

Inhibitory effects of ginkgolides on nitric oxide production in neonatal rat microglia *in vitro*

DU Ze-Ying, LI Xiao-Yu¹ (Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China)

KEY WORDS ginkgolides; platelet-activating factor; nitroarginine; nitric oxide; microglia; lipopolysaccharides; cultured cells; apafant

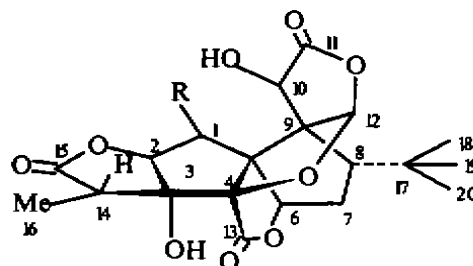
AIM: To study the effects of ginkgolide A (GA) and B (GB), apafant (Apa), and *N* ω -nitro-*L*-arginine (*L*-NA) on nitric oxide (NO) production in cultured neonatal rat microglia.

METHODS: NO concentration was represented by nitrite which was determined by Griess reaction. **RESULTS:** In resting microglia, no inhibitory effects of GA, GB, and Apa were observed. *L*-NA inhibited NO production, its IC₅₀ value (95% confidence limits) being 3.4 (0.8-14.9) $\mu\text{mol}\cdot\text{L}^{-1}$. GA, GB, and *L*-NA inhibited NO production in LPS-stimulated microglia, and their IC₅₀ values (95% confidence limits) were 5.7 (1.8-18.1), 1.1 (0.3-4.4), and 0.5 (0.1-2.8) $\mu\text{mol}\cdot\text{L}^{-1}$, respectively. Apa did not inhibit NO production. **CONCLUSION:** GA and GB inhibited NO production in LPS-stimulated microglia.

Nitric oxide (NO) is considered as a new class of signaling molecule that acts primarily on the nervous and cardiovascular systems. In rodent, Ca²⁺-independent inducible NO synthase (iNOS) is found in monocyte/macrophage series, including microglia, the resident macrophage of the central nervous system^[1]. β -Amyloid protein, interferon- γ , interleukin-1 (IL-1), tumor necrosis factor- α (TNF- α), and lipopolysaccharide (LPS) activate microglia to produce high level of NO^[2-4]. Activated microglia mediate neuron injury through NO mechanism^[4]. This highly reactive free radical has cytotoxic properties and is implicated in neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, AIDS dementia, and stroke. Thus, to inhibit NO production

from microglia may be a new way to counteract neuronal injury by therapeutic agents.

Ginkgolides, from *Ginkgo biloba*, are platelet-activating factor (PAF) receptor antagonists^[5,6]. Ginkgolide A (GA, BN52020) and B (GB, BN52021) reduced excitotoxic damage to cultured neurons and protected rat hippocampus and neocortex from ischemic injury *in vivo*^[5]. GB counteract tissue damage and enhanced neurological recovery after traumatic brain injury^[6]. GB inhibited *D*-[³H] aspartate release in rat hippocampal slice after ischemia *in vitro*^[7]. However, apafant (Apa), a PAF antagonist of the triazolobenzodiazepine type, did not protect neuron from excitotoxic damage in cultured neurons from embryonic chick telencephalon^[5]. In this paper, the effects of ginkgolides on NO production from LPS-stimulated neonatal rat microglia *in vitro* were investigated.



R = H ginkgolide A (GA, C₂₀H₂₄O₉)

R = OH ginkgolide B (GB, C₂₀H₂₄O₁₀)

MATERIALS AND METHODS

Reagents Crystals of GA and GB were generous gifts from Prof CHEN Zhong-Liang (Department of Phytochemistry of our institute). LPS (*E coli* O55:B5) and *N* ω -nitro-*L*-arginine (*L*-NA) were purchased from Sigma Co. Apa was kindly offered by Dr YU Xiang-Bin (Department of Clinical Pharmacology, Dongfang Hospital, Fuzhou, China). Dulbecco's modified Eagle medium (DMEM, high glucose, Gibco BRL) was supplemented with 10% newborn bovine serum, *L*-glutamine 2 mmol·L⁻¹, sodium

