Brain stroke is often accompanied by a high fever, which is insensitive to a blockade with classic antipyretic drugs known to inhibit the synthesis of prostaglandin E$_2$ (PGE$_2$), a proximal mediator of fever associated with infection. The molecular mechanism of fever associated with stroke is mostly unknown, and has not been thoroughly investigated. One characteristics of the stroke is an extravasation of the erythrocytes into the brain tissue followed by a release of hemoglobin and free heme. In the present study we have tested the hypothesis that free heme itself can induce fever after releasing into the brain. The study was conducted on Sprague Dawley rats instrumented with biotelemetry devices to monitor deep body temperature, and implanted with brain cannulae projected to the lateral ventricle. We demonstrate that heme-L-lysinate microinfused intraventricularly (icv) induces a dose-dependent fever lasting ca. 8 hours. Injection of heme-L-lysinate provoked a significant elevation of PGE$_2$ in the rat cerebro-spinal fluid collected 3 hours post-injection. The fever induced by heme-L-lysinate was blocked by an icv injection of anti- PGE$_2$ antibody. It was not affected, however, by intraperitoneal administration of indomethacin, a cyclooxygenase inhibitor. We conclude that heme-induced fever may underlie the stroke fever.

Key words: Biotelemetry, rats, body temperature, heme, prostaglandin E$_2$, indomethacin

INTRODUCTION

Fever is regarded as part of the acute-phase response to infection, inflammation and trauma. Generation of fever involves a number of the host cell-derived molecules. Among these factors are cytokines such as interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, and interferon-γ (IFN-γ), collectively ascribed to as endogenous pyrogens (1 - 3). Cytokines trigger liberation of the
arachidonic acid from membrane phospholipids, activation of cyclooxygenase (COX), and subsequent production of prostanoids. It is thought that induction of the expression of COX-2 and generation of prostaglandin E$_2$ (PGE$_2$) play a critical role in affecting the thermoregulatory centers to start the fever (4). This train of molecular events is initiated upon a contact of the host with non-self immunostimulatory agents termed as exogenous pyrogens, which originate typically from the pathogenic microbes (1). However, there are also fevers which are not accompanied with traces of infections. One of them is a traumatic brain injury – the stroke (5, 6).

Stroke is defined as any head injury with traumatic etiology. It can manifest as diffuse axonal injury, intraparenchymal contusions or intracranial hematomas. Mechanical injury is often followed by secondary physiologic and biochemical events that generate further damage (7). Hemorrhage accompanies most of subtypes of stroke and its appearance and volume is correlated to a survival rate of patients (8, 9). During hemorrhage blood is released from the ruptured cerebrovasculature and is pooled into the surrounding brain tissue (10 - 12). Within hours after the hemorrhage, the lysis of blood cells take a place (13 - 15) which leads to exposure of the central nervous system (CNS) to free blood constituents – hemoglobin and subsequently heme (13, 16). Heme iron, a hemoglobin degradation product, plays a key role in neurodegeneration (17, 18).

It has been found that heme infused into the rat brain lateral ventricle (icv) provoked a fever (19). The heme-induced increase in body temperature ($T_b$) of rats was unaffected by intraperitoneal (ip) injection of indomethacin, an inhibitor of COX. Based on these data a hypothesis has been put forward that fever provoked by heme is a prostaglandin-independent phenomenon (19). It is known, however, that free heme is a potent inducer of the PGE$_2$ production in the endothelial cells (20). Therefore, to further test the hypothesis that fever triggered by heme does not depend on prostaglandins, in the present study we demonstrate that administration of heme into the rat brain provokes: (i) a fever-like elevation in $T_b$, and (ii) increase of PGE$_2$ in the cerebro-spinal fluid. The increase in $T_b$ was, indeed, unaffected by an ip injection of indomethacin. It was inhibited, however, by an icv pretreatment of the rat with antibody against PGE$_2$.

MATERIALS AND METHODS

**Animals**

Male Sprague-Dawley rats (Charles River) weighing 250 to 300 g were used throughout the study. They were housed individually in cages in a temperature-controlled room at 25 ± 1°C on 12:12-hour light-dark cycle with lights on at 07:00. The animals had free access to commercial food for rodents (Teklad Rodent Diet) and drinking water. All experiments were approved by the Institutional Animal Care and Use Committee.
Surgical Procedures

The rats were anaesthetized with a mixture of ketamine/xylazine (87 mg/kg and 13 mg/kg, respectively) injected intramuscularly. An incision was made in the abdomen, and a miniature battery-operated, temperature-sensitive telemetry transmitter was placed into the abdominal cavity for continuous monitoring of body temperature ($T_b$). The muscle level of the abdomen and the skin were sutured closed. The wound area was swabbed with Furacin, and the following surgical step was an implantation of the cannula into the brain lateral ventricle.

Stainless-steel (22 gauge, 5 mm long), thin-walled cannulae (Plastic Products, Roanoke, VA) were implanted stereotaxically into the lateral cerebral ventricle according to the atlas developed by Paxinos and Watson (21). The coordinates were 1.0 mm posterior to the bregma and 1.5 mm lateral of the midline. All surgical procedures were done at least 10 days before the start of experiment. After the completion of experiments, the animals were sacrificed and then dye was injected through the canulas to mark the ventricular space. The brain sections were visually examined to verify that the tip of the cannula was located in the lateral cerebral ventricle.

