COX-2 gene expression as well as the enzyme activity in C6 cells, this natural product is suggested to be valuable for drug development for the treatment of these brain diseases and inflammations.

In conclusion, we for the first time demonstrated that γ-mangostin, a tetraoxygenated diprenylated xanthone isolated from mangosteen, suppresses IKK activity to inhibit LPS-induced NF-κB activation without affecting cell viability in C6 glioma cells and thereby decreases COX-2 induction.

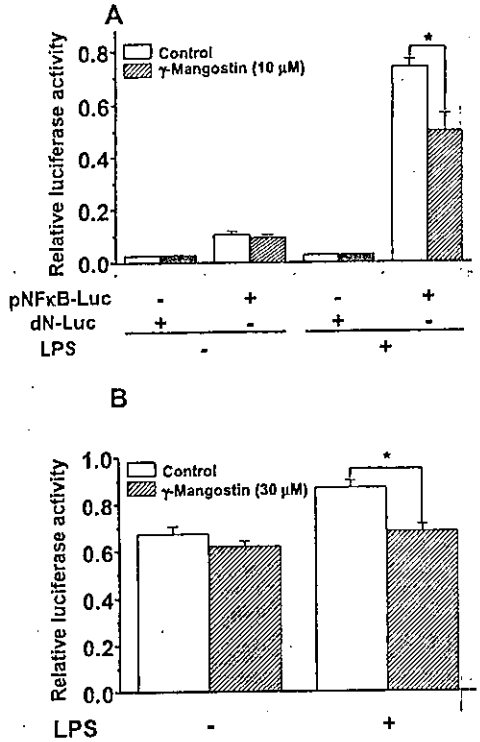

Fig. 7. Inhibitory effect of γ-mangostin on LPS-induced enhancement of luciferase activity of pNFκB-Luc (A) or phPES2(−327/+59)-Luc (B) in C6 cells. Cells were transfected with 0.475 μg/well pNFκB-Luc or dN-Luc (the NF-κB–responsive element-deficient pNFκB-Luc), or 0.4 μg/well phPES2(−327/+59)-Luc, and 0.025 μg/well of pRG-TK plasmid. After transfection, cells were preincubated with the indicated concentration of γ-mangostin or without this compound for 3 h and then incubated in the absence or presence of 1 μg/ml LPS for 8 h. Cells were harvested, and thereafter the luciferase activity was determined as described under *Materials and Methods*. Relative luciferase activity was calculated as the ratio of firefly luciferase activity to *R. reniformis* luciferase activity. Each column represents the mean ± S.E.M. ($n = 3$). *, $P < 0.05$ compared with the value for cotransfected cells with pNFκB-Luc (A), or phPES2(−327/+59)-Luc (B) and pRG-TK plasmid, which were treated with LPS alone (0.1% DMSO).

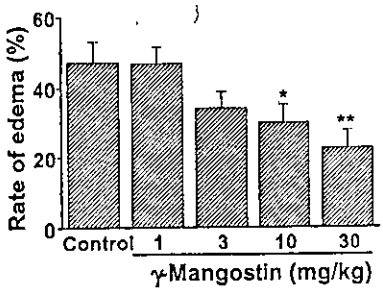

Fig. 8. Concentration-dependent inhibition of rat carrageenan-induced paw edema by γ-mangostin. Rats were injected i.p. with the indicated doses of γ-mangostin 30 min before carrageenan injection. Each column represents the mean ± S.E.M. ($n = 6$). *, $P < 0.05$ and **, $P < 0.01$ compared with the value for the vehicle (DMSO).

This study also demonstrated that γ-mangostin had an anti-inflammatory activity in vivo. These anti-inflammatory properties of this natural compound revealed by our present study using the combined methods of biochemistry and molecular biology sufficiently account for the anti-inflammatory action of the fruit hull of mangosteen. The chemical structure of this natural product is totally different from those of the NSAIDs reported so far, including aspirin and sodium salicylate. These findings thus suggest that γ-mangostin serves not only as a new attractive pharmacological tool for studying the molecular mechanism underlying inflammation but also as a new lead compound for drug development for the prevention and/or treatment of inflammation and brain diseases, including amyotrophic lateral sclerosis and brain tumors.

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Temporal and topographic profiles of cyclooxygenase-2 expression during 24 h of focal brain ischemia in rats

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Abstract

Substantial increases in cyclooxygenase-2 (COX-2) mRNA and protein levels were demonstrated in the peri-infarct and focal ischemic areas after 3–24 and 12–24 h, respectively, in rats. In the ischemic core, significant increases in COX-2 mRNA followed 6 h of ischemia, though the peak level was about one-third of that in the peri-infarct area. Increases in COX-2 protein in the ischemic core were not observed during ischemic periods. Diffuse, neuronal COX-2 staining was found in peri-infarct areas as well as in discrete, immunoreactive neurons in the ischemic core. Robust increases in prostaglandin E_2 levels in the peri-infarct area were demonstrated following 24 h of ischemia. Prostaglandin production as well as COX-2 expression in ischemic tissues depended on the degree and duration of the reduction in cerebral blood flow.

