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Aspirin May Exert Its Antipyresis by Inhibiting the *N*-Methyl-D-aspartate Receptor-Dependent Hydroxyl Radical Pathways in the Hypothalamus

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Abstract. Recent findings have suggested that the *N*-methyl-D-aspartate (NMDA) receptor-dependent hydroxyl radical pathway in the hypothalamus of rabbit brain may mediate the fever induced by lipopolysaccharide (LPS). The aim of this study was to investigate whether aspirin exerts its antipyresis by suppressing hypothalamic glutamate and hydroxyl radicals in rabbits. The microdialysis probes were stereotaxically and chronically implanted into the preoptic anterior hypothalamus of rabbit brain for determination of both glutamate and hydroxyl radicals in situ. It was found that intravenous (i.v.) injection of LPS, in addition to inducing fever, caused increased levels of both glutamate and hydroxyl radicals in the hypothalamus. Pretreatment with aspirin (10 – 60 mg/kg, i.v.) one hour before an i.v. dose of LPS significantly reduced the febrile response and attenuated the LPS-induced increased levels of both glutamate and hydroxyl radicals in the hypothalamus. The increased levels of prostaglandin E_2 (PGE_2) in the hypothalamus induced by LPS could be suppressed by aspirin pretreatment. The data indicate that systemic administration of aspirin, in addition to suppressing PGE_2 production, may exert its antipyresis by inhibiting the NMDA receptor-dependent hydroxyl radical pathways in the hypothalamus during LPS fever.

Keywords: fever, lipopolysaccharide, glutamate, hydroxyl radical, aspirin

Introduction

Recently, we have demonstrated that the rise in both the fever and increased hypothalamic 2,3-dihydroxybenzoic acid (2,3-DHBA) could be induced by direct injection of glutamate into the cerebroventricular fluid system (1). Either the early or the late phase of fever rise and increased hypothalamic levels of 2,3-DHBA, a marker of free radical generation, following systemic injection of lipopolysaccharide (LPS) were significantly antagonized by pretreatment with injection of α-lipoic acid (an antioxidant), *N*-acetyl-L-cysteine (an antioxidant), MK-801 (an *N*-methyl-D-aspartate [NMDA]-receptor antagonist), or LY235959 (an NMDA-receptor antagonist) 1 h before LPS injection. The increased levels of prostaglandin E_2 (PGE_2) in the hypothalamus induced by LPS could be suppressed by α-lipoic acid or *N*-acetyl-L-cysteine pretreatment. These findings suggest that an NMDA-receptor-dependent hydroxyl radical pathway in the rabbit hypothalamus may mediate LPS fever (1, 2).

Another line of evidence has also shown that both the core temperature elevation and the augmented glutamate release in the hypothalamus after an intravenous (i.v.) dose of staphylococcal enterotoxin A (SEA) were significantly reduced by pretreatment with i.v. administration of aspirin. Systemic injection of aspirin one hour before an intrahypothalamic dose of glutamate also significantly suppressed the glutamate-induced fever in rabbits (3, 4). In rabbits, i.v. administration of aspirin was also shown to prevent LPS-induced fever by inhibiting oxygen radical formation in plasma (5). This raises

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the possibility that aspirin, when administered systemically, exerts its antipyresis by suppressing the NMDA-receptor-dependent hydroxyl radical pathway in rabbit hypothalamus.

To deal with the question, the present study was performed to assess the changes in body temperature and the hypothalamic concentrations of glutamate and hydroxyl radical during LPS administration in rabbits with or without systemic pretreatment of aspirin.