Reagents and Injections

**Heme-L-lysinate.** Heme-L-lysinate was prepared using the methods described by Tehunen et al. (22). Briefly, hemin (3.85 mmol; Frontiers Scientific) was added to a solution containing ethanol (10 ml), 1,2-propanediol (40 g), lysine (11.5 mmol), and double distilled water to bring the total volume of solution to 100 ml. The solution was filtered (0.2-µm pore size membrane filter), and aliquots protected from light were stored at -20°C. One µl of this solution contains 38.5 µmol of heme-L-lysinate. Resultant heme-L-lysinate was dissolved 100x and 1000x in artificial cerebrospinal fluid (aCSF; see below) for injections. Heme-L-lysinate was administered intracerebroventricularly (icv) in a volume of 4 µl: 0.154 µM and 1.54 µM per rat per injection. For control injections, heme-free preparation of L-lysine (dissolved with aCSF as above) was used. All injections were made at 09:00 AM, i.e., two hours after the light was on in a 12:12 light:dark cycle.

**Artificial cerebrospinal fluid.** The aCSF used for icv injections consisted of 145 mM NaCl, 3.3 mM KCl, 1.3 mM CaCl$_2$, and 1.0 mM MgCl$_2$ dissolved in pyrogen-free water. The solution was filtered through a 0.2-µm membrane filter and stored in aliquots at -20°C.

**Indomethacin.** Indomethacin (Sigma, lot no. I-7378) was prepared as an aqueous sodium solution in 0,01 M anhydrous sodium carbonate as described elsewhere (23). Indomethacin was injected intraperitoneally (ip) at doses of 5 mg/kg and 10 mg/kg (vehicle was used as control injections). These doses of indomethacin inhibited a fever in rats treated ip with lipopolysaccharide (LPS) at a dose of 50 µg/kg (a fever-producing dose of LPS).

**PGE2 antibody.** PGE$_2$ antibody (rabbit IgG anti-PGE$_2$; Assay Designs, Inc., MI; cat #905-013) was diluted 10x and 100x with aCSF from the stock and infused icv at a volume of 5 µl/rat 2 h prior to pyrogens. Rabbit IgG was used as control injection. Amount of PGE$_2$ antibodies were determined empirically based on the inhibition of fever induced by peripheral injection of LPS (50 µg/kg; ip injections) in rats.

**Evaluation of PGE$_2$ in cerebro-spinal fluid**

Samples of the cerebrospinal fluid for PGE$_2$ analyses were collected from lightly anesthetized (halothane) rats by cisternal puncture as described elsewhere (2). Prostaglandin levels were determined using a highly sensitive colorimetric assay kit from R&D Systems for rats (Minneapolis,
MN; cat. no. DE2100; sensitivity of assay 3 pg/ml). Wells were read at 670 nm with an ELISA reader (model EL 312; Biotek Instruments, Winooski, USA) 30 min after addition of substrate.

Data analysis

Results, expressed as mean ± SEM, were analyzed by analysis of variance followed by the Newman-Keuls Student's t test with the level of significance set at P < 0.05.

RESULTS

Effect of icv injection of heme on body temperature of rats

Injection of heme-L-lysinate into the lateral cerebral ventricle of the rat triggered a significant rise in T\(_b\) lasting at least 8 h post-injection (Fig. 1). Body temperature of control vehicle-injected rats, after a brief 30-min elevation at the time of injection and handling, returned to a pre-injection level. Heme-injected rats, on the other hand, revealed a fever-like increase of T\(_b\) for the period of 8.5 h shown in the figure. The increase of T\(_b\) was dose-dependent. Heme-L-lysinate at a dose of 0.154 µM induced

![Graph showing changes in body temperature over time for different treatments.](image)

*Fig. 1. Changes of body temperature (°C) over time (min) of rats injected intracerebroventricularly (icv) at 0 time with heme-L-lysinate at a dose of 0.154 µM (closed circles) and 1.54 µM (open circles), and with vehicle as control (closed triangles). Values are means ± SE at 15-min averages; n indicates sample size in a respective group.*
an elevation of $T_b$ to $38.54 \pm 0.19^\circ C$ (measured at 180 min post-injection), whereas dose of 1.54 $\mu M$ provoked an increase in $T_b$ to $39.1 \pm 0.22^\circ C$ at the same time ($P<0.05$). Moreover, the higher dose of heme-L-lysinate induced a longer-lasting elevation of $T_b$, which did not tend to decrease during the period of $T_b$ monitoring (Fig. 1). The lower dose of the heme-L-lysinate was selected for further experiments.

