSH	=	glutathione
GSSH	=	glutathione disulfide
H_2O_2	=	hydrogen peroxide
HBSS	=	Hank's balanced salt solution
hr	=	hour
IFN-γ	=	interferon-gamma
IL-1	=	interleukin-1
IL-6	=	interleukin-6
iNOS	=	inducible nitric oxide synthase
kg	=	kilogram
L	=	liter
LA	=	lipoic acid
LD_{50}	=	Median lethal dose
LDH	=	lactate dehydrogenase
LPS	=	lipopolysaccharide
M	=	molar (mole per liter)
mg	=	milligram

mg/kg	=	milligram per kilogram
min	=	minute
ml	=	milliliter
mM	=	millimolar (millimole per liter)
MPP^+	=	1-methyl-4-phenylpyridinium
MPTP	=	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MS	=	multiple sclerosis
MTT	=	3-[4,5-dimethiazol-2-yl]-2,5,diphenyl tetrazoliumbromide
MW	=	molecular weight
NAC	=	N-acetyl cysteine
NADH	=	reduced nicotinamide adenine dinucleotide
NADPH	=	reduced nicotinamide adenine dinucleotide phosphate
NBT	=	nitroblue tetrazolium
NGF	=	nerve growth factor
NMDA	=	N-methyl-D-aspartate
NO	=	nitric oxide
NSAIDs	=	non-steroidal antiinflammatory drugs
O_2^-	=	superoxide anion
OD	=	optical density
$ONOO^-$	=	peroxynitrite
PBS	=	phosphate-buffered saline
PD	=	Parkinson's disease
PGE_2	=	prostaglandin E_2
pH	=	potential of hydrogen
ROS	=	reactive oxygen species
rpm.	=	revolutions per minute

s	=	second
SEM	=	standard error of mean
SOD	=	superoxide dismutase
TNF-α	=	tumor necrosis factor-alpha
vs.	=	versus

CHAPTER I

INTRODUCTION

Oxidative stress is possibly involved in the aging process and is one of the pathogenic mechanisms of a variety of neurodegenerative diseases, such as Alzheimer's disease (AD), amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD) (Beal et al., 1993; Reiter, 1998; Cassarino and Bennett Jr, 1999). In the central nervous system (CNS), neuroglial cells are known to play an important role in maintaining normal homeostasis as well as regulation of inflammatory response. They provide physical and nutritional support to neurons (Merrill, 1987; Tsacopouloa and Magistretti, 1996). In addition, they play a pivotal role in providing neurotrophic factors which are crucial to neuronal health and function (Hou et al., 1997; Sawada et al., 2000; Nakajima and Kohsaka, 2001). Glial cells also protect neurons against toxicity of various compounds. One mechanism for the protective action of glial cells is supplying neurons with precursors necessary for reduced glutathione (GSH) synthesis and helping neurons maintain their GSH synthesis (Sagara et al., 1993; Bains and Shaw, 1997; Iwata-Ichikawa et al., 1999; Dringen et al., 2000).

Furthermore, neuroglial activation is one of the normal beneficial activities of glial cells. In response to injury glial cells become activated and increase their number and size, and change their pattern of gene expression. Activation of glial cells produces the potentially detrimental factors such as pro-inflammatory cytokines as well as reactive oxygen/nitrogen species. However, chronic glial activation or an abnormally high proportion of activated glial cells has the potential to initiate or exacerbate neuronal dysfunction in various diseases of the CNS. There are several studies showing that large numbers of activated astrocytes and microglia are a common pathological feature of many neurodegenerative disorders, including AD, sustained brain trauma, vascular insufficiency, acquired immunodeficiency syndrome (AIDS) and Down's syndrome (DS) (Griffin et al., 1998; Cotter et al., 2001).

Besides affecting neighboring neurons, the activated glial-derived factors also inhibit their own mitochondrial and cellular function (Bolanos et al., 1994; Noack et al., 2000). Recently, accumulating evidence suggests that the occurrence of

oxidative stress or mitochondrial dysfunction might originate in glial cells rather than in neurons and alterations in glial functions may be important contributors to the pathogenesis of neuronal degeneration. Numerous experiments indicated that a defective release of neurotrophic protective agents such as small antioxidants with free radical scavenging properties or neurotrophic factors may be involved in the pathogenesis of neurodegenerative disorders (Jenner and Olanow, 1998; Cotter et al., 2001).

Several lines of recent evidence suggest the potential of antiinflammatory compounds and antioxidative agents for modulation of chronic inflammatory processes as a therapeutic approach to a neurodegenerative disease (Schwartz et al., 1998; Van Eldik, 2001; Gonzalez-Pelez et al., 2002). Therefore, the ability of a single compound to exhibit antiinflammatory and antioxidant effects is attractive pharmacological treatments to modulate glial function in brain inflammatory response.

Piperine is the principal alkaloid present in the *Piper* species of Piperaceae family. This compound was found to possess a wide variety of pharmacological properties such as anticonvulsant, CNS depressant, respiratory stimulant, antipyretic, and analgesic activities (Kulshrestha et al., 1971; Piyachaturawat et al., 1981, 1982; Liu et al., 1984; Lee et al., 1984). Interestingly, some results revealed that piperine exerted the anti-inflammatory and antioxidant effects (Mujumdar et al., 1990; Koul and Kapil, 1993, Khajura et al., 1998; Mittal and Gupta, 2000).

Consequently, lipopolysaccharide (LPS) was used as a model stimulus to stimulate glial cells in order to investigate effects of piperine on injuries and oxidative changes in LPS-exposed glial cell cultures in this study. Our pharmacological standard of comparison was trolox, an antioxidant compound which was demonstrated to protect astrocytes from mitochondrial damage (Heales et al., 1994).

Hypothesis

Piperine exerts protective and/or toxic effects on lipopolysaccharide-induced injuries and oxidative changes in cultured rat brain glial cells.

Significance

The results from this study would be preliminary data of whether the treatment with piperine inhibits and/or induces injuries and oxidative changes in cultured rat brain glial cells mediated by lipopolysaccharide. Therefore, this information would be helpful in considering the possibility of piperine to increase and/or decrease endotoxin-induced inflammation and/or oxidative stress in glial cells in the CNS.

CHAPTER II

LITERATURE REVIEWS

PIPERINE

Piperine (1-piperoyl piperidine) is the principal alkaloid present in the *Piper* species of Piperaceae family, including black pepper (*Piper nigrum* Linn.) and long pepper (*Piper longum* Linn.) which are the most common species consumed by a large number of people throughout the world. It is also present in the leaves of *Rhododendron fauriae* and in the fruits of *Xylopia brasilliensis* (Verghese, 2002). This compound has the composition of $C_{17}H_{19}O_3N$ and molecular weight of 285.16. Piperine is a neutral or slightly alkaline, crystalline substance. It is insoluble in water but readily soluble in alcohol

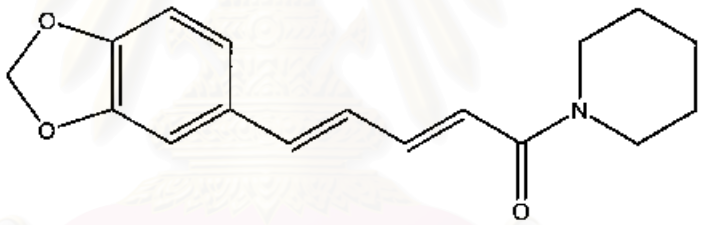


Fig. 1 Chemical Structure of Piperine

Pharmacological effects of piperine

Piperine has been used not only as food additive (as pungent agent) but also medicinally. It is considered to be the active principle of various *Piper* species which have been employed in folklore medicine for treatment of asthma, bronchitis, pyrexia, insomnia and abdominal disorder (Atal et al., 1975).

Pharmacological studies indicated that piperine possesses the central nervous system (CNS) depressant property. It was found that this compound and several of its derivatives protect experimental animals against seizures and convulsions induced by maximal electroshock, leptazol, picrotoxin and strychnine (Pei, 1983). Piperine and its derivative were reported to affect the central serotonergic system and this action

might be related to the anticonvulsant activity of these compounds (Pei, 1983; Liu et al., 1984; Mori et al., 1985). They also showed sedative, hypnotic, tranquilizing, and muscle-relaxing actions and could potentiate the depressive action of other depressive agents. For example, piperine was shown to increase barbiturate-induced sleeping time in mice (Mujumdar et al., 1990b). Additionally, pretreatment with this compound prolonged hexobarbital sleeping-time and increased zoxazolamine-induced muscle relaxation time. This potentiation of pharmacological activity by piperine is most likely caused by its inhibitory effect on the liver microsomal enzyme systems. This inhibition prevented prompt biodegradation and inactivation of the tested neurotropic compounds (Atal et al., 1985). Moreover, piperine was found to significantly block convulsions induced by intracerebroventricular injection of kainate. Nevertheless, it did not appear to act as a kainate receptor antagonist. It displayed no or only slight effects on convulsions induced by L-glutamate, N-methyl-D-aspartate (NMDA) (D′Hooge et al., 1996).

Conversely, some neurochemical studies revealed that piperine, especially at high doses, can stimulate CNS activity in various laboratory animals. For example, it was effective in counteracting respiratory depression induced in mongrel dogs by barbiturates (Singh et al., 1973). The main site of its analeptic (CNS-stimulating) activity has been postulated to be in the brain stem (Kulshrestha et al., 1971).

Further investigations demonstrated the pain-relieving action of piperine. It appeared that administration of piperine to rat cause an increase in the levels of pain-relieving β-endorphins in the brain. Moreover, this compound also reduced the level of substance P in the rat spinal cord (Micevych et al., 1983).

Besides its prominent effects on the CNS, piperine was shown to exert various interesting pharmacological activities such as antifertility activity (Piyachaturawat et al., 1982, 1991; Malini et al., 1999); antiamoebic activity (Ghoshal et al., 1996); antiulcer activity (Bai et al., 2000). It was also reported in one study to possess antipyretic, analgesic and anti-inflammatory activity (Lee et al., 1984).

Results from numerous experimental studies were in support of the anti-inflammatory action of piperine. It was found that treatment with piperine or oxyphenylbutazone, a common non-steroidal anti-inflammatory drug, significantly

inhibited the carrageenan-induced inflammation. Both of these treatments reduced the accumulation of lipid peroxides in the livers of the rats treated with carrageenan. In this connection, administration of either piperine or oxyphenylbutazone was shown to lower the levels of acid phosphatase, an enzyme which is known to leak from injured cells (Sharma et al., 1972). It also showed significant anti-inflammatory activity both in acute and chronic models of inflammation in experimental animals (Majumdar et al., 1999a).

Interestingly, one study indicated that piperine did not act as hepatotoxic agent but it functioned as a chemopreventive substance by enzyme modulation (Dalvi and Dalvi, 1991). Moreover, the compound was found to exert significant protection against chemically induced hepatotoxicity by reducing both *in vitro* and *in vivo* lipid peroxidation, enzymatic leakage of glutamate pyruvate transaminase (GPT) and alkaline phosphatase (AP), and by preventing the depletion of reduced glutathione and total thiols in the intoxicated mice (Koul and Kapil, 1993).

Most recently, systematic pharmacological studies on piperine revealed its antioxidant and free-radical scavenging properties. In DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging assay, the methanolic extracts of the fruits from some *Piper* species in the Piperaceae family (*P. chaba* Hunt, *P. longum* Linn and *P. nigrum* Linn.) exhibited markedly antioxidant activity with EC_{50} values of 47.8, 45.1 and 48.7 μg/ml, respectively, while those of aqueous extracts were 57.6, 69.4 and 56.9 μg/ml, respectively. In contrast, the volatile oils of these plants showed slight effect on DPPH scavenging with the EC_{50} values greater than 100 μg/ml (Tewtrakul, 1998). Additionally, piperine was found to modulate the oxidative changes by inhibiting the peroxidation and mediating enhanced synthesis or transport of GSH in chemical carcinogen-induced oxidative stress in rat intestinal model (Khajura et al., 1998). Piperine also acted as a hydroxyl radical scavenger at low concentrations, but at higher concentrations, it activated the fenton reaction resulting in increased generation of hydroxyl radicals. Furthermore, this compound was found to act as a powerful superoxide scavenger (Mittal and Gupta, 2000). However, several studies indicated that piperine contained very weak antioxidant and free-radical scavenging activities comparing to other spice principles. In one *in vitro* study, it was indicated that higher concentrations of piperine were required to completely inhibit superoxide

anion (O_2^-) and hydrogen peroxide (H_2O_2) released by activated macrophages when compared with curcumin (from turmeric) and capsaicin (from red chilies) (Joe and Lokesh, 1994). There is also scientific evidence indicating that subacute treatment with piperine for 14 days is only partially effective as an antioxidant therapy in streptozotocin-induced diabetic rats. This compound reversed the diabetic effects on oxidized glutathione (GSSG) concentrations in brain; on renal glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities; and on cardiac glutathione reductase (GRx) activity and lipid peroxidation but it did not reverse the effects of diabetes on hepatic GSH concentrations, lipid peroxidation, or GPx or catalase activities; on renal SOD activity; or on cardiac GPx or catalase activities (Rauscher et al., 2000). Moreover, piperine failed to scavenge O_2^- in nitrobluetetrazolium (NBT) reduction assay (Krishnakantha and Lokesh, 1993).

Effects of Piperine on Drug Metabolism

Various studies on piperine attribute its role as a bioavailability enhancer of many traditional and modern drugs such as hexobarbital, zoxazolamine, phenytoin, propanolol. Both *in vitro* and *in vivo* experiments demonstrated that piperine is a non-specific inhibitor of enzymes participating in the biotransformation of drug, thus slowing or partially preventing the inactivation and elimination of drugs (Atal et al., 1985; Singh et al., 1986; Bano et al., 1987; Mujumdar et al., 1990b; Kang et al., 1994; Reen et al., 1993; 1996). Another mechanism for piperine-increased bioavailability of drugs and nutrients may involve in non-specific mechanisms promoting rapid absorption of drugs and nutrients such as increased blood supply to the gastrointestinal tract and increased levels of enzymes such as γ-glutamyl transpeptidase (γGT), which participate in active and passive transport of nutrients to the intestinal cells (Johri and Zutshi, 1992).

Metabolism of Piperine

Upon administration of piperine to male albino rats at a dose of 30 mg (170 mg/kg) by gavage or 15 mg (85 mg/kg) intraperitoneally, about 97% was absorbed irrespective of the mode of dosing. Three percent of the administered dose was excreted as piperine in the feces. It was not detectable in urine and only trace (less than 0.15%) of piperine was detected in serum, kidney and spleen from 30 min to 24

hr. About 1-2.5% of the intraperitoneally administered piperine was detected in the liver during 0.5-6 hr after administration as contrasted with 0.1-0.25% of the orally administered dose. Four metabolites of piperine, piperonylic acid, piperonyl alcohol, pipseronal and vanillic acid were identified in the free form in 0-96 hr urine whereas only piperic acid was detected in 0-6 hr bile. The increased excretion of conjugated uronic acids, conjugated sulphates and phenols indicated that removal of the methylenedioxy group of piperine, glucuronidation and sulfation appeared to be the major steps in the disposition of piperine in the rat (Bhat and Chandrasekhara, 1986, 1987).

Toxicological effects of Piperine

Acute toxicity of piperine was investigated in different species of animals including mouse, rat and hamster. After receiving a fatal dose of piperine, they immediately exhibited an increase in locomotor activity. This activity was progressively increased from running, jumping, to convulsion and finally the death was occurred with whole body muscle spasm. The cause of death may possibly be the certain types of neurotoxicity and respiratory paralysis. It was reported that the LD_{50} values for single intravenous and intragastric administration of piperine are 15.1 and 330 mg/kg body weight respectively (Piyachaturawat et al., 1983).

Moreover, piperine pretreatment in rats potentiated carbon tetrachloride (CCl_4)-induced hepatotoxicity by interacting with liver cells and accelerated biotrans-formation of CCl_4, thereby increasing lipid peroxidation and enhancing hepatotoxicity (Piyachaturawat et al., 1995). *In vitro* studies demonstrated the cytotoxic effect of piperine on embryonic rat brain neurons in culture (Unchern et al., 1994a). It was shown to suppress both neuronal survival and neurite extension of viable neurons (Unchern et al., 1994b). In this connection, piperine-induced cytotoxicity was relatively selective for cultured neurons in comparison with cultured astrocytes. From these finding, Unchern and others (1997) suggested that cultured astrocytes are more tolerant and may respond to piperine in a different manner.

Glial cells

The central nervous system (CNS) consists of neurons and glial cells. Neuroglial cells provide structural and nutritional support and protection for neurons. They are known as the "supporting cells" of the nervous system. There are three types of glial cells in the CNS, the astroglia, oligodendroglia and microglia. Astroglia or astrocytes are the most abundant glial cell type in the brain. They provide physical support to neurons and clean up debris within the brain. Astrocytes also supply neurons with some of the chemicals needed for proper functioning and help control the chemical composition of fluid surrounding neurons. Moreover, they play a crucial role in providing nourishment to neurons. In addition to astroglia, microglia act as phagocytes that clean up CNS debris. Furthermore, they protect the brain from invading microorganisms. Oligodendroglia or oligodendrocytes produce the myelin sheath to insulate neurons (Merrill, 1987; Tsacopoulos and Magistretti, 1996).