Keywords: Cyclooxygenase-2; Focal brain ischemia; Prostaglandin E_2; 6-keto-PG $F_{1\alpha}$; Cerebral blood flow; Rat

Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in prostaglandin synthesis, is rapidly induced by proinflammatory cytokines in vitro and has been shown to mediate the induction of prostaglandin synthesis during the inflammatory response in vivo [17]. Accumulating evidence suggests that inflammatory processes play a role in the development and progression of atherosclerosis [2,8,14] and COX-2 in particular has become the focus of attention as a therapeutic target enzyme in acute coronary syndromes [1] and Alzheimer's disease [16]. We previously reported that neuronal COX-2 was induced within potentially viable hypoperfused brain areas after a 24 h ischemic period in non-human primates [20]. The role of neuronal COX-2 within such peri-infarct areas, however, is still unclear. Several reports using various rodent models suggested that COX-2 played a role in the development of ischemic injury [3,4,12]. A few postmortem reports suggested that the production of prostanoids by COX-2 after acute ischemia could contribute to the remodeling of neural networks that is seen after focal infarction [15]. The objective of the present study was to elucidate the topography and time course of COX-2 expression and prostaglandin (PG) E_2 (the major prostanoid involved in inflammation) production, as well as the production of the prostacyclin metabolite 6-keto-PG $F_{1\alpha}$ [11,13] during 24 h of focal brain ischemia.

Male Sprague–Dawley rats (300–350 g, $n = 40$) were used in this study. All procedures were approved by our Institutional Animal Research Committee and were performed in accordance with the standards published by the National Research Council. Rats were anesthetized with chloral hydrate (400 mg/kg body weight i.p.) and focal brain ischemia was produced by the intraluminal occlusion of the ostium of the right middle cerebral artery with nylon monofilaments, as previously described [7,9]. Rectal temperatures were monitored and maintained at around 37

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°C with the aid of heating pads. Rats were sacrificed under chloral hydrate anesthesia at time 0 and at different times points after arterial occlusion (1, 2, 3, 4, 6, 8, 12, and 24 h, $n = 4$-5/time point) and their brains immediately immersed in ice-cold saline. The brains were then cut into four coronal sections (blocks A–D) as shown in Fig. 1A. Several blocks were frozen in isopentane-dry ice and stored at −80 °C until use, whereas others (from C) were embedded in paraffin for immunohistochemistry. Analysis of COX-2 expression (mRNA, protein), and measurement of the concentrations of PGE_2 and the prostacyclin metabolite 6-keto-PG $F_{1\alpha}$ in the peri-infarct areas and the ischemic core were performed using blocks A and C, respectively.

In some animals, *N*-isoproryl-*p*-[^{125}I]-iodoamphetamine ([^{125}I]IMP) (2.22 MBq/kg body weight) was injected into the femoral vein 5 min before sacrifice and ex vivo autoradiography was performed to measure cerebral blood flow (CBF) using blocks B and D. For each frozen block, tissues that were adjacent to block C were serially sectioned (20 μm). Exposure was carried out for 7 days in order to visualize the distribution of [^{125}I]IMP. The autoradiograms were analyzed using a computerized imaging analysis system (Bio-imaging Analyzer BAS-5000, Fuji Photo Film, Tokyo, Japan). A total of four regions of interest (ROIs), as shown in Fig. 1B, were bilaterally and symmetrically positioned in the cerebral cortices in each coronal slice of blocks B and D. Asymmetry indices (AIs) were defined as the ratios of values for ROIs in the hemisphere ipsilateral to the arterial occlusion (right) to those of the contralateral homologous ROIs. AIs of the ischemic core were defined as a/d, whereas the AIs of the peri-infarct area were defined as b/c (Fig. 1B). An average AI value from blocks B and D was calculated for the CBF in each area of the ischemic core and peri-infarction areas.

RNA preparation and blot analysis were performed using cortices from blocks A (peri-infarct area) and C (ischemic core) as previously described [6]. For the immunoblot analyses, right cortical samples from block A (peri-infarct area) were obtained from each animal at time 0, and 3, 6, 12, and 24 h after ischemia ($n = 4$-5 for each period). The sample volumes, which were about 20 mg for each animal, were pooled together for each ischemic period. Right

