ELSEVIER

Pharmacology, Biochemistry and Behavior 69 (2001) 469–474

PHARMACOLOGY BIOCHEMISTRY AND BEHAVIOR

www.elsevier.com/locate/pharmbiochembeh

Effect of nordihydroguaiaretic acid on behavioral impairment and neuronal cell death after forebrain ischemia

Yoshiyuki Shishido*, Masayoshi Furushiro, Shusuke Hashimoto, Teruo Yokokura

Yakult Central Institute for Microbiological Research, 1796 Yaho, Kunitachi, Tokyo 186-8650, Japan

Received 7 September 2000; received in revised form 8 March 2001; accepted 22 March 2001

Abstract

The purpose of this study was to evaluate the neuroprotective effect of nordihydroguaiaretic acid (NDGA), an antioxidant and/or 5-lipoxygenase inhibitor, on ischemia–reperfusion injury behavioral pharmacologically and histologically in vivo. First, the antioxidant activity of NDGA was evaluated in vitro by measuring the production of thiobarbituric acid reactive substances (TBARS) in rat brain homogenate. Second, the effect of NDGA on learning and memory impairment induced by rat four-vessel occlusion transient ischemia was investigated with the Morris water-maze task. Third, the effect of NDGA on pyramidal cell loss in the hippocampus after transient ischemia was examined. NDGA inhibited the production of TBARS with an IC_{50} of 0.1 μM, and significantly attenuated postischemic learning and memory impairment at 10 mg/kg. Furthermore, consecutive 4-day administration of NDGA at 10 mg/kg significantly reduced the postischemic neuronal death. NDGA was found to be potent and effective as an anti-ischemia–reperfusion injury agent in terms of behavioral pharmacology and histology. The present results suggest that NDGA has beneficial effects on behavioral deficits and histological injury caused by ischemia–reperfusion.

Keywords: Nordihydroguaiaretic acid (NDGA); Antioxidant; Thiobarbituric acid reactive substances (TBARS); Lipid peroxidation; Ischemia–reperfusion injury; Postischemic neuronal death

1. Introduction

The tissue injury that follows ischemia and reperfusion (ischemia–reperfusion injury) is a main cause of disorders of the brain, heart, liver, and kidney. Although the pathophysiological mechanisms underlying ischemia–reperfusion injury are not well-understood, they are likely multifactorial and interdependent, involving hypoxia, hyperoxia, free radical damage, and inflammatory responses (Haba et al., 1991; Paller, 1994). It is important to clarify these mechanisms and to develop a new drug for prevention of the injury. It is known that activated neutrophils can cause tissue injury by releasing cytotoxic oxygen free radicals and/or proteases, and indeed, the role of oxygen free radicals in ischemia–reperfusion injury has received a great deal of attention. Oxygen free radical-mediated lipid peroxidation is, therefore, one of the most important reactions in the progression of ischemia–reperfusion injury (De Vecchi et al., 1998; Haba et al., 1991).

Ischemia–reperfusion injury in the brain induced by stroke, cardiac arrest, and brain injury causes neuronal death and dementia. Recent evidence suggests that the cellular damage induced by cerebral ischemia is at least partly due to oxidative damage caused by free radicals and lipid peroxidation (Haba et al., 1991; Kogure et al., 1985). Inhibition of free radical formation or lipid peroxidation prevents the progression of neuronal damage (Hara et al., 1990; Kondo et al., 1998). Antioxidants such as α-tocopherol (vitamin E), U-92032, and indomethacin have neuroprotective effects on the cell loss after ischemia (Hara et al., 1990; Ito et al., 1994; Kondo et al., 1998). Although there are several reports that antioxidant and/or the 5-lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), has a protective effect on the toxicity of *N*-methyl-D-aspartate (NMDA) and amyloid β (Aβ) in cell cultures (Goodman et al., 1994; Rothman et al., 1993), little is known about the effect of NDGA on behavioral deficits and histological injury caused by ischemia–reperfusion in vivo.

