

*Short Communication***Cyclooxygenase Inhibitors Attenuate Augmented Glutamate Release in Organum Vasculosum Laminae Terminalis and Fever Induced by Staphylococcal Enterotoxin A**Wu-Tein Huang<sup>1</sup>, Jhi-Joung Wang<sup>2</sup>, and Mao-Tsun Lin<sup>2,\*</sup><sup>1</sup>Department of Health Care Administration, Di Wan College of Management, Madou, Tainan, Taiwan 721<sup>2</sup>Department of Medical Research, Chi-Mei Medical Center, Tainan, Taiwan 710

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**Abstract.** Both the hyperthermia and augmented glutamate release in the organum vasculosum laminae terminalis (OVLT) after an intravenous dose (30 ng/kg) of staphylococcal enterotoxin A (SEA) were significantly reduced by pretreatment with intravenous administration of cyclooxygenase inhibitors such as aspirin (1–10 mg/kg), sodium salicylate (1–10 mg/kg), or diclofenac (10 mg/kg). Intra-OVLT administration of 50–200  $\mu$ g in 1.0  $\mu$ l of either aspirin or sodium salicylate 60 min before or 120 min after an intra-OVLT dose (50  $\mu$ g in 1.0  $\mu$ l) of glutamate also significantly suppressed the glutamate-induced hyperthermia. These findings suggest that inhibition of cyclooxygenase receptor mechanisms suppresses SEA fever by inhibition of glutamate release in the OVLT of rabbit brain.

**Keywords:** glutamate, fever, diclofenac

Our previous findings have demonstrated that the glutamatergic pathways in the organum vasculosum laminae terminalis (OVLT) are involved in pyrogenic fever genesis (1). The OVLT is a site through which signals that induce hyperthermia are transferred from the blood to the brain in animals (2, 3). For example, intravenous administration of staphylococcal enterotoxin A (SEA) produced fever accompanied by increased release of glutamate in the OVLT of rabbit brain (1). Treatment with glutamatergic receptor antagonists significantly attenuated the SEA-induced augmenting glutamate release in the OVLT and fever. The SEA induced fever could be mimicked by direct administration of glutamate into the OVLT area. Furthermore, the hyperthermia induced by intra-OVLT injection of glutamate was greatly reduced by pretreatment with an intra-OVLT dose of glutamatergic receptor antagonists.

Evidence has accumulated to indicate that chronic non-steroidal anti-inflammatory drugs reduce glutamate excitotoxicity (4), thought to underlie several neurodegenerative diseases (5). Redox mechanisms at the glutamate synapse are believed to play an important

role in inducing neurotoxic effects (6). In addition, aspirin reduced nociceptive behavior induced by glutamate administered intrathecally in a dose-dependent manner in the mouse (7). A more recent report (8) demonstrated that inhibition of glutamate release accounted for a neuroprotective effect of aspirin in rat cortical neurons exposed to oxygen-glucose deprivation. Recent data further show that salicylates act not only as inhibitors of cyclooxygenase (COX), but also as powerful antioxidants (9, 10). This raises the possibility that COX inhibitors inhibit the augmented glutamate release in the OVLT after SEA fever by more than a single mechanism.

To deal with the matter, experiments were carried out in the present study in rabbits to assess the effect of COX inhibitors such as salicylates and/or diclofenac on both the fever and the increased levels of glutamate release in the OVLT following SEA injection. In addition, the effects of intra-OVLT injection of salicylates or diclofenac on the hyperthermia induced by intra-OVLT injection of glutamate were also assessed.

Adult male New Zealand-derived white rabbits, weighing between 2.1 and 2.8 kg, were used. Thermal experiments were carried out in unanesthetized animals restrained in rabbit stocks. Between experiments, the

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animals were housed individually at an ambient temperature ( $T_a$ ) of  $22 \pm 1^\circ\text{C}$  with a 12:12 h light-dark cycle, with the lights being switched on at 0600 h. Animal chow and water were allowed ad libitum. All experimental animals were obtained from the Animal Center of National Yang-Ming University (Taipei, Taiwan).