Several lines of evidence suggest that both astroglia and microglia play an important role in maintaining normal homeostasis and regulation of inflammatory responses in the CNS (Mucke and Eddleston, 1993; Kreutzberg, 1996; Streit et al, 1999). It was shown that both of them synthesize and release many neurotrophic factors such as basic fibroblast growth factor (bFGF), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and nerve growth factor (NGF) to support neuronal growth and function in the developing and adult brain (Shimojo et al., 1991; Hyman et al., 1991; Knusel et al., 1991; Araujo and Cotman, 1992; Lin et al., 1993; Verity et al., 1998). In this connection, apoptosis in dopaminergic neuronal cultures induced by serum deprivation or by the withdrawal of astrocytic support was prevented by BDNF or GDNF incorporation (Thajeb et al., 1997; Burke et al., 1998). In addition, withdrawal of these factors was associated with vulnerability to cell damage or death (McNaught and Jenner, 2000a). *In vitro* studies indicated that the toxic effect of a variety of reactive oxygen species (ROS)-generating compounds on primary cultured neurons or a neuronal cell line was completely prevented by BDNF, GDNF as well as NGF (Tiffany-Castiglioni and Perez-Polo, 1981; Gong et al., 1999). Interestingly, several culture experiments reported that the glial-derived neurotrophic factors protect cultured neurons against ROS by an induction of free radical detoxifying mechanisms such as upregulation of

the concentration of glutathione and/or the activities of enzyme involved in the defense against ROS (Spina et al., 1992; Mattson et al., 1995; Pan and Perez-Polo, 1993; Hou et al., 1997; Sawada et al., 2000). Similarly, conditioned media derived from glia [glial conditioned medium (GCM)] enhanced neuronal resistance to oxidative stress by increasing transcription of γ-glutamyl-cysteine (γ-GluCys) synthase, the rate-limiting enzyme in glutathione synthesis (Iwata-Ichikawa et al., 1999).

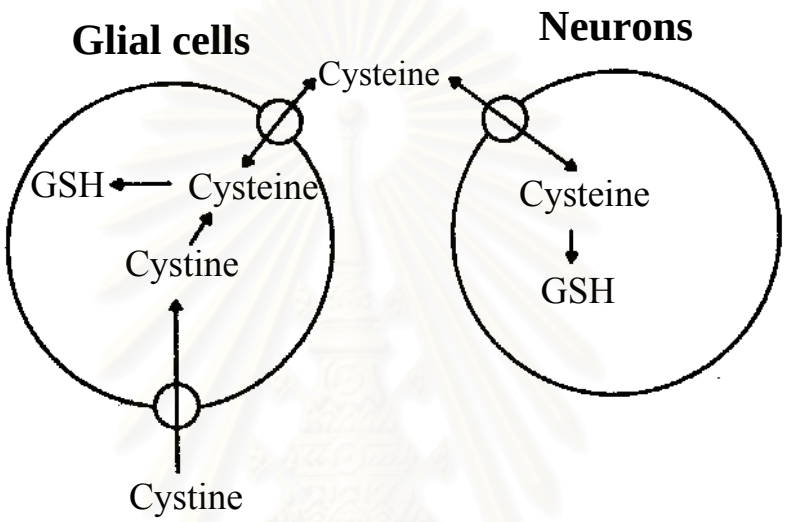


Fig. 2 Supply of cysteine in neurons by glial cells (Sagara et al., 1993)

In brain, glutathione system plays an important role in the detoxification of ROS. It is essential for cell survival and it is involved in protection against oxidative stress and conjugation of various toxic substances (Sagara et al., 1993; 1996; Makar et al., 1994; Bains and Shaw, 1997; Wullner et al., 1999). It was reported that glutathione deficiency induced in newborn rats by application of an inhibitor of γ-GluCys synthase, buthionine sulfoximine (BSO), led to mitochondrial damages in brain (Jain et al., 1991). Moreover, reduction of the brain glutathione content by BSO was found to enhance the toxic effects of insults that are associated with elevated production of ROS, i.e., ischemia or treatment with 1-methyl-4-phenylpyridinium (MPP^+) or 6-hydroxydopamine (6-OHDA) (Mizui et al., 1992; Wullner et al., 1996; Dringen, 2000). Furthermore, loss of glutathione was suggested to constitute signaling events in apoptotic cell death (Sato et al., 1995). Biochemical and histochemical analyses showed that the reduced glutathione (GSH) content was higher in glial cells than in neurons (Slivka et al., 1987; Philbert et al., 1991). In addition, the rapid

turnover of glutathione in cultured astrocytes was also reported in one study (Yudkoff et al., 1990). Further investigation indicated that glial cells provide neurons with cysteine, a precursor necessary for GSH synthesis, and help neurons maintain their GSH synthesis [Fig. 2] (Sagara et al., 1993). Moreover, glial cells were reported to support the survival of neurons during oxidative stress induced by exposure to glutamate (Rosenberg and Aizenman, 1989), H_2O_2 (Desagher et al., 1996; Iwata-Ichikawa et al., 1999), L-DOPA (Han et al., 1996), or 6-OHDA (Hou et al., 1997; Iwata-Ichikawa et al., 1999). In three of these studies (Han et al., 1996; Hou et al., 1997; Iwata-Ichikawa et al., 1999), the neuroprotective effect of glial cells was attributed to increase in the content of GSH. In this connection, glia-mediated protection from H_2O_2 toxicity was abolished by depletion of GSH (Drukarch et al., 1997). These results suggest the pivotal role of glial GSH in the support of neuronal survival.

Although, glial cells have several protective functions in the CNS, there is increasing interest in the possibility that glial cells may be a major contributor to the pathogenesis of normal aging and several CNS disorders, including inflammatory, traumatic, infectious and degenerative diseases. Neuroglial activation is one of the normal beneficial activities of glial cells, serving mechanisms for brain to respond to signals that are potentially deleterious by maintaining ionic and chemical balance, cleaning up debris and restoring homeostasis and neuronal survival. These activated glial cells express some of the potentially detrimental factors, including pro-inflammatory cytokines and chemokines, oxidative stress-related enzymes, and components of the immunologic and complement cascades to initiate and maintain immunological defense to protect the brain. When these restorative functions are complete, the normal feedback loops that keep the amplitude and duration of glial activation responses in check lead to a return of the glia to a non-activated state. However, some reports claimed that in a number of neurodegenerative diseases, this orderly progression is inoperative or overwhelmed. It showed that this more intense or prolonged glial activation could potentially lead to neurotoxicity through propagation of a localized inflammation and oxidative stress cycle (Minghetti and Levi, 1998; Stollg and Jander, 1999; Raivich et al., 1999; Van Eldik, 2001; Nakajima and Kohsaka, 2001).

Increasing evidence suggests that the occurrence of oxidative stress or mitochondrial dysfunction might originate in glial cells rather than in neurons and alteration of glial functions may be an important factor contributing to the neurodegeneration [Fig. 3] (Hirsch et al., 1998; Jenner and Olanow, 1998; Hirch and Hunot, 2000; McNaught and Jenner, 2000a, 2000b; Cotter et al., 2001). There was a 40% decrease in the levels of the GSH (Sian et al., 1994), an increase in iron levels (Dexter et al., 1991), and a 37% decrease of complex I activity of mitochondrial respiratory chain (Mizuno et al., 1989; Schapira et al., 1990) in homogenates of post-mortem Parkinson's disease (PD) nigral tissues. The magnitude of the nigral alterations is too great to take place solely in dopaminergic neurons, which make up only 1-2% of the total cell population in the substantia nigra in brain. Indeed, some neurochemical studies indicated that most GSH in brain is present in glial cells (Sagara et al., 1993), and only glial cells stain positive for elevated non-heme iron in substantia nigra in PD (Morris and Edwardson, 1994). Significantly higher activity of complex I in astrocytes compared to neurons was also reported in one study (Stewart et al., 1998). From these findings, McNaught and Jenner suggested that the GSH depletion and complex I inhibition, for example, occur predominantly in glial cells which involved in the pathogenesis of PD (McNaught and Jenner, 1999, 2000a, 2000b).

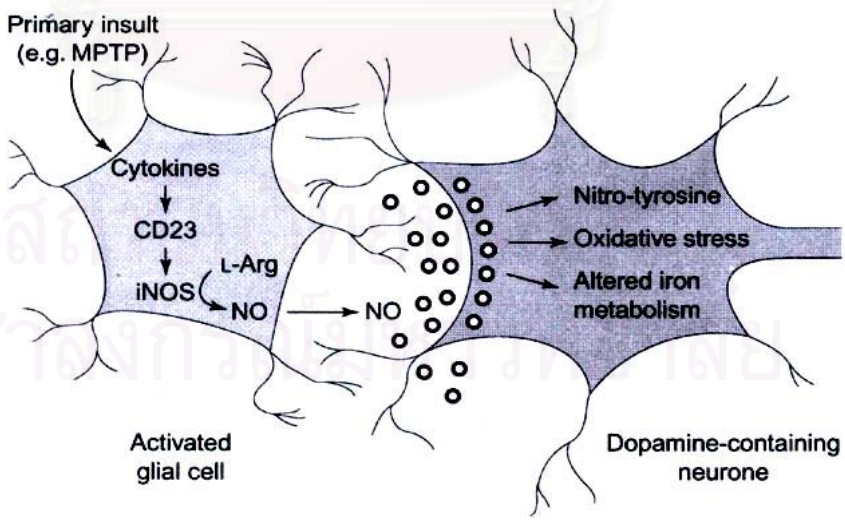


Fig. 3 Possible contribution of the glial reactions to the degeneration of dopamine-containing neurons in Parkinsonian syndromes (Hirsch and Hunot, 2000)

In addition, the post-mortem findings from patients with PD indicated that the loss of dopamine-containing neurons in the substantia nigra is associated with a massive astrogliosis and the presence of activated microglial cells (McGeer et al., 1988). Similarly, a massive gliosis in the substantia nigra was also observed in animal models in which the PD is induced by intoxication with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Kurkowska-Jastrzebska et al., 1999). Like PD, activated glia cells were closely associated with amyloid plaques in Alzheimer's disease (AD) brain (Griffin et al., 1998). Additionally, it was found that neuroglial activation is initially localized to the region of neuronal death after cerebral ischemia or traumatic brain injuries (Apelt and Schliebs, 2001).

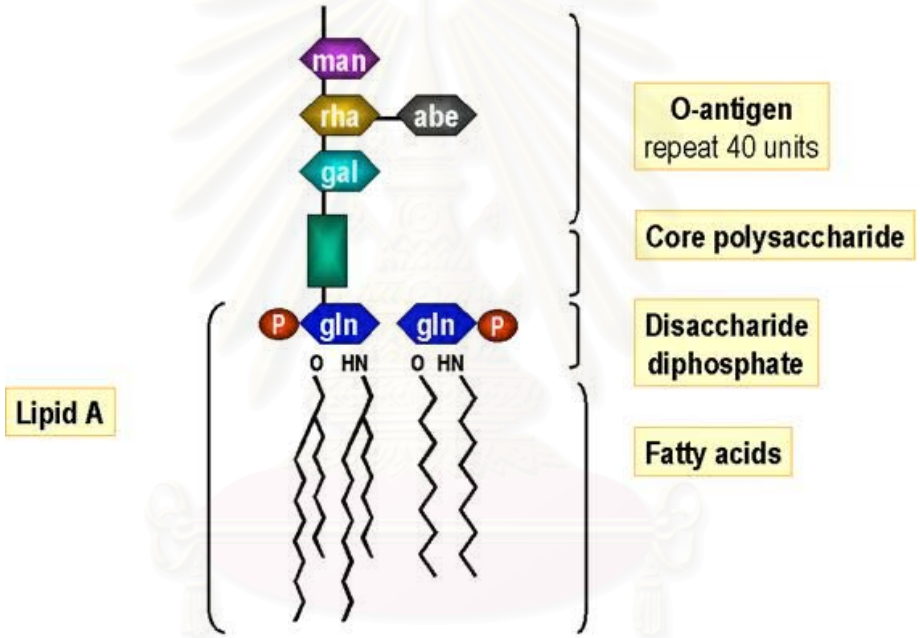


Fig. 4 Structure of lipopolysaccharide (http://www.med.sc.edu: 85/fox.lps.jpg)

Numerous neurochemical studies indicated that activated glia can kill neurons in co-culture (Hu et al., 1997; Kingham et al., 1999; Tanabe et al., 1999), and this may occur *in vivo* during brain trauma, inflammation, post-ischemia, and infection, and in neurodegenerative diseases (Bolanos and Almeida, 1999; Liberatore et al., 1999). The mechanisms by which activated glia kill neurons in culture was suggested to include the release of toxic compounds such as nitric oxide (NO) (Merrill et al., 1993; Hu et al., 1997; Hirsch et al., 1998; Hirsch and Hunot, 2000; Bal-Price and Brown, 2001), ROS (Tanaka et al., 1994), glutamate (Barger and Basile et al., 2001)

and cytokines (Robbins et al., 1987; Selmaj and Raine, 1988; Hirsch et al., 1998). Some investigations reported that glial cells become activated by inflammatory mediators in a wide range of CNS pathologies such as β-amyloid protein (Aβ) [the primary plaque component in AD], HIV-coated protein gp120 as well as microbial products like bacterial lipopolysaccharide (LPS) (Akama et al., 1998; Jeohn et al., 2000a). Also, in several *in vitro* experiments, these cells were induced by cytokines such as interleukin-1 (IL-1), interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) or by a combination of the cytokines and LPS (Libermann et al., 1989; Lee et al., 1993; Meda et al., 1995).

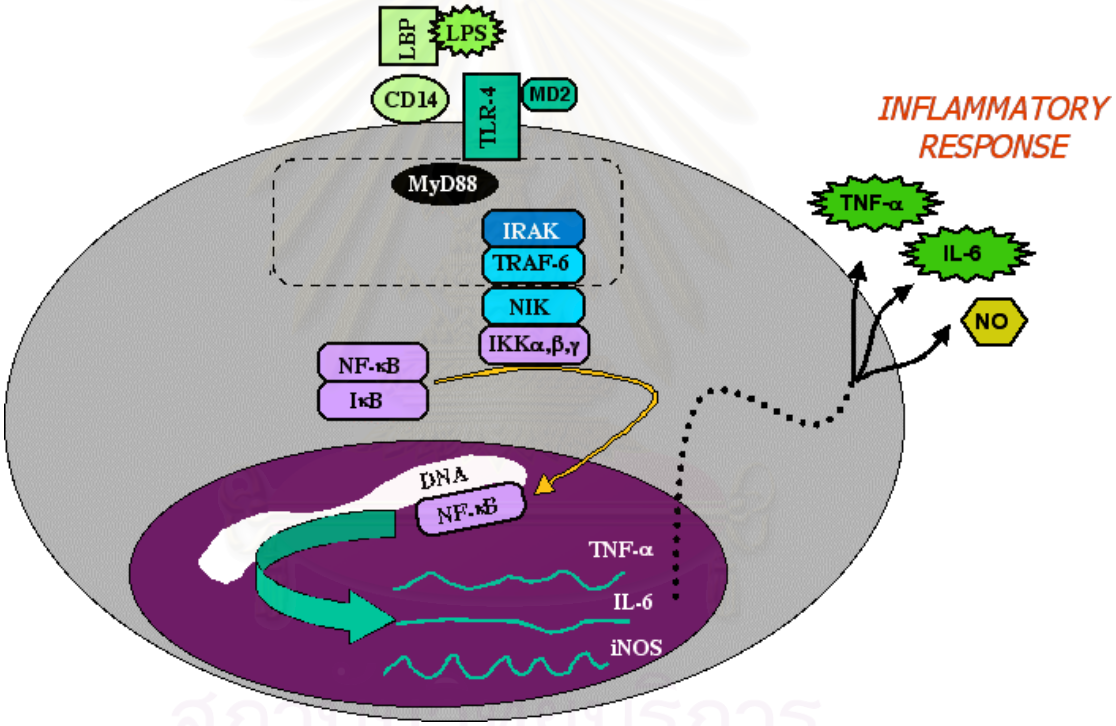


Fig. 5 Some of intracellular LPS-signaling pathways (http://www.med.sc.edu: 85/fox/lps.jpg.)

Accumulating evidence suggests that TLR4 serves as a major pattern recognition receptors (PRR) for signaling initiated by LPS. It was shown that TLR4 is activated by LPS after it binds to LPS-binding protein (LBP) and CD14. After activation, the TLR4 homodimer signals the adaptor protein MyD88 through the TIR domain, and this in turn recruits the IL-1R-associated kinase (IRAK) to the receptor complex. IRAK then autophosphorylates and activates TRAF6, which through

intermediate kinases leads to phosphorylation and degradation of the NF-κB inhibitor, IκB. NF-κB is now free to translocate to the nucleus to activate target genes [Fig. 5]. However, some studies indicated that protein kinases such as protein tyrosine kinase and the mitogen-activated protein kinases (MAPKs, ERK and p38 kinase) cascade also regulate the induction of iNOS/NO in brain glia (Feinstein et al., 1994; Bhat et al., 1998; Chen et al., 1998; Jeohn et al., 2000b). In addition to NO, pro-inflammatory cytokines such as TNF-α, IL-6 were also induced by LPS (Lee et al., 1993; Kong et al., 1997). The pro-inflammatory cytokines, in combination, can re-stimulate glia in an autocrine fashion to further increase the level of NO production (Romero et al., 1996), which may exacerbate the inflammatory response.

Within the brain, astrocytes and microglial cells may be a major source of NO because these cells were found to have the highest concentration of the NO precursor L-arginine and possess the inducible form of NO synthase (Galea et al., 1992; Simmons and Murphy, 1992; Murphy et al., 1993; Feinstein et al., 1994; Tholey, 1998). Several investigations revealed that the induction of iNOS play a role in neuronal damage in chronic neurodegenerative disorders as well as inflammatory diseases of the CNS. In AD, activated microglial and astrocytes expressing iNOS were found in the amyloid plaques surrounded by dead and dystrophic neurites (Wa et al., 1996; Wallace et al., 1997). Moreover, the post-mortem findings from patients with PD demonstrated that the production of NO by glial cell might participate in the cascade of events that leads to degeneration of dopamine-containing neurons in parkinsonism syndromes (Liberatore et al., 1999). Similarly, an increased number of glial cells that express iNOS and of the content of proteins that contain nitro-tyrosine were observed at autopsy in the substantia nigra of patients with PD (Hirsch and Hunot, 2000). Furthermore, there are several studies indicating that transcriptional induction of iNOS in the CNS is associated with autoimmune reactions, acute infection, and traumatic injury (Koprowski et al., 1993; Bagasra et al., 1995; Adamson et al., 1996; Oleszak et al., 1997; Samdani et al., 1997). In addition, NO was synthesized by microglial cells in the MPTP-intoxicated mice (Liberatore et al., 1999).