* Corresponding author. Tel.: +81-42-577-8970; fax: +81-42-577-3020.

E-mail address: yoshiyuki-shishido@yakult.co.jp (Y. Shishido).

0091-3057/01/$ – see front matter
PII: S0091-3057(01)00572-X

In the present study, after the antioxidant activity of NDGA was evaluated by measuring the production of thiobarbituric acid reactive substances (TBARS), the effect of NDGA, using the rat four-vessel occlusion transient ischemia model, on learning and memory, as assessed with the Morris water-maze task, was investigated. Furthermore, the effect of NDGA on pyramidal cell loss in the hippocampus after transient ischemia was evaluated.

2. Materials and methods

2.1. Animals

Eight-week-old male rats of the Wistar strain (Nippon Clea, Tokyo, Japan) were housed in groups of three per cage at a constant temperature (24 ± 1°C) and humidity (60 ± 5%) with a 12-h light–dark cycle (light period: 0830–2030). The rats were given food and water ad libitum. The animal experimentation guidelines of our institute were followed.

2.2. Measurement of TBARS

Antioxidant activity was evaluated by measuring rat brain-derived TBARS, an indicator of lipid peroxidation (Agar et al., 1999). In brief, a rat was decapitated, and its brain was removed rapidly. The whole brain besides the cerebellum was homogenized in 50 mM potassium phosphate buffer (pH 7.4) and the concentration adjusted to 1 g wet weight of brain per 60 ml. NDGA original solutions of various concentrations were prepared with dimethylsulfoxide (DMSO). They were diluted 500-fold with ethanol [0.2% (v/v) DMSO-ethanol] and dispensed to 1.5-ml polyethylene tubes (Eppendorf, Germany), 50 μl in each. Then, 250 μl of the homogenate was introduced into the polyethylene tubes, and the mixtures were incubated in a water bath for 1 h at 37°C. After incubation, 400 μl of 35% perchloric acid was added to stop peroxidation. We obtained 600 μl of supernatant by centrifugation at 1400 × *g* for 10 min, and added it to 200 μl of 1.2% sodium 2-thiobarbiturate solution. The mixture was then placed in a water bath and heated for 30 min at 95–100°C. After the solution cooled, the absorbance was measured with a microplate reader (SPECTRA max 250; Molecular Devices, CA) at a wavelength of 532 nm. The protein concentration of the original homogenate was determined by using Bio-Rad Protein Assay (Bio-Rad, USA).

2.3. Surgery and experimental procedures

Transient forebrain ischemia was produced by the method of Pulsinelli and Brierley (1979). Briefly, the rats were anesthetized with sodium pentobarbital (Nembutal Sodium solution; Abbott Laboratory, North Chicago, IL), and the vertebral arteries were cauterized bilaterally with a bipolar coagulator (MICRO-ID; Mizuho Ikakogyo, Tokyo). At the same time, threads were placed loosely around each common carotid artery, but carotid blood flow was not interrupted. On the following day, the rats, under light anesthesia with ether, were fixed ventral-side upwards on boards, and their common carotid arteries were exposed by pulling the threads. By occluding the bilateral carotid artery with Sugita's aneurysm clips (No. 52; Mizuho Ikakogyo), 5-min forebrain ischemia was produced in behavioral studies, and 10-min forebrain ischemia in histological studies. Body temperature during the ischemia was maintained at 37°C by a heating mat (Animal Blanket Controller; Nihon Koden, Tokyo). The criteria for forebrain ischemia were bilateral loss of the righting reflex and paw extension. Only the animals that showed continuous loss of the righting reflex for over 10 min after 5 min of ischemia, or over 15 min after 10 min of ischemia were selected. There was no significant difference in duration between the NDGA-injected group and saline-injected group. The control (sham-operated) rats had their vertebral arteries cauterized, but did not have their carotid arteries occluded.