Materials and Methods

Animals and pyrogen assay

Adult male New Zealand White rabbits, weighing between 2.1 and 3.0 kg at the start of the experiment, were used. The pyrogen assay was performed with unanesthetized animals restrained in rabbit stocks. Between experiments, the animals were housed individually at an ambient temperature of 22 ± 1°C with a 12-h light-dark cycle, with the lights being switched on at 06:00 h. Animal chow and water were allowed ad libitum. Experiments were conducted between 09:00 and 19:00 h, with each animal being used at an interval of not less than 7 days. Each rabbit has been used for three times in this study. Throughout the experiment, core temperature (Tco) was measured every 5 min with a copper constantan thermocouple inserted into the rectum and connected to a thermometer (HR1300; Yokogawa, Tokyo). The Tco of each animal was allowed to stabilize for at least 120 min before any injections. Only animals whose Tco were stable and in the range of 38.5°C – 39.2°C were used to determine the effect of drug application. All experimental animals were obtained form the animal center of Chimei Medical Center (Tainan, Taiwan). The animal protocol described here was approved by the Animal Care Committee of Chimei Medical Center.

Surgical techniques

An intracerebral probe guide cannula was implanted into each animal under general anesthesia (sodium pentobarbital, 30 mg/kg, i.v.). Standard aseptic techniques were employed. The stereotaxic atlas and coordinates of Sawyer et al. (6) were used. The cannula was located in the left preoptic anterior hypothalamus (POAH) (A: 2.5 mm, L: 2 mm, and V: 15 mm). The animal was placed in the stereotaxic apparatus, and the frontal and parietal bones were exposed by a midline incision into the scalp. After the appropriately located craniotomy had been trephined, two self-tapping screws were inserted into the parietal or frontal bones, and the cannula was inserted to the depth through the craniotomy hole. The cannula was anchored with dental acrylic cement to the calvarium surface, which had been scraped clean of periosteum. The reflected muscles and skin were replaced around the acrylic mound containing the cannula and screws and were sutured with surgical silk sutures. After surgery, the guide cannula was plugged with a stylet, and animals were returned to their cages for a minimal recovery of 1 week.

Drugs

All drug solutions were prepared in pyrogen-free glassware that was heated for 5 h at 180°C before use. All drug solutions were prepared in pyrogen-free sterile saline and passed through 0.22-μm Millipore bacterial filters. The LPS used in this study, which was derived from *Escherichia coli* serotype 026:B6 (2 μg/kg; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) was dissolved in sterile saline. Aspirin was dissolved in 10% $NaHCO_3$ solution.

Experimental groups

At least 1 h before an experiment, the indwelling stylet of the guide cannula was replaced by a CMA-12 microdialysis probe (CMA/Microdialysis; Ros-Lagsvägen, Stockholm, Sweden) so that its dialysis membrane tip protruded exactly 1.5-mm beyond the guide tube. The microdialysis probes and perfusion procedures (1.2 μl/min) used in this study have been described previously (1, 2). One hour before an i.v. dose of sterile saline or LPS (2 μg/kg), an i.v. dose of $NaHCO_3$ or aspirin (10 – 60 mg/kg) was randomly administered into rabbits (n = 16), and their effects on both Tco and hypothalamic levels of glutamate and 2,3-DHBA were assessed.

Microdialysis for detection of extracellular glutamate and hydroxyl radicals

For measurement of extracellular levels of glutamate in POAH of rabbit brain, the dialysates were collected every 20 min in a CMA/140 fraction collector. Aliquots of dialysates (2 μl) were injected onto a CMA600 Microdialysis analyzer for measurement of glutamate. 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 quimonediimine. The rate of formation is measured photometrically at 546 nm and is proportional to the glutamate.

For measurement of extracellular levels of hydroxyl radicals in the POAH, a probe guide cannula was planted in the POAH. The morning before an experiment, after insertion of a microdialysis probe into the POAH, it was perfused with artificial cerebrospinal fluid (aCSF:

149 mM $NaCl_2$, 2.8 mM KCL, 1.3 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.125 mM ascorbic acid, and 5.4 mM D-glucose, pH 7.2 – 7.4) containing 10 mM salicylic acid by a high pressure pump (CMA/Microdialysis, RosLagsvägen) at a flow rate of 1.2 μl/min (1). The dialysis probe is a CMA12 microdialysis probe.

