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Effects of Heme Oxygenase System on the Cyclooxygenase in the Primary Cultured Hypothalamic Cells

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Endogenous carbon monoxide (CO) shares with nitric oxide (NO) a role as a putative neural messenger in the brain. Both gases are believed to modulate CNS function via an increase in cytoplasmic cGMP concentrations secondary to the activation of soluble guanylate cyclase (sGC). Recently CO and NO were proposed as a possible mediator of febrile response in hypothalamus. NO has been reported to activate both the constitutive and inducible isoform of the cyclooxygenase (COX). Thus, we investigated whether CO arising from heme catabolism by heme oxygenase (HO) is involved in the febrile response via the activation of COX in the hypothalamus. PGE_2 which is a final mediator of febrile response released from primary cultured hypothalamic cells was taken as a marker of COX activity. PGE_2 concentration was measured with EIA kits. Exogenous CO (CO-saturated medium) and hemin (a substrate and potent inducer of HO) evoked an increase in PGE_2 release from hypothalamic cells, and these effects were blocked by methylene blue (an inhibitor of sGC). And membrane permeable cGMP analogue, dibutyryl-cGMP elicited significant increases in PGE_2 release. These results suggest that there may be a functional link between HO and COX enzymatic activities. The gaseous product of hemin through the HO pathway, CO, might play a role through the modulation of the COX activity in the hypothalamus.

Key words: Heme oxygenase, Carbon monoxide, Cyclooxygenase, Hypothalamic cells

INTRODUCTION

Inflammation or administration of exogenous pyrogens (e.g., bacterial endotoxin) leads to production of endogenous pyrogenic cytokines (LeMay *et al.*, 1990; Kluger, 1991; Van Zee *et al.*, 1991; Roth *et al.*, 1993; Jansky *et al.*, 1995; Zeisberger, 1999) and subsequent stimulation of PGs production in the central nervous system (CNS) (Cao *et al.*, 1995; 1996; Quan *et al.*, 1998; Yermakova & O'Banion, 2000). Although there are some exceptions in which fever develops independently from PGs (Zampronio *et al.*, 1994a, 1994b; Szelenyi *et al.*, 1997; Fabricio *et al.*, 1998), the action of PGE_2 in the preoptic area of the anterior hypothalamus is accepted as the ending step in febrile response (Coceani, 1991; Matsuda *et al.*, 1992; Zeisberger, 1999). Various substances (interleukin (IL)-1β, IL-6, interferons, and tumor necrosis factor) have been suggested as being involved in the thermoregulatory pathways within the brain (Klunger, 1991; Klir *et al.*, 1994; Dinarello, 1999) but the various steps are still unknown.

Recently nitric oxide (NO) was proposed as a possible mediator of febrile response in the hypothalamus (Minghetti *et al.*, 1996; Lin & Lin, 1996; Scammell *et al.*, 1996; Roth *et al.*, 1998; Jung *et al.*, 2001). NO has been reported to activate the heme enzyme, COX, in ex vivo and in vitro models (Salvemini *et al.*, 1993; 1994; Molina-Holgado, 1995).

Endogenous CO shares with NO a role as a putative neural messenger in the brain (Verma *et al.*, 1993; Gosta *et al.*, 1996). Like NO, CO is believed to modulate CNS function via an increase in cytoplasmic cGMP concentraions (Verma *et al.*, 1993; Kharitonov *et al.*, 1995).

Heme oxygenase (HO) catalyzes the metabolism of heme to biliverdin, free iron and CO. And this gasous compound, CO, stimulates soluble guanylate cyclase activity and promotes an increase in cGMP (Maines, 1997).

Three heme oxygenase isoforms have been identified, which are HO-1, HO-2 and HO-3 (Maines, 1997;

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McCoubrey *et al.*, 1997). Among which the HO-1 and HO-2 were shown to be present in several areas of the rat central nervous system (CNS), most of the heme catabolism in the brain is accounted for by HO-2 (Sun *et al.*, 1990). HO-1 activity in the brain is usually low, but it can be induced by stimuli, such as oxidative stress or endotoxin. CO was also suggested to be involved in febrile response (Steiner *et al.*, 1999; Steiner & Branco, 2000; 2001).