¹ Correspondence to Prof LI Xiao-Yu. Phn 86-21-6431-1833, ext 317. Fax 86-21-6437-0269. E-mail xyli@server.shnc.ac.cn
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pyruvate $1 \text{ mmol} \cdot \text{L}^{-1}$, benzylpenicillin $100 \text{ kU} \cdot \text{L}^{-1}$, and streptomycin $100 \text{ mg} \cdot \text{L}^{-1}$. Sulfanilamide, *N*-(1-naphthyl) ethylenediamine dihydrochloride (NEDA) and phosphoric acid were domestic AR grade products. Griess reagent was composed of 1 % sulfanilamide and 0.1 % NEDA in 4 % phosphoric acid.

Primary neonatal rat microglial culture

Mixed glial cultures were prepared from cerebra of neonatal Sprague-Dawley rats (within 24 h)^[8]. Brain tissues were minced and dissociated by gently pipetting in phosphated buffered saline (PBS, pH 7.4), then passed through 80 and 120 μm diameter stainless steel meshes successively. Cells ($5 \times 10^9 \cdot \text{L}^{-1}$) were suspended in DMEM and seeded on glass tissue flasks precoated with rat tail collagen. The flasks were incubated at $36.5 \text{ }^\circ\text{C}$ in a humidified air with 5 % CO_2 . At time of primary cell confluence (d 14), microglia were harvested by gentle shaking flasks 3 - 4 h on a horizontal platform (LAB-LINE/DINOFF Shaker). In each subsequent week, microglia were harvested from the very same astrocyte (feeder) cultures^[9]. The supernatants were centrifuged at $200 \times g$ for 5 min. Cells were resuspended on DMEM and seeded in 48-well plastic plates (Costar) at the concentration of $2 \times 10^8 \cdot \text{L}^{-1}$ for adherence for 40 min. After the cells attached, they were supplied with fresh media and cultured for 1 d to allow them recovered from mechanical stimulation. The cells were evaluated^[10] by nonspecific esterase staining >99 %, by rat macrophage specific antigen ED1 staining >95 %, and astroglial specific protein GFAP staining <5 %.

NO production in rat microglia induced by medication Primary rat microglia were seeded on 48-well plates (Costar) at $2 \times 10^8 \cdot \text{L}^{-1}$. GA, GB, Apa, or *L*-NA ($1 - 10\,000 \text{ nmol} \cdot \text{L}^{-1}$) were added separately with or without LPS $1 \text{ mg} \cdot \text{L}^{-1}$. All treatments were incubated for 24 h. The supernatants were stored at $-20 \text{ }^\circ\text{C}$ until assay.

Assay for NO production Nitrite concentration was determined by Griess reaction^[11]. Culture supernatants $100 \mu\text{L}$ were mixed with $100 \mu\text{L}$ of Griess reagent. After chromophore was formed at $25 \text{ }^\circ\text{C}$ for 15 min,

absorbance was determined at 570 nm using EIA reader, and nitrite concentration was determined with reference to a standard curve of sodium nitrite using concentrations from 3.125 to $200 \mu\text{mol} \cdot \text{L}^{-1}$.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed by *t*-test. IC_{50} values and their 95 % confidence limits were calculated by Bliss method.

RESULTS

In resting microglia GA, GB, and Apa at $0.001 - 10 \mu\text{mol} \cdot \text{L}^{-1}$ did not inhibit NO production. However, *L*-NA ($0.01 - 10 \mu\text{mol} \cdot \text{L}^{-1}$) decreased NO production in a concentration-dependent manner ($r = 0.92$), its IC_{50} value (95 % confidence limits) being $3.4 (0.8 - 14.9) \mu\text{mol} \cdot \text{L}^{-1}$ (Tab 1).

Tab 1. Effects of GA, GB, *L*-NA, and Apa on NO production in resting and LPS-stimulated microglia. $n = 4$ experiments ($2 \times 10^8 \text{ cells} \cdot \text{L}^{-1}$ from 12 - 14 neonatal rats, 0.5 mL/well). $\bar{x} \pm s$. ^a $P > 0.05$, ^c $P < 0.01$ vs resting microglia $0 \text{ nmol} \cdot \text{L}^{-1}$. ^d $P > 0.05$, ^e $P < 0.05$, ^f $P < 0.01$ vs LPS-stimulated microglia $0 \text{ nmol} \cdot \text{L}^{-1}$.