The concentrations of hydroxyl radicals were measured by a modified procedure based on the hydroxylation of sodium salicylate by hydroxyl radicals, leading to the production of 2,3-DHBA and 2,5-DHBA (7, 8). A Ringer solution containing 0.5 mM sodium salicylate was perfused through the microdialysis probe at a constant flow rate (1.2 ml/min). After 2 h of stabilization, the dialysates from the hypothalamus were collected at 10-min intervals. An Alltima reverse-phase C18 column (Bio-analytical Systems, Lafayette, IN, USA; 150 × 1-mm inside diameter, particle size of 5 mm) was used to separate the DHBAS, and the mobile phase consisted of a mixture of 0.1 M chloroacetic acid, 26.87 nM disodium EDTA, 688.76 nM sodium octyl sulfate, and 10% acetonitrite (pH 3.0). The retention time of 2,3-DHBA and 2,5-DHBA were 8.1 and 6.0 min, respectively.

Measurement of PGE_2 in the hypothalamus

For measurement of hypothalamic PGE_2, the dialysis system was connected to a microdialysis pump and perfused with artificial cerebrospinal fluid at a flow rate of 1.2 μl/min. The unanesthetized animals were restrained in rabbit stocks for at least 90 min to achieve a stable dialysis level of PGE_2. Dialysis samples from the hypothalamus were collected into a microdialysis vial at 60-min intervals for 8 h, and they were stored at –80°C until analyzed within 7 days. Immunoreactive PGE_2 concentrations in dialysates were determined using commercially available enzyme immunoassay kits (Cayman Chemicals Co., Ann Arbor, MI, USA). Triplicate aliquots of 50 μl sample were added to each well of plate and each sample was assayed at a minimum of two dilutions. The limit of quantitation for PGE_2 was 20 pg/ml.

Data presentation

At the end of an experiment, the animals were killed with an overdose of anesthetics and decapitated with a guillotine. Their brains were removed and stored in 10% phosphate-buffered formalin for histological verification of the placement of the dialysis probe tips. Only experiments in which the preoptic localization of the microdialysis probes was confirmed histologically were included in the results.

Statistical analysis

Temperature response was assessed as changes from pre-injection values (Δ°C) and the fever index (FI), which was the area under the curve produced in the 5-h period after the injection of LPS in term of degrees centigrade per 5 h, were calculated (1, 2). The glutamate and 2,3-DHBA levels of samples were expressed as a percentage of three consecutive mean baseline values. Results were expressed as the mean ± S.E.M. for n experiments. Two way analysis of variance (ANOVA) for repeated measurements (in the same animals) was used for the factorial experiment, whereas Dunnett's test was used for post hoc multiple comparisons among means. A P value less than 0.05 was considered to indicate a statistically significant difference.

Results

The top panel of Fig. 1 shows that an i.v. dose (2 μg/kg) of LPS causes a biphasic febrile response, with the Tco maxima at about 80 and 200 min post-injection. Each Tco rise was accompanied by a distinct wave of cellular levels of both glutamate (middle panel of Fig. 1) and 2,3-DHBA (bottom panel of Fig. 1) in the hypothalamus. Both the Tco and hypothalamic levels of glutamate and 2,3-DHBA returned to their preinjection levels about 7 h after an i.v. dose of 2 μg/kg of LPS. In addition, as can be seen from Fig. 1 and Table 1, the temperature elevations of both early and late phase and the fever index induced by LPS (2 μg/kg, i.v.) were dose-dependently reduced by pretreatment with aspirin (10 – 60 mg/kg, i.v.) 1 h before the LPS injection. The time course changes of Tco and hypothalamic levels of glutamate and 2,3-DHBA induced by aspirin 1 h before the LPS injection are shown in Fig. 1.

Figure 2 shows the effects of pretreatment with aspirin (10 – 60 mg/kg, i.v.) on the increased hypothalamic PGE_2 level produced by an i.v. dose of LPS (2 μg/kg) in rabbits. It can be seen from the figure that LPS-induced PGE_2 overproduction is suppressed significantly and dose-dependently by aspirin pretreatment.

