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SIGNALING THE BRAIN IN INFLAMMATION: THE ROLE OF ENDOTHELIAL CELLS

Kiyoshi Matsumura and Shigeo Kobayashi

Department of Intelligence Science and Technology, Graduate School of Informatics, Kyoto University, Kyoto 606-8501, Japan

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1. ABSTRACT

Peripheral inflammation signals the brain primarily via blood-borne proinflammatory cytokines, released from activated immune cells. In addition to these cytokines, immune-brain signaling is known to involve another key mediator, prostaglandin E_2 (PGE_2), the level of which is elevated in the brain during various inflammatory states and which acts to influence the central neuronal activity to evoke some, but not all, of the sickness behavior including fever and the activation of hypothalamo-pituitary-adrenal axis. Studies over the last decade have indicated that brain endothelial cells are the major source of PGE_2 under various inflammatory states. In this review, we highlight the significance of the endothelial mechanism in immune-brain signaling mediated by PGE_2, but discuss also the possible influence of other mechanisms on brain PGE_2 elevation.

2. INTRODUCTION

Peripheral inflammation activates various components of the acute phase response including fever, hyperalgesia, anorexia, excessive slow wave sleep, behavioral suppression, and the activation of hypothalamo-pituitary-adrenal (HPA) axis (1). Many of these responses are mediated through immune-brain signaling, primarily via blood-borne proinflammatory cytokines released from activated immune cells. These cytokines are proteins of approximately 15-20 kDa that hardly penetrate into the brain from the blood due to barrier functions of brain capillaries, called blood brain barrier (BBB). Brain capillaries are made up of non-fenestrated endothelial cells that are connected each other with tight junctions. This special feature of brain capillaries prevents water-soluble molecules including proteins from diffusing into the brain implying that some special mechanisms enable cytokines to signal the brain. To date, 4 possible mechanisms have been proposed for this immune-brain signaling: (i) cytokines act on their receptors in brain endothelial cells and/or perivascular microglial cells, (ii) cytokines act on the circumventricular organs (CVOs) that lack a BBB, (iii) cytokines enter the brain through transporters, and (iv) vagal afferent nerves convey cytokine signals to the brain (1, 2). In addition to cytokines, immune-brain signaling is known to involve another key mediator, prostaglandin E_2 (PGE_2), the level of which is elevated in the brain during various inflammatory states and which acts to influence the central neuronal activity to evoke some, but not all, of the sickness behavior including fever and activation of the HPA axis (3). A further understanding of the mechanism of immune-brain signaling could therefore be elucidated, at least in part, if we understand how these peripherally released cytokines elevate brain PGE_2 levels. Studies over the last decade have indicated that, among the 4 mechanisms, the mechanism that involves cytokines binding to their receptors in endothelial cells best explains the elevation of brain PGE_2 levels under inflammatory states. In this review, we highlight the significance of the endothelial mechanism in immune-brain signaling mediated by PGE_2, but discuss also the possible influence of other mechanisms on brain PGE_2 elevation.

3. IMMUNE-BRAIN SIGNALING MEDIATED BY PGE_2

3.1. Enzymes involved in PGE_2 biosynthesis in fever

Before detailing the mechanisms for immune-brain signaling, we review very briefly the enzymes involved in PGE_2 biosynthesis during fever. A more comprehensive review on this topic is presented in another chapter of this issue by Ivanov and Romanovsky (4). PGE_2 is biosynthesized from membrane phospholipids through