All drug solutions were prepared in pyrogen-free glassware that was heated at  $210^\circ\text{C}$  for 5 h before use. All solutions were passed through Millipore bacterial filters (pore size of  $0.22 \mu\text{m}$ ). Sterile SEA (Sigma Chemical, St Louis, MO, USA) was made up in 0.9% NaCl solution. Glutamate (Sigma) was dissolved in 0.9% NaCl solution. All the SEA solutions used in this study did not induce gelation in the *Limulus* amoebocyte lysate test, so any contamination with endotoxin was below the level of 25 ng/ml. Aspirin was dissolved in 10%  $\text{NaHCO}_3$  solution, while sodium salicylate was dissolved in 0.9% NaCl solution. Diclofenac was dissolved in 30% alcohol solution.

Experiments were conducted between 09:00 and 20:00 h, with each animal being used at an interval of no less than seven days. Throughout the experiment, colonic temperatures ( $T_{co}$ ) were measured every minute with a copper constantan thermocouple connected to a thermometer (HR 1300; Yokogawa, Tokyo).  $T_{co}$  of each animal was allowed to stabilize for at least 90 min before any injections. Only animals whose  $T_{co}$  were stable and in the range of  $39 - 40^\circ\text{C}$  were used to determine the effect of drug application.

The following drugs were used for systemic administration: sterile SEA (30 ng/kg, i.v.), aspirin (1–10 mg/kg, i.v.), sodium salicylate (1–10 mg/kg, i.v.), and diclofenac (10 mg/kg, i.v.). Drugs used for intra-OVLT injection included aspirin (50 or 100  $\mu\text{g}$  in 1.0  $\mu\text{l}$ ), sodium salicylate (50 or 100  $\mu\text{g}$ ), and glutamate (Sigma; 10, 50, or 100  $\mu\text{g}$ ). As shown in our previous results, intra-OVLT injection of glutamate produced dose-dependent fever over the dose range of 10–100  $\mu\text{g}$  (1). An intra-OVLT dose of 50  $\mu\text{g}$  glutamate was chosen for antipyretic experiments.

An intra-OVLT cannula (0.81-mm o.d.) was implanted into each animal under general anesthesia (pentobarbital sodium, 30 mg/kg, i.v.). Standard aseptic techniques were employed. The stereotaxic atlas and coordinates of Sawyer et al. (11) were used. The cannula was located in the OVLT (A, 4.5 mm; L, 0.0 mm; V, 14 mm).

For measurement of extracellular glutamate in OVLT, a CMA/12 microdialysis probe (CMA/Microdialysis, Solna, Sweden) was inserted into the guiding cannula. According to the methods described by Berger et al (12), an equilibrium period of 60 min without sampling was allowed after probe insertion. The microdialysis was

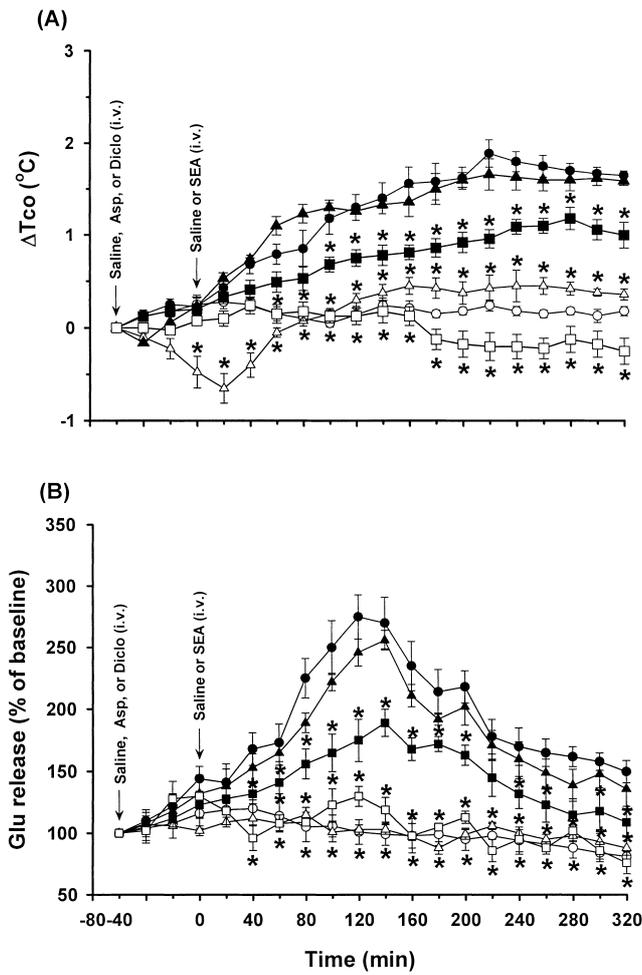
perfused at 2.0  $\mu\text{l}/\text{min}$ , and the dialysates were sampled in microvials. The dialysates were collected every 10 min in a CMA/140 fraction collector. Aliquots of dialysates (5  $\mu\text{l}$ ) were injected onto a CMA 600 Microdialysis analyzer for measurement of glutamate. The thermal experiments were started after showing stabilization in four consecutive samples. In the present results, an equilibrium period of 2–3 h assured a stable level of the extracellular substance tested. Glutamate is enzymatically oxidized by glutamate oxidase. The hydrogen peroxide formed reacts with *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine and 4-amino-antipyrine. This reaction is catalyzed by peroxidase and yields the red-violet colored quinonediimine. The rate of formation is measured photometrically at 546 nm and is proportional to the glutamate.