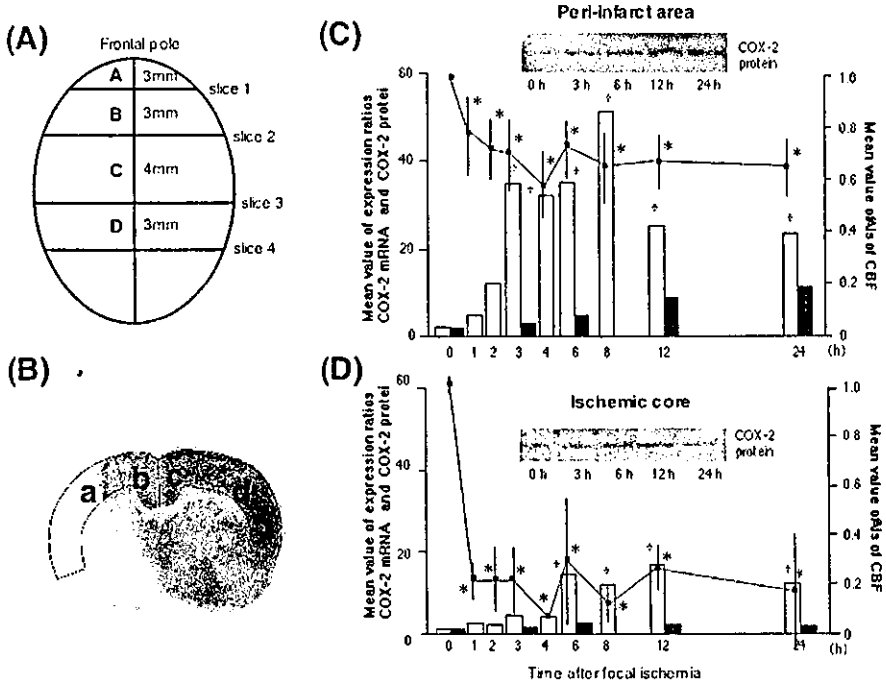

Fig. 1. Temporal profile of COX-2 expression associated with changes in CBF during 24 h of ischemia. (A) The brain was stereotaxically divided, on ice, into four coronal sections using a brain matrix. The first slice was made 3 mm from the frontal pole (block A), while the other three were cut at 3 mm (block B), 7 mm (block C), and 10 mm (block D) intervals posterior to the first slice. Determination of COX-2 expression levels (mRNA, protein) in the peri-infarct area and ischemic core was performed using blocks A and C, respectively. (B) To measure CBF in each animal, four regions of interest (ROIs) were bilaterally symmetrically placed on the cerebral cortices using coronal frozen slices from blocks B and D. Asymmetry indices (AIs) of the ischemic core were defined as a/d, whereas the AIs of the peri-infarct area were defined as b/c. (C,D) Lines indicate the mean AI values of CBF. The open and solid columns correspond to the mean expression ratios of COX-2 mRNA and COX-2 protein, respectively. Figures C and D show the time course of COX-2 expression in the peri-infarct area and ischemic core, respectively. A one-way ANOVA and post-hoc Fisher's tests were used to assess the differences in AIs and expression ratios of COX-2 mRNA between the different ischemic time points. CBF values in the peri-infarct area and ischemic core were significantly reduced compared to controls immediately after arterial occlusion ($^*P < 0.05$). The mean CBF values in the ischemic core and peri-infarct area were 0.19 ± 0.07 (mean ± SD) and 0.67 ± 0.06, respectively. The time course of COX-2 expression in the peri-infarct area was different from that in the ischemic core. Thus, the expression ratios of COX-2 mRNA increased significantly after 3 h of ischemia ($^\dagger P < 0.05$), with COX-2 protein also increasing with time in the peri-infarct area. On the other hand, significant increases in COX-2 mRNA were found 6 h after ischemia ($^\dagger P < 0.05$), and increases in COX-2 protein were not observed during the ischemic period in the ischemic core.

cortical samples from block C (ischemic core) for each ischemic period were also pooled together in this manner. Immunoblot analyses were then performed on each pooled sample as previously described [19]. COX-2 expression (mRNA, protein) in the ischemic cortices was calculated as expression ratios, defined as the ratio of the COX-2 mRNA or protein signals in the ischemic samples to their mean values in the corresponding control areas.

For immunohistochemistry, a mirror sectioning technique was used to colocalize COX-2 and microtubule-associated protein 2 (MAP-2), a neuronal skeletal protein, in sections from block C as previously described [19]. Negative controls consisted of sections that were incubated overnight without the primary antibody and processed as above.

Tissue concentrations of PGE_2 and 6-keto-PG $F_{1\alpha}$ in the right (ischemic) cortices of blocks A (peri-infarct area) and C (ischemic core) were determined using radioimmunoassay kits (Perkin-Elmer Life Sciences, Inc. MA, USA), and values were normalized for protein content.

Significant reductions in AIs for CBF in the peri-infarct area and ischemic core were demonstrated in animals at each ischemic time point compared to controls (Fig. 1C,D). The expression ratios of COX-2 mRNA increased significantly between 3 and 24 h of ischemia in the peri-infarct area compared to controls (Fig. 1C). In the ischemic core, significant increases in COX-2 mRNA were seen following 6 h of ischemia, which remained through 24 h (Fig. 1D). The peak value of the expression ratio of COX-2 protein in the peri-infarct area was 10.7 at 24 h of ischemia, while the peak expression ratio in the ischemic core was 2.0 at 6 h of ischemia.

COX-2 immunoreactive neurons were found predominantly in the peri-infarct area, though elevations in the immunohistochemical staining of discrete neuronal populations were also observed in the ischemic core (Fig. 2). Both COX-2 and MAP-2 immunoreactivity were abolished when the primary antibody was omitted.