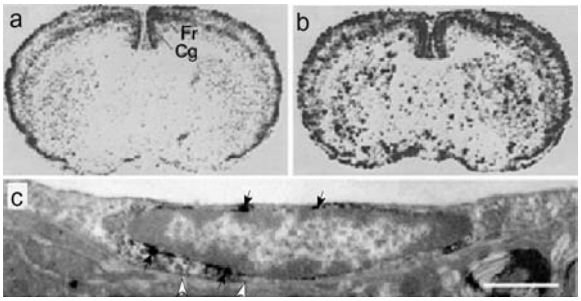

Figure 1. Distribution of COX-2 mRNA (a, b) and COX-2-lile immunoreactivity (c) in the rat brain. (a) COX-2 mRNA in the cerebral cortex under the control condition (3 h after i.p. injection of saline) (b) Spot-like COX-2 mRNA signals appeared throughout the brain 3 h after i.p. injection of LPS. (c) Immunoelectron microgram showed COX-2-like immunoreactivity was located in the nuclear envelop (black arrows) of a brain endothelial cell 5 h after i.p. injection of LPS. Scale bar in (c) represents 1 micrometer. Fr: frontal cortex, Cg: cingulated cortex, white arrowheads in (c) indicate the basement membrane.

three enzymatic steps. First, arachidonic acid is cleaved from the membrane phospholipids by the action of phospholipase A_2 (PLA_2). Arachidonic acid is then converted to PGH_2 by cyclooxygenase (COX). Finally, PGH_2 is isomerized to PGE_2 by PGE synthase (PGES). Thus, PGE_2 is produced through three enzyme steps of PLA_2, COX, and PGES. PLA_2 enzymes are classified into 2 groups, cytosolic PLA_2 ($cPLA_2$) and secretory PLA_2 ($sPLA_2$), the latter of which includes over ten isozymes. At present, it is unclear which PLA_2 enzyme is responsible for the biosyntheis of PGE_2 in fever. Two isoforms of COX have been characterized, i.e., COX-1 and COX-2. COX-1 is constitutively expressed in various tissues and seems to play a house-keeping role. On the other hand, COX-2, generally, is not expressed under the normal conditions, but is induced strongly by various stimuli including proinflammatory cytokines, endotoxin lipopolysaccharide (LPS), and tumor promoters (3, 5, 6). Several lines of evidence indicate that COX-2, rather than COX-1, plays a major role in PGE_2 biosynthesis during fever: (i) a COX-2 specific inhibitor (NS398) blocks fever as well as the elevation of PGE_2 in the brain in response to intraperitoneal (i.p.) injection of LPS (7, 8); and (ii) COX-2 gene deficient mice, unlike COX-1 gene deficient mice, do not develop fever to systemic injection of LPS (9) or interleukin (IL)-1beta (10). COX-1 might also play a role in fever. Administration of a COX-1 specific inhibitor in rats resulted in a profound hypothermic response to intravenous (i.v.) injection of LPS suggesting that COX-1 prevents hypothermic response to LPS and, makes COX-2-driven fever more evident (11). PGE synthase (PGES) activity, which catalyzes the conversion of PGH_2 to PGE_2, has been recognized in various tissues. So far 4 proteins possessing PGES activity have been identified including microsomal-PGES (mPGES)-1 (12), mPGES-2 (13), cytosolic PGES (cPGES) (14), and the mu class glutathione-S-transferase (15). Among these, mPGES-1 likely plays the essential role in PGE_2 biosynthesis for fever since mice lacking the mPGES-1 gene do not develop fever in response to i.p. injection of LPS (16). At present, colocalized expression of COX-2 and mPGES-1 is the most reliable index of PGE_2 biosynthesis for fever.

3.2. Endothelial cells as the dominant source of PGE_2 during fever

The role of PGE_2 in fever was established in the early 1970s (17, 18), however, it was unclear for a long time as to how PGE_2 is elevated in the brain under inflammatory/infectious states. One possibility was that blood-borne PGE_2 enters the brain. The molecular size of PGE_2 is much smaller than that of cytokines (350 Da vs 15000-20000 Da) and some fraction of it may enter the brain from the circulation. Although this possibility is not ruled out, and might explain some parts of fever response, the fact that injection of a COX inhibitor directly into the brain suppresses fever clearly indicates the importance of brain-derived PGE_2 in evoking fever (19, 20).