Evidences that would seem to support a role of glial-derived NO in neuronal degeneration have been obtained from numerous culture experiments. It was shown

that activation of astrocytes and/or microglial cells by LPS or cytokines causes neuronal death in mixed glial/neuronal co-cultures by a mechanism involving glial iNOS mRNA expression and production of NO from glial cells (Chao et al., 1992; Dawson et al., 1994; Goureau et al., 1999; McNaught and Jenner, 1999; Jeohn et al., 2000a). In addition, it appeared that microglial cell cytotoxicity of oligodendrocytes is mediated through NO (Merrill et al., 1993). In one study reported that the neurotoxicity of LPS activated astrocytes is inhibited by the inducible nitric oxide synthase inhibitor aminoguanidine, by the nitric oxide scavenger 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylmidazoline-1-oxyl-3-oxide, and by GSH (McNaught and Jenner, 1999). Beside LPS and/or cytokines, some observations also indicated that β-amyloid can stimulate NO production from glial cells (Simmons and Murphy, 1992; Rossi and Bianchini, 1996; Akama et al., 1998; Akama and Van Eldik, 2000). The molecular mechanism by which NO could involve in oligodendrocyte and neuronal cell death is still a matter of debate, however, there are several possible explanations that might account for its deleterious role. For example, NO was shown to release iron from ferritin leading to accumulation of free iron, which can induce oxidative damages to cells via the fenton reaction (Aisen et al., 1999). Alternatively, NO also reacted with the O_2^- to form peroxynitrite ($ONOO^-$), an extremely potent oxidizing agent, which can inhibit mitochondrial respiratory chain on neighboring neurons (Bolanos et al., 1995). Currently, NO from activated glia was reported in one study to inhibit neuronal respiration resulting in glutamate release which subsequently causes excitotoxic death of the neurons [Fig. 6] (Bal-Price and Brown, 2001).

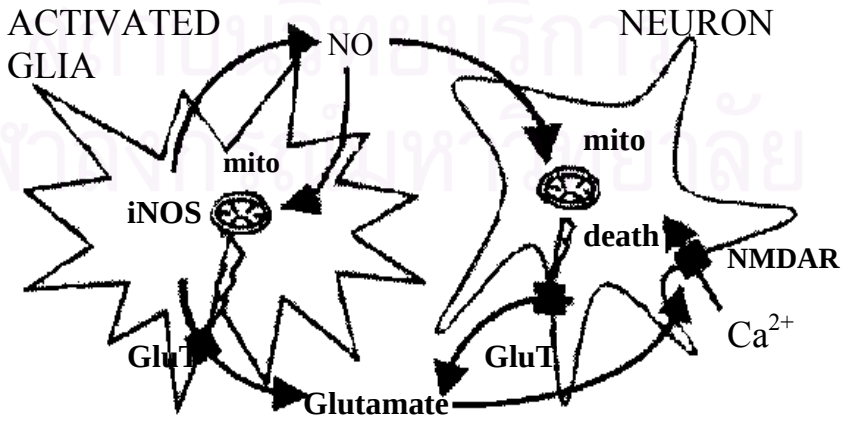


Fig.6 Proposed scheme of inflammatory neurodegeneration mediated by activated glia (Bal-Price and Brown, 2001)

Beside NO, LPS-induced activation of cultured glial cells was found to release glutamate and H_2O_2 (McNaught and Jenner, 2000b; Barger and Basile, 2001). The mechanisms of extracellular glutamate and H_2O_2 accumulation in astrocytic cultures are still unclear, but have been purposed that glutamate and H_2O_2 may be released from astrocytes secondary to impairment of respiratory chain by LPS-generated NO. Moreover, experimental evidence revealed that activated glial cells can release O_2^- (Colton and Gilbert, 1987; Pou et al., 1992). Additionally, one neurochemical study reported that activated microglial-derived O_2^- is toxic to PC12 cells (Tanaka et al., 1994).

Apart from free radicals and excitotoxin, pro-inflammatory cytokines may contribute to the deleterious role of neuroglial cells. The nervous system, by itself, can produce cytokines such as TNF-α, IFN-γ, IL-1 and IL-6. In the normal intact CNS, these cytokines exert neurotrophic effect and/or neuroprotective actions such as restoration of homeostasis, promotion of neuritic process outgrowth and differentiation of CNS neurons (Giulian and Lachman, 1985; Munoz-Fernandez and Fresno, 1998). However, many studies showed that the exacerbated production of these cytokines which occurs during inflammation or prolonged infection may lead to CNS damages in various pathological conditions (Robbins et al., 1987; Lieberman et al., 1989; Chung et al., 1991; Henderson and Blake, 1992; Meda et al., 1995). For example, elevated levels of TNF-α, IL-1 or IL-6 have been detected in the brain of patients with ischemic or traumatic injuries, multiple sclerosis (MS), AIDS, PD and AD (Tyor et al., 1992; Mogi et al., 1994; Kong, et al., 1997). Under *in vitro* condition, IL-1 receptor antagonists markedly reduced the infarct volume in an animal model of focal ischemia (Relton and Rothwell, 1992). In addition, TNF was shown to cause oligodendrocyte injury, which contributes to myelin damage and/or the demyelination process observed in AIDS and MS (Selmaj and Raine, 1988). Similarly, systemic administration of IFN-γ was reported in one study to worsen the disease in MS patients (Panitch et al., 1987) and *in vitro* it is able to induce oligodendrocyte death by apoptosis (Vartanian et al., 1995).

The major source of pro-inflammatory cytokines in the brain appears to be activated microglia and astrocytes (Lieberman et al, 1989; Chung and Benveniste, 1990; Lee et al., 1993; Mrak et al., 1995). It was shown that many glial cells

displaying IL-1β immunoreactivity are detected in the substantia nigra of patients with PD (Hirsch et al., 1998). Additionally, chronic microglial activation with overexpression of IL-1β was also observed in Alzheimer's and Down's syndrome patients (Cacabelos et al., 1994; Griffin et al., 1998). Moreover, glial cells were reported in one study to express TNF-α in the substantia nigra of patients with PD (Boka et al., 1994) and in the MS plaque (Hofman et al., 1989). Recently, immunohistochemical detection of IL-1β, IL-6 and TNF-α were localized to glia surrounding Aβ deposit in Tg2576 mice (Sly et al., 2001). In addition, under conditions of oxidative stress, the IL-1β released by activated glia could contribute to neurodegeneration. Accordingly, it was shown that exposure of mesencephalic cultures to IL-1β increases BSO-induced damage (Kramer et al., 2002).

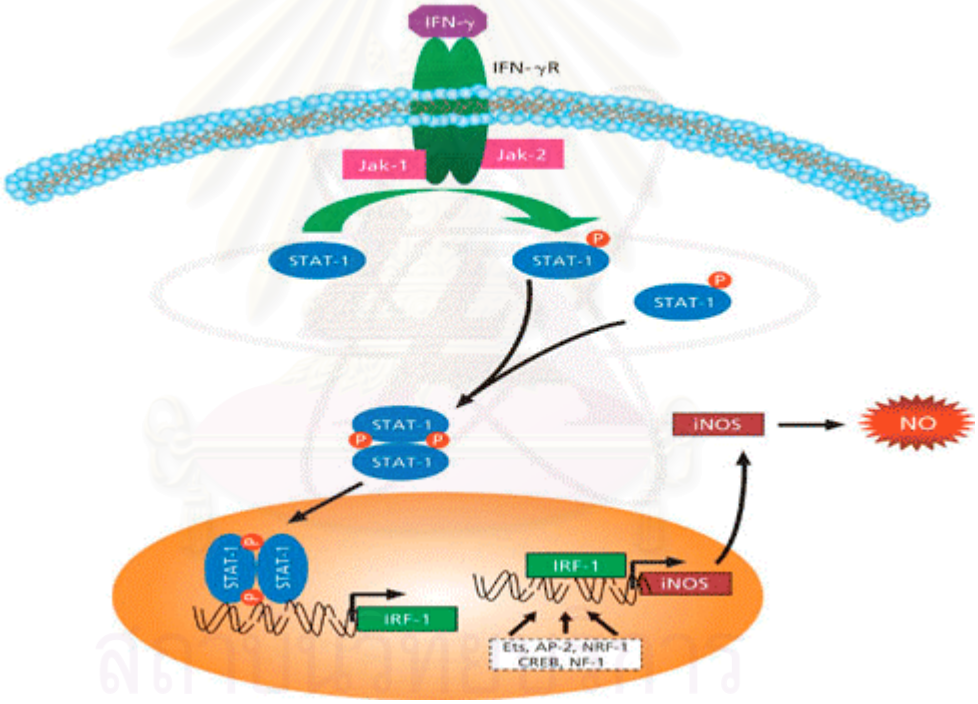


Fig. 7 Cytokine-signaling pathway
(http://www.sigmmaldrich.com/img/assets/6460.inos.gif.)

The mechanism by which cytokines may play a detrimental role for the neurons and oligodendrocytes is not known. Several lines of evidence suggest that these cytokines may act synergistically to induce the inflammatory reaction by stimulating their own and other cytokine synthesis as well as NO synthesis [Fig. 7] (Meda et al., 1995; Hirsch et al., 1998). Moreover, some experiments indicated that

activation of TNF-α transduction pathway involved a transient production of oxygen free radicals and translocation of the transcription factor NFκB, and that these phenomena ended with almost complete neuronal degeneration by apoptosis (Hunot et al., 1997).

Like pro-inflammatory cytokines, several lines of evidence suggest that S100β is a glia-derived cytokine which involves in the progression of CNS diseases. At low concentrations, this molecule was shown to have neurotrophic functions that include promotion of neurite outgrowth and promotion of neuronal survival and differentiation *in vivo* (Barger et al, 1992). However, overexpression of S100β was observed in AD, DS and epilepsy. Moreover, it was reported that elevated S100β levels in AD coincide with regions of high neuropathology, such as regions around amyloid plaques (Griffin et al., 1995; 1998) and mice that express S100β were found to have defects in brain functions (Reeves et al., 1994). The deleterious effects of S100β were suggested through its ability to induce pro-inflammatory cytokines and oxidative stress enzymes in glial cells, and to enhance other stimuli to activate glia (Donato, 1999; Lam et al., 2001). This molecule was shown to induce neuronal cell death through nitric oxide release from astrocytes (Hu et al., 1997).

Compared to neurons, astrocytes are enriched in antioxidant enzymes [Fig. 8] (Makar et al., 1994; Desagher et al., 1996). However, the potentially activated glia-derived toxic mediators also alter their own mitochondrial and cellular functions, which create additional oxidative stress in brain. It was found that LPS/IFN-γ mediated NOS induction causes inhibition of mitochondrial respiratory chain without apparent cell death in cultured glial cells (Bolanos et al., 1994). Nitrosative stress also occurred in primary glial cultures after induction of iNOS by LPS and IFN-γ (Noack et al., 2000). Currently, LPS treatment was shown to cause significant reduction in glutathione content in both astrocytes and mixed neuronal cultures. Furthermore, LPS potentiated the effect of BSO on GSH depletion, which suggests that the inflammatory response of glial cells causes oxidative stress and has the potential to cause oxidative damage (Kramer et al., 2002). Moreover, in the presence of LPS-treated astroglial cells the toxicity of 6-hydroxydopamine to tyrosine hydroxylase-positive neurons was increased (Bronstein et al., 1995).

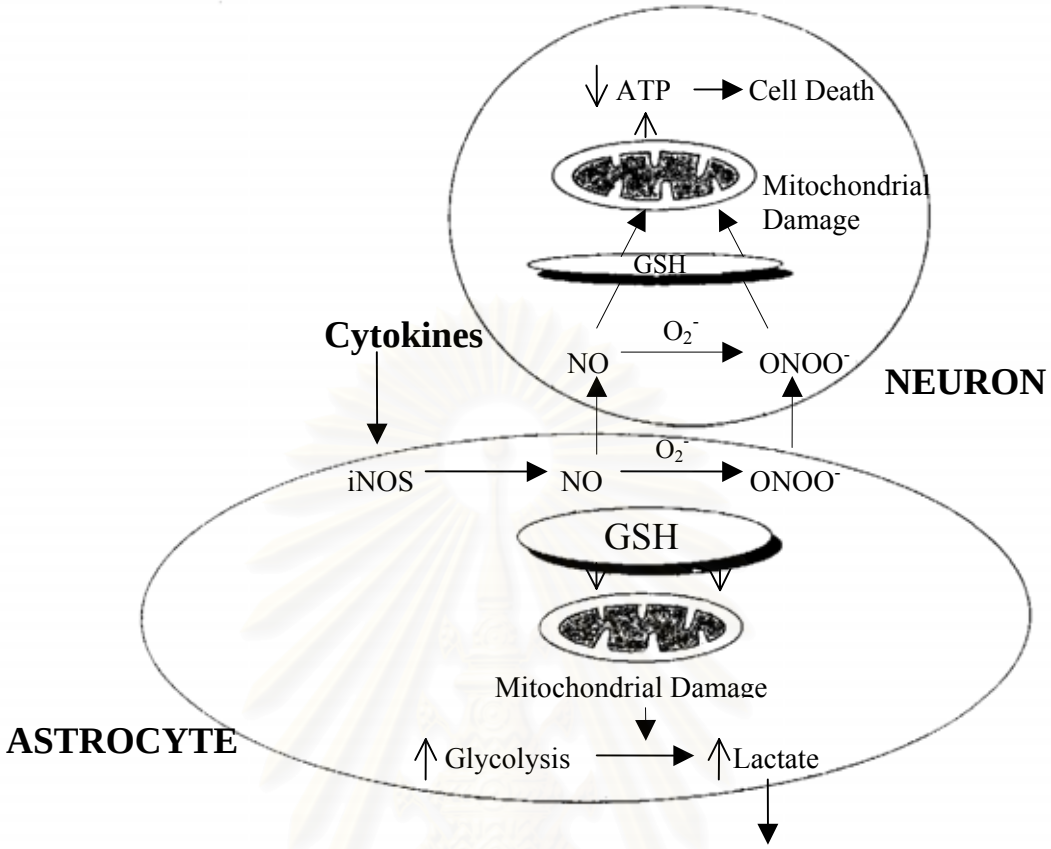


Fig.8 Neuronal death as a result of astrocyte generation of NO and $ONOO^-$ (Heales et al., 1999).

Beside LPS-activated glial cells, alteration of glial function by depletion of glutathione levels with L-BSO, or inhibition of complex I activity with MPP^+ in cell cultures was shown to cause the extracellular accumulation of NO, H_2O_2 and glutamate (McNaught and Jenner, 2000b). Additionally, LPS activation or inhibition of complex I activity stimulated TNF-α release while glutathione depletion or complex I inhibition impaired GDNF release in primary cultures of rat brain astrocytes (McNaught and Jenner, 2000a). Furthermore, LPS-activated or glutathione-depleted glial cells potentiated the ability of MPP^+ or 6-OHDA to cause neurotoxicity in astrocytic/ventral mesencephalic co-cultures (McNaught and Jenner, 1999). In this connection, it was reported in one study that glutathione-depleted glial cells generate ROS which leads to degeneration of co-cultured neurons (Mytilineou et al., 1999). These findings suggest that altered glial functions can lead to further oxidative stress

in themselves, which contribute to initiation or exacerbation of the pathology in various diseases of the CNS.

Interestingly, there have been many scientific evidences indicated that anti-inflammatory compounds can modulate detrimental glial response. For example, dexamethasone, clexan and pentoxifylline were shown to inhibit production of inflammatory mediators by glial cells (Schwartz et al., 1998). Moreover, it was reported that carbamazepine, antiepileptic drug, which possesses both anti-inflammatory and anticonvulsive properties inhibits NO and plostaglandin-E_2 (PGE_2) production by stimulated glial cells (Matoth et al., 2000). Currently, anti-inflammatory drug K2529, an inhibitor of calmodulin regulated protein kinases (CaMKs), was reported in one study to block induction of both the oxidative stress related enzymes iNOS and the proinflammatory cytokine IL-1β in primary cortical glial cultures and the microglial BV-2 cell line (Watterson et al., 2001). Additionally, clinical and epidemiological studies revealed that there is a lower AD incidence in individuals on chronic non-steroidal anti-inflammatory drugs (NSAIDs) (Van Eldik, 2001).

Similarly, the antioxidant trolox was shown to protect mitochondrial complex IV from nitric oxide-mediated damage in astrocytes (Heales et al., 1994). Furthermore, N-acetyl cysteine (NAC), a potent antioxidant, inhibited the induction of NO production by endotoxin (LPS) or cytokines (IL-1β, IFN-γ, TNF-α) in peritoneal macrophages, C6 glial cell line, and primary astrocytes (Pahan et al., 1998). Moreover, one previous experiment demonstrated that membrane-permeable antioxidants including estrogen derivatives (e.g. 17β-estradiol), thiol antioxidants e.g. (R+)-alpha-lipoic acid) and *Gingko bioloba* extract Egb761 attenuate the expression of iNOS mediated by advanced glycation endproducts (AGEs) in murine microglia (Wong et al., 2001). Moreover, estrogen was shown to prevent the production of inflammatory mediators: iNOS, PGE_2 and metalloproteinase-9 (MMP-9) (Vegoto et al., 2001). Recently, alpha-lipoic acid (LA), an antioxidant with broad neuroprotective capacity, induced detoxification enzymes [NADPH quinone oxidoreductase (NQO1)] and glutathione-S-transferase (GST) in cultured astroglial cells (Flier et al., 2002). In addition to culture system, α-LA and vitamin E mixture reduced glial reactivity and

enhanced neuronal remodeling in cerebral tissues of adult rats injured by ischemic embolism (Gonzalez-Perez et al., 2002).

CHAPTER III

MATERIALS AND METHODS

Experimental Animals

Timed pregnant Wistar rats of embryonic day 18 (E18) were supplied by the National Laboratory Animal Center, Mahidol University, Salaya, Nakornpathom, Thailand. They were allowed free access to food [F.E. Zeulig, Thailand] and water in the Central Animal House, Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Chemicals

5-5'-dithiobis-(2-nitrobenzoic acid) [Sigma, U.S.A.]

Deoxyribonuclease I (DNase I) [Sigma, U.S.A.]