In this study, to determine the mechanism of CO-induced febrile response we investigated the effects of hemin, a substrate of HO and a potent inducer of HO-1 (Maines, 1997), on COX in the rat primary cultured hypothalamic cells.

MATERIALS AND METHODS

Chemicals

Hemin chloride, zinc protoporphyrin IX(ZnPP-IX), dibutyryl -cGMP (db-cGMP), methylene blue and indomethacin were purchased from Sigma, and minimal essential medium (MEM) and fetal bovine serum from Gibco BRL.

Hemin and ZnPP-IX were dissolved in 100 mM NaOH solution and diluted in artificial CSF solution (aCSF : 138 mM NaCl, 50 mM KCl, 11 mM $NaHCO_3$, 1 mM KH_2PO_4, 1.1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.4) or in MEM. The pH of the final dilutions of hemin and ZnPP-IX was neutral. Indomethacin was dissolved with 4% sodium bicarbonate solution. Methylene blue and db-cGMP were dissolved in MEM. CO-saturated MEM was prepared by bubbling MEM with CO gas for 1 h.

Surgery

Adult male Sprague-Dawley rats, weighing 250-300 g, were used and were housed individually at an ambient temperature of 22 ± 1°C with a 12 h light-dark cycle. Animal water and food *ad libitum* were allowed. After anesthetia with secobarbital sodium (30 mg/kg, i.p), a cannula (0.8 mm o.d.) was stereotaxically implanted in the lateral ventricle (P: 0.9 mm, L: 1.5 mm, V: 3.5 mm) for intracere-broventricular (i.c.v.) injection of drugs, according to Paxinos and Watson (Paxinos & Watson, 1997). The cannula was anchored with dental cement to the calvarium surface. The reflected muscles and skin were replaced around the mound containing the cannula and were sutured. These animals were used for experiment after the recovery period for 3-5 days.

Determination of effects on body temperature

Experiments were conducted between 10:00 a.m. and 7:00 p.m.. The rectal temperature of each rats was measured at every 30 min in conscious state. Only animals whose body temperature was stable and in the range of 36.5-37.5°C were used to determine the effect of drug applications.

Hypothalamic mixed cell cultures

Primary cultures were created from 1- to 3-day-old Sprague-Dawley rat pups (Clark & Gillies, 1988; Tolias *et al.*, 1999). Pubs were sterilized by dipping them into 70% ethanol solution for several seconds and then decapitated by razor. The whole brain was quickly removed and the hypothalamic tissue dissected.

The cells were prepared by mechanoenzymatic dissociation using trypsin and cultured in minimal essential medium (MEM) supplemented with 2 mM glutamine, 10% fetal bovine serum, penicillin 50 U/ml, streptomycin 50 g/ml and amphotericin 2.5 g/ml. Cells were plated at a density between 2×10^6 and 2.5×10^6 in 60-mm dishes pre-treated with poly-L-lysine (10 g/ml). After 2 days the medium was changed to fresh medium, and then twice a week. The cells were subcultured twice before the experiments were performed. All experiments were performed seven days after second subculture.

Hypothalamic incubation

After 3 h pre-incubation period, the medium was replaced with fresh medium. The cells were incubated to assess basal PGE_2 release for 3 h. It was followed by consecutive 3 h incubations in medium containing test substances. Medium samples were collected and stored at -70°C until PGE_2 assays were perfomed.

Assay for PGE_2

PGE_2 were measured with enzymeimmunoassay (EIA) kits from Amersham (UK). Briefly, for each assay, 100 µl of prostaglandin E_2 standard or sample was incubated in a refrigerator at 2-8°C for 3 h and incubated with prostaglandin E_2 conjugate in the same condition for 1 h. After 4 times of washing the wells, 150 µl of enzyme substrate was added into all wells and for 30 min at room temperature. The optical density (OD) was read at 450 nm within 30 min.