3.2.1. Histochemical localization of PGE_2 and COX-2

In 1993, van Dam and colleagues (21) reported that PGE_2-like immunoreactivity was present in the brain blood vessels of rats injected systemically with LPS. This study provided the first evidence for a role of brain blood vessels as the source of PGE_2 during fever. Unfortunately, however, immunohistochemical detection of PGE_2 *in situ* generally was difficult, probably because PGE_2 is released very quickly upon synthesis and its cellular content is, therefore, low. Subsequent studies, therefore, were directed towards localization of the enzymes responsible for PGE_2 generation or their mRNA. In 1995, Cao and colleagues (22) demonstrated, for the first time, that COX-2 mRNA was strongly induced in blood vessels in brain parenchyma and subarachnoidal space after i.p. injection of LPS (Figure 1a, b). This finding has been confirmed also in mice injected with LPS (23), and in rats given other inflammatory agents including IL-1beta (24), tumor necrosis factor (TNF)-alpha (25), turpentine oil (26) and carrageenan (27).

3.2.2. Are COX-2 expressing cells endothelial cells or perivascular cells?

To determine the identity of COX-2-expressing cells in the brain blood vessels, double immunostaining of COX-2 with some cell-specific marker was performed. Elmquist et al. (28) demonstrated that the COX-2-positive cells, activated in response to i.v. injection of LPS (5 – 125 microgram/kg), were perivascular microglia since they were reactive also toward a monoclonal antibody for activated microglia. On the other hand, Matsumura et al. (29) found that in response to i.p. injection of LPS (100 microgram/kg) COX-2-like immunoreactivity was colocalized with von Willebrand factor, a marker for endothelial cells. An immunoelectron microscopic study further supported this finding (Figure 1c). Then, Schiltz and Sawchenko (30) reported something in between the preceding two studies: a distinct population of vascular cells expressed COX-2, depending on the type and dose of the immune stimulant. I.v. injection of IL-1beta (1.87-30 microgram/kg) or a low dose of LPS (0.1 microgram/kg) induced COX-2 expression in perivascular cells (ED2-positive cells), which seemed identical to those called perivascular microglia by Elmquist et al. (28). Higher

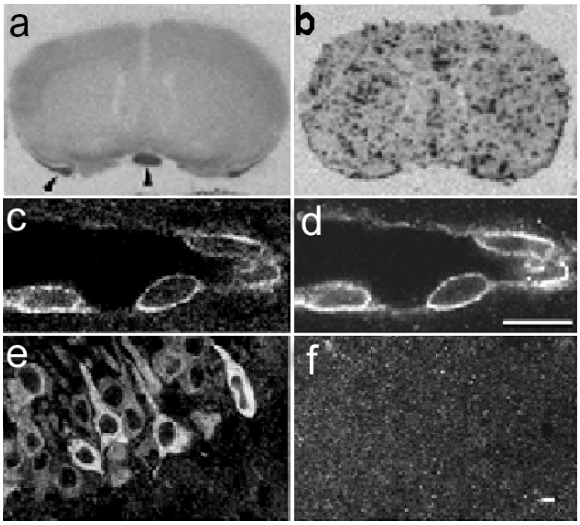

Figure 2. Distribution of mPGES-1 mRNA (a, b), COX-2 protein (c, e), and mPGES-1 protein (d, f) in the rat brain. (a) mPGES-1 mRNA was not detectable in the rat brain 3 h after i.p. injection of saline. (b) Spot-like mPGES mRNA signals appeared throughout the brain 3 h after i.p. injection of LPS. Double immunostaining of COX-2 (c, e) and mPGES-1 (d, f) revealed almost identical distribution of them in brain endothelial cells 5 h after i.p. injection of LPS. On the other hand, in the cortical neurons, only COX-2 was expressed. Scale bars indicate 10 micrometer.

doses of LPS (2-100 microgram/kg), however, induced COX-2 in cells positive for endothelial markers. Finally, Konsman et al. (31) showed recently that both i.v. injection of IL-1beta (3 microgram/kg) and i.p. injection of LPS (250 microgram/kg) induced COX-2 mainly in endothelial cells of brain venules and in a smaller number of perivascular cells. What might be the reasons for the inconsistent findings above?