Dimethyl sulfoxide (DMSO) [BDH, England]

Dulbecco's Modified Eagle's Medium (DMEM) [Sigma, U.S.A.]

Dulbecco's Phosphate Buffered Saline (DPBS) [Sigma, U.S.A.]

Equine serum [Hyclone, U.S.A.]

Ethylenediaminetetraacetic acid (EDTA) [Sigma, U.S.A.]

Fetal Bovine Serum (FBS) [Hyclone, U.S.A.]

Glutathione (reduced form) (GSH) [Sigma, U.S.A.]

Glutathione reductase (GRx) [Sigma, U.S.A.]

Griess reagent [Sigma, U.S.A.]

Hank's balanced salt solution (HBSS) [Sigma, U.S.A.]

Human transferrin [Sigma, U.S.A.]

In vitro toxicology assay kit (Lactate Dehydrogenase Based) [Sigma, U.S.A.]

Insulin from bovine pancrease [Sigma, U.S.A.]

Lipopolysaccharide (LPS) (from *E. coli* Serotype 0127: B8) [Sigma, U.S.A.]

Penicillin G sodium [Sigma, U.S.A.]

Poly-D-lysine hydrobromide [Sigma, U.S.A.]

Progesterone [Sigma, U.S.A.]

Putrescin dihydrochloride [Sigma, U.S.A.]

Sodium nitrite [Sigma, U.S.A.]

Streptomycin sulfate [Sigma, U.S.A.]

Sulfosalicylic acid [Sigma, U.S.A.]

Triton X-100 [Sigma, U.S.A.]

Trypan blue [Sigma, U.S.A.]

Trypsin [Sigma, U.S.A.]

Piperine and Trolox

In all experiments, piperine [Sigma, U.S.A.] and trolox [Sigma, U.S.A.] were dissolved in DMSO for treatment of cells. The final concentration of DMSO was ≤ 0.5%. All the solutions were sterilized through a 0.22 μm Millipore (Bedford, MA) filter. Control samples were treated with the corresponding amount of DMSO.

Culture Media

Dulbecco's modified Eagle's medium (DMEM, high glucose) was supplemented with 99 μg/ml sodium pyruvate, 3.7 mg/ml sodium bicarbonate, 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate, 10% (v/v) fetal bovine serum (FBS). While serum-free DMEM was composed of normal DMEM supplemented with 30 nM selenium, 100 μg/ml human transferrin, 10 μg/ml bovine insulin and 20 nM progesterone.

Materials

24-well cell culture plate [Nunc, Denmark]

96-well microtiter plate [Nunc, Denmark]

Cell culture dish: diameter 35-mm and 100-mm [Nunc, Denmark]

Cell culture flask: 25 cm^2, 75 cm^2 [Nunc, Denmark]

Conical tube: 15 ml, 50 ml [Nunc, Denmark]

Sterile Milled – GV (0.22 μm filter unit) [Millipore, U.S.A.]

Sterivex – GS (0.22 μm filter unit with filling bell) [Millipore, U.S.A.]

Instruments

Autoclave [Hirayama, Japan]

Carbon dioxide incubator [Forma Scientific, U.S.A.]

Centrifuge [Kokusan, Japan]

Inverted microscope [Zeiss, Germany]

Laminar air flow hood [Hepaco, U.S.A.]

Microliter pipette : 10–100 μl [Labsystems, Findland]

Microliter pipette : 200–1000 μl, 1–5 ml [Labsystems, Findland]

Microplate reader [Anthos htl, Australia]

Microplate reader [Biorad model 3550, U.S.A.]

pH meter [Beckman Instruments, U.S.A]

Sonicator [Elma, Germany]

Surgical equipments

Vortex mixer [Clay adams, U.S.A.]

Water bath [Thelco, U.S.A.]

Methods

Glial cell cultures

Primary mixed glial cell cultures were prepared from the cerebral cortex of embryonic day 21 Wistar rats (Matoth et al., 2000). After removal of the meninges and blood vessels the tissue was cut into pieces and incubated with 0.25% trypsin and 0.01% DNase I in Ca^{++}/Mg^{++}-free Hank's balanced salt solution for 20 min at 37 °C. Trypsinization was terminated by the addition of an equal volume of equine serum and tissue fragments were centrifuged at 3,000 rpm for 10 min. The tissue pellet was gently resuspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The single cells were dissociated by gently passing the suspension through a flame-polished Pasteur pipette 15 to 20 times. The cell suspension was filtered through two sheets of nylon net [50 μm-mesh] to remove cell lumps and diluted with DMEM containing 10% FBS. The cell suspension was plated into poly-D-lysine coated 75 cm^2 plastic culture flasks and grown in DMEM containing 10% FBS in a humidified incubator maintaining at 37 °C and containing 5% CO_2-95% air atmosphere. When the cultures reached confluency [approximately 7-10 days], the cells were removed from the flasks by trypsinization. The culture medium was aspirated and further incubated with 0.05% trypsin-EDTA solution for 15 min with gentle agitation, followed by the addition of an equal volume of DMEM containing 10% FBS. The cell suspension was centrifuged at 3,000 rpm for 10 min and the pellet was suspended in fresh DMEM containing 10% FBS. The cells were dissociated by passing through a flame-polished Pasteur pipette and then filtered through two sheets of nylon net. The cells were counted on hemocytometer using trypan blue exclusion. After diluting with culture medium, aliquots of 0.8 ml were plated at a density of 1×10^5 cells/cm^2 in poly-D-lysine-coated 24-well culture plates and cultures were incubated in a humidified atmosphere of 5% CO_2-95% air at 37 °C. After growth to confluency [approximately 5-7 days after plating], the cells were used in experiments.

Fig. 9 Preparation of primary glial cells cultures

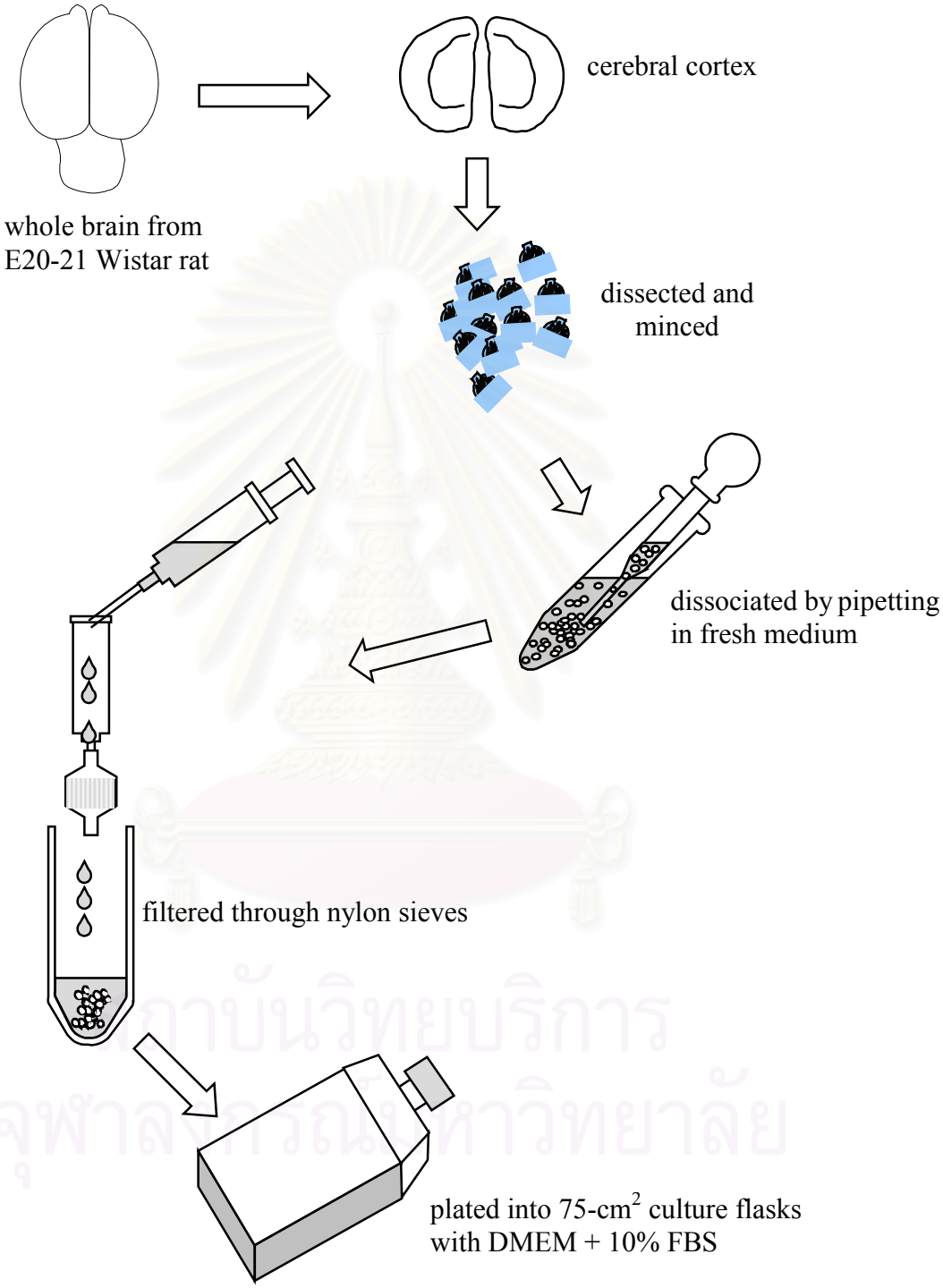
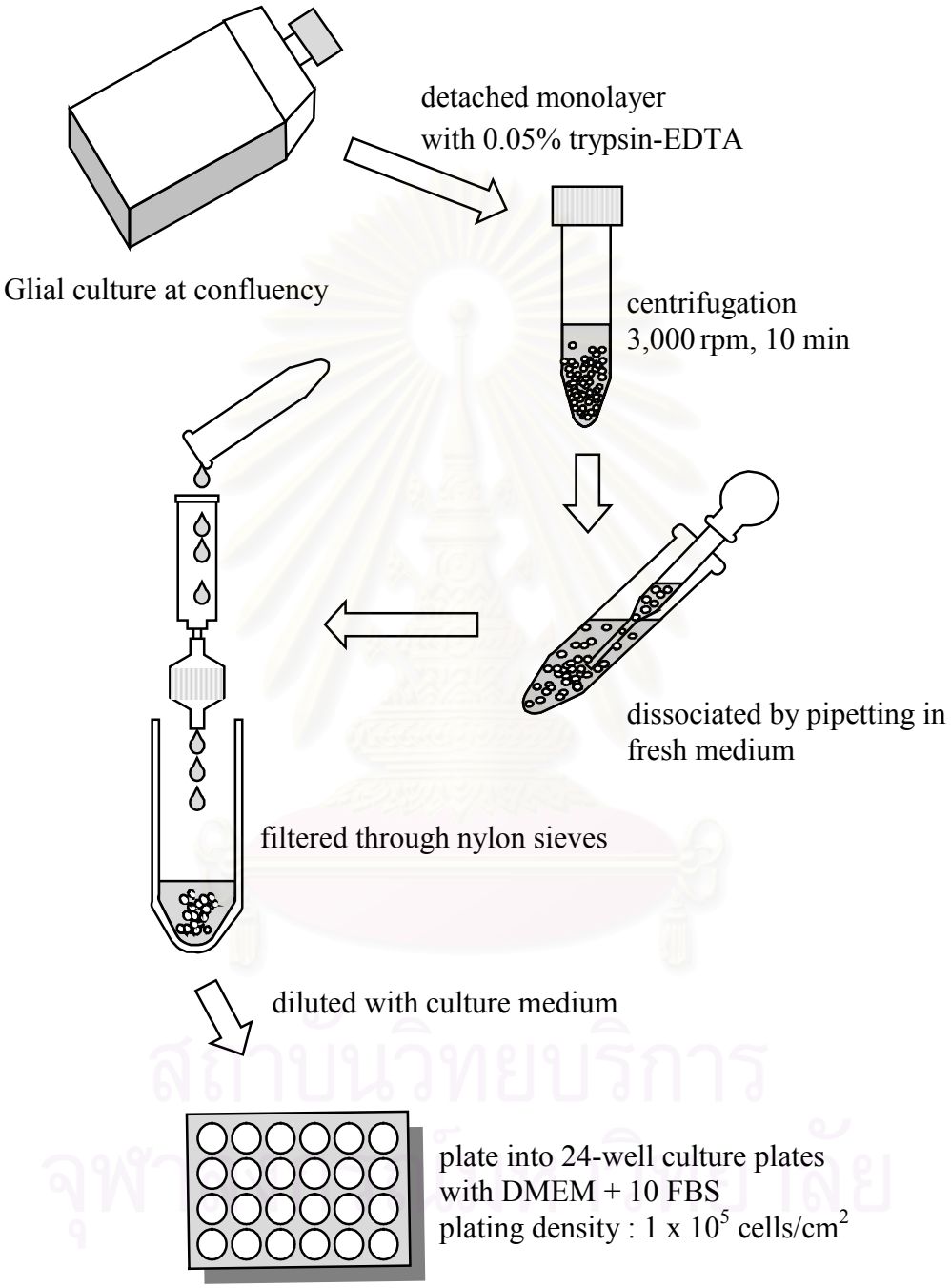


Fig. 10 Subculture of glial cells cultures



Culture Conditions

1. Treatment of cultured glial cells with piperine

Cultures were treated with 1, 5, 10, 25, 50, 75 and 100 μM piperine for 6, 12, or 24 hr in serum-free, DMEM containing N2 media supplements.

2. Exposure of cultured glial cells to LPS

Cultures were exposed to LPS at final concentrations of 0.01, 0.1, 1, 10 and 100 μg/ml in serum-free, N2-supplemented DMEM for 96 hr.

3. Treatment of LPS-exposed glial cells with piperine.

3.1 Pre-treatment with piperine or trolox

Cultures were first incubated with 1, 2.5, 5, 7.5 and 10 μM of piperine or 100 μM of trolox in serum-free DMEM containing N2 media supplements for 24 hr. At the indicated time, the medium of the culture was replaced with fresh serum-free, N2-supplemented DMEM containing 1 μg/ml of LPS and further incubated for 96 hr.

3.2 Co-treatment with piperine or trolox for a period of 24 hr

Cultures were incubated with piperine at concentrations of 1, 2.5, 5, 7.5 and 10 μM or trolox at a concentration of 100 μM in combination with LPS at a concentration of 1 μg/ml in serum-free DMEM containing N2 media supplement for 24 hr of incubation.

3.3 Co-treatment with piperine or trolox for a period of 96 hr

Cultures were incubated with 1, 2.5, 5, 7.5 and 10 μM of piperine or 100 μM of trolox in combination with 1 μg/ml of LPS in serum-free DMEM containing N2 media supplement for 96 hr of incubation.

3.4 Post-treatment with piperine

Cultures were incubated with 1 μg/ml of LPS in DMEM containing N2 media supplements for 96 hr. Subsequently, the exposure medium was replaced with fresh serum-free, N2 supplemented DMEM containing piperine at concentrations ranging from 1, 2.5, 5, 7.5 and 10 μM or trolox at a concentration of 100 μM and further incubated for 24 hr.

Analytical Methods

MTT reduction colorimetric assay

Cellular dehydrogenase activity, which is considered to reflect mitochondrial activity, was measured by the reduction of tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide to formazan. Mitochondrial dehydrogenase in viable cells converts the soluble yellow form of the salt into an insoluble, intracellular purple formazan which is quantitated spectrophotometrically by solubilization in an organic solvent (Desagher et al., 1996).

MTT reduction was analyzed by adding 16 μl of the MTT stock solution [5 mg/ml in phosphate-buffered saline (PBS)] to the medium in each well (final concentration was 100 μg/ml). Cultures were incubated in a CO_2 incubator at 37°C for 1 hr and the medium in each well was aspirated off without disturbing the formazan precipitate. Then 400 μl of DMSO was added to each well in order to solubilize the formazan crystals. Following through formazan solubilization, 200 μl aliquots of soluble formazan were transferred to a 96-well microtiter plate. The cellular reduction of MTT was determined by measuring the absorbance at 595/655 nm with a BIO-RAD Model 3550 dual wavelength microplate reader. The results were expressed as percentage of MTT reduction relative to that of control culture.

Lactate dehydrogenase release assay

Released LDH is a biologically stable enzymatic marker that correlates linearly with cell death. Cell viability was determined by assaying the medium from each well for lactate dehydrogenase (LDH) activity using an *in vitro* toxicology assay kit [Sigma, U.S.A.] according to the manufacture's instructions. The assay is based on the reduction of NAD by the LDH-catalyzed conversion of lactate to pyruvate. Then reduced NAD (NADH) is then utilized in the conversion of a tetrazolium dye. The resulting colored compound is measured spectrophotometrically.

Briefly, medium LDH was assayed by pipetting 100 μl of culture medium from each well into a 96-well microtiter plate. Cellular LDH was measured by carefully aspirated off the remaining culture medium and solubilized cells with 1 ml

of 0.5% Triton X-100 in PBS after which 100 µl aliquots were pipetted into a 96-well microtiter plate. The reaction was started by adding 50 µl of lactate dehydrogenase assay mixture into each well. The plate was covered with an aluminum foil to protect from light and incubated at room temperature for 30 min. The reaction was terminated by the addition 50 µl of 1N HCl into each well. The light absorbance in each well was measured at 490/655 nm with a BIO-RAD Model 3550 dual wavelength microplate reader. The total activity of LDH in control cultures was considered to represent 100% cell viability. The LDH release which reflects cell death was presented as percentage of total LDH activity by the following formula:

$$\% \text{ LDH release} = \frac{\text{LDH activity in medium} \times 100}{\text{LDH activity in medium + LDH activity in cells}}$$

In most cases, comparative LDH release in test conditions was expressed as the percentage of that in control conditions.

Glutathione assay

Cellular GSH levels were measured enzymatically by using a modification of the procedure of Tietze. This method is based on the determination of a chromophoric product, 2-nitro-5-thiobenzoic acid, resulting from the reaction of 5-5'-dithiobis-(2-nitrobenzoic acid) with GSH. In this reaction, GSH is oxidized to GSSG, which is then reconverted to GSH in the presence of GSH reductase (GRx) and NADPH. The rate of 2-nitro-5-thiobenzoic acid formation is followed at 450 nm (Tietze, 1969; Baker et al., 1990).