An appropriate control injection of aspirin or $NaHCO_3$ caused an insignificant change in both Tco and hypothalamic release of both glutamate and 2,3-DHBA.

Discussion

It has been shown that in the rat, a small dose (2 – 8 μg/kg, i.v.) of LPS causes biphasic febrile responses, whereas a large dose (10 – 10,000 μg/kg, i.v.) of LPS causes a triphasic fever response (9). However, in the rabbit, previous (1, 10) and present results showed that a

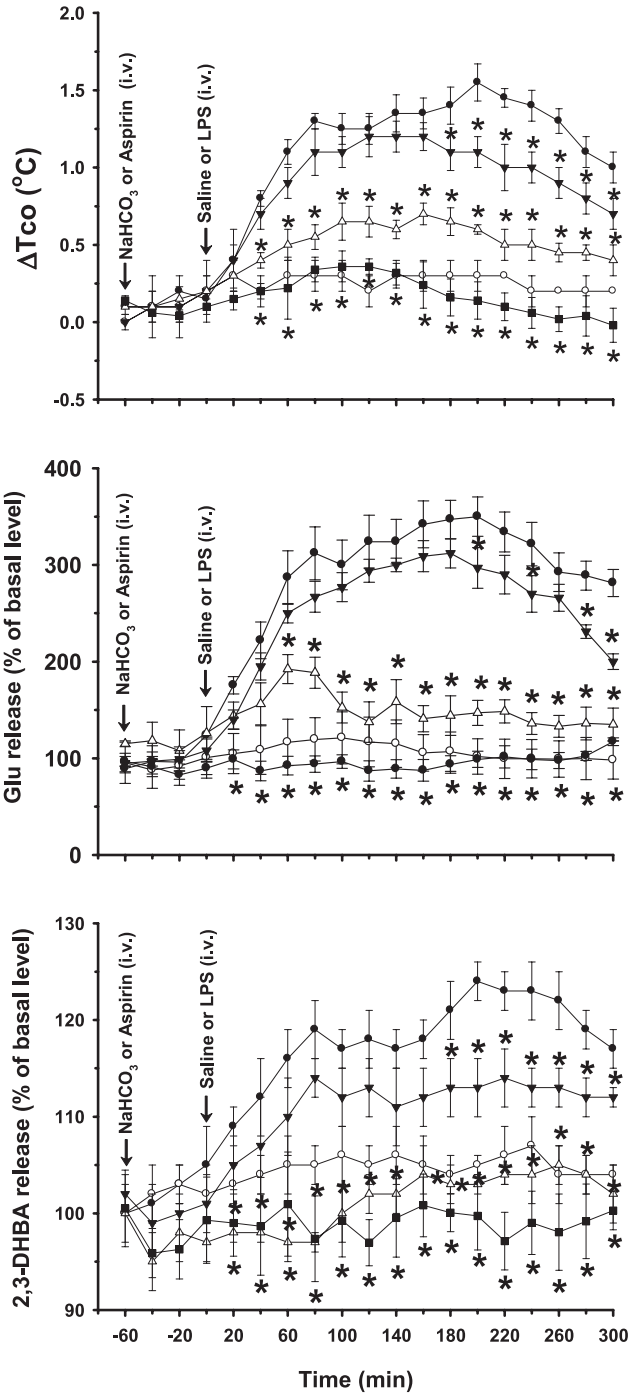

Fig. 1. Mean ± S.E.M. changes in core temperature (Tco) and glutamate (Glu) and 2,3-DHBA generation in POAH with intravenous administration of either $NaHCO_3$ plus saline (open circle) (n = 8), $NaHCO_3$ plus LPS at 2 μg/kg (closed circle) (n = 8), aspirin (10 mg/kg) plus LPS (2 μg/kg) (closed triangle) (n = 8), aspirin (30 mg/kg) plus LPS (2 μg/kg) (open triangle) (n = 8), or aspirin (60 mg/kg) plus LPS (2 μg/kg) (closed square) (n = 8). *P<0.05, significantly different from corresponding control values ($NaHCO_3$ plus LPS group) (ANOVA followed by Dunnett's test).