After the completion of the experiments, an aliquot of India ink (0.5  $\mu\text{l}$ ) was injected into the cannula to measure the spread of the injected solution. Then the fixed brain was cut in 50- $\mu\text{m}$  sections so that stereotaxic coordinates of the injection site in each rabbit were verified as described in our previous studies (1).

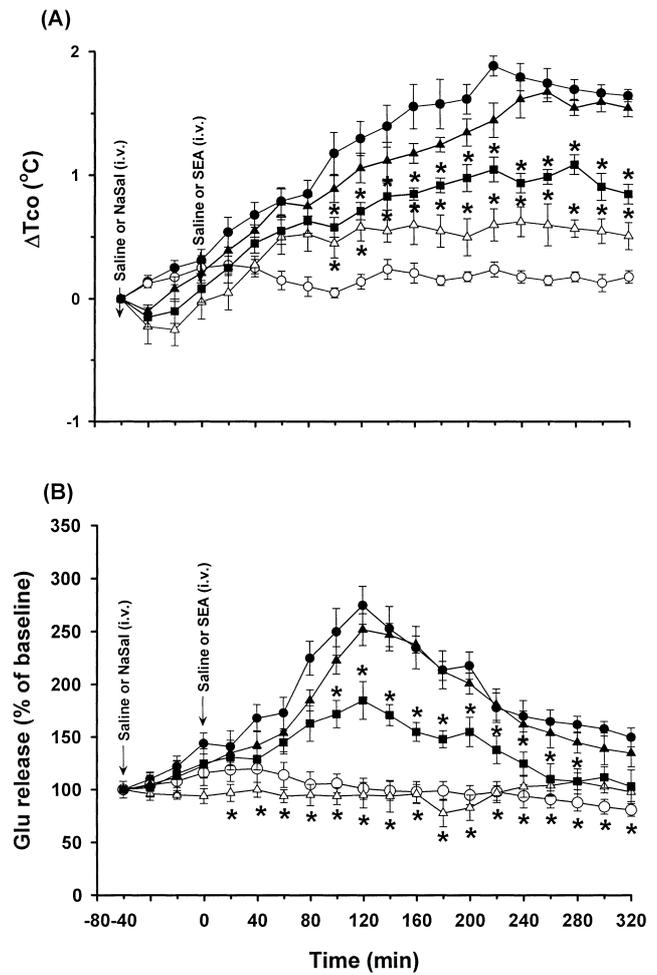
Temperature responses were assessed as changes from the pre-injection value ( $\Delta^\circ\text{C}$ ) after the injection of SEA. Results are expressed as the mean  $\pm$  S.E.M. of the indicated number of experiments; statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the post-hoc Scheffe's multiple range test.