Cells were washed with ice-cold PBS and immediately collected by scraping with 0.3 ml of 1% (wt/vol) sulfosalicylic acid. Cell lysates were transferred to 1.5-ml Eppendorf tubes and centrifuged at 12,000 rpm for 5 min at 4 °C. Following cell extraction, 10 µl aliquots of supernatant were transferred into 96-well microtiter plate and the volume was made up to 100 µl with 0.1 M phosphate buffer containing 1 mM EDTA (pH 7.5). The reaction was started by adding 100 µl of reaction mixture [0.3

mM 5,5′-dithio-bis (2-nitrobenzoic acid), 0.4 mM NADPH, and 1 U/ml glutathione reductase in 0.1 M sodium phosphate buffer (pH 7.5)]. The formation of 2-nitro-5-benzoic was monitored at 30-s intervals for 10 min at 450/620 nm by using a microplate reader [Anthos htl]. The slope of the initial rate of reaction was used for calculating GSH content from a standard curve obtained by plotting known amount of GSH (25-150 μM). The cellular content of GSSG was typically less than 2% of the GSH level and was not considered.

Nitrite assay

The production of NO was assessed as nitrite accumulated in the culture medium by using a colorimetric reaction with Griess reagent, which converts nitrite into a colored azo compound. This method is based on the reaction that sulfanilic acid is quantitatively converted to a diazonium salt in the presence of nitrite in acid solution. The diazonium salt is then coupled to *N*-(1-naphthyl)ethylenediamine, forming an azo dye that can be quantitated spectrophotometrically (Grisham et al., 1996; Chang et al., 2000).

Briefly, 100 μl of the medium was mixed with an equal volume of the Griess reagent [0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% H_3PO_4]. After 10-min at room temperature, the absorbance at 510/620 nm was measured with a microtiter plate reader [Anthos htl]. The nitrite concentration was determined from a sodium nitrite standard curve.

STATISTICAL ANALYSIS

All results were presented as the means ± SEM values from 6 to 8 independent cultures, with duplicate replications in each experiment. Differences among means were analyzed using one-way analysis of variance (ANOVA). One-way ANOVA followed by Student-Newman-Keuls test was used for planned multiple comparisons. All statistical analysis was performed according to the instruction for the statistical program SPSS. A value of $P < 0.05$ was considered to be significant.

CHAPTER IV

RESULTS

1. Effects of lipopolysaccharide on mitochondrial activity and viability of cultured glial cells

Upon stimulation with 0.01 to 100 μg/ml of LPS for 96 hr, mitochondrial metabolic activity of cultured glial cells was decreased by approximately 20-30% when compared with control cultures (Fig. 11). However, LPS did not affect cell survival (Fig. 12).

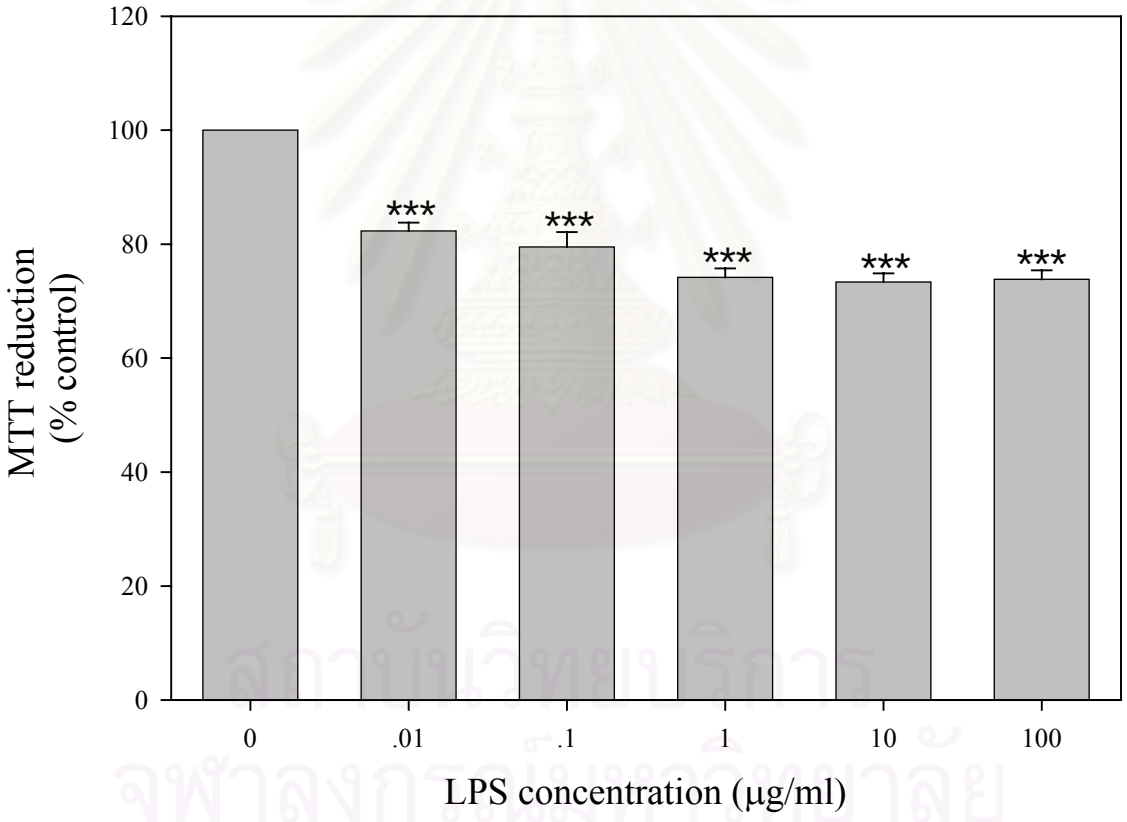


Fig. 11 Inhibitory effects of LPS on mitochondrial activity in cultured glial cells. Cultures were exposed to LPS for 96 hr before determination of MTT reduction activity. Values are mean ± S.E.M. of 6 samples from duplicate experiments. ***P< 0.001 compared with untreated control (One-way ANOVA followed by Student-Newman-Keuls test for multiple comparison).

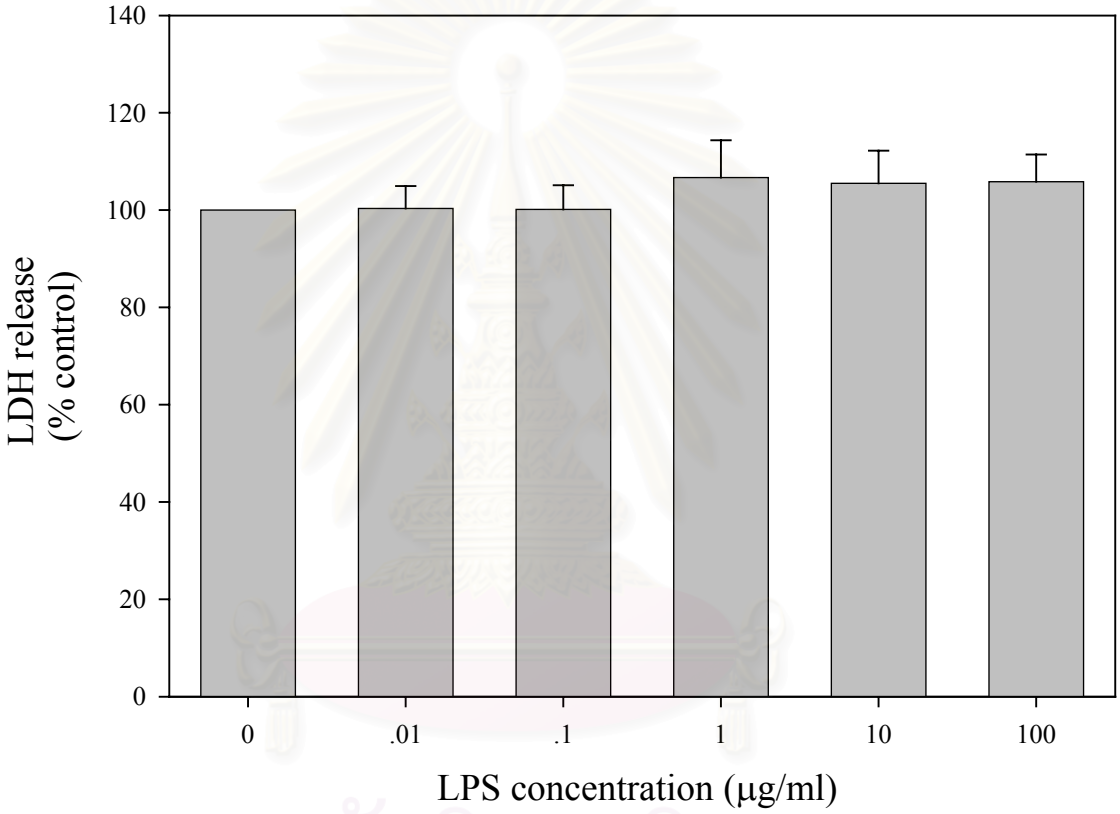


Fig. 12 Effects of LPS on viability of cultured glial cells. Cultures were exposed to LPS for 96 hr before determination of LDH release. Values are mean ± S.E.M. of 6 samples from duplicate experiments. Statistical analysis for differences among means was made by one-way ANOVA.

2. Effects of piperine on mitochondrial activity and viability of cultured glial cells

Treatment of glial cells with a range of piperine concentrations for various time intervals showed that low concentrations of piperine (1-10 μM) significantly increased mitochondrial metabolic activity after 12 and 24 hr of incubation. In contrast, mitochondrial activity was markedly decreased at higher concentrations of piperine (25-100 μM) after 6, 12 and 24 hr of incubation in a concentration and time-dependent manner (Fig. 13). In addition, the number of surviving glial cells was decreased after 6, 12 and 24 hr of treatment with 25-100 μM of piperine. While low piperine concentrations (1-10 μM) did not affect cell survival (Fig. 14).

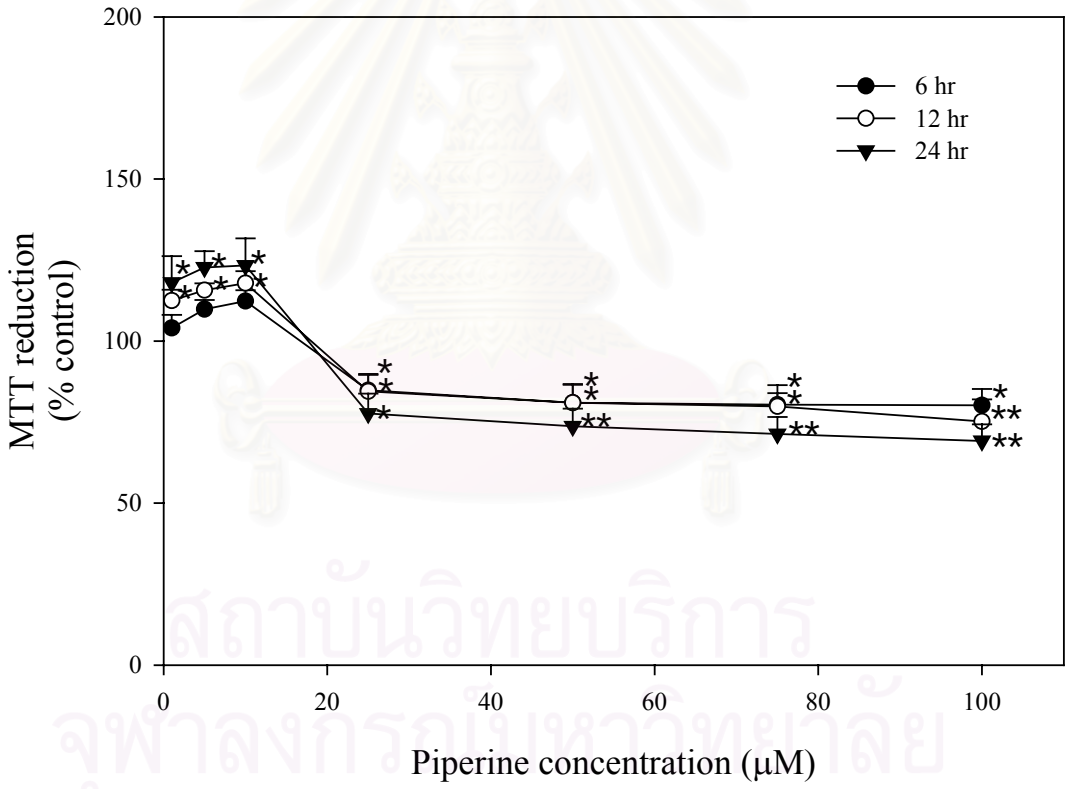


Fig. 13 Bimodal effects of piperine on mitochondrial activity in cultured glial cells. Cultures were treated with increasing concentrations of piperine. Mitochondrial metabolic activity was estimated 6, 12 and 24 hr later by using the MTT colorimetric assay. Values are mean ± S.E.M. (N=6). *P< 0.05, **P< 0.01 compared with untreated control (One-way ANOVA and Student-Newman-Keuls test).

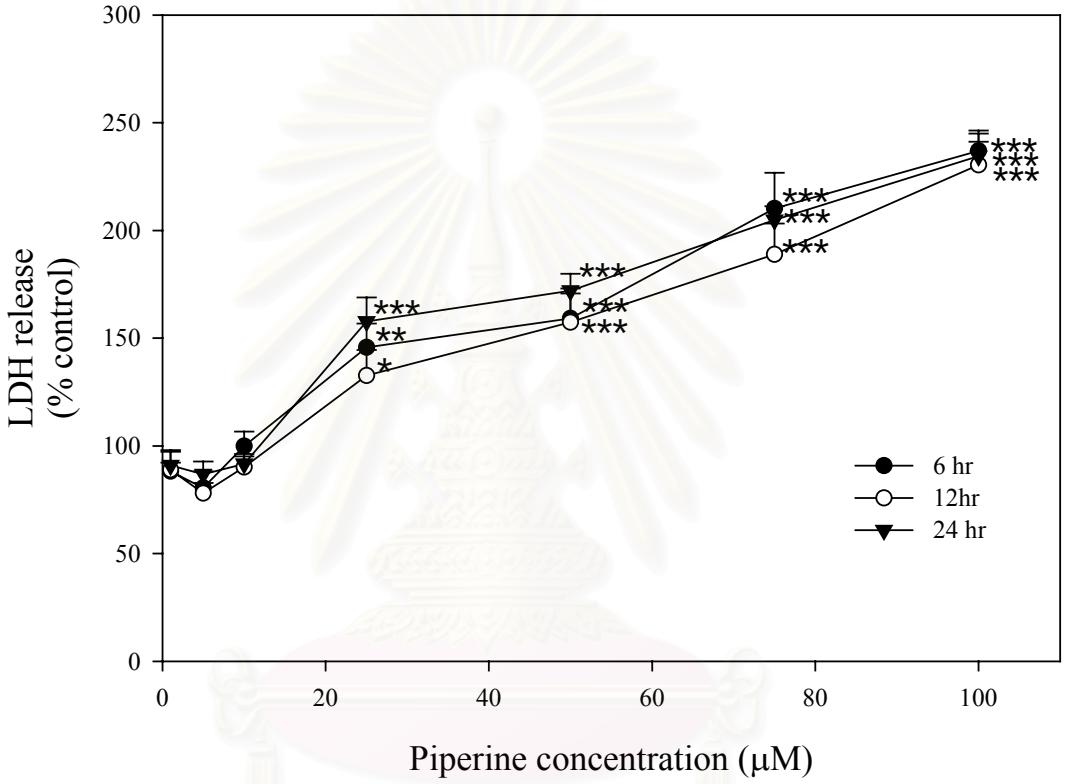


Fig. 14 Effects of piperine on viability of cultured glial cells. Cultures were treated with increasing concentrations of piperine. Cell viability was estimated 6, 12 and 24 hr later by using the LDH release assay. The leakage of LDH which is an indicator of cell death was assessed by using an *in vitro* toxicology assay kit (Sigma, U.S.A.). Values are mean ± S.E.M. (N=6). *P< 0.05, **P< 0.01, ***P< 0.001 compared with untreated control (One-way ANOVA and Student-Newman-Keuls test).

3. Effects of pre-treatment with piperine on mitochondrial activity and viability of LPS-exposed cultured glial cells.

Exposure of glial cells to LPS at a concentration of 1 μg/ml for 96 hr reduced mitochondrial activity but did not affect cell survival. However, the mitochondrial damage caused by LPS was significantly blocked by pre-incubation with 1-10 μM of piperine or 100 μM of trolox (Fig. 15). Under this condition, neither piperine nor trolox affected cell viability of LPS-exposed glial cells. (Fig. 16).