Table 1. Effect of systemic administration of aspirin 1 h before the LPS injection on the febrile response to intravenous injection of LPS in rabbits

Treatments	Fever index (FI, °C h)
$NaHCO_3$ + Saline	1.30 ± 0.08
$NaHCO_3$ + LPS (2 μg/kg)	5.86 ± 0.09
Aspirin (10 mg/kg) + LPS (2 μg/kg)	4.72 ± 0.12*
Aspirin (30 mg/kg) + LPS (2 μg/kg)	2.60 ± 0.08*
Aspirin (60 mg/kg) + LPS (2 μg/kg)	0.92 ± 0.11*

The values are the mean ± S.E.M. of six rabbits per group. FI represents the fever index for 5-h experimental observation. *Significantly different from the corresponding control value ($NaHCO_3$ plus LPS group) (P<0.05, two way analysis of variance of variance followed by Dunnett's test).

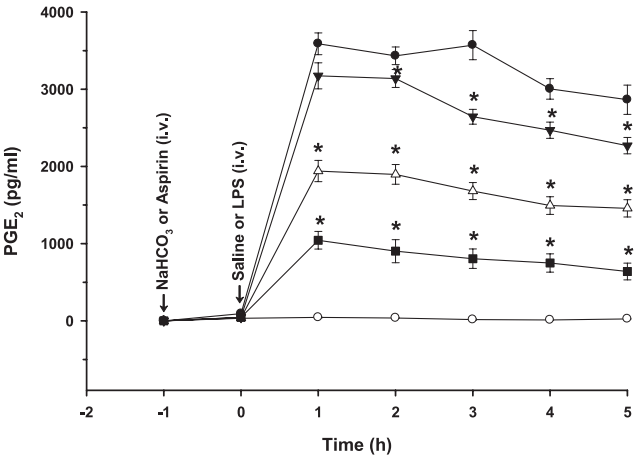

Fig. 2. Mean ± S.E.M. changes in the hypothalamic concentration of PGE_2 in rabbits injected with either 8% $NaHCO_3$ plus LPS (2 μg/kg) (closed circle) (n = 8), aspirin at 10 mg/kg (i.v.) plus LPS (2 μg/kg) (i.v.) (closed triangle) (n = 8), aspirin (30 mg/kg, i.v.) plus LPS (2 μg/kg) (i.v.) (open triangle) (n = 8), aspirin (60 mg/kg, i.v.) plus LPS (2 μg/kg, i.v.) (closed square) (n = 8), or $NaHCO_3$ (i.v.) plus saline (i.v.) (open circle) (n = 8). *P<0.05, significantly different from corresponding control values (8% $NaHCO_3$ + LPS group) (ANOVA followed by Dunnett's test).

small dose (0.5 μg/kg, i.v.) of LPS caused a monophasic fever, while larger doses (2 – 10 μg/kg) of LPS caused a biphasic fever. Evidence has accumulated to suggest that the steps that lead to fever genesis is that they start with the production of pyrogenic cytokines by mononuclear cells, their release into the peripheral blood stream and transport to the hypothalamus, and their formation of cyclooxygenase-2 (COX-2)-dependent PGE_2 (11). It postulates that the early phase of the LPS-induced fever denotes an antioxidant-sensitive PGE_2 rise, while the late phase of the LPS-induced fever represents a delayed PGE_2-dependent Tco rise. Both our previous (1) and present results provide evidence indicating that an

NMDA-receptor-dependent hydroxyl radical pathway in the hypothalamus may mediate both the first and late phases of LPS fever. In addition, the present findings demonstrate that both the first and late phases of the LPS-induced fever are suppressed by aspirin. As demonstrated in the current results, aspirin may exert its antipyresis by reducing the production of glutamate and hydroxyl radical in the hypothalamus.