$\text{nmol} \cdot \text{L}^{-1}$	GA	$\text{NO}_2^- / \mu\text{mol} \cdot \text{L}^{-1}$		
		GB	<i>L</i> -NA	Apaant
Resting microglia				
0	23.8 ± 1.7	20.7 ± 3.2	25.2 ± 4.1	17.2 ± 2.6
1	26.6 ± 2.4^a	23.6 ± 3.0^a	25.8 ± 0.7^a	16.9 ± 1.9^a
10	22.7 ± 5.6^a	19.8 ± 4.1^a	20.2 ± 1.5^c	16.2 ± 3.0^a
100	24.2 ± 7.2^a	22.4 ± 6.2^a	16.8 ± 1.5^c	15.8 ± 2.1^a
1 000	21.8 ± 3.3^a	22.8 ± 0.7^a	14.6 ± 2.8^e	14.6 ± 2.2^a
10 000	20.9 ± 5.4^a	20.2 ± 1.4^a	12.4 ± 1.8^e	15.9 ± 2.3^a
LPS-stimulated microglia				
0	35.1 ± 0.7	35.1 ± 0.7	32.6 ± 2.3	32.6 ± 1.3
1	35.8 ± 2.1^d	31.5 ± 1.0^e	26.8 ± 2.1^f	32.1 ± 4.8^d
10	35.1 ± 4.9^d	22.9 ± 6.1^f	20.3 ± 1.2^f	30.4 ± 4.1^d
100	26.5 ± 1.0^f	19.3 ± 3.0^f	17.8 ± 1.1^f	31.9 ± 3.3^d
1 000	22.4 ± 1.0^f	18.6 ± 1.1^f	14.3 ± 2.8^f	29.8 ± 6.4^d
10 000	18.0 ± 2.1^f	15.0 ± 1.4^f	13.4 ± 1.8^f	30.4 ± 5.7^d

In LPS ($1 \text{ mg} \cdot \text{L}^{-1}$)-stimulated microglia GA ($0.1 - 10 \mu\text{mol} \cdot \text{L}^{-1}$), GB ($0.01 - 10 \mu\text{mol} \cdot \text{L}^{-1}$), and *L*-NA ($0.001 \text{ nmol} \cdot \text{L}^{-1} - 10 \mu\text{mol} \cdot \text{L}^{-1}$) reduced NO production in dose-dependent manner ($r = 0.96, 0.85, \text{ and } 0.95$, respectively), and IC_{50} values (95 % confidence limits) were $5.7 (1.8 - 18.1)$, $1.1 (0.3 - 4.4)$, and $0.5 (0.1 - 2.8) \mu\text{mol} \cdot \text{L}^{-1}$,

respectively. However, Apa did not inhibit NO production from LPS-stimulated neonatal rat microglia (Tab 1).

DISCUSSION

The synthesis of NO in neurons, endothelial cells, and platelets is initiated by a receptor or less commonly by ion-channel receptor influx of calcium, resulting in activation of the calcium/calmodulin-dependent NOS, ie, constitutive NOS (cNOS). By contrast, the synthesis of NO by cells of the immune system is calcium-independent and is induced in the presence of LPS and various cytokines. It was suggested that NO production in rat microglia was dependent on Ca^{2+} -independent iNOS which could be induced to produce sustained high level of NO by LPS and interferon- γ . In the present study, high level of NO production in LPS-activated microglia was observed. Moreover, resting microglia produced NO ($17.2 - 25.2 \mu\text{mol} \cdot \text{L}^{-1}$). The IC_{50} value of NOS inhibitor L-NA in resting microglia was similar with K_i ($1.5 \mu\text{mol} \cdot \text{L}^{-1}$) for bovine cerebrum cNOS which is dependent on Ca^{2+} and calmodulin^[12]. The results might suggest that there exist cNOS, besides iNOS in rat neonatal microglia. Moreover, the IC_{50} value of L-NA in LPS-stimulated microglia was lower. It suggested that L-NA showed relative selectivity in inhibition of activated microglia to produce NO.