Closer inspection of the photographs in the studies by Elmquist et al. (28) and by Schiltz and Sawchenko (30) indicates that two types of morphologically-distinct vascular cells expressed COX-2 in both studies. One type of cells had clear processes and distributed COX-2-like immunoreactivity in their processes and cytoplasm but not in the perinuclear region. This type of cells was termed as perivascular microglia in the former study, or as perivascular cell in the latter; these two terms probably representing the identical cell population of monocytic lineage. Another type of vascular cells displayed COX-2-like immunoreactivity in a round or oval shape, probably representing perinuclear distribution of COX-2. Schiltz and Sawchenko (30) showed that these cells were also stained with endothelial cell markers. On the other hand, Elmquist et al. (28) did not use endothelial markers in their study. Therefore, it is possible that Elmquist et al. (28) might have identified only one population of COX-2-expressing cells while leaving another type unidentified in spite of that the latter type of cells with round-shaped COX-2 staining appeared to be more numerous than those with processes in their photographs. Thus, all four studies above seem to agree that endothelial cells are the dominant cell population that expresses COX-2 in response to higher doses of LPS (5 microgram/kg or above). The question still remains, however, as to why IL-1beta injected i.v., in the study by Schiltz and Sawchenko (30), induced COX-2 only in perivascular cells, whereas a similar dose of IL-1beta induced COX-2 mainly in endothelial cells in the study by Konsman et al. (31). As pointed out by Konsman et al. (31), one possible reason for the discrepancy may be the time points studied. They detected COX-2 at 60 min after IL-1beta injection whereas Schiltz and Sawchenko used 120 min as their earliest time point (30).

3.2.3. Localization of mPGES-1

In situ hybridization studies reveal that mPGES-1 mRNA, seldom expressed in the brain under normal conditions, is vigorously induced in brain blood vessels in response to systemic administration of IL-1beta (32) or LPS (Figure 2a, b) (8). The expression pattern is very similar to that of COX-2 except that COX-2 but not mPGES-1 is constitutively expressed in telencephalic neurons (Figure 2e, f). As in the case of COX-2, double immunostaining of mPGES-1 and von Willebrand factor indicated that the vascular cells expressing mPGES-1 were mostly endothelial cells (8). Double immunostaining for COX-2 and mPGES-1 demonstrated almost identical localization of these 2 enzymes in the nuclear envelope of brain endothelial cells implying a tight functional coupling of these two enzymes (Figure 2c, d) (8). COX-2 and mPGES-1 were evident in endothelial cells of veins and venules in the central nervous system but not in those of arteries or arterioles. A similar result was obtained in rats after systemic injection of poly I:C (a model for viral infection), after subcutaneous injection of carrageenan (our unpublished observation) or complete Freund's adjuvant (a model for localized inflammation) (33), or after burn injury (34). On the other hand, no telencephalic neurons showed mPGES-1 immunoreactivity suggesting that COX-2 in the neurons couples with downstream enzymes other than mPGES-1 (Figure 2f). Thus, within the brain, endothelial cells are the sole cell type expressing both COX-2 and mPGES-1 in response to various inflammatory stimuli, and are, therefore, the dominant source of PGE_2 under the inflammatory states.