Although no significant increases in PGE_2 and prostacyclin levels were observed in the peri-infarct and ischemic core areas following 3 h of ischemia, significant increases in prostaglandin levels were found in the ischemic hemisphere

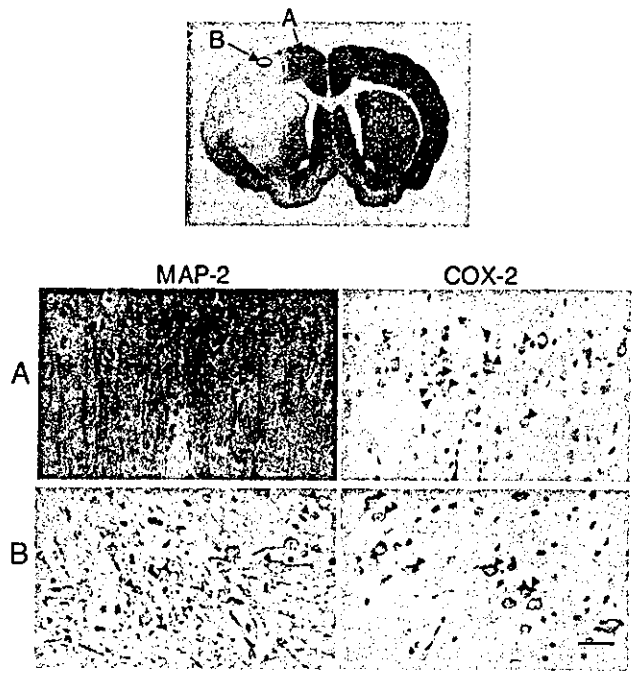

Fig. 2. Immunohistochemical analysis of COX-2. The single top figure shows a coronal slice of the brain of an animal that had undergone 3 h of ischemia, which was immunostained for microtubule-associated protein 2 (MAP-2). The bottom figures are sections that were immunostained for COX-2 and MAP-2 that were derived from either the peri-infarct area (region A in the top figure) or the ischemic core (B). Immunoreactive COX-2 and MAP-2 were localized in the same neurons in the ischemic core and peri-infarct area (arrow heads). Scale bar: 100 μm.

following 24 h of ischemia. In particular, PGE_2 levels in the peri-infarct area increased significantly (Table 1).

We previously demonstrated, in a small number of non-human primates, that post-ischemic COX-2 expression was regulated by the extent of CBF reduction [20]. In the present study, serial changes in the expression of COX-2 during focal ischemia were evaluated more closely in relation to the degree and duration of CBF reduction, and COX-2 reaction products (PGE_2 and prostacyclin), which were not analyzed in our previous study, were also examined. The time course of COX-2 expression in the ischemic core, characterized by a CBF of $<20\%$ of baseline values, was different from that seen in the peri-infarct area, where 70–80% of control CBF was observed following 24 h of ischemia. The upregulation

Table 1
Prostaglandin production (pg/mg total protein) in the right (ischemic) hemisphere

Prostaglandin	Duration of ischemia (h)	Peri-infarct area	Ischemic core
PGE_2	0	60.8 ± 16.6	21.4 ± 11.4
	3	156.6 ± 70.1	54.4 ± 22.3
	24	2609.0 ± 2522.0*†	414.6 ± 226.3*†
Prostacyclin metabolite (6-keto-PG $F_{1\alpha}$)	0	122.3 ± 47.6	47.6 ± 23.0
	3	200.8 ± 59.7	93.4 ± 43.5
	24	1143.0 ± 623.7*†	341.6 ± 84.5*†

*$P < 0.05$ vs. 0 h (control); †$P < 0.05$ vs. 3 h ischemia by ANOVA. The values are the mean ± SD.

of COX-2 mRNA in the peri-infarct area persisted for at least 24 h after ischemia, as did the production of COX-2 protein, which led to significant increases in prostacyclin as well as PGE_2 levels following 24 h of ischemia. In the ischemic core, increases in COX-2 mRNA persisted during the 24 h of ischemia, though significant increases in COX-2 protein were not observed. This latter finding was considered to be due to the severe ischemic injury that was caused by reduced CBF, which likely affected protein synthesis [5]. This assertion is supported by the work of Xie et al. [18] who reported that a CBF of $<70\%$ of controls suppressed protein synthesis. In spite of these effects on COX-2 protein, significant increases were seen in the concentration of prostaglandins in the ischemic core 24 h after ischemia. Local increases in neuronal COX-2 expression in the ischemic core, as determined by immunohistochemical analysis, could have accounted for this increase in prostaglandin concentration. Increases in PGE_2 in ischemic cortices after 24 h of ischemia, particularly in peri-infarct areas, were probably due to the upregulation of membrane-associated PGE_2 synthase (mPGES) activity as well as the induction of COX-2, which were reported to be essential components for delayed PGE_2 biosynthesis [10].

The induction of neuronal COX-2 is important for the regulation of prostaglandin signaling in post-ischemic regions, and the magnitude of COX-2 activity and prostaglandin production is determined by the degree and duration of CBF reduction. Before novel therapeutic options for stroke patients can be developed, further clarification of the effects of COX-2 during and after ischemia will be required.