However, GA and GB decreased NO production in LPS-stimulated rat microglia only. EGb 761, a mixed extract which consists of flavonoids, terpenoids (ginkgolides and bilobalides), and some organic acid, from *Ginkgo biloba* leaves, exhibits to scavenge the superoxide anion, hydroxyl radicals, peroxy radical species, and NO directly, inhibit NOS activity directly in intact cells and the activity of iNOS from macrophage cytosolic fraction, and inhibits iNOS mRNA expression at high concentration ($200 \text{ mg} \cdot \text{L}^{-1}$)^[13,14]. The various constituents of EGb 761 may contribute to these inhibitory effects by their additive or synergistic roles. Our results showed that GA and GB inhibited NO production. However, another PAF antagonist Apa did not show any inhibitory effect on NO production in rat microglia, it was parallel with the negative effect

on excitotoxic damage to neurons^[8]. It supported that NO is a key factor mediated neuron damage. Recently, GB has been used in the treatment of multiple sclerosis patients and got encouraging results^[15]. Thus, inhibition of NO production from microglia may be an effective way to protect neuron injury from trauma, Alzheimer's disease, multiple sclerosis, and stroke.

REFERENCES

- 1 Broenan CF, Battistini L, Raine CS, Dickson DW, Casadevall A, Lee SC. Reactive nitrogen intermediates in human neuropathology: an overview. *Dev Neurosci* 1994; 16: 152 - 61.
- 2 Meda L, Cassatella MA, Szendrei GI, Ottos L Jr, Baron P, Villalba M, *et al.* Activation of microglial cells by β -amyloid protein and interferon- γ . *Nature* 1995; 374: 647 - 50.
- 3 Chao CC, Hu S, Molitor TW, Shaskan EG, Peterson PK. Activated microglia mediated neuronal cell injury via a nitric oxide mechanism. *J Immunol* 1992; 149: 2736 - 41.
- 4 Boje KM, Arora PK. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. *Brain Res* 1992; 587: 250 - 6.
- 5 Prehn JHM, Kriegstein J. Platelet-activating factor antagonists reduce excitotoxic damage in cultured neurons from embryonic chick telencephalon and protect the rat hippocampus and neocortex from ischemic injury *in vivo*. *J Neurosci Res* 1993; 34: 179 - 88.
- 6 Faden AI, Tzetzaljian PA. Platelet-activating factor antagonists limit glycine changes and behavioral deficits after brain trauma. *Am J Physiol* 1992; 263: R909 - 14.
- 7 Zahocka B, Domanska-Janik B. PAF antagonist, BN52021, inhibits [^3H] D-aspartate release after ischaemia *in vitro*. *Neuroreport* 1994; 6: 85 - 8.
- 8 Keller M, Jackisch R, Seregi A, Hertting G. Comparison of prostanoid forming capacity of neuronal and astroglial cells in primary cultures. *Neurochem Int* 1985; 7: 655 - 65.
- 9 Appel K, Honegger P, Gebicke-Haerter PJ. Expression of interleukin-3 and tumor necrosis factor- β mRNAs in cultured microglia. *J Neuroimmunol* 1995; 60: 83 - 91.
- 10 Hayes GM, Woodroffe MN, Cuzner ML. Characterization of microglia isolated from adult human and rat brain. *J Neuroimmunol* 1988; 19: 177 - 89.
- 11 Kondo Y, Takano F, Hojo H. Inhibitory effect of bisbenzylisoquinoline alkaloids on nitric oxide production in activated macrophages. *Biochem Pharmacol* 1993; 46: 1887 - 92.
- 12 Ohshima H, Oguchi S, Adachi H, Iida S, Suzuki H, Sugimura T, *et al.* Purification of nitric oxide synthase from bovine brain: Immunological characterization and tissue distribution. *Biochem Biophys Res Commun* 1992; 183: 238 - 44.
- 13 Kobuchi H, Droy-Lefaix MT, Christen Y, Packer L. *Ginkgo biloba* extract (EGb 761): inhibitory effect on nitric oxide production in the macrophage cell line RAW 264.7.