2.4. Morris water maze

2.4.1. Apparatus

The swim tank was monotone blue, 150 cm in diameter and 45 cm in height, with a hidden platform 10 cm in diameter and 30 cm in height made of clear plexiglass and placed 37.5 cm from the wall in the middle of one of the quadrants. The platform was submerged 3 cm below the surface of water kept at 20 ± 1°C (Fig. 1). The location of the hidden platform remained unchanged throughout the experiment. The animal's performance was recorded using an overhead video camera (Sony, Tokyo) and AXIS 30 (Neuroscience, Tokyo). The time taken to reach the platform (latency) and the swim path length were analyzed by TARGET/2 (Neuroscience). Swimming speed (cm/s) was calculated by dividing the swim path length (cm) by the latency (sec).

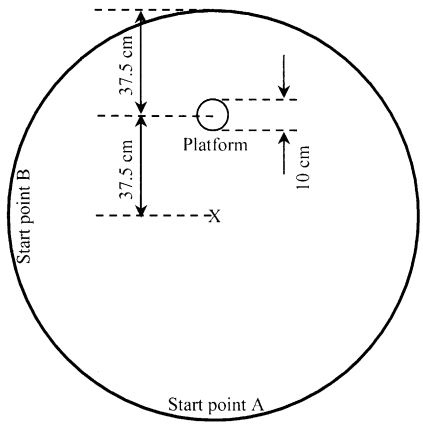

Fig. 1. Schematic drawing of the Morris water-maze apparatus.

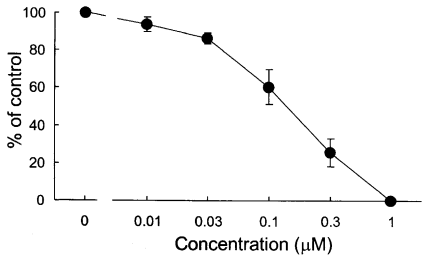

Fig. 2. Inhibitory effect of NDGA on TBARS production in rat brain homogenate. Rat brain homogenate (1.8 mg protein/ml) containing indicated concentrations of NDGA was incubated at 37°C for 1 h. TBARS were measured by the method described in the Materials and Methods section. Data are the mean ± S.D. for three individual experiments.

2.4.2. Procedure

The Morris water-maze task was begun 1 day after carotid occlusion. The method was essentially based on the technique described by Morris (1984). Trials were started by placing the animals into the pool, close to and facing the wall at one of two equally spaced starting locations, A and B, around the perimeter of the pool (Fig. 1). Animals were allowed to swim freely until they found the submerged platform or until 180 s elapsed. If an animal found the platform, it was allowed to remain there for 30 s and then returned to its home cage. If an animal failed to find the platform in this allotted time, the trial was terminated and a maximum score of 180 s was assigned. The animal was placed on the platform by hand and was forced to stay there for 30 s to learn its location. Each rat received two trials in one session a day. The data in each trial were combined as the mean of each session.

2.5. Evaluation of neuronal death

Serial hippocampal sections were examined to assess the brain damage by transient ischemia. In brief, 5 days after the ischemia, each rat was decapitated, and its brain was removed rapidly and embedded in Tissue Mount (Shiraimatsu Kikai, Tokyo) using standard procedures. Coronal sections, 5-μm thick, were fixed with 4% paraformaldehyde and stained with Toluidine blue (Sigma, St. Louis, MO). Representative sections of the dorsal hippocampus were used for the quantification of neuronal degeneration. The criteria for hippocampus CA1 damage were based upon findings of Buchan and Pulsinelli (1990), Pulsinelli and Brierley (1979), Pulsinelli et al. (1982a,b), that is, 0 (*normal hippocampus*); 1 (*< 10% of neurons damaged*); 2 (*10–50% of neurons damaged*); and 3 (*> 50% of neurons damaged*).