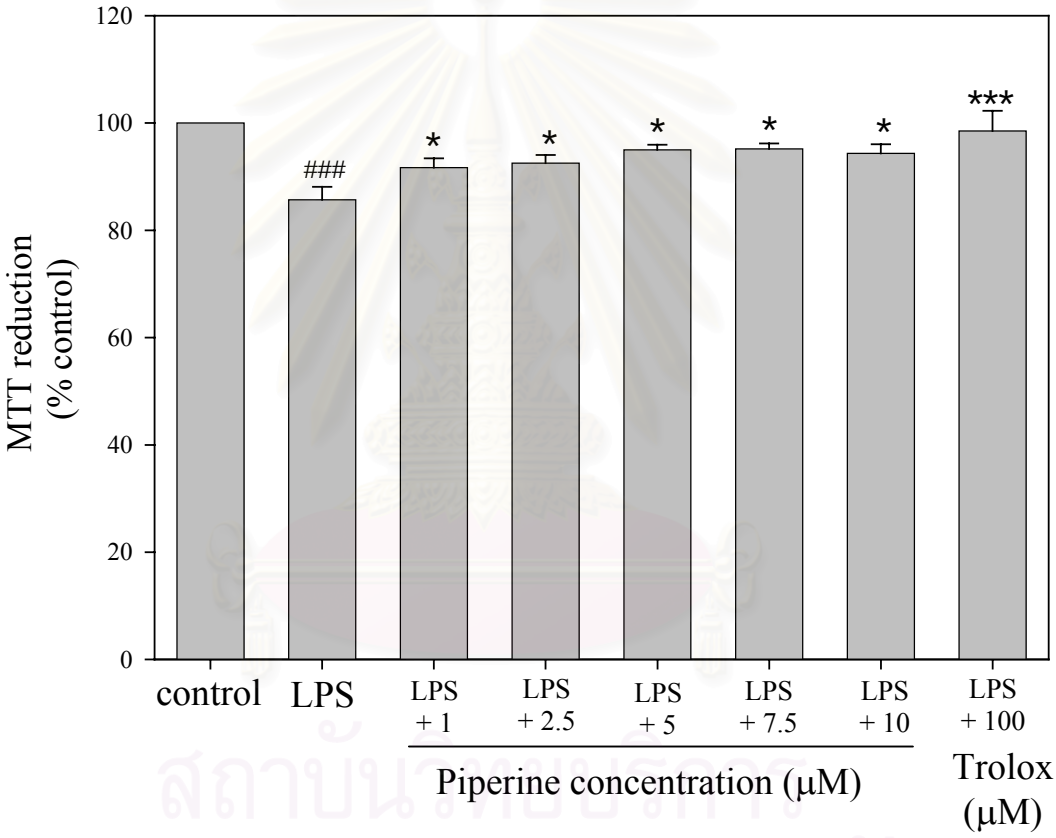


Fig. 15 Effects of pre-exposure to piperine on mitochondrial activity in cultured glial cells exposed to LPS. Cultures were incubated with 1-10 μM piperine or 100 μ M trolox for 24 hr and further incubated with fresh medium containing 1 μg/ml LPS for 96 hr after which mitochondrial activity was determined. Values are mean ± S.E.M. (N = 6). $^{###}$P< 0.001 vs control group, *P< 0.05, ***P< 0.001 vs LPS-treated group (One-way ANOVA and Student-Newman-Keuls test).

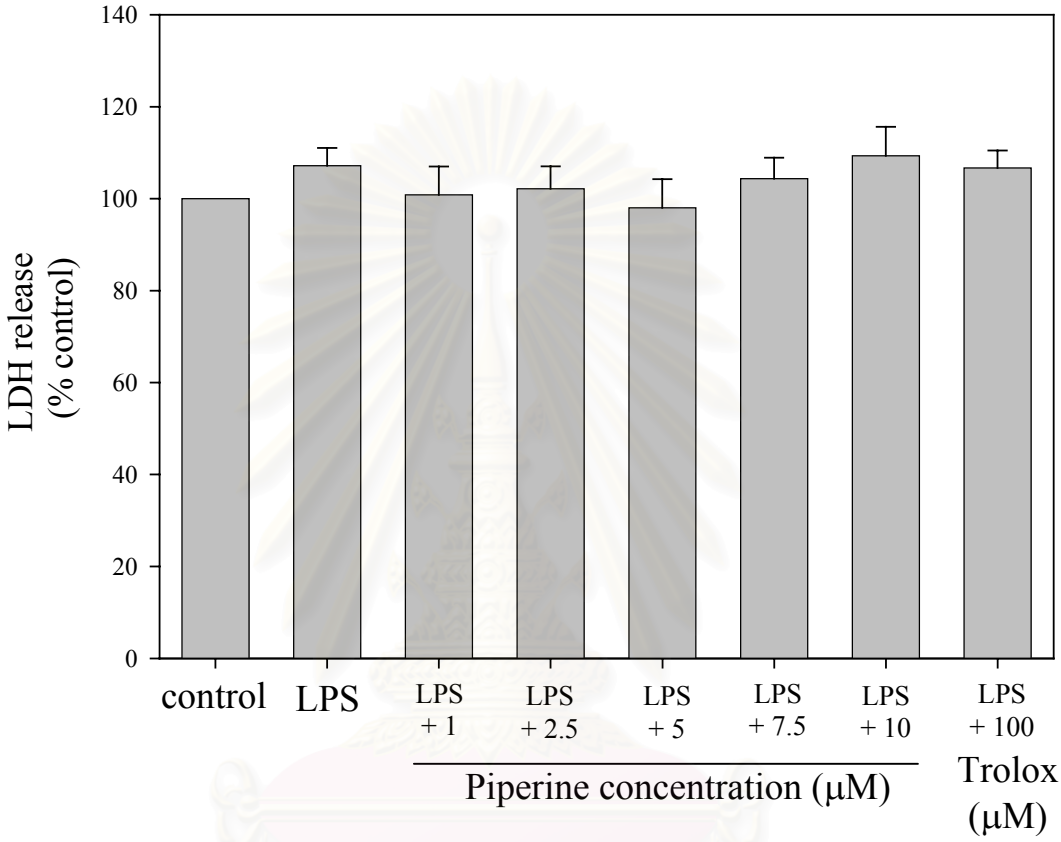


Fig. 16 Effects of pre-exposure to piperine on viability of cultured glial cells exposed to LPS. Cultures were incubated with 1-10 μM piperine or 100 μM trolox for 24 hr and further incubated with fresh medium containing 1 μg/ml LPS for 96 hr after which cell viability was determined. Values are mean ± S.E.M. (N = 6). Statistical analysis for differences among means was made by one-way ANOVA.

4. Effects of co-treatment with piperine and LPS for 24 hr on mitochondrial activity and viability of cultured glial cells.

Mitochondrial metabolic activity and cell viability of glial cells were unaffected after exposure of glial cells to LPS (1 μg/ml) for 24 hr. While significant increase of MTT reduction was observed after treatment with 10 μM of piperine or 100 μM of trolox in combination with LPS (Fig. 17). However, cell survival was unaffected under the same condition (Fig. 18).

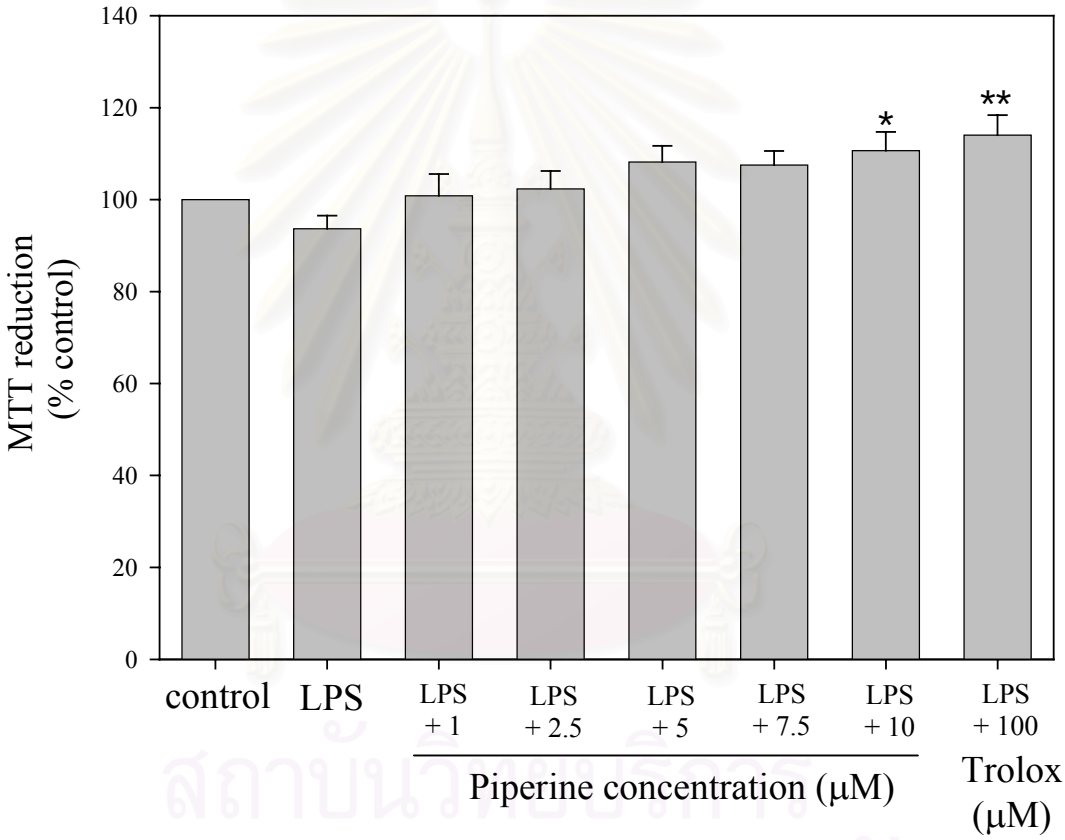


Fig. 17 Effects of 24-hr co-exposure to piperine and LPS on mitochondrial activity in cultured glial cells. After a co-incubation with 1-10 μM piperine or 100 μ M trolox and 1 μg/ml LPS for 24 hr, cellular MTT reduction activity was determined. Values are mean ± S.E.M. (N = 6). *P< 0.05, **P< 0.01 vs LPS-treated group (One-way ANOVA and Student-Newman-Keuls test).

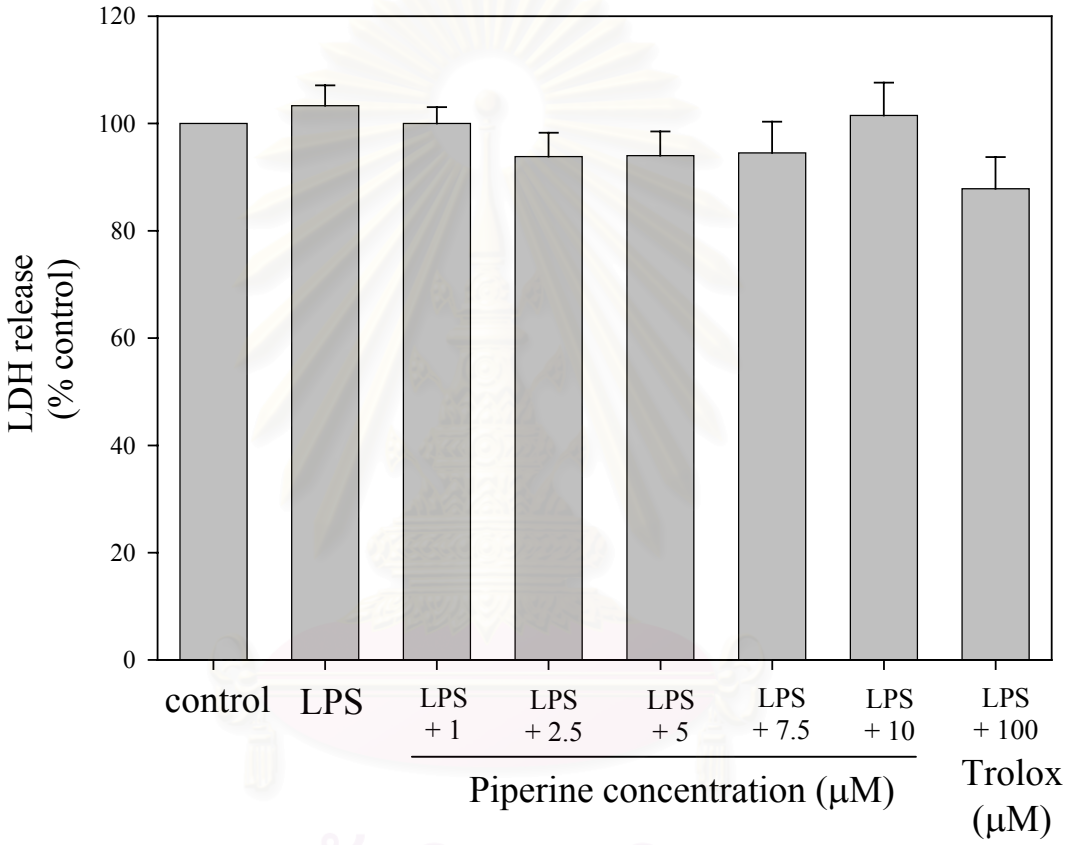


Fig. 18 Effects of 24-hr co-exposure to piperine and LPS on viability of cultured glial cells. After a co-incubation with 1-10 μM piperine or 100 μM trolox and 1 μg/ml LPS for 24 hr, cytoplasmic LDH release was determined. Values are mean ± S.E.M. (N = 6). Statistical analysis for differences among means was made by one-way ANOVA.

5. Effects of co-treatment with piperine and LPS for 96 hr on mitochondrial activity and viability of cultured glial cells.

Exposure to LPS at a concentration of 1 μg/ml for 96 hr induced mitochondrial metabolic damage without apparent cell death. However, treatment with piperine at concentrations of 1 to 10 μM or trolox at a concentration of 100 μM in combination with LPS for 96 hr had no effect on mitochondrial metabolic activity and cell viability of LPS-exposed glial cells (Fig. 19, 20).

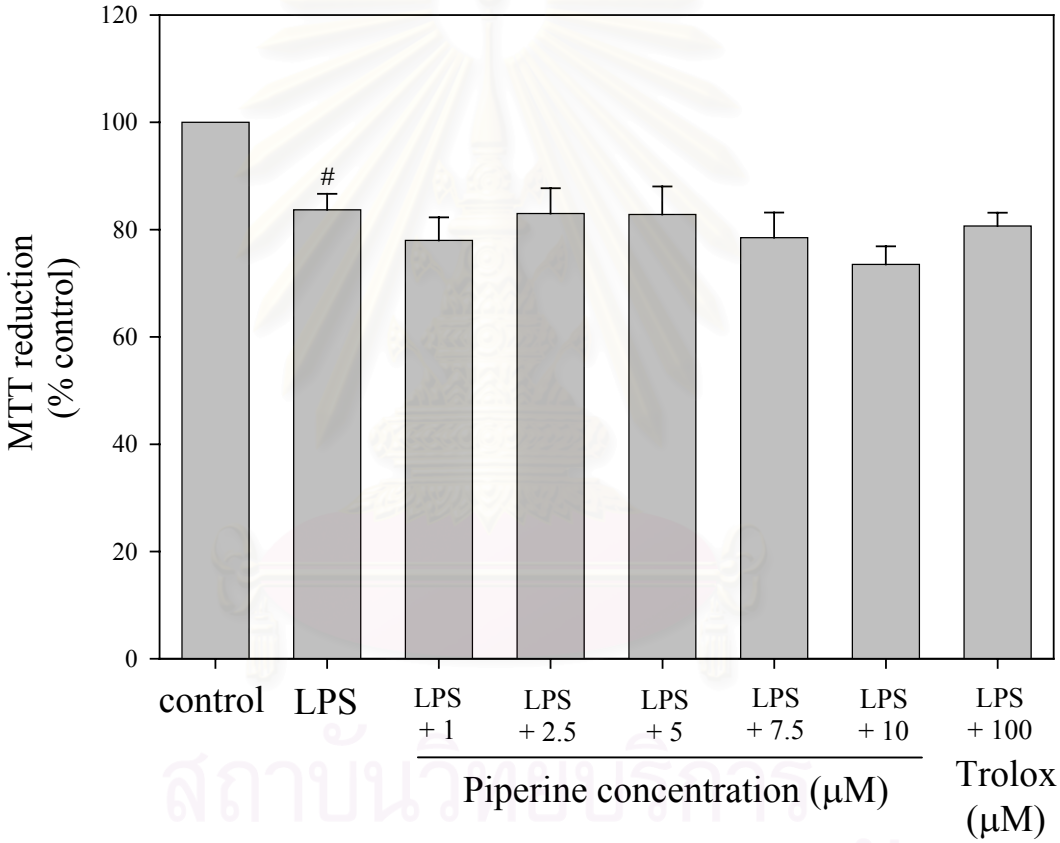


Fig. 19 Effects of 96-hr co-exposure to piperine and LPS on mitochondrial activity in cultured glial cells. Cultures were incubated with piperine (1-10 μM) or trolox (100 μM) in combination with LPS (1 μg/ml) for 96 hr before determination of cellular MTT reduction. Values are mean ± S.E.M. (N = 6). $^{\#}P<0.05$ vs control group (One-way ANOVA and Student-Newman-Keuls test).

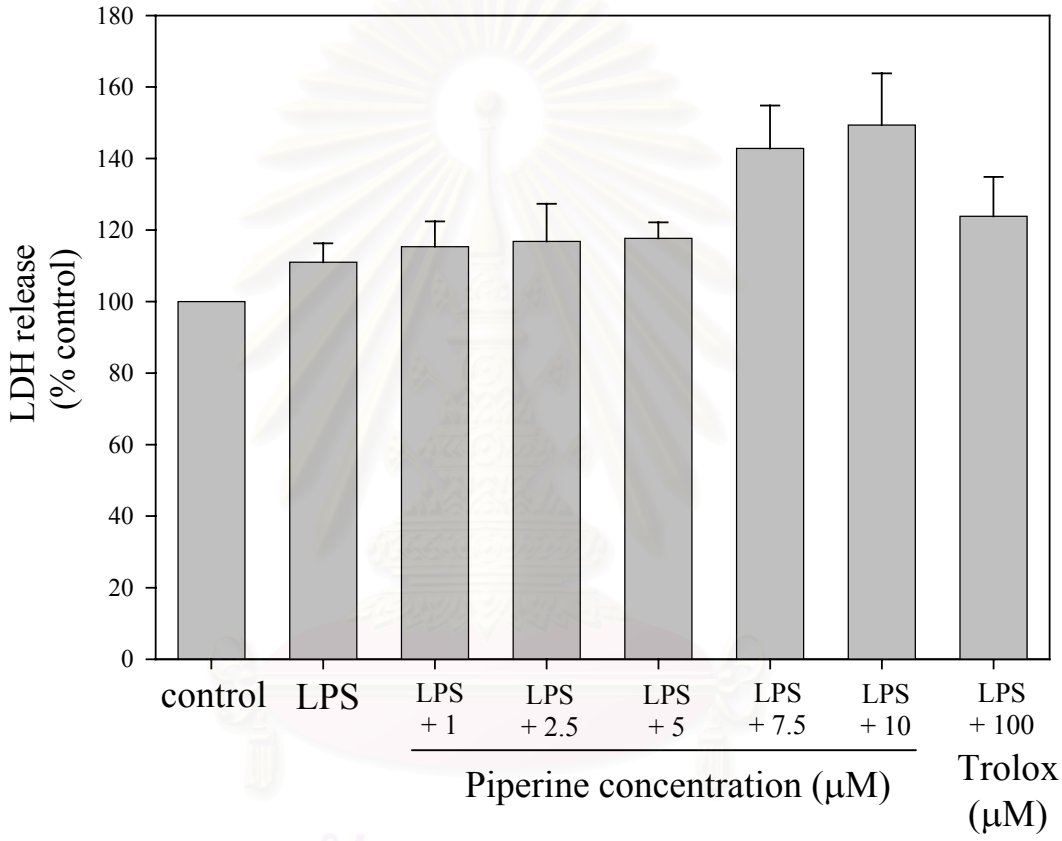


Fig. 20 Effects of 96-hr co-exposure to piperine and LPS on viability of cultured glial cells. Cultures were incubated with piperine **(**1-10 μM) or trolox (100 μM) in combination with LPS (1 μg/ml) for 96 hr before determination of cytoplasmic LDH released. Values are mean ± S.E.M. (N = 6). Statistical analysis for differences among means was made by one-way ANOVA.