3.2.4 PLA_2

Although it is not yet resolved which PLA_2 enzyme is involved in brain PGE_2 biosynthesis under inflammatory states, we know of at least two PLA_2 enzymes that possibly play a role in brain endothelial cells. One is $cPLA_2$, the mRNA of which is constitutively expressed in brain blood vessels of the rats (35), with more pronounced expression in venules, as opposed to arterioles. Another is $sPLA_2$ type IIA ($sPLA_2$IIA). Whereas this enzyme is little expressed in brain blood vessels under both normal and inflammatory states, it is abundantly expressed in circulating platelets in rats (36). When platelets are activated, $sPLA_2$IIA is released into the blood, and may easily act on brain endothelial cells expressing COX-2 and mPGES.

3.2.5. Cytokine receptors in brain endothelial cells

Brain endothelial cells constitutively express IL-1 receptor type1 (IL-1R1) (31) and TNF-alpha receptor p55

(TNFR1) (37). IL-1R1 is located both the luminal and basolateral surfaces of brain endothelial cells, suggesting that IL-1 can act on the cells from the blood and brain sides (38). Subcellular localization of TNFR1 in brain endothelial cells has not yet been reported. Both IL-1R1 and TNFR1 are upregulated in response to inflammatory stimuli. IL-1R1 is also expressed in perivascular cells (30, 31). IL-6 receptor (IL-6R), absent in brain endothelial cells under normal states, also was induced in response to inflammatory stimuli (39). Even in the absence of membrane bound IL-6R, however, soluble IL-6R in the blood may participate in IL-6 signaling together with gp130, the signal transducer for IL-6 receptor, constitutively expressed in endothelial cells. Systemic as well as intracerebroventricular (i.c.v.) injection of IL-1beta or TNF-alpha induced fever and COX-2 and/or mPGES expression in brain endothelial cells in rats (24, 25, 32). In contrast, systemic injection of IL-6 alone induced neither fever nor the enzyme expression in rats. When injected i.c.v., however, IL-6 effectively evoked fever and COX-2 induction in brain endothelial cells (38). Since systemic injection of LPS elevates these cytokines, the inductions of fever and COX-2/mPGES by LPS are likely mediated through the actions of these cytokines on brain endothelial cells. It is unclear, however, whether LPS also has a direct action on the endothelial cells. The LPS receptor, toll-like receptor 4 (TLR4), is little expressed in brain endothelial cells in the rat (40).

3.2.6. Physiological relevance

As far as we have tested, all inflammatory stimuli known to be pyrogenic induce COX-2 in brain endothelial cells, the induction of mPGES was studied only in a few cases. The inflammatory stimuli tested include LPS, IL-1beta, TNF-alpha, poly I:C, carrageenan, turpentine, and burn injury. On the other hand, rats at near term pregnancy (41), or those which are LPS tolerant (7) show reduced fever and COX-2 expression in brain endothelial cells. The time courses of COX-2 and mPGES in brain endothelial cells appears to correlate closely with that of the fever and the elevation of PGE_2 in the CSF, until 3 h after i.p. injection of LPS (42). However, at 5 h after administration of LPS, the PGE_2 level decreases significantly although the enzyme levels remained elevated. Thus, the PGE_2 level is not a simple reflection of the enzyme levels, suggesting the presence of additional factors that influence the PGE_2 level in the CSF. There are three possible mechanisms that can best explain the dissociation between PGE_2 and enzyme levels: (i) reduced supply of the COX-2 substrate (arachidonic acid); (ii) inhibition of COX-2 and/or mPGES activity; and (iii) stimulated degradation of PGE_2 or excretion of it from the brain to the circulation. We found that mRNA for prostaglandin transporter (PGT) is upregulated in the arachnoidal membrane and subarachnoidal endothelial cells (43). Since PGT is involved in the uptake of PGE_2 from the extracellular space (44), the increase of its level may result in facilitated excretion of PGE_2.