Statistical analysis

PGE_2 levels were expressed as pg/ml of incubation medium. And the effect of drug on COX activity was expressed as a percentage to the basal PGE_2 level. All results were expressed as the means ± S.E.M. for experiments. The data were analyzed by means of Student's *t*-test.

RESULTS

Effects of hemin on body temperature

I.c.v. administration of aCSF produced no significant

change in body temperature. But, i.c.v. injection of hemin 10 μg markedly increased the body temperature, and produced the maximal effect within 3 h. The hemin-induced febrile response was completely attenuated by pretreatment with indomethacin (10 mg/kg, i.p.) or ZnPP IX (10 μg, i.c.v.) 30 min before injection of hemin (Fig. 1).

Effect on the PGE_2 release.

The rate of PGE_2 release for 1 h was expressed as the concentration of PGE_2 of incubation medium. The basal rate was 9.37 ± 2.44 pg/ml and the incubation with vehicle produced no significant change (Fig. 2). The PGE_2 release was significantly increased by incubation with CO for 90 min, but not by incubation for 30 min (Fig. 3).

To test the effect of endogenous CO, we used hemin which is a specific substrate of HO and potent inducer of HO-1. Incubation with hemin (50 μM) for 3 h significantly stimulated PGE_2 release. This effect was attenuated by pretreatment with ZnPP-IX (Fig. 4).

Soluble guanylate cyclase inhibitor, methylene blue (10 μM) inhibited heminstimulated PGE_2 release from rat hypothalamic cells. The membrane permeable analogue of cyclic GMP, dibutyryl cGMP (1 mM) significantly increased PGE_2 levels (Fig. 5). CO-induced stimulation of

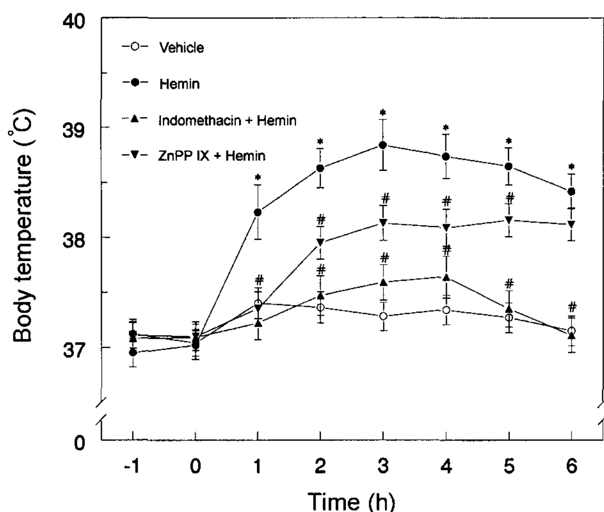

Fig. 1. Effects of i.c.v. injected hemin on the body temperature. Each value represents the mean S.E.M. of 4-6 experiments. Indomethacin (10 mg/kg, i.p.) and ZnPP-IX (10 μg, i.c.v.) were injected 30 min before hemin administration.Vehicle: 3 μl of artificial cerebrospinal fluid *P<0.05 vs vehicle, #P< 0.05 vs hemin alone group

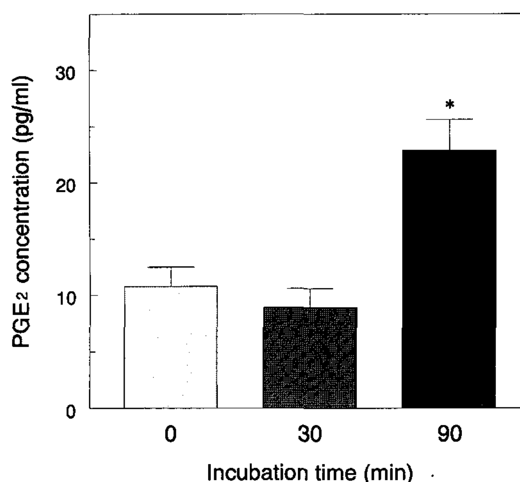

Fig. 3. The effect of exogenous CO on the PGE_2 release in the primary cultured hypothalamic cells. All results are expressed as a PGE_2 concentration of the basal release. Each value represents the mean ± S.E.M. of 6 wells. * P<0.05