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Neuronal cyclooxygenase-2 induction associated with spreading depression and focal brain ischemia in primates

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Abstract. We investigated pathophysiology of a primate model eliciting spreading depression (SD), as well as a primate thromboembolic model, by using PET. Immediately after the first SD, focal cortical hyperemia was demonstrated without being followed by spreading or persistent hypoperfusion. Cyclooxygenase-2 (COX-2) induction was detected in SD animals by microarray analysis. Immunoreactive neurons were observed in SD animals. In the thromboembolic model, cerebral blood flow (CBF) following 24 h of ischemia reduced to 20–40% in the ischemic temporal cortex as well as ischemic basal ganglia, while the reduction was 40–60% in the ischemic parietal cortex. Upregulation of COX-2 mRNA expression was observed after 2 h of ischemia, but disappeared by 24 h in the ischemic temporal cortex. In the ischemic parietal cortex, where CMRglc was preserved, COX-2 expression persisted even after 24 h of ischemia. In conclusion, we showed unique features of CBF changes associated with SD in primates. Neuronal COX-2 induction was demonstrated in SD animals as well as within potentially viable hypoperfused brain areas in primates.

Keywords: Primate; Spreading depression; Focal brain ischemia; Cyclooxygenase 2; PET

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1. Introduction

Cortical spreading depression (SD) [1] has been suggested to play a significant role in the development of ischemic injury under conditions of focal brain ischemia in rat models [2,3]. As proposed by the Stroke Therapy Academic Industry Roundtable [4], nonhuman primate studies are required to clarify the pathophysiology of ischemic stroke and to verify the safety and efficacy of newly developed drugs that show promising results in rodents. In order to investigate the pathophysiology of acute ischemic stroke, we have developed a primate model eliciting SD, as well as a primate thromboembolic stroke model.

All procedures in this study were approved by our Institutional Animal Research Committee and were performed in accordance with the standards published by the National Research Council (Guide for the Care and Use of Laboratory Animals).

2. Material and methods

2.1. Spreading depression in a primate model

We used nine adult male cynomolgus monkeys. Animals were anesthetized with pentobarbital (0.1 mg/kg, i.p.). Anesthesia was maintained with an N_2O/O_2 (70%:30%) gas mixture inhalation under artificial ventilation through an experimental period. They were divided into two groups, such as normal control (group C, $n=3$) and SD-evoked animals (group SD, $n=6$).

SD was elicited by applying 3.3 mol/l potassium chloride (KCl) through a burr hole made in the left parietal skull [5]. Two other burr holes were made rostral to the hole for KCl application. DC potentials were monitored with microelectrodes inserted into the cortex to a depth of 1 mm through the burr holes, except the hole for KCl application.

Cerebral blood flow (CBF) was measured with PET and the ^{15}O-labeled water bolus injection method. A baseline CBF measurement was done once prior to application of KCl solution. CBF measurements were repeated five times, beginning 3 min after the first SD at intervals of approximately 15 min. After completion of the PET studies (at 120 min after KCl application), the brain tissues in group SD were quickly removed after exsanguination following perfusion with cold saline. Samples of brain tissues in group C were also obtained in the same manner as those in group SD. We investigated the gene expression profile associated with SD by a cDNA array system containing 9182 human elements, which was confirmed by RNA blot, immunoblot, and immunohistochemical analyses [6].

2.2. Thromboembolic stroke model in primates

Thromboembolic stroke was produced in male cynomolgus monkeys ($n=4$) as described previously [7]. CBF was measured with ^{15}O-labeled water before and 1, 2, 4, 6, and 24 h after embolization. CMRglc was measured with [^{18}F] FDG methods 24 h after embolization [8]. Lesion size and location was determined 24 h after embolization by the 2,3,5-triphenyl-tetrazolium chloride (TTC)-staining method.

For biochemical analyses of brain tissues in the thromboembolic stroke model, we used nine adult male cynomolgus monkeys; three monkeys served as normal control and the remaining six as ischemic animals [9]. Two hours after a single autologous blood clot

injection in three monkeys, or after the completion of the PET studies in the other monkeys with 24-h ischemia, brain tissues were perfused with cold saline, and the animals were sacrificed. Three normal controls were also sacrificed in the same manner. Expression ratios of cyclooxygenase-2 (COX-2) mRNA were calculated as ratios of COX-2 mRNA against those of normal brains. Cell injury was evaluated by incorporation of digoxigenin deoxy-uridine-5′ -triphosphate (dUTP) with the use of DNA polymerase I [10].

3. Results

3.1. SD in primates

SD waves were recorded in eight of the nine monkeys. Single episodes in three monkeys, twice in two, and six episodes in one were recorded in the rostral sites. In two of three animals with the caudal hole, one had eight episodes, and another had one in the caudal sites for chemical stimulation, while there were no SD waves in the rostral sites. The remaining one had two episodes in the rostral and six episodes in the caudal sites. Focal hyperemia was demonstrated adjacent to the site of the KCl application immediately after the first SD. Average cortical CBF in the ipsilateral hemisphere increased significantly immediately after the chemical stimulation ($p<0.05$ by paired t-test), and the significant increase in CBF persisted throughout the experimental period of 2 h. In the contralateral hemisphere, no significant changes in CBF were observed.