3.3. Other immune-brain signaling mechanisms and PGE_2 in the brain

3.3.1. CVOs and brain PGE_2

Since the role of CVOs in immune-brain signaling is thoroughly reviewed elsewhere in this issue (45), we will restrict the issue to a possible role of CVOs in the production of PGE_2. Amongst the CVOs, the organum vasculosum laminae terminalis (OVLT) has attracted the most attention by researchers working towards defining a fever mechanism primarily, because it is located adjacent to the preoptic area (POA), the thermoregulatory center. Moreover, the rostral part of the POA surrounding the OVLT expresses high levels of PGE_2 receptor, consistently demonstrated by ^{3}H-PGE_2 binding in the earlier studies (46, 47), and by *in situ* hybridization (3, 48) and immunohistochemistry for the EP3 receptor in later studies (49, 50). Histochemical studies clearly demonstrated that the OVLT is the target of circulating cytokines or LPS. First, the OVLT expresses receptors for cytokines including IL-1alpha/beta (24, 51-53), TNF-alpha (37), and IL-6 (39), although the type of cells expressing these cytokine receptors are non-neuronal. Second, cells in the OVLT respond to systemic administration of pyrogens with nuclear translocation of NFkB (54) and STAT3 (55, 56), the transcriptional control factors downstream of IL-1alpha/beta, TNF-alpha, or LPS, and of IL-6, respectively. Third, cells in the OVLT respond to systemic administration of LPS with an expression of FOS (57), an immediate early gene product and a transcriptional control factor. Fourth, macrophage-like cells in the OVLT express IL-1beta in response to systemic administration of LPS indicating that cytokines are also produced within OVLT (58, 59). Fifth, the OVLT constitutively expresses the LPS receptors, TLR4 and CD14, possibly being the molecular bases for the responses to LPS (40). Whilst these findings are also the case for some other CVOs, such as the subfornical organ and area postrema, they are not evident in the brain parenchyma suggesting that CVOs are the privileged sites for immune-brain signaling.

In contrast to the apparent responses of the OVLT (and other CVOs) to inflammatory stimuli, its physiological significance in brain PGE_2 biosynthesis and fever is unclear. *In situ* hybridization study using ^{35}S-labeled cRNA probes show the rat OVLT to constitutively express low levels of COX-2 mRNA under control states, which is not upregulated in response to administration of LPS (7, 60). Moreover, immunohistochemical studies reveal COX-2 and mPGES-1 levels to be lower than the detection limits in the OVLT (8). From histochemiacl point of view, therefore, it is unlikely that the OVLT is the primary site for PGE_2 biosynthesis for fever. This is in contrast to an earlier physiological study by Komaki et al. (61), in which microdialysis technique was used to measure local PGE_2 levels in the brain. Komaki and colleagues (61) found that i.v. injection of IL-1beta elevated interstitial PGE_2 most sharply in the OVLT, among a few other brain regions tested, and this elevation was almost completely suppressed if the dialysis probe was perfused with a nonspecific-COX inhibitor, indomethacin, indicating that PGE_2 is biosynthesized in the OVLT. It is plausible, however, that the microdialysis probe might have picked up PGE_2 produced in the tissue surrounding the OVLT. LPS stimulation did indeed induce COX-2 and mPGES-1 in blood vessels running dorsal and ventral to the OVLT. In particular, the subarachnoidal space just below the OVLT is rich in blood vessels expressing these enzymes under the

inflammatory states. PGE_2 synthesis near the OVLT seems to be involved in fever because localized injection of a nonselective COX inhibitor into the anteroventral preoptic region attenuates LPS fever (20). Electrolytic lesion of the OVLT resulted in profound but inconsistent effects on fever. Destruction of the OVLT completely or partially suppressed LPS-induced fever in guinea pigs (62), rats (63), rabbits (64), and sheep (65), whereas, in another study, a small lesion in the OVLT enhanced LPS-induce fever in rats and rabbits (66). The reason for the discrepancy is unclear.