2.6. Drugs

NDGA was purchased from Sigma. It was dissolved in DMSO. The solution was diluted with saline five times [20% (v/v) DMSO-saline solution] and administered intraperitoneally in a volume of 0.1–0.5 ml per 100 g body weight. In behavioral studies, the drug was administered once at 30 min before the ischemia. In histological studies, it was administered 30 min before and once a day for 3 consecutive days after the ischemia (× 4 treatment).

2.7. Data analysis

The significance of differences between groups was determined using Wilcoxon's test, Schally–Williams' test and Dunnett's test.

3. Results

3.1. Antioxidant activity of NDGA

The production of TBARS after 1-h incubation at 37°C in 0.03% DMSO, 17% ethanol, and 42 mM potassium phosphate buffer (pH 7.4) (control) was equivalent to 4 nmol malondialdehyde/1.8 mg protein/ml. The effects of various concentrations of NDGA on the production are shown in Fig. 2. The production in the control buffer was regarded as 100%. TBARS production was inhibited by NDGA (0.03–1 μM), in a concentration-dependent manner. The IC_{50} of NDGA for the production was 0.1 μM, similar to that of α-tocopherol (0.2 μM).

3.2. Effects of NDGA on postischemic impairment of learning and memory

Ischemia-rats (vehicle) required significantly more time than sham-operated controls (Fig. 3). Corresponding to the prolongation of escape latency, the swim path length for ischemia-rats was significantly longer than that of sham-operated controls except for the first session, but the swim speed of ischemia-rats did not differ from that of sham-

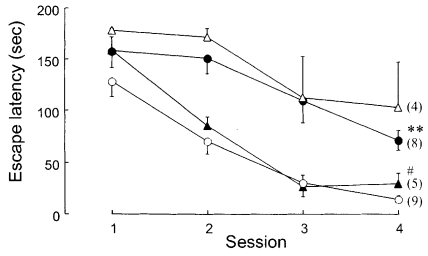

Fig. 3. Effect of NDGA on the cerebral ischemia-induced increase in latency in the Morris water-maze task. The water-maze task was performed 24 h after carotid artery occlusion (○, sham; ●, 5 min of ischemia, vehicle). NDGA was administered intraperitoneally 30 min before the occlusion (△, 5 mg/kg; ▲, 10 mg/kg). Each point and bar represent the mean ± S.E.M. of escape latency in each session. Numbers in parentheses indicate the number of rats. Statistical significance: ** $P < .01$ vs. sham (Wilcoxon's test), # $P < .05$ vs. vehicle (Schally–Williams' test).