As a result of microarray analysis, increases in normalized signals of gene expression above 1.5-fold was seen in the cyclooxygenase-2 (COX-2) gene (1.6-fold), and 265 genes were different by at least 1.3-fold between the two groups. COX-2 induction was

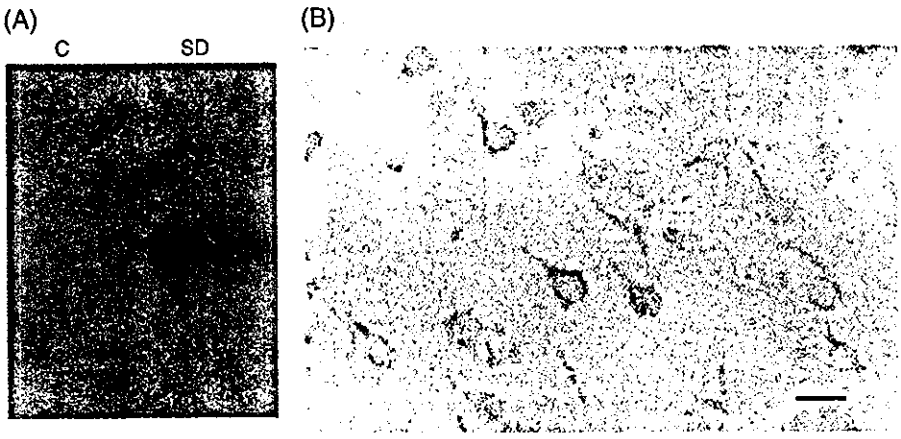

Fig. 1. Induction of COX-2 protein. (A) Immunoblot analysis shows a 70–72-kDa COX-2-immunoreactive band that is barely detectable in the group C (control), but clearly seen in the group SD. (B) Representative image of intense, immunoreactive neurons are shown in the animals with 6 SD episodes in the site rostral to the site of chemical stimulation. In these immunoreactive neurons, cell bodies and apical dendrites showed intense immunoreactivity. Scale bars: 100 μm.

confirmed by RNA blot, immunoblot (Fig. 1), and immunohistochemical analyses (Fig. 1). Intense immunoreactive neurons were induced in the animals with SDs.

3.2. Focal brain ischemia in primates

CBF in the temporal cortex and the basal ganglia decreased to <40% of the contralateral values 1 h after embolization, following further decline in CBF as well as CMRglc at 24 h of ischemia. These regions were consistently unstained with TTC, indicating that both temporal cortex and basal ganglia ipsilateral to the arterial embolization were regarded as the ischemic core. CBF was >40% of the contralateral values 1 h after embolization and recovered gradually with time in the parietal cortex ipsilateral to the embolization. No obvious TTC-unstained lesions were demonstrated in these regions, which implicated that the parietal cortex ipsilateral to the embolization was regarded as the ischemic penumbra. An increase in CMRglc at 24 h of ischemia compared with those in the contralateral regions, an uncoupling of CBF and CMRglc, was demonstrated in these regions.

The upregulation of COX-2 mRNA expression was observed at 2 h (expression ratio, 7.4), but disappeared by 24 h in the ischemic temporal cortex (Fig. 2), where cell injury was apparent by incorporation of dUTP. In the ischemic parietal cortex, where flow-metabolism uncoupling was observed, COX-2 mRNA was persistently induced even at

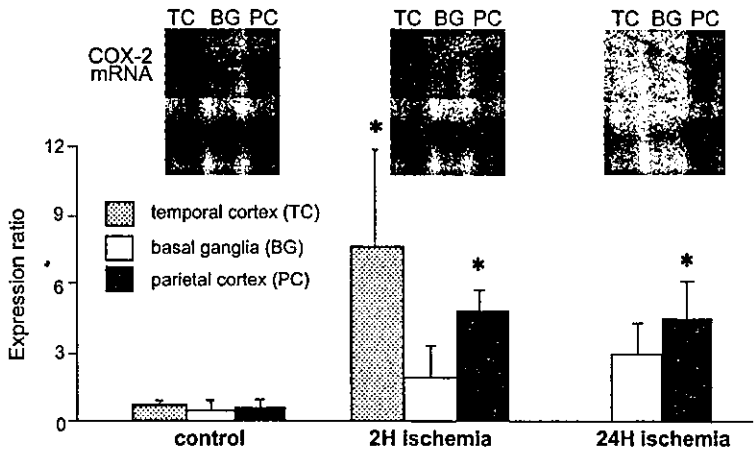

Fig. 2. RNA blot analysis of COX-2 expression. Autoradiograms of COX-2 (top) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (bottom) from the following samples: normal control, 2-h ischemia on the ipsilateral side, 24-h ischemia on the ipsilateral side relative to the arterial embolization. Note that induced COX-2 expression is prominent in ischemic temporal and parietal cortices in 2-h ischemia. Upregulated expression of COX-2 mRNA was also shown in the ischemic parietal cortex in 24-h ischemia, while detection of COX-2 mRNA was faint in control. The diagram shows the expression ratio of COX-2 in each region. The expression ratio of the ischemic temporal cortex in 2-h ischemia was significantly higher than that in control ($*p<0.05$). Neither COX-2 nor GAPDH mRNA levels were determined in the ischemic temporal cortex in 24-h ischemia because of a reduction in both COX-2 and GAPDH mRNA levels. The expression ratio of the ischemic parietal cortex in 24-h ischemia was the same as that in 2-h ischemia, and both were significantly higher than in the normal control ($*p<0.05$).