**Effect of heme icv injections on cerebrospinal fluid levels of PGE$_2$ of rats**

Rats were injected icv with heme-L-lysinate at a dose of 0.154 $\mu M$ and control solutions. Three hours post-injection the rats were anesthetized with inhaled halothane and cerebrospinal fluid was collected to measure levels of PGE$_2$. As shown in Fig. 2 injection of apyrogenic water and L-lysinate solution did not induce significant difference in PGE$_2$ levels ($7.8 \pm 2.6$ pg/ml and $13.4 \pm 4.7$ pg/ml, respectively; $P>0.05$ between the two groups). In contrast, however, injection of heme-L-lysinate into the lateral cerebral ventricle triggered a significant increase in the PGE$_2$ contents of CSF at 3 h post-injection (to an average of $132.6 \pm 21.9$ pg/ml; $n = 6$, $P<0.001$ between heme-L-lysinate-treated group and control groups).

**Effect of anti-PGE$_2$ immunoglobulin and indomethacin on heme-induced increase of $T_b$ in rats**

To determinate whether the PGE$_2$ is involved in a heme-induced rise of $T_b$, in separate experiments we have used anti-PGE$_2$ immunoglobulin (Fig. 3) and

![Fig. 2. Cerebrospinal fluid levels of PGE$_2$ (pg/ml) at 3 h post-injection of heme-L-lysinate (black bar) and/or control solutions (open and hatched bars). Values are means ± SE; $n$ depicts sample sizes for each group.](image-url)
indomethacin (*Fig. 4*). Rats were treated icv with an anti-PGE$_2$ IgG 2 h prior to the injection of heme-L-lysinate. *Fig. 3* demonstrates the results of this experiment. A stock solution of a goat-anti-rat PGE$_2$ antibody, obtained from the Assay Designs, Inc., was diluted 10x and 100x with aCSF for icv injections. As can be seen from *Fig. 3*, both dilutions of antibody inhibited the elevation of T$_b$ provoked by the injection of heme-L-lysinate, with greater reduction seen following a pre-treatment with anti-PGE$_2$ diluted 10x.

Indomethacin at doses 5 mg/kg and 10 mg/kg did not affect the rise in T$_b$ induced by icv injection of heme-L-lysinate (*Fig. 4*). The drug was injected ip immediately before icv infusion of heme-L-lysinate. As can be seen, elevation of T$_b$ in rats treated with indomethacin and injected with doses of indomethacin did not differ from that of treated with vehicle and heme-L-lysinate.

*Fig. 3*. Changes of body temperature of rats treated icv with anti-PGE2 at –120 min (left arrowhead) and/or control IgG, and then at time 0 (right arrowhead) injected with heme-L-lysinate at a dose of 0.154 µM and/or with aCSF as shown. Values are means ± SE at 15-min averages; n indicates sample size in a respective group.
In the present report we demonstrate that icv injection of heme-L-lysinate causes a dose dependent elevation of $T_b$ in rats. The rise in $T_b$ was accompanied by elevation of PGE$_2$ level in CSF, and was prevented by an icv infusion of antibody against PGE$_2$. However, administration of indomethacin did not affect a heme-induced fever-like response. These data indicate that heme-induced fever is, in fact, a PGE$_2$-dependent response. However, unlike to the fever induced by LPS, a classical exogenous pyrogen used in the laboratory settings (2), fever provoked by heme-L-lysinate appeared to be not sensitive to a COX inhibitor. The latter is in line with previous findings reported by Steiner and Bronco (19).

Production of prostaglandins is attributed mostly to the action of cyclooxygenases. However, there are also studies suggesting a non-COX related oxygenation of arachidonic acid yielding PGE-like products. For example, Zilletti
et al. (24) have demonstrated that hemoglobin incubated with purified arachidonic acid under aerobic conditions gives rise to the formation of PGE$_2$, and this effect was not inhibited by indomethacin. Accordingly, it has been reported that heme did not activate cyclooxygenases (25, 20). Nevertheless, Ciuffi et al. (26) have observed upregulation of PGE$_2$ in the corticocerebral homogenates incubated with hemoglobin, which is in line with data by Haider et al. (20) demonstrating the PGE$_2$ production in the endothelial cells co-cultured with heme. Furthermore, injection of hemoglobin into the subarachnoid space has also elicited release of PGs (27, 28). Based on these data we hypothesize that heme can trigger fever by inducing a PGE$_2$ synthesis which is independent of COX.

We assume that data presented in our report have important clinical value, especially with regard to the clinics of the brain stroke. Although Sare et al. (29) have reported that after ischemia/reperfusion period the concentration of PGE$_2$ in the brain is elevated, the stroke is often associated with a specific type of fever in patients, which cannot be reduced by treatment with non-steroidal anti-inflammatory drugs (5, 6). Azzimondi et al. (30) have postulated that there is a strait correlation between body temperature of the stroke patients and survival rate. They estimated that temperature over the 37.9 °C significantly correlated with poor outcome. Also Hindfelt (31) indicated that 37.5 °C during brain stroke is correlated with poor prognosis in 2 months after the stroke. Terent and Anderson (32) have established that border temperature at 38 °C, and suggested using a fever-abolishing regimen targeting cyclooxygenases in the stroke patients. Our data indicate that applying COX inhibitors alone in such regimen may appear insufficient, if the fever is in part triggered by free heme penetrating the brain tissue.

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