The contention that an NMDA-receptor-dependent hydroxyl radical pathway in the rabbit hypothalamus may mediate aspirin antipyresis is supported by many previous observations. For example, both the fever and augmented release of glutamate in the hypothalamus after an i.v. dose of SEA were significantly reduced by pretreatment with i.v. administration of cyclooxygenase inhibitors such as aspirin, sodium salicylate, acetaminophen, and diclofenac (3, 4). Intrahypothalamic administration of either aspirin or sodium salicylate significantly suppressed the glutamate-induced fever (3, 4). It was also found that inhibition of oxygen radical formation in the plasma by aspirin (12) prevented LPS-induced fever in rabbits (5). In vivo findings further showed that glutamate neuron discharge promoted the extracellular release of hydroxyl radicals (13). These findings suggest that aspirin acts upstream, in addition to downstream, of glutamate release in the NMDA-receptor-radical pathways mediating LPS-induced rise in fever. In that sense, the interrelationships between radical production and glutamate release should be defined clearly in future studies.

Evidence has also accumulated to indicate that tumor necrosis factor-α (TNF-α) overproduction may be related to LPS fever genesis. Intravenous administration of LPS caused fever accompanied by increased levels of TNF-α in both the plasma and cerebrospinal fluid in rats and guinea pigs (2, 14 – 16). The circulating TNF-α can reach the central nervous system by crossing at leaky areas in the blood-brain-barrier through the organum vasculosum laminae terminalis (17 – 19). Administration of neutralizing antibodies to TNF-α into the brain causes attenuation of the febrile response to systemic injection of LPS (20). The rise in both the core temperature and hypothalamic glutamate and hydroxyl radicals could also be induced by direct injection of TNF-α into the lateral cerebral ventricle of rabbit brain (2). However, according to the findings of Riedel et al. (5), treating rabbits with aspirin does not alter the secretion of circulating TNF-α following systemic administration of LPS but does prevent LPS from eliciting fever. Thus, it is likely that aspirin might reduce fever solely by its inhibitory action on the NMDA-receptor-dependent hydroxyl radical pathway in the hypothalamus. Our previous findings (21, 22) have demonstrated that LPS, SEA, or interleukin-1 may act through the nitric oxide synthase (NOS) – COX pathways in the hypothalamus to induce fever in rabbits. The fever induced by LPS or SEA has also been shown to be attenuated by nuclear-kappa B (NF-κB) inhibitors in rabbits (23, 24). In fact, NF-κB activation can be modulated by hydroxyl radicals (25). Aspirin has recently been demonstrated to function as an antioxidant mainly by means of its ability to scavenge hydroxyl radicals, thereby inhibiting LPS-induced NF-κB activation (12). Aspirin can be the most widely used antipyretic as it possesses a combination of activities as an antioxidant and a potent inhibitor of COX.

Catalase is an enzyme containing free thiol groups essential for enzyme action (26). The decreased catalase activity following LPS and the restoration of catalase activity by α-lipoic acid (an antioxidant) suggests that oxidative stress occurs in fever (5). Both inhibition of catalase activity and production of hydroxyl radicals may be involved in LPS fever. Indeed, it was found that aspirin prevented the LPS-induced inhibition of catalase and contributed to the prevention of fever (5). Both the fever rise and increased hypothalamic levels of 2,3-DHBA following systemic injection of LPS were significantly antagonized by pretreatment with injection of α-lipoic acid 1 h before LPS injection (1). Therefore, aspirin may exert its antipyresis by restoring catalase activities as well as reducing hydroxyl radical production.

It is believed that fever genesis begins with the production of pyrogenic cytokines by mononuclear cells, their release into the peripheral blood stream and transport to the hypothalamus, and their production of PGE_2 (10, 11). The present results further demonstrated that the increased levels of PGE_2 in the hypothalamus induced by LPS could be suppressed by aspirin pretreatment.

In summary, as demonstrated in the present results, pretreatment with aspirin significantly reduced the LPS-induced fever and increased levels of glutamate and hydroxyl radicals. These results indicate that aspirin, in addition to suppressing PGE_2 production, may exert its antipyresis by inhibiting the NMDA-receptor-dependent hydroxyl radical pathway in the hypothalamus.

Acknowledgments

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