24 h after ischemia (expression ratio, 4.7), and few damaged cells could be detected by incorporation of dUTP as well as in each region from the hemisphere contralateral to the clot injection. Intense COX-2 immunoreactivity was found in discrete neurons in the ischemic parietal cortex, although no significant increases in COX-2 protein level were shown either in the ischemic temporal or parietal cortices.

4. Discussion

The CBF pattern obtained in the SD model of primates differed from those obtained in other studies using rat and cat SD models [11,12]. The focal hyperemia was not followed by prolonged hypoperfusion. The changes in CBF during the SD phenomenon in primates also differed from those in patients with migraine [13,14]. These unique features of SD in this primate demand reappraisal of the hypothesis that SD contributes to the pathogenesis of human brain diseases. In biochemical analyses for brain tissues, COX-2 was induced in the cortices where SD was recorded, in accordance with previous observations in rodent models [15,16]. COX-2 was reported to play a role in mediating the increase in CBF produced by synaptic activity in the somatosensory cortex of mice [17].

We observed COX-2 expression during focal brain ischemia in a primate thromboembolic stroke model. In the ischemic core, in which a significant decrease in CBF was accompanied by reduced CMRglc, we observed upregulated COX-2 mRNA at 2-h ischemia with a decrease by 24 h. Disappearance of COX-2 at 24-h ischemia was parallel to a housekeeping GAPDH-mRNA reduction, indicating that ischemic injury was already apparent at 24-h ischemia in the temporal cortex and the basal ganglia. In the peri-infarct area, on the contrary, induced expression of COX-2 mRNA was still found at 24-h ischemia in the parietal cortex with a mild CBF reduction and maintained CMRglc. The results suggested that COX-2 expression might be regulated by the depth and duration of CBF reduction that is highly associated with local metabolic conditions.

The induction of neuronal COX-2 in brain tissues where SDs were elicited as well as in the ischemic cortices may participate in activity-dependent neural plasticity [18], because the metabolites of the arachidonic acid cascade are considered to play an important role in neuronal signaling [19,20]. Further studies describing the time course and topography of COX-2 expression, effects of a selective COX-2 antagonist given at various time-points after ischemia on ischemic brain damage, and prostanoids production downstream from COX-2 are required to clarify the role of COX-2 expression in ischemic brain injury.

Acknowledgements

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Neuronal cyclooxygenase-2 expression during spreading depression and focal brain ischemia

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Go Kito[7], Nagara Tamaki[8] and Kazuo Minematsu[3]

Abstract

In order to clarify the pathophysiology of ischemic stroke, we examined a primate model eliciting SD, a primate thromboembolic model, and a rat model of focal brain ischemia. Immediately after the first SD, focal cortical hyperemia was demonstrated without being followed by spreading or persistent hypoperfusion. Cyclooxygenase-2 (COX-2) induction was detected in SD monkeys by microarray analysis. Immunoreactive neurons were observed in SD animals. In the thromboembolic model, upregulation of COX-2 mRNA expression was observed after 2 h of ischemia, but disappeared by 24 h in the ischemic core. In peri-infarct areas, where flow-metabolism uncoupling was observed, COX-2 expression persisted even after 24 h of ischemia. In focal ischemic rats, diffuse, neuronal COX-2 staining was found in peri-infarct areas as well as in discrete, immunoreactive neurons in the ischemic core. Robust increases in prostaglandin E_2 levels in the peri-infarct areas were demonstrated following 24 h of ischemia. In conclusion, neuronal COX-2 induction was observed in SD animals as well as within potentially viable hypoperfused brain areas. COX-2 expression and prostaglandin production in ischemic tissues depended on the degree and duration of the reduction in cerebral blood flow.

Key words: spreading depression, focal brain ischemia, cyclooxygenase 2, cerebral blood flow

1. Introduction

Cortical spreading depression (SD)[1] has been suggested to play a significant role in the development of ischemic injury under conditions of focal brain ischemia in rat models[2,3]. As proposed by the Stroke Therapy Academic Industry Roundtable[4], nonhuman primate studies are required to clarify the pathophysiology of ischemic stroke, and to verify the safety and efficacy of newly developed drugs that show promising results in rodents. In order to investigate the pathophysiology of acute ischemic stroke, we have developed a primate model eliciting SD and a primate thromboembolic stroke model.

Cyclooxygenase-2 (COX-2), a rate-limiting en-

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zyme in prostaglandin synthesis, was induced associated with either eliciting SD or focal brain ischemia in the cortex ipsilateral to the SD elicitation or brain ischemia in the nonprimate cortex[5]. Therefore we examined COX-2 expression and its reaction products during SD and focal brain ischemia in primates as well as rats.

All procedures in this study were approved by our Institutional Animal Research Committee and were performed in accordance with the standards published by the National Research Council (Guide for the Care and Use of Laboratory Animals).

2. Material and methods

2. 1. Spreading depression in a primate model

We used nine adult, male cynomolgus monkeys. Animals were anesthetized with pentobarbital (0.1 mg/kg, i.p.). Anesthesia was maintained with a N_2O/O_2 (70% : 30%) gas mixture inhalation under artificial ventilation through an experimental period. They were divided into 2 groups, such as normal control (group C, n = 3) and SD evoked animals (group SD, n = 6).