- Biochem Pharmacol 1997; 53: 897-903.
- 14 Maitra I, Marcocci L, Droy-Lefaix MT, Packer L. Peroxyl radical scavenging activity of *Ginkgo biloba* extract EGb 761. *Biochem Pharmacol* 1995; 49: 1649-55.
- 15 Guinot W, Braquet P. Effects of the PAF antagonists, ginkgolides (BNS2063, BNS2021), in various clinical indications. *J Lipid Cell Signalling* 1994; 10: 141-6.

目的: 观察银杏内酯 A 和 B, PAF 拮抗剂阿帕泛 (Apa) 和 NOS 抑制剂 L-NA 对新生大鼠小胶质细胞 (Mi) 产生 NO 的影响. 方法: 以 Griess 反应测定亚硝酸盐含量表示 NO 量. 结果: 在静息 Mi, GA, GB 和 Apa 在 1-10 000 nmol·L⁻¹ 范围对 Mi 产生 NO 没有影响, 但 L-NA 可浓度依赖性地抑制 NO 产生, 其 IC₅₀ (95% 可信限) 值为 3.4 (0.8-14.9) μmol·L⁻¹. 而在激活的 Mi, GA, GB 和 L-NA 可浓度依赖性地抑制 NO 产生, 其 IC₅₀ (95% 可信限) 值分别为 5.7 (1.8-18.1), 1.1 (0.3-4.4) 和 0.5 (0.1-2.8) μmol·L⁻¹, 但 Apa 不能抑制 NO 产生. 结论: GA 和 GB 抑制 LPS 诱导 Mi 产生 NO.

467-470 银杏内酯抑制新生大鼠小胶质细胞产生一氧化氮

杜泽英, 李晓玉¹

(中国科学院上海药物研究所, 上海 200031, 中国)

关键词 银杏内酯; 血小板激活因子; 硝基精氨酸; 一氧化氮; 小胶质细胞; 脂多糖; 培养的细胞; 阿帕泛

R 2 8 5 5

Nitric oxide and soman poisoning

WANG Yu-Xia, SUN Man-Ji¹ (*Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, Beijing 100850, China*)

KEY WORDS soman; acetylcholine; nitric oxide; arginine; nitric-oxide synthase; N^G-nitro-L-arginine methyl ester

AIM: To examine whether nitric-oxide (NO) is involved in the toxicity of soman. **METHODS:** With pretreatments of icv L-arginine (Arg, the substrate of nitric-oxide synthase NOS), N^G-nitro-L-arginine methyl ester (NAME, the inhibitor of NOS), the latency of seizure, and the mortality of mice induced by soman poisoning were examined. The activities of brain NOS in soman-intoxicated mice were measured. **RESULTS:** In case of Arg pretreatments, the latency decreased ($P < 0.05$) from (5.2 ± 1.8) min (control) to (4.3 ± 0.8) min (Arg 160 nmol), and the mortality increased ($P < 0.05$) from 50% (control) to 81% (Arg 160 nmol). In case of NAME pretreatment, the latency increased ($P < 0.01$) from (4.0 ± 1.1) min (control) to (14.5 ± 5.0) min (NAME 2.20 μmol), and the mortality decreased ($P < 0.05$)

from 87% (control) to 50% (NAME 2.20 μmol). The toxicity of soman in mice was enhanced by Arg and reduced by NAME all in a dose-dependent fashion. NAME antagonized the enhancement of soman poisoning by Arg. Intoxication of mice with soman increased the NOS activity in cerebrum, cerebellum, and hippocampus from 100% to 104% ($P < 0.05$), 115% ($P < 0.01$), and 111% ($P < 0.01$), respectively. **CONCLUSION:** The onset of seizure and death of mice induced by soman poisoning are related to the NO messenger system.

Soman is a potent inhibitor of acetylcholinesterase. The enzyme loses its activity after being phosphorylated, hence the released acetylcholine accumulates at the synaptic cleft, consecutively stimulates the cholinergic receptors causing so-called hypercholinergy.

Being a highly reactive free radical, nitric-oxide (NO) manifests cytotoxic action in neurons intoxicated by many compounds^[1-3]. We are intriguing in whether NO is also involved in soman intoxication.

¹ Correspondence to Prof SUN Man-Ji. Pbn 86-10-6688-2639, ext 67691. Fax 86-10-6821-1656. E-mail Sunmj@nic.bmi.ac.cn
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