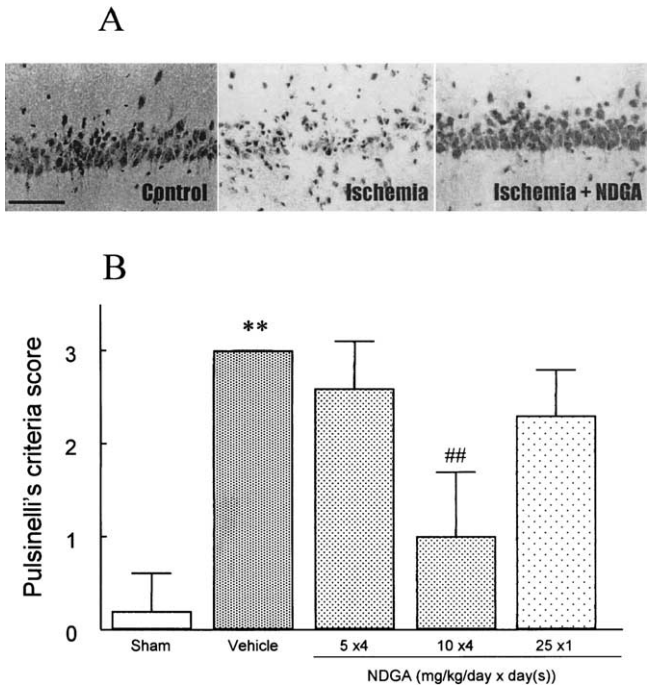

Fig. 4. Effect of NDGA on hippocampal CA1 neuronal cell loss evoked by 10-min global ischemia in rats. (A) Histological changes in pyramidal cells in the CA1 area of rat hippocampus at 5 days postischemia. Sham: sham-operated; Ischemia: ischemia + saline; Ischemia + NDGA: ischemia + NDGA (10 mg/kg × 4). Scale bar: 100 μm (Toluidine blue stain). (B) Evaluation of the effect of NDGA by Pulsinelli's criteria score. Data are the mean ± S.D. for five rats. Statistical significance: ** $P < .01$ vs. sham-operated group (sham) (Wilcoxon's test), ## $P < .01$ vs. ischemia + saline (vehicle) (Dunnett's test).

operated rats (data is not shown). NDGA at 5 mg/kg had no effect, but NDGA at 10 mg/kg significantly reduced the prolongation of escape latency induced by ischemia (Fig. 3). Corresponding to the reduction of escape latency, treatment with NDGA resulted in a decrease in the exaggeration of the swim path length induced by ischemia, but the swim speed was not influenced by NDGA (data not shown).

3.3. Effects of NDGA on postischemic neuronal death

Brain sections from the anterior hippocampus of a sham-operated (control) rat, a rat given 10 min of transient forebrain ischemia and a rat administered 10 mg/kg NDGA, 30 min before the ischemia and once a day for 3 consecutive days after the ischemia (10 mg/kg/day × 4 days) are shown in Fig. 4. Histological examination of the brains of sham-operated animals revealed no cell damage at all. In rats exposed to 10 min of transient forebrain ischemia (vehicle), extensive cell loss in the CA1 subfield was observed. In the NDGA-administered rats, almost no cells were damaged. The severity of CA1 neuronal cell loss in each group, as assessed by Pulsinelli's criteria, is shown in Fig. 4. The difference between the 10-min ischemia (vehicle) group and the sham-operated group was significant. Ten minutes of ischemia significantly led to damage of pyramidal cells. Although single treatment with NDGA at 25 mg/kg, 30 min before the ischemia, had no effect, consecutive 4-day treatment with NDGA significantly reduced the postischemic neuronal death.

4. Discussion

In the first experiment, NDGA inhibited the production of TBARS derived from rat brain homogenate. The IC_{50} was 0.1 μM, less than that of α-tocopherol (0.2 μM). This result indicates that NDGA is an extremely potent inhibitor of lipid peroxidation. In the second experiment, 5-min forebrain ischemia affected escape latency and swim path length, but not swimming speed. These results show that the ischemia–reperfusion induces learning and memory impairment but not motor dysfunction. The impairment is prevented by NDGA. In the third experiment, consecutive 4-day treatment with NDGA attenuated 5-day postischemic neuronal death in the hippocampal CA1 subfield. In the histological study, we produced ischemia-rats with 5-min occlusion and 10-min occlusion. In the 5-min ischemia-rats, the histological damage was not severe. Thus, we chose 10 min of ischemia for the histological study. Since the behavioral experiments and the histological experiments were performed under different conditions, it is not possible to draw a direct relationship between the two beneficial effects of NDGA in the behavioral and the histological studies. However, considering that several previous reports suggesting lipid peroxidation as a physiologic process in

postischemic neuronal damage (Aktan et al., 1993; Haba et al., 1991; Hall et al., 1988), the present results demonstrate that NDGA significantly protects against postischemic histological and functional damage in the brain by its antioxidative activity. The ability of NDGA to inhibit central nervous system tissue lipid peroxidation appears to involve multiple mechanisms including an α-tocopherol-like scavenging of lipid hydroperoxides (Aktan et al., 1993) and inhibitory actions for key enzymes in arachidonic acid metabolism (Aktan et al., 1993; Rothman et al., 1993).