SEA administration (30 ng/kg, i.v.;  $n = 8$  for each group) caused a rise in both the  $T_{co}$  and glutamate release in the OVLT of rabbit brain (as depicted in Fig. 1). Glutamate release in OVLT rose earlier than  $T_{co}$ . Fever was maximal about 4 h after SEA injection, while OVLT glutamate release was maximal about 2 h after SEA injection. Both  $T_{co}$  and OVLT glutamate levels returned to pre-injection levels about 16 h after an intravenous dose of SEA. In addition, it was found that both fever and increased levels of glutamate in OVLT induced by i.v. injection of SEA were significantly attenuated by pretreatment with i.v. injection of aspirin (1–10 mg/kg) (Fig. 1), diclofenac (10 mg/kg) (Fig. 1) or sodium salicylate (1–10 mg/kg) (Fig. 2) 60 min before SEA injection. An appropriate control injection of aspirin (10 mg/kg, i.v.), diclofenac (10 mg/kg, i.v.), sodium salicylate (10 mg/kg, i.v.), or saline produced a slight decrease in either  $T_{co}$  or OVLT glutamate release. Suppression of glutamate release in OVLT in SEA-treated rabbits started earlier than  $T_{co}$  decline after an i.v. dose of aspirin, diclofenac, or sodium salicylate.

Direct injection of glutamate (50  $\mu\text{g}$ ) into the OVLT of rabbit brain caused a rise in  $T_{co}$  (Fig. 2). The hyper-



**Fig. 1.** Mean changes ( $\pm$ S.E.M.) in both the peak elevations of colonic temperature ( $\Delta T_{co}$ ) (A) and the release of glutamate (Glu) (B) in the OVLT in 8 rabbits treated with saline (i.v.) plus SEA (30 ng/kg, i.v.) (closed circle), aspirin (1 mg/kg, i.v.) plus SEA (30 ng/kg, i.v.) (closed triangle), aspirin (5 mg/kg, i.v.) plus SEA (30 ng/kg, i.v.) (closed square), aspirin (10 mg/kg, i.v.) plus SEA (30 ng/kg, i.v.) (open triangle), saline (ml/kg, i.v.) plus saline (i.v.) (open circle), or diclofenac (10 mg/kg, i.v.) plus SEA (30 ng/kg, i.v.) (open square). \*Significantly different from the corresponding control values (saline + SEA group) ( $P < 0.05$ , ANOVA followed by Duncan's test).



**Fig. 2.** Mean changes ( $\pm$ S.E.M.) in both the peak elevations of colonic temperature ( $\Delta T_{co}$ ) (A) and the release of glutamate (Glu) (B) in the OVLT in 8 rabbits treated with saline (i.v.) plus SEA (30 ng/kg, i.v.) (closed circle), sodium salicylate (NaSal; 1 mg/kg, i.v.) plus SEA (30 ng/kg, i.v.) (closed triangle), NaSal (5 mg/kg, i.v.) plus SEA (30 ng/kg, i.v.) (closed square), NaSal (10 mg/kg, i.v.) plus SEA (30 ng/kg, i.v.) (open triangle), or saline (ml/kg, i.v.) plus saline (i.v.) (open circle). \*Significantly different from the corresponding control values (saline + SEA group) ( $P < 0.05$ , ANOVA followed by Duncan's test).

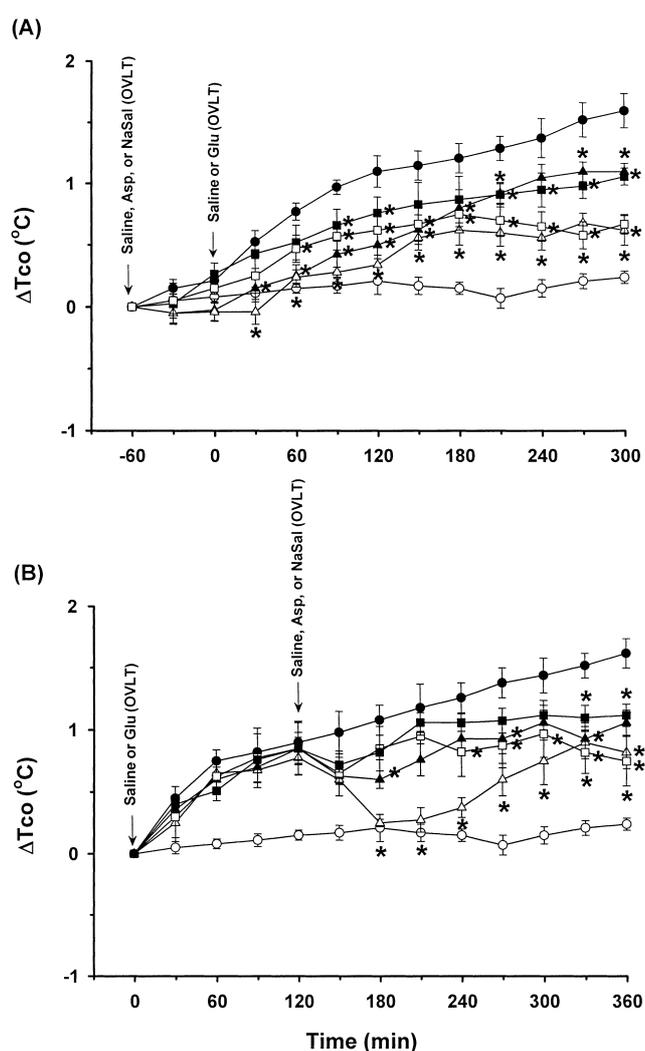
thermia induced by intra-OVLT injection of glutamate was significantly attenuated by pretreatment with intra-OVLT injection of aspirin (50–200  $\mu$ g) or sodium salicylate (50–200  $\mu$ g) when given 1 h before or 2 h after glutamate injection (Fig. 3). Control injection of normal saline had an insignificant effect on glutamate-induced hyperthermia.