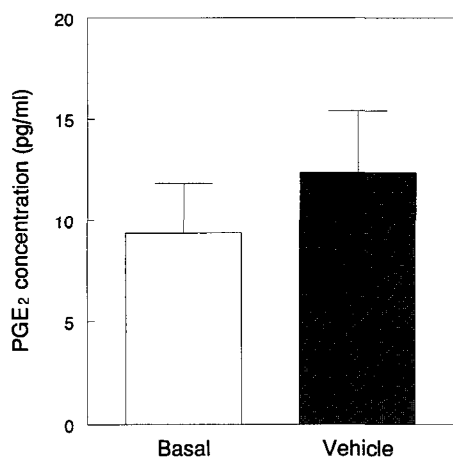

Fig. 2. PGE_2 release of basal level (first 3 h) and vehicle (second 3 h). All results are expressed as a PGE_2 concentration of the basal release. Each value represents the mean ± S.E.M. of 6 wells.

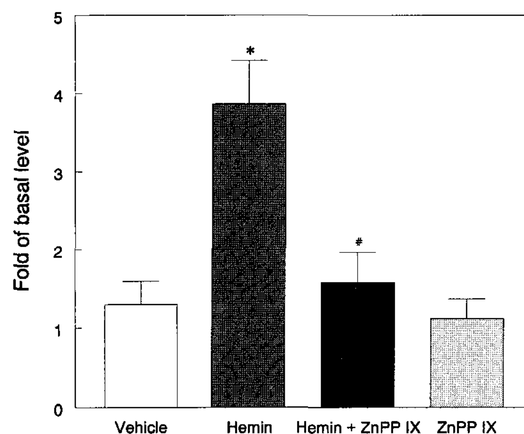

Fig. 4. The effect of hemin (50 μM) on the PGE_2 release in the primary cultured hypothalamic cells.. All results are expressed as a percentage of the basal release of PGE_2. Each value represents the mean ± S.E.M. of 8 wells. ZnPP IX: 100 μM zinc protoporphrine IX. *P<0.05 vs vehicle, # P<0.05 vs hemin alone group.

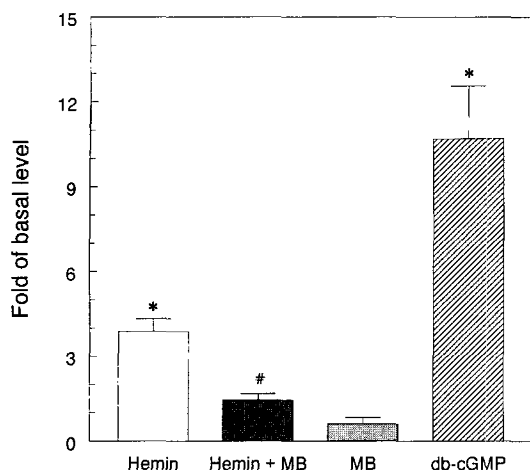

Fig. 5. The effect of methylene blue on the hemin-induced stimulation of PGE_2 release. All results are expressed as a percentage of the basal release of PGE_2. Each value represents the *mean* ± S.E.M. *of 6 wells. MB: 10 μM methylene blue,* db-cGMP: 1 mM dibutyryl cGMP, *P<0.05 vs vehicle, #P<0.05 vs hemin alone group

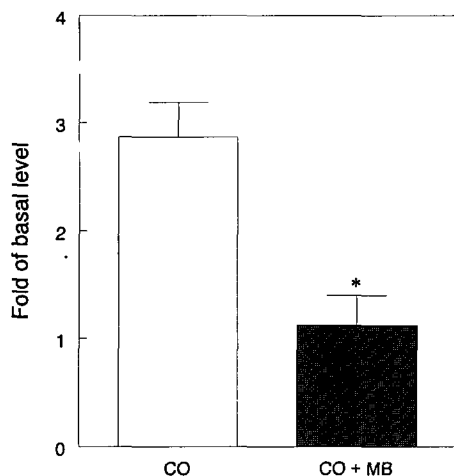

Fig. 6. The effect of methylene blue on the hemin-induced stimulation of PGE_2 release. All results are expressed as a percentage of the basal release of PGE_2. Each value represents the mean ± S.E.M. of 6 wells. *P<0.05 vs CO alone group.