The mechanisms of ischemia–reperfusion injury are multifactorial, and it is possible that other actions of NDGA are effective in preventing the injury. In brief, NDGA is known as an inhibitor of 5-lipoxygenase (Chang et al., 1984). Since potentially deleterious arachidonic acid metabolites accumulate markedly in gerbil tissue during postischemic reperfusion (Gaudet and Levine, 1979), a reduction of arachidonic acid release by NDGA in the central nervous system membrane may play a partial role in the neuroprotective effect of NDGA. Keeping the body temperature low is known to prevent ischemia–reperfusion-induced neuronal death. Arachidonic acid metabolism modulates body temperature. In the present study, the body temperature of rats was maintained at 37°C by a heating mat to avoid hypothermia during ischemia and the first 15 min after reperfusion. But we did not monitor temperature until a few hours after the reperfusion. It is thus necessary to investigate the influence of the NDGA on temperature, too.

Activation of NMDA receptors is a typical model for ischemia–reperfusion-like stress on neurons in vitro. The liganded NMDA receptor promotes the release of arachidonic acid and induces nitric oxide production (Rordorf et al., 1991; Sanfeliu et al., 1990). Some investigators have suggested that NMDA-induced production of nitric oxide is partially responsible for the neurotoxicity (Dawson et al., 1991). Since NDGA appears to interfere with nitric oxide production, that might explain the protective effect against the neurotoxicity of NMDA or ischemia–reperfusion (Forstermann et al., 1988). On the other hand, NDGA enhances nitric oxide production in cultured endothelial cells and this action might have beneficial effects for hypoxic conditions (Ramasamy et al., 1999).

NDGA also protected cultured rat hippocampal neurons against the toxicity of Aβ by suppressing Aβ-induced accumulation of reactive oxygen species and intracellular calcium (Goodman et al., 1994). Considering further that NDGA strongly diminished cytokine secretion by dendritic cells (Ramoner et al., 1998) and that NDGA interfered with TNF-induced NF-κB-mediated transactivation (Van Puijenbroek et al., 1999), more detailed studies are needed to provide conclusive evidence of an in vivo effect of NDGA on lipid peroxidation and/or arachidonic acid release. Until such data are provided, other protective mechanisms cannot be excluded.

The current study has demonstrated that NDGA is potent and effective as an anti-ischemia–reperfusion injury agent in terms of behavioral pharmacology and histology. For these reasons, antioxidants such as NDGA may prevent brain damage due to ischemic attack and become an agent for the treatment of cerebrovascular disease.

References

Agar E, Bosnak M, Amanvermez R, Demir S, Ayyildiz M, Celik C. The effect of ethanol on lipid peroxidation and glutahione level in the brain stem of rat. NeuroReport 1999;10:1799–801.

Aktan S, Aykut C, Yegen BC, Okar I, Ozkutlu U, Ercan S. The effect of nordihydroguaiaretic acid on leukotriene C_4 and prostaglandin E_2 production following different reperfusion periods in rat brain after forebrain ischemia correlated with morphological changes. Prostaglandins, Leukotrienes Essent Fatty Acids 1993;49:633–41.

Buchan AM, Pulsinelli WA. Septo-hippocampal deafferentation protects CA1 neurons against ischemic injury. Brain Res 1990;512:7–14.

Chang J, Skowronek MD, Cherney ML, Lewis AL. Different effects of putative lipoxygenase inhibitors on arachidonic acid metabolism in cell-free and intact cell preparations. Inflammation 1984;8:143–55.

Dawson VL, Dawson TM, London ED, Bredt DS, Snyder SH. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. PNAS 1991;88:6368–71.

De Vecchi E, Lubatti L, Beretta C, Ferrero S, Rinaldi P, Kienle MG, Trazzi R, Paroni R. Protection from renal ischemia–reperfusion injury by the 2-methylaminochroman U83836E. Kidney Int 1998;54:857–63.

Forstermann U, Alheid U, Frolich JC, Mulsch A. Mechanism of action of lipoxygenase and cytochrome *P*-450-monooxygenase inhibitors in blocking endotherium-dependent vasodilation. Br J Pharmacol 1988;93:569–78.