Our previous (1) and present results have demonstrated that the glutamate release in the OVLT of rabbit brain is attributable to the fever induced by SEA. The present results further demonstrated that COX inhibitors such as aspirin, sodium salicylate, or diclofenac cause

antipyresis by reducing excessive accumulation of glutamate in OVLT after SEA injection.

Evidence has accumulated to indicate that aspirin, in addition to inhibition of the COX-dependent pathway (13), reduces the activation of the transcription factor nuclear factor- $\kappa$ B (4). It is understood that activation of nuclear factor- $\kappa$ B will result in transcription of pro-inflammatory and inflammatory mediators such as inducible nitric oxide synthase (iNOS) and COX-2. Its inhibition would translate into anti-inflammatory or antipyretic effects.

Previous results showed that aminoguanidine (an



**Fig. 3.** Mean changes ( $\pm$ S.E.M.) of the peak elevations of colonic temperature ( $\Delta T_{co}$ ) in 8 rabbits treated with intra-OVLT injection of saline plus glutamate (Glu, 50  $\mu$ g) (closed circle), aspirin (50  $\mu$ g) plus Glu (50  $\mu$ g) (closed triangle), aspirin (200  $\mu$ g) plus Glu (50  $\mu$ g) (open triangle), Na Sal (50  $\mu$ g) plus Glu (50  $\mu$ g) (closed square), NaSal (200  $\mu$ g) plus Glu (50  $\mu$ g) (open square), or saline plus saline (open circle). \*Significantly different from the corresponding control values (saline + SEA group) ( $P < 0.05$ , ANOVA followed by Duncan's test). Saline, aspirin, or NaSal was administered into the OVLT either 60 min before (A) or 120 min after (B) an intra-OVLT injection of SEA.

iNOS inhibitor) suppressed the expression of iNOS induced by lipopolysaccharide (LPS) or cytokines (14). Pretreatment of rabbits with intra-OVLT administration of aminoguanidine attenuated the fever induced by either intra-OVLT or i.v. administration of SEA, LPS, or interleukin-1 $\beta$  in rabbits (15–17). It was also shown that activation of NMDA receptors with glutamate triggered the opening of a cation permeable channel that resulted in stimulation of NOS activity (18). These results suggest that SEA acts on the OVLT which, in

turn, stimulates glutamatergic neurons of OVLT. From these glutamatergic neurons in the OVLT, nitric oxide and prostaglandin-E (PGE) might be released into the brain.

It should be noted that the excitation of NMDA receptors by glutamate opens the  $Ca^{2+}$  channel only if the redox-sensitive site attached to it is in the reduced state. Oxidation by NO or oxygen radicals of the redox-sensitive site prevents the postsynaptic neuron from becoming overstimulated. It has been shown for the rat and the rabbit that application of reducing drugs activate the NMDA receptor and cause a heat loss effector response, and these drug effects (including salicylates) are blocked by NMDA receptor antagonists (9, 19). The antipyretic effect of COX inhibitors could thus be explained by action on the redox-sensitive site, blocking the (neuroprotective) feedback mechanism on one hand, and increased levels of NO under these conditions could diminish glutamate release from presynaptic neurons. The hyperthermia induced by injection of glutamate into the OVLT could similarly be interpreted as a strong (pharmacological) stimulus that induces NO synthesis and oxidizes the redox-sensitive site, thereby blocking the heat loss pathway. That salicylates injected prior to glutamate are apparently more effective than when they are given 60 min after glutamate is clearly shown in Fig. 3 and would support such a conclusion.