6. Effects of post-treatment with piperine on mitochondrial activity and viability of LPS-exposed cultured glial cells.

Exposure of glial cells to LPS (1 μg/ml) for 96 hr inhibited mitochondrial metabolic activity but did not affect cell survival. However, post-treatment with 5 and 7.5 μM of piperine or 100 μM of trolox attenuated mitochondrial damage that resulted from LPS-induced cytotoxicity (Fig. 21). Under the same condition, the number of cell survived was unaffected either by piperine or trolox (Fig. 22).

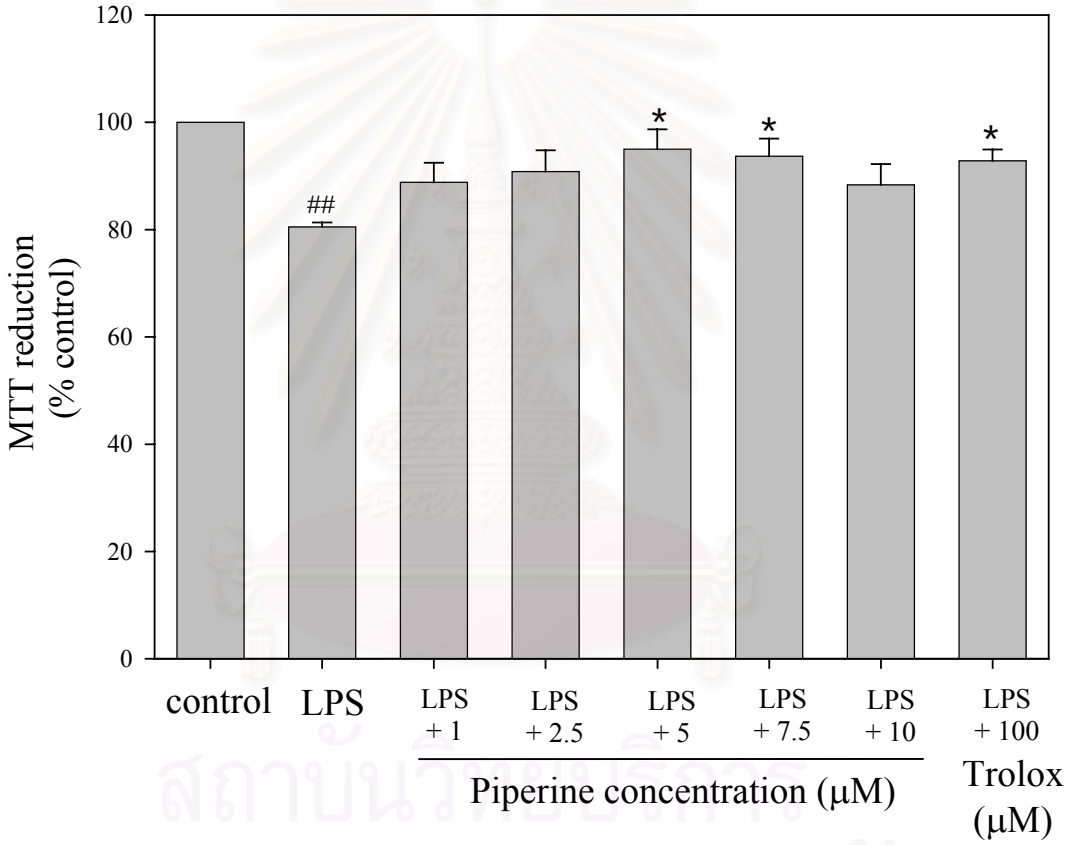


Fig. 21 Effects of post-exposure to piperine on mitochondrial activity in cultured glial cells exposed to LPS. Cultures were incubated with 1-10 μM piperine or 100 μ M trolox for 24 hr following exposure to 1 μg/ml LPS for 96 hr after which mitochondrial activity was determined. Values are mean ± S.E.M. (N = 6). $^{\#\#}P< 0.01$ vs control group, *P< 0.05 vs LPS-treated group (One-way ANOVA and Student-Newman-Keuls test).

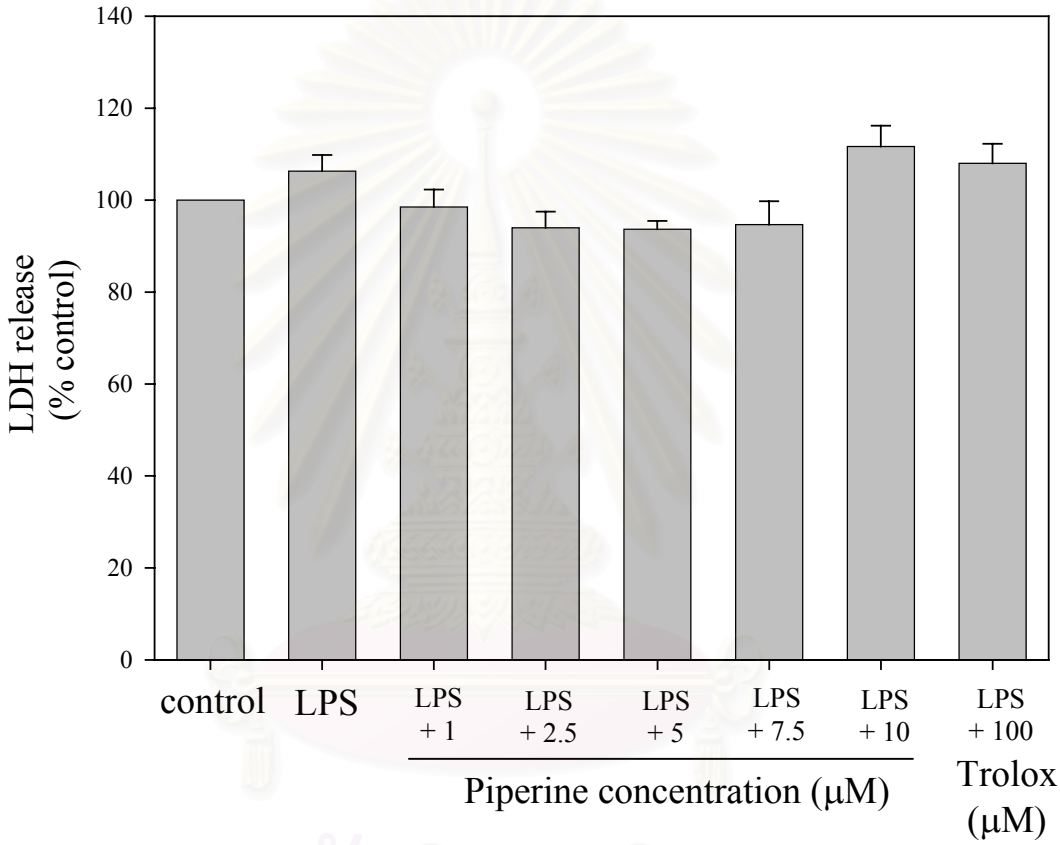


Fig. 22 Effects of post-exposure to piperine on viability of cultured glial cells exposed to LPS. Cultures were incubated with 1-10 μM piperine or 100 μM trolox for 24 hr following exposure to 1 μg/ml LPS for 96 hr after which cell viability was determined. Values are mean ± S.E.M. (N = 6). Statistical analysis for differences among means was made by one-way ANOVA.

7. Effects of post-treatment with piperine on glutathione level in LPS-exposed cultured glial cells.

Post-treatment with 5 μM of piperine or 100 μM of trolox for 24 hr reversed LPS-induced glutathione content diminution in cultured glial cells (Fig. 23). After 96 hr, exposure to LPS decreased glutathione content. This effect of LPS was significantly attenuated when treated glial cells with piperine or trolox.

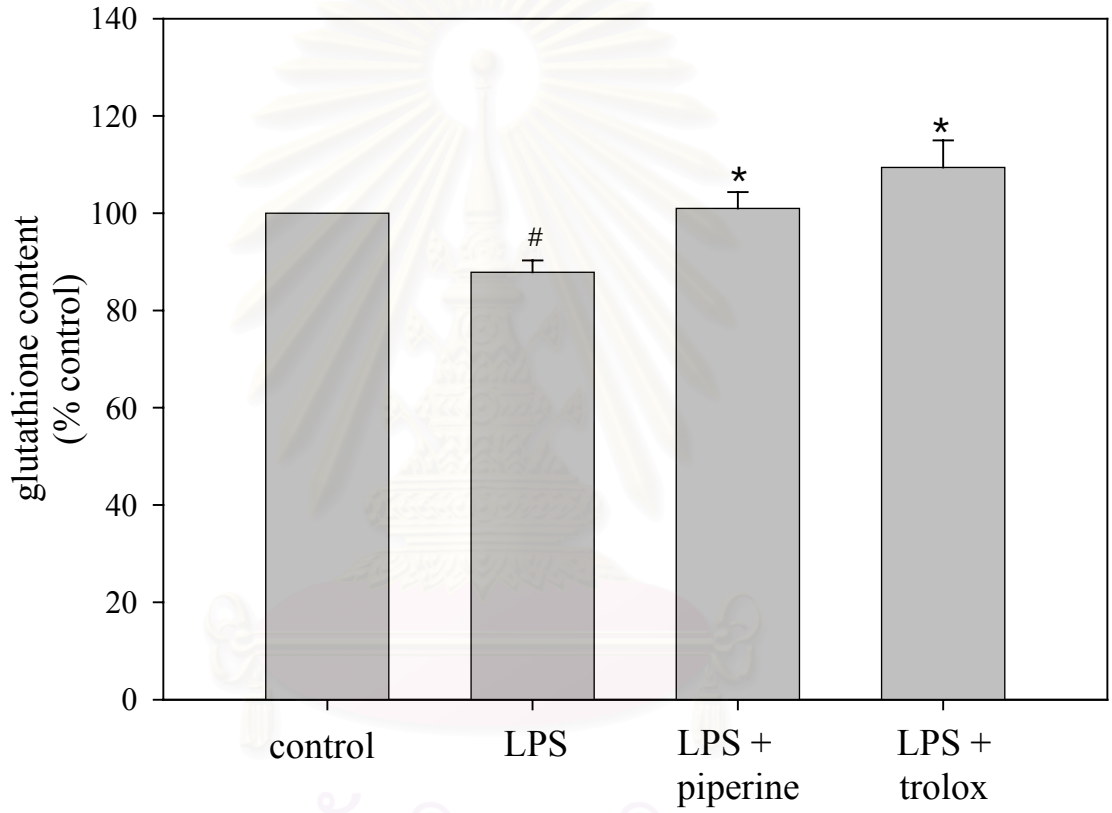


Fig. 23 Effects of post-exposure to piperine on LPS-induced gluthathione diminution in cultured glial cells. Cultures were incubated with 1 μg/ml LPS for 96 hr and further incubated with 5 μM piperine or 100 μM trolox for 24 hr before glutathione content determination. Values are mean ± S.E.M. (N = 6). $^{\#}P<0.05$ vs control group, *P< 0.05 vs LPS-treated group (One-way ANOVA and Student-Newman-Keuls test).

8. Effects of post-treatment with piperine on the release of nitrite from LPS-exposed glial cells cultures.

Post-incubation with piperine or trolox for 24 hr did not change LPS-induced nitrite accumulation in cultured glial cells (Fig. 24). After 96 hr of incubation with 1 μg/ml LPS, nitrite content was increased in the medium of cultured glial cells. However, neither piperine (5 μM) nor trolox (100 μM) affected the increase of nitrite accumulation in the supernatant of LPS-exposed glial cells.

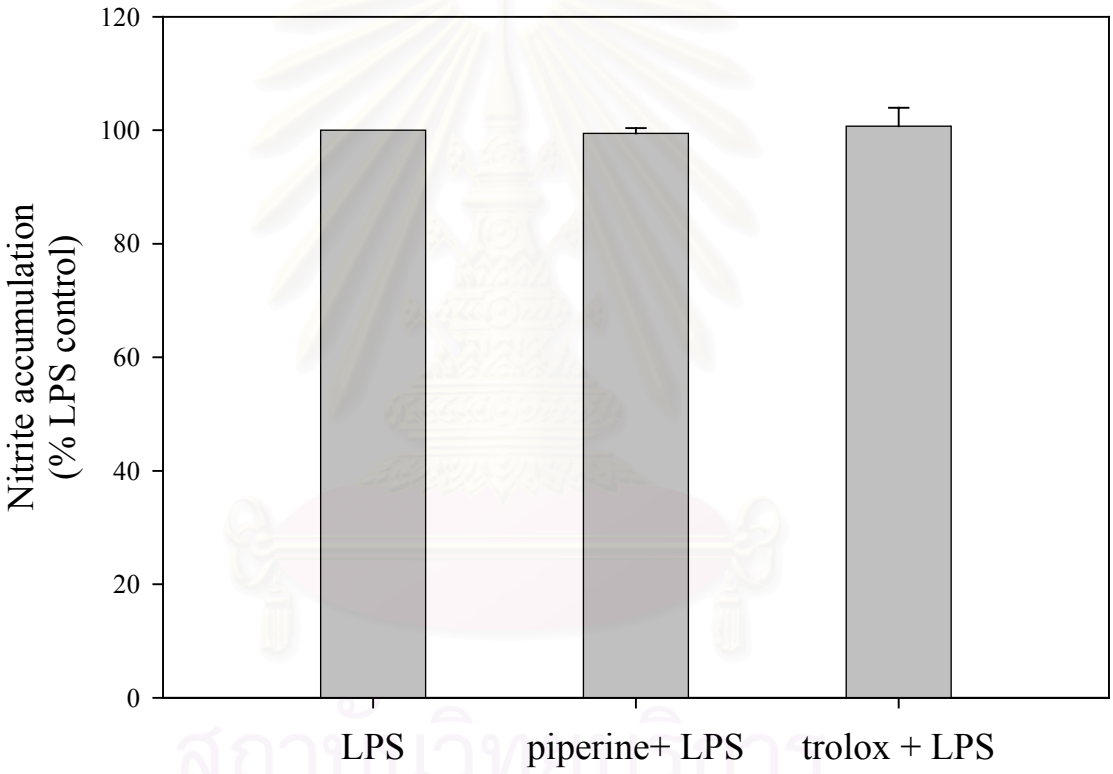


Fig. 24 Effects of post-exposure to piperine on LPS-induced nitrite accumulation in cultured glial cells. Cultures were incubated with 1 μg/ml LPS for 96 hr and then further incubated with 5 μM piperine or 100 μM trolox for 24 hr after which nitrite accumulation in the medium was determined by Griess reagent. Values are mean ± S.E.M. (N = 6). Statistical analysis for differences among means was made by one-way ANOVA.

CHAPTER V

DISCUSSION AND CONCLUSION

Neuroinflammation is a process that results primarily from the presence of chronic activated glial cells in the brain, and is a common feature of several neurodegenerative disorders. Activation of glial cells leads to induction of proinflammatory cytokines such as IL-1β and TNF-α and oxidative stress-related enzymes such as iNOS. Chronic glial activation or an abnormally high proportion of activated glia could potentially lead to toxicity to themselves and neighboring neurons through propagation of a localized inflammation and oxidative stress cycle. Therefore, the ability of pharmacological treatments to modulate detrimental glial responses may be an effective therapeutic approach to delay the onset or progression of neurodegeneration.

The bacterial endotoxin lipopolysaccharide (LPS) has long been used as a model stimulus for studying inflammation in different biological systems, including the CNS. LPS-stimulated glial cells produce a mixture of various cytokines, free radicals and nitric oxide. This is similar to the response reported after brain injury (Chang et al., 2000). In this culture system, LPS-exposure did not cause apparent cell death (Fig. 12) but it resulted in oxidative stress which was implied by the reduction of mitochondrial metabolic activity (Fig. 11) and the reduction of glutathione content (Fig. 23) in cultured glial cells. Several previous investigations indicated that one possible mechanism of LPS-induced oxidative change in glial cells may involve a release of NO. Accumulation of nitrite (NO_2^-), a stable metabolite of NO was also observed after the exposure to 1 μg/ml of LPS for 96 hr in this experiment. The finding is in agreement with previous studies showing that nitric oxide formation after induction of iNOS by LPS and IFN-γ increased oxidative load in microglial cells, as observed by the oxidation rate of ROS- and peroxynitrite indicator dichlor-dihydrofluorescein (DCF-H) in these cells (Noack et al., 2000). In addition, LPS/ IFN-γ-mediated NOS induction in astrocytes causes marked inhibition of mitochondrial respiratory chain (Bolanos et al., 1994). Furthermore, LPS-treatment also reduced GSH content in astrocytes and mix neuronal/glial cultures (Kramer et al.,

2002). From these experiments, cell viability was not affected by nitric oxide formation. Some studies suggest that glial cells are more resistant to oxidative stress than neurons. It was shown that exposure of astrocytes to cytokines/LPS leads to an induction of iNOS and marked generation of NO and $ONOO^-$. Consequently, mitochondrial damage may occur in these cells but ATP levels are maintained due to an increase in glycolytic flux. Further, cellular damage is minimized due to the relatively high glutathione concentration. In contrast to glial cells, diffusion of NO and/or $ONOO^-$ into neighboring neurons may lead to mitochondrial damage. Since these cells are unable to compensate by increasing glycolysis, a cellular energy deficiency state ensues which may lead ultimately to cell death (Heales et al., 1999; Dringen et al., 1999). Like GSH, in one study reported that the concentration of vitamin E is higher in cultured chick astrocytes than in neurons (Makar et al., 1994).