3.3.2. Vagus and brain PGE_2

Since the effects of vagotomy on fever and sickness behavior are thoroughly reviewed elsewhere (67) in this issue, we will restrict the issue to a possible role of vagus in brain PGE_2 elevation. Vagal afferents also are considered to transmit peripheral immune signals to the brain (68). This transmission appears to involve COX-1 derived prostaglandins since a COX-1, but not COX-2, ihbibitor suppressed the c-FOS response in the nucleus tractus solitarius (NTS) of rats injected i.v. with LPS (11). In agreement, a subset of vagal afferent neurons have been shown to express COX-1-like immunoreactivity (69), and their terminals in the NTS also express high levels of receptors for PGE_2 and PGI_2 (70). Thus, PGE_2 or PGI_2 may be released from the central terminals of vagal afferents via the COX-1 dependent mechanism, and may act on the terminals in an autocrine or paracrine manner to modulate transmitter release. We examined if the vagus has some impact on the levels of PGE_2 in the CSF (69). The experiments were conducted in urethane anesthetized rats under the acute condition, in which the vagi were cut bilaterally at the cervical level proximal to the nodose ganglia. In both sham-operated rats and vagotomized rats, i.p. injection of LPS (400 microgram/kg) resulted in higher levels of PGE_2 in the CSF than did injection of saline. Surprisingly, the LPS-induced elevation of PGE_2 was more significant in vagotomized rats than in sham-operated ones. In both groups, LPS induced COX-2 in brain endothelial cells. These results indicate that the vagal afferents are not essential to LPS-induced COX-2 expression in brain endothelial cells and elevation of PGE_2 in the CSF. Perhaps, COX-1 in the vagal afferent terminals elevates local levels of prostanoids within the NTS, but has little influence on PGE_2 levels in the CSF. The enhanced PGE_2 response in the CSF in the vagotomized rats is in line with the fact that acetylcholine released from the vagal efferent terminals exerts anti-inflammatory actions on peripheral macrophages via the nicotinic acetylcholine receptor alpha7 subunits (71). Since vagotomy eliminates both afferent and efferent signals experimental results derived from vagotomized animals need to be interpreted with caution.

3.3.3. Cytokine transporters and brain PGE_2

Banks and his colleague demonstrated transport of blood borne cytokines into the brain by saturable mechanisms (72, 73). All pyrogenic cytokines when injected via the i.c.v. induce COX-2 in brain endothelial cells and fever. In addition, the i.c.v. injection of IL-1 receptor antagonist partially suppresses fever in the rats injected i.p. or intra-airpouch with LPS (74-76). These results strongly indicate that central IL-1 participates in fever. It is uncertain, however, if the central IL-1 derives from the circulation or is newly synthesized within the brain. Until cytokine transporters are molecularly identified, and their specific inhibitors or mice deficient in those are developed, we cannot conclude how cytokine transporters are involved in fever and the brain PGE_2 response under the inflammatory states.

4. CONCLUSION AND PERSPECTIVES

Of the 4 mechanisms for immune-brain signaling presented so far, the mechanism that involves cytokine receptors in endothelial cells best explains the elevation of brain PGE_2 levels under inflammatory states. Fever, hyperalgesia, and the activation of the HPA axis all are evoked through this mechanism. However, several issues remain still to be elucidated: (i) the type of PLA_2 enzyme upstream of COX-2, (ii) the mechanism of PGE_2 secretion from the endothelial cells, and (iii) the mechanism of exclusion of PGE_2 for the termination of PGE_2 signaling. Furthermore, other components of sickness behavior such as excessive slow wave sleep and behavioral suppression are resistant to COX inhibitors, and likely mediated through other immune-brain signaling mechanisms. Such mechanisms need to be determined in the future for a more thorough understanding of immune-brain signaling.

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Send correspondence to: Dr Kiyoshi Matsumura, Ph.D., Associate professor, Division of Biological Information, Department of Intelligence Science and Technology, Graduate School of Informatics, Kyoto University, Sakyo-ku, Kyoto, 606-8501, Japan, Tel: 81-75-753-9134, Fax: 81-75-753-3145, E-mail: matsu@i.kyoto-u.ac.jp