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#### References

- Huang WT, Tsai SM, Lin MT. Involvement of brain glutamate release in pyrogenic fever. *Neuropharmacology*. 2001;41:811–818.
- Stitt JT. Evidence for the involvement of the organum vasculosum laminae terminalis in the febrile response of rabbits and rats. *J Physiol (Lond)*. 1985;368:501–511.
- Hashimoto M, Ueno T, Iriki M. What roles does the organum vasculosum laminae terminalis play in fever in rabbits? *Pfluegers Arch Eur J Physiol*. 1994;429:50–57.
- Grilli M, Pizzi M, Memo M, Spano F. Neuroprotection by aspirin and sodium salicylate through blockade of NF- $\kappa$ B activation. *Science*. 1996;274:1383–1385.
- Ginsberg MD, Globus MYT, Martinez E, et al. In: Kriegelstein J, Oberpichler-Schwenk H, editors. *Pharmacology of cerebral ischemia*. Stuttgart: Wissenschaftliche Verlagsgesellschaft; 1994. p. 255–268.
- Smythies J. Redox mechanisms at the glutamate synapse and their significance: a review. *Eur J Pharmacol*. 1999;370:1–7.
- Choi SS, Lee JK, Suh HW. Antinociceptive profiles of aspirin

- and acetaminophen in formalin, substance P and glutamate pain models. *Brain Res.* 2001;921:233–239.
- 8 Cristóbal JD, Cárdenas A, Lizasoain I, et al. Inhibition of glutamate release via recovery of ATP levels accounts for a neuroprotective effect of aspirin in rat cortical neurons exposed to oxygen-glucose deprivation. *Stroke.* 2002;33:261–267.
  - 9 Riedel W, Lang U, Oetjen U, Schlapp U, Shibata M. Inhibition of oxygen radical formation by methylene blue, aspirin, or  $\alpha$ -lipoic acid, prevents bacterial-lipopolysaccharide-induced fever. *Mol Cell Biochem.* 2003;247:83–94.
  - 10 Shi X, Ding M, Dong Z, et al. Antioxidant properties of aspirin: characterization of the ability of aspirin to inhibit silica-induced lipid peroxidation, DNA damage, NF- $\kappa$ B activation, and TNF- $\alpha$  production. *Mol Cell Biochem.* 1999;199:93–102.
  - 11 Sawyer GH, Everett JW, Green JD. The rabbit diencephalons in stereotaxic coordinates. *J Comp Neurol.* 1954;101:801–824.
  - 12 Berger C, Schäbitz WR, Georgiadis D, Steiner T, Aschoff A, Schwab S. Effects of hypothermia on excitatory amino acids and metabolism in stroke patients. A microdialysis study. *Stroke.* 2002;33:519–524.
  - 13 Vane JR. Inhibition of prostaglandin synthesis as a mechanism of action for aspirin-like drugs. *Nat New Biol.* 1971;231:232–235.
  - 14 Misko TP, Moore WM, Kasten TP, et al. Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur J Pharmacol.* 1993;233:119–125.
  - 15 Lin JH, Lin MT. Inhibition of nitric oxide synthase or cyclooxygenase pathways in organum vasculosum laminae terminalis attenuates interleukin-1 $\beta$  fever in rabbits. *Neurosci Lett.* 1996;208:155–158.
  - 16 Lin JH, Lin MT. Nitric oxide synthase-cyclooxygenase pathways in organum vasculosum laminae terminalis: possible role in pyrogenic fever in rabbits. *Br J Pharmacol.* 1996;118:179–185.
  - 17 Huang WT, Lin MT, Won SJ. Mechanisms and sites of pyrogenic action exerted by staphylococcal enterotoxin A in rabbits. *Neurosci Lett.* 1997;236:53–56.
  - 18 Snyder SH. Nitric oxide: first in a new class of neurotransmitters. *Science.* 1992;257:494–496.
  - 19 Canini F, Bréjot T, d'Ateo P, Mercier S, Bourdon L. NMDA receptors are involved in dithiothreitol-induced hypothermia. *Eur J Pharmacol.* 2001;426:179–183.