Piperine was shown to possess various pharmacological activities. This study primarily investigated effects of piperine in cultured glial cells. The preliminary results revealed that at low concentrations, piperine exhibited protective effect by increasing mitochondrial metabolic activity (Fig. 13). While at higher concentrations, it exerted cytotoxic effect to glial cells in cultures by reducing cellular MTT reduction and increasing cytoplasmic LDH release (Fig. 14). The toxic effect of piperine in culture experiments was previously reported that exposure to high concentrations of piperine induced injuries on cultured glial cells. However, piperine cytotoxicity on glial cells was found to be much lower than that on hippocampal neurons (Unchern et al., 1997).

Because of apparent protective effects at low concentrations of piperine in the preliminary study, the concentrations of 1-10 μM were selected to investigate the effects of piperine on LPS-exposed glial cells. These observations demonstrated that the inhibition of metabolic activity by LPS could be attenuated by pre-treatment with 1-10 μM of piperine (Fig. 15) or post-treatment with 5 and 7.5 μM of piperine (Fig. 21). However, mitochondrial metabolic activity and cell viability were unaffected after exposure of glial cells to LPS for 24 hr. In addition, co-treatment with 10 μM of piperine and LPS also increased mitochondrial metabolic activity (Fig. 17) while did not show significant effect on cell survival under these conditions (Fig.16, 18, 22).

These observations suggest the beneficial action of piperine at low concentrations on metabolic activity of glial cells in the inflammatory responses.

Several experiments indicated that one mechanism of compound that can modulate chronic inflammation and oxidative stress process is by scavenging free radicals such as nitric oxide. In this experiment, no alteration of LPS-induced nitrite accumulation was observed after treatment with piperine (Fig. 24). The results suggest that reduction of mitochondrial damage by piperine does not appear to be mediated through inhibition of nitric oxide production. On the other hand, glial NO generation during LPS exposure might diffuse to the medium and was washed out by a medium change to a new one with piperine. In this connection, the generation of NO by induced iNOS in activated glial cells during 24 hr of piperine exposure may be insufficient for accurate detection by the assay used in this study.

In previous studies, piperine was shown to modulate the oxidative change by enhancing synthesis or transport of GSH in chemical carcinogen-induced oxidative stress in rat intestinal model (Khajura et al., 1998). In addition, it reversed the diabetic effect on glutathione system in brain, kidney and heart in streptozotocin-induced diabetic rats (Rauscher et al., 2000). In the present experiments, piperine, used at a concentration of 5 μM that produced maximal stimulatory effect on glial cells metabolic activity, reversed the glutathione diminution from LPS toxicity (Fig. 23). These observations suggest that up-regulation of glutathione is one important factor which could preserve the mitochondrial function. It is in accordance with a previous study showing that induction of glucose-6-phosphate dehydrogenase by LPS contributes to preventing nitric oxide-mediated glutathione depletion in cultured rat astrocytes (Garcia-Nogales et al., 1999). Furthermore, depletion of astrocytic glutathione rendered the cells more susceptible to the insults, with mitochondrial complex I and II/III being decreased in activity by 80% and 64%, respectively, after peroxynitrite exposure. In addition, cell death was significantly increased in the glutathione depleted astrocytes exposed to peroxynitrite (Barker et al., 1996).

Like piperine, trolox, a soluble antioxidant compound which is the pharmacological standard of this study for comparison, exerted beneficial effects under the same condition (Fig. 15, 17, 21). Trolox also attenuated the LPS-induced glutathione diminution in this experiment (Fig. 23). While it did not alter LPS-

induced NO accumulation in cultured glial cells in this experiment (Fig. 24). Previously, the antioxidants ascorbic acid and superoxide dismutase/catalase but not trolox resulted in decreased iNOS activity in cultured human endothelial cells (Galley et al., 1996). However, trolox was shown in one previous study to protect mitochondrial complex IV from nitric oxide-mediated damage in astrocytes by inhibiting lipid peroxidation (Heales et al., 1994) Therefore, the inhibition of glutathione diminution may not be the sole mechanism underlying the advantageous action of piperine and trolox. Reports by other investigators indicated that piperine may exert antiinflammatory and antioxidant effects as well as hepatoprotective action by inhibiting or reducing *in vivo* and *in vitro* lipid peroxidation (Koul and Kapil, 1993; Khajura et al., 1998; Rauscher et al., 2000). Recently, prior incubation of glial cells with trolox was shown to down-regulate IL-1β expression and completely prevented neuronal cell death in rat hippocampal neurons/glial co-cultures (Viviani et al., 2001).

Minghetti and Levi (1998) revealed that activation of glial cells causes up-regulation and secretion of a number of pro- and anti-inflammatory cytokines, growth factors, as well as potential toxins. This line of evidence is supported by a study indicating that in mixed neuronal-glial culture, pre-exposure to 10 μg/ml LPS for 24 hr decreased the toxicity of BSO treatment. One of the suggested mechanisms for the protective effect of LPS is an increase in the protein content and the activity of Mn-superoxide dismutase (MnSOD) (Kramer et al., 2002). Moreover, it was shown that priming with small doses of LPS can protect mice against ischemia (Ahmed et al., 2000). Consequently, LPS may potentiate the beneficial effect of piperine in the case of co-treatment of cultured glial cells with piperine and LPS for 24 hr.

In contrast, co-incubation of piperine with LPS for 96 hr did not reverse the LPS-induced mitochondrial damage (Fig. 19). However, prolonged exposure of glial cell cultures to piperine may lead to deleterious effects (Unchern et al., 1997). Furthermore, the nitrosation reaction of piperine was observed in the human stomach after ingested piperine and nitrite (Wakabayashi et al., 1989).

In conclusion, experimental results from this preliminary study suggested that piperine, especially at low concentrations, might have stimulatory effect on glial cell metabolic activity and facilitate glial cell functions in brain inflammatory responses.

Additional mechanisms, besides the induction of mitochondrial metabolic activity and glutathione contents are likely to be responsible for neuroprotective effects of piperine. Further studies and understanding on the mechanisms of action of piperine may provide insights into potential therapeutic interventions for inflammation-related neurodegenerative diseases.

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APPENDICES

Table 1. Effects of piperine on MTT reduction in cultured glial cells

Table 1.1 : 6 hr of incubation								
	Control	1μM	5μM	10μM	25μM	50μM	75μM	100μM
group 1	100	87	102	112	68	75	76	74
group 2	100	113	107	121	76	60	68	72
group 3	100	104	105	100	85	84	79	71
group 4	100	102	109	110	87	94	63	72
group 5	100	103	120	109	100	75	98	97
group 6	100	115	116	122	93	98	98	95
mean	100.00	104.00	109.83	112.33	84.33	81.00	80.33	80.17
S.E.M	0.000	4.066	2.798	3.353	4.700	5.715	6.048	5.029

Table 1.2 : 12 hr of incubation								
	Control	1μM	5μM	10μM	25μM	50μM	75μM	100μM
group 1	100	124	123	127	94	82	80	53
group 2	100	110	112	117	80	74	74	74
group 3	100	109	113	115	87	80	77	67
group 4	100	119	118	120	80	81	85	74
group 5	100	101	109	102	64	64	67	79
group 6	100	112	119	126	102	105	96	104
mean	100.00	112.50	115.67	117.83	84.50	81.00	79.83	75.17
S.E.M	0.000	3.294	2.214	3.719	5.365	5.526	4.061	6.848

Table 1.3 : 24 hr of incubation								
	Control	1μM	5μM	10μM	25μM	50μM	75μM	100μM
group 1	100	102	113	104	97	88	76	67
group 2	100	120	122	128	66	59	56	59
group 3	100	122	127	133	88	85	83	68
group 4	100	104	124	127	88	81	74	79
group 5	100	155	143	152	65	72	84	88
group 6	100	105	107	96	62	57	55	54
mean	100.00	118.00	122.67	123.33	77.67	73.67	71.33	69.17
S.E.M	0.000	8.815	5.077	8.309	6.136	5.426	5.251	5.134

Table 2. Effects of piperine on LDH release in cultured glial cells

Table 2.1 : 6 hr of incubation								
	Control	1μM	5μM	10μM	25μM	50μM	75μM	100μM
group 1	100	105	81	94	122	152	229	260
group 2	100	62	88	93	187	179	236	247
group 3	100	70	63	109	162	187	262	261
group 4	100	68	63	78	113	110	168	226
group 5	100	107	113	127	139	177	208	203
group 6	100	117	75	98	151	150	158	225
mean	100.00	88.17	80.50	99.83	145.67	159.17	210.17	237.00
S.E.M	0.000	9.816	7.650	6.789	11.068	11.603	16.546	9.356

Table 2.2 : 12 hr of incubation								
	Control	1μM	5μM	10μM	25μM	50μM	75μM	100μM
group 1	100	93	77	90	131	173	195	216
group 2	100	81	67	93	185	228	253	253
group 3	100	101	97	114	124	144	194	273
group 4	100	94	85	95	113	132	156	218
group 5	100	84	66	78	102	123	171	204
group 6	100	80	76	71	141	144	164	218
mean	100.00	88.83	78.00	90.17	132.67	157.33	188.83	230.33
S.E.M	0.000	3.439	4.761	6.096	11.851	15.722	14.370	10.859

Table 2.3 : 24 hr of incubation								
	Control	1μM	5μM	10μM	25μM	50μM	75μM	100μM
group 1	100	97	104	104	174	183	197	214
group 2	100	63	60	98	164	171	183	207
group 3	100	95	91	87	155	173	195	230
group 4	100	86	91	93	198	202	212	279
group 5	100	108	85	80	126	153	220	235
group 6	100	97	90	88	129	150	222	242
mean	100.00	91.00	86.83	91.67	157.67	172.00	204.83	234.50
S.E.M	0.000	6.288	5.952	3.490	11.203	7.891	6.353	10.382

Table 3. Effects of exposure to LPS in cultured glial cells

Table 3.1 : MTT reduction assay						
	Control	0.01 μg/ml	0.1 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml
group 1	100	85	74	71	67	77
group 2	100	77	70	68	71	78
group 3	100	84	79	76	74	68
group 4	100	86	85	75	75	76
group 5	100	83	86	77	76	73
group 6	100	79	83	78	77	71
mean	100.00	82.33	79.50	74.17	73.33	73.83
S.E.M	0.000	1.453	2.617	1.579	1.520	1.579

Table 3.2 : LDH release assay						
	Control	0.01 μg/ml	0.1 μg/ml	1 μg/ml	10 μg/ml	100 μg/ml
group 1	100	109	111	130	123	124
group 2	100	92	95	88	98	91
group 3	100	117	115	128	115	111
group 4	100	102	104	108	117	110
group 5	100	87	93	89	78	88
group 6	100	95	83	97	102	111
mean	100.00	100.33	100.17	106.67	105.50	105.83
S.E.M	0.000	4.587	4.915	7.649	6.717	5.594

Table 4. Effects of pre-treatment with piperine in LPS-exposed cultured glial cells

Table 4.1 : MTT reduction assay								
	control	LPS +						
		0 μM	1 μM piperine	2.5 μM piperine	5 μM piperine	7.5 μM piperine	10 μM piperine	100 μM trolox
group 1	100	78	90	90	94	91	92	83
group 2	100	92	95	98	93	95	99	106
group 3	100	90	87	89	95	94	98	108
group 4	100	79	92	89	93	96	89	100
group 5	100	85	98	95	96	97	97	93
group 6	100	90	88	94	99	98	91	101
mean	100.00	85.67	91.67	92.5	95.00	95.17	94.33	98.50
S.E.M.	0.000	2.458	1.726	1.522	0.931	1.014	1.706	3.766

Table 4.2 : LDH release assay								
	control	LPS +						
		0 μM	1 μM piperine	2.5 μM piperine	5 μM piperine	7.5 μM piperine	10 μM piperine	100 μM trolox
group 1	100	112	109	108	104	102	122	98
group 2	100	105	120	116	107	119	129	108
group 3	100	121	92	83	80	96	104	122
group 4	100	111	113	109	108	118	116	111
group 5	100	97	86	94	77	96	94	104
group 6	100	97	85	103	112	95	91	97
mean	100.00	107.17	100.83	102.17	98.00	104.33	109.33	106.67
S.E.M.	0.000	3.833	6.140	4.854	6.266	4.595	6.302	3.792

Table 5. Effects of co-treatment with piperine and LPS in cultured glial cells for 24 hr of incubation

Table 5.1 : MTT reduction assay								
	control	LPS +						
		0 μM	1 μM piperine	2.5 μM piperine	5 μM piperine	7.5 μM piperine	10 μM piperine	100 μM trolox
group 1	100	88	94	102	99	103	102	103
group 2	100	87	84	95	105	102	104	104
group 3	100	87	103	96	103	105	105	111
group 4	100	101	119	121	124	122	129	130
group 5	100	99	101	99	110	104	111	113
group 6	100	100	104	101	108	109	113	123
mean	100.00	93.67	100.83	102.33	108.17	107.5	110.67	114.00
S.E.M.	0.000	2.848	4.743	3.896	3.535	3.063	4.055	4.351

Table 5.2 : LDH release assay								
	control	LPS +						
		0 μM	1 μM piperine	2.5 μM piperine	5 μM piperine	7.5 μM piperine	10 μM piperine	100 μM trolox
group 1	100	101	105	107	90	101	101	94
group 2	100	88	93	95	97	99	107	90
group 3	100	99	93	79	91	81	106	91
group 4	100	109	103	105	107	113	112	109
group 5	100	113	95	86	76	74	72	74
group 6	100	110	111	91	103	99	111	69
mean	100.00	103.33	100.00	93.83	94.00	94.50	101.50	87.83
S.E.M.	0.000	3.783	3.044	4.430	4.502	5.852	6.114	5.907

Table 6. Effects of co-treatment with piperine and LPS in cultured glial cells for 96 hr of incubation

Table 6.1 : MTT reduction assay								
	control	LPS +						
		0 μM	1 μM piperine	2.5 μM piperine	5 μM piperine	7.5 μM piperine	10 μM piperine	100 μM trolox
group 1	100	88	75	70	71	76	68	85
group 2	100	78	69	75	71	61	67	83
group 3	100	86	76	91	95	85	70	82
group 4	100	85	82	93	91	83	76	87
group 5	100	73	69	73	72	72	71	71
group 6	100	94	97	96	97	94	89	76
mean	100.00	83.67	78.00	83.00	82.83	78.50	73.50	80.67
S.E.M.	0.000	2.974	4.290	4.712	5.205	4.682	3.354	2.459

Table 6.2 : LDH release assay								
	control	LPS +						
		0 μM	1 μM piperine	2.5 μM piperine	5 μM piperine	7.5 μM piperine	10 μM piperine	100 μM trolox
group 1	100	100	87	102	118	122	127	115
group 2	100	106	125	128	127	133	128	128
group 3	100	122	102	104	126	173	178	110
group 4	100	95	121	94	97	100	100	87
group 5	100	128	124	164	122	172	185	136
group 6	100	115	133	109	116	157	178	167
mean	100.00	111.00	115.33	116.83	117.67	142.83	149.33	123.83
S.E.M.	0.000	5.254	7.055	10.515	4.492	12.010	14.495	11.038

Table 7. Effects of post-treatment with piperine in LPS-exposed cultured glial cells

Table 7.1 : MTT reduction assay								
	control	LPS +						
		0 μM	1 μM piperine	2.5 μM piperine	5 μM piperine	7.5 μM piperine	10 μM piperine	100 μM trolox
group 1	100	79	85	87	95	92	89	102
group 2	100	79	86	87	87	86	76	90
group 3	100	79	82	80	87	90	79	87
group 4	100	84	93	99	106	101	99	94
group 5	100	82	105	106	106	106	98	91
group 6	100	80	82	86	89	87	89	93
mean	100.00	80.50	88.83	90.83	95.00	93.67	88.33	92.83
S.E.M.	0.000	0.847	3.628	3.945	3.679	3.293	3.862	2.088

Table 7.2 : LDH release assay								
	control	LPS +						
		0 μM	1 μM piperine	2.5 μM piperine	5 μM piperine	7.5 μM piperine	10 μM piperine	100 μM trolox
group 1	100	92	84	85	91	87	98	102
group 2	100	113	113	89	89	87	124	106
group 3	100	109	99	92	89	109	120	107
group 4	100	100	99	89	97	79	98	107
group 5	100	113	100	102	97	97	114	128
group 6	100	111	96	107	99	109	116	98
mean	100.00	106.33	98.50	94.00	93.67	94.67	111.67	108.00
S.E.M.	0.000	3.480	3.784	3.502	1.838	5.097	4.544	4.250

Table 8. Effects of post-treatment with piperine on glutathione level in LPS-exposed cultured glial cells

	control	LPS	piperine + LPS	trolox + LPS
group 1	100	81.63	102.04	95.92
group 2	100	91.43	114.29	131.43
group 3	100	84.31	98.04	105.88
group 4	100	94.74	105.26	102.63
group 5	100	93.18	90.91	120.45
group 6	100	81.82	95.45	100.00
mean	100.00	87.85	101.00	109.39
S.E.M.	0.000	2.424	3.353	5.585

Table 9. Effects of post-treatment with piperine on the release of nitrite from LPS-exposed cultured glial cells

	LPS	piperine + LPS	trolox + LPS
group 1	100	98.82	94.12
group 2	100	100.00	92.95
group 3	100	102.50	111.25
group 4	100	101.28	107.69
group 5	100	96.67	94.44
group 6	100	97.40	103.90
mean	100.00	99.45	100.72
S.E.M	0.000	0.918	3.230

CURRICULUM VITAE

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