PGE_2 release was also inhibited by methylene blue (Fig. 6).

DISCUSSION

In this study, we conformed that CO is a putative mediator of febrile response in hypothalamus because the results shown hemin-induced febrile response was blocked by *HO inhibitor and indomethacin. Blockade by indome*thacin suggests that hemin-induced pyresis was mediated through the mechanim of thermoregulation in hypothalamus. So, we investgated the effects of CO on the COX in the cultured hypothalamic cells.

To test the effects of exogenous CO on cyclooxygenase, CO-saturated incubation media was added to the incubation medium of hypothalamic cells. CO-saturated medium stimulated PGE_2 release from the hypothalamic cells.

Also, hemin (50 μM) elicited the significant increase in PGE_2 production. The PGE_2 release from hypothalamic cells reached peak within 3 hrs of incubation (data is not shown). This effect was blocked by methylene blue which is an inhibitor of soluble guanylate cyclase. And methylene blue also reduced basal PGE_2 release. Membrane permeable cGMP analogue, db-cGMP significantly increased PGE_2 production.

Hemin can bind the apoenzyme with a stoichiometry of approximately one hemin molecule per COX subunit (Smith & Marnett, 1991). As a consequence, COX catalytic activity is increased by the addition of hemin in a concentration-dependent manner to purified enzyme preparation in vitro (Vander Ouderaa *et al.*, 1979). But in this study, blocking of hemin-induced stimulation by a HO inhibitor (ZnPP-IX) and methylene blue excludes the possibility of direct effect of hemin

Prostaglandin formation occurs in the majority of mammalian organs and tissues, but not in all cell types of each organ. And the amounts produced by different cell types may vary (Smith & Marnett, 1991). In the brain, COX protein immunoreactivity was shown to be associated with neurons and to a lesser extent, glial cells in most areas (Tsubokura *et al.*, 1991). But findings on cultured neurons and astrocytes showed that the latter produce and release far larger amount of PGE_2 (Katsuura *et al.*, 1989). And prostanoid production was significantly increased by interleukin-β1 in astrocytes but not in neurons These results suggest that PG biosynthesis can be induced in the glial cell line.

The cellular source of prostaglandin in the generation of the febrile response has been investigated (Coceani, 1991). Several studies have supported a role of the cerebral microvasculature. LPS stimulates cultured cerebral microvessels to release PGE_2 (Bishai *et al.*, 1987). And neurons are thought to be alternative cellular source for PGE_2 production (Breder *et al.*, 1995).

Hypothalamus is known as a thermoregulatory center. The anteroventral region around the third ventricle is the site most likely to contain the prostaglandin-synthesis and responsive elements involved in the generation of the febrile response. This region is one of the most sensitive in the generation of PGE_2-induced hyperthermia (Stitt, 1991). The organum vasculosum of the lamina terminalis (OVLT), a circumventricular organ at the rostral end of the third ventricle, is the most sensitive site for the antipyretic effects of salicylates, which act through inhibition of PGE_2 synthesis (Morimoto *et al.*, 1988). Furthermore the preoptic area (POA), which are directly adjacent to the OVLT, contain the densest concentration of PGE_2 binding sites in the rat brain (Matsumura *et al.*, 1990)

These evidences suggest that there may be a functional link between HO and COX enzymatic activities. And the gaseous product of hemin through the HO pathway, CO, might play a role in febrile response through the modulation of the COX activity in the hypothalamus, possibly stimulating COX via sGC.

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