Gaudet RJ, Levine L. Transient cerebral ischemia and brain prostaglandins. Biochem Biophys Res Commun 1979;86:893–901.

Goodman Y, Steiner MR, Steiner SM, Mattson MP. Nordihydroguaiaretic acid protects hippocampal neurons against amyloid β-peptide toxicity, and attenuates free radical and calcium accumulation. Brain Res 1994;654:171–6.

Haba K, Ogawa N, Mizukawa K, Mori A. Time course of changes in lipid peroxidation, pre- and postsynaptic cholinergic indices, NMDA receptor binding and neuronal death in the gerbil hippocampus following transient ischemia. Brain Res 1991;540:116–22.

Hall ED, Pazara BA KE, Braughler JM. 21-Aminosteroid lipid peroxidation inhibitor U74006F protects against cerebral ischemia in gerbils. Stroke 1988;19:997–1002.

Hara H, Kato H, Kogure K. Protective effect of α-tocopherol on ischemic neuronal damage in the gerbil hippocampus. Brain Res 1990;510:335–8.

Ito C, Im WB, Takagi H, Takahashi M, Tsuzuki K, Liou SY, Kunihara M. U-92032, a T-type Ca^{2+} channel blocker and antioxidant, reduces neuronal ischemic injuries. Eur J Pharmacol 1994;257:203–10.

Kogure K, Arai H, Abe K, Nakano M. Free radical damage of the brain following ischemia. Prog Brain Res 1985;63:237–59.

Kondo F, Kondo Y, Gomez-Vargas M, Ogawa N. Indomethacin inhibits delayed DNA fragmentation of hippocampal CA1 pyramidal neurons after transient forebrain ischemia in gerbils. Brain Res 1998;791:352–6.

Morris RGM. Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 1984;11:47–60.

Paller MS. The cell biology of reperfusion injury in the kidney. J Invest Med 1994;42:632–9.

Pulsinelli WA, Brierley JB. A new model of bilateral hemispheric ischemia in the unanesthetized rat. Stroke 1979;10:267–72.

Pulsinelli WA, Brierley JB, Plum F. Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol 1982a;11:491–8.

Pulsinelli WA, Levy DE, Duffy TE. Regional cerebral blood flow and glu-

cose metabolism following transient forebrain ischemia. Ann Neurol 1982b;11:499–509.

Ramasamy S, Drummond GR, Ahn J, Storek M, Pohl J, Parthasarathy S, Harrison DG. Modulation of expression of endothelial nitric oxide synthase by nordihydroguaiaretic acid, a phenolic antioxidant in cultured endothelial cells. Mol Pharmacol 1999;56:116–23.

Ramoner R, Rieser C, Bartsch G, Thurnher M. Nordihydroguaiaretic acid blocks secretory and endocytic pathways in human dendritic cells. J Leukocyte Biol 1998;64:747–52.

Rordorf G, Uemura Y, Bonventre JV. Characterization of phospholipase A_2 (PLA_2) activity in gerbil brain: enhanced activities of cytosolic, mitochondrial, and microsomal forms after ischemia and reperfusion. J Neurosci 1991;11:1829–36.

Rothman SM, Yamada KA, Lancaster N. Nordihydroguaiaretic acid attenuates NMDA neurotoxicity — action beyond the receptor. Neuropharmacology 1993;32:1279–88.

Sanfeliu C, Hunt A, Patel AJ. Exposure to *N*-methyl-D-aspartate increases release of arachidonic acid in primary cultures of rat hippocampal neurons and not in astrocytes. Brain Res 1990;526:241–8.

Van Puijenbroek AAFL, Wissink S, Van der Saag PT, Peppelenbosch MP. Phospholipase A_2 inhibitors and leukotriene synthesis inhibitors block TNF-induced NF-κB activation. Cytokine 1999;11:104–10.