SD was elicited by applying 3.3 mol/L potassium chloride (KCl) through a burr hole made in the left parietal skull[6]. Two other burr holes were made rostral to the hole for KCl application. DC potentials were monitored with microelectrodes inserted into the cortex to a depth of 1 mm through the burr holes except the hole for KCl application.

Cerebral blood flow (CBF) was measured with PET and the ^{15}O-labeled water bolus injection method. A baseline CBF measurement was done once prior to application of KCl solution. CBF measurements were repeated 5 times, beginning 3 minutes after the first SD at intervals of approximately 15 minutes. After completion of the PET studies (at 120 min after KCl application), the brain tissues in the group SD were quickly removed after exsanguination following perfusion with cold saline. Samples of brain tissues in the group C were also obtained as the same manner as those in the group SD. We investigated the gene expression profile associated with SD by a cDNA array system containing 9,182 human elements, which was confirmed by RNA blot, immunoblot, and immunohistochemical analyses[7].

2. 2. Thromboembolic stroke model in primates

Thromboembolic stroke was produced in male cynomolgus monkeys (n = 4) as described previously[8]. CBF was measured with ^{15}O-labeled water before and 1, 2, 4, 6, and 24 hours after embolization. Cerebral glucose metabolic rate (CMRglc) was measured with [^{18}F] FDG methods 24 hours after embolization[9]. Lesion size and location 24 hours after embolization was determined by the 2, 3, 5-triphenyl-tetrazolium chloride (TTC) staining method.

For biochemical analyses for brain tissues in thromboembolic stroke model, we used 9 adult male cynomolgus monkeys ; 3 monkeys were served as normal control and the remaining 6 were as ischemic animals[10]. Two hours after a single autologous blood clot injection in 3 monkeys or after the completion of the PET studies in the other monkeys with 24 h-ischemia, brain tissues were perfused with cold saline and the animals were sacrificed. Three normal controls were also sacrificed as the same manner. Expression ratios of COX-2 mRNA were calculated as ratios of COX-2 mRNA against those of normal brains. Cell injury was evaluated by incorporation of digoxigenin deoxy-uridine-5'-triphosphate (dUTP) with the use of DNA polymerase I.

2. 3. Focal brain ischemia in rats

Male Sprague-Dawley rats (300-350 g, n = 40) were used. Focal brain ischemia was produced by the intraluminad occlusion of the ostium of the right middle cerebral artery with nylon monofila-

ments, as previously described[11]. Rats were sacrificed at time 0 and at different times points after arterial occlusion (1, 2, 3, 4, 6, 8, 12, and 24 h, n = 4-5/time point) and their brains immediately immersed in ice-cold saline. Several blocks were frozen in isopentane-dry ice and stored at −80℃ until use, whereas others were embedded in paraffin for immunohistochemistry. Analysis of COX-2 expression (mRNA, protein), and measurement of the concentrations of PGE_2 and the prostacyclin metabolite, 6-keto-$PGF_{1\alpha}$ in the peri-infarct areas and the ischemic core were performed. In some animals, N-isoproryl-*p*-[^{125}I]-iodoamphetamine ([^{125}I] IMP) (2.22 MBq/kg body weight) was injected into the femoral vein 5 min before sacrifice and *ex-vivo* autoradiography was performed to measure cerebral blood flow (CBF) as described previously[12].

3. Results

3. 1. SD in primates

SD waves were recorded in eight of the 9 monkeys. Single episode in three monkeys, twice in two, and six episodes in one were recorded in the rostral sites. In two of three animals with the caudal hole, one had eight episodes and another did once in the caudal sites for chemical stimulation while they did no SD waves in the rostral sites. The remaining one had twice episodes in the rostral and six episodes in the caudal sites. Focal hyperemia was demonstrated adjacent to the site of KCl application immediately after the first SD. Average cortical CBF in the ipsilateral hemisphere increased significantly immediately after the chemical stimulation ($p < 0.05$ by paired t-test), and the significant increase in CBF persisted throughout the experimental period of 2 hours. In the contralateral hemisphere, no significant changes in CBF were observed.

As a result of microarray analysis, increases in normalized signals of gene expression above 1.5-fold was cyclooxygenase-2 (COX-2) gene (1.6-fold), and signal levels in 265 genes were different by at least 1.3-fold between the 2 groups. COX-2 induction was confirmed by RNA blot, immunoblot, and immunohistochemical analyses. Intense immunoreactive neurons were induced in the animals with SDs.

3. 2. Focal brain ischemia in primates

CBF in the temporal cortex and the basal ganglia decreased to < 40% of the contralateral values 1 hour after embolization, following further decline in CBF as well as CMRglc at 24 hour of ischemia. These regions were consistently unstained with TTC, being indicated that both temporal cortex and basal ganglia ipsilateral to the arterial embolization were regarded as the ischemic core. While CBF was > 40% of the contralateral values 1 hour after the embolization and recovered gradually with time in the parietal cortex ipsilateral to the embolization. No obvious TTC-unstained lesions were demonstrated in these regions, implicated that the parietal cortex ipsilateral to the embolization was regarded as the ischemic penumbra. Increased in CMRglc at 24 hours of ischemia compared with those in the contralateral regions, an uncoupling of CBF and CMRglc, were demonstrated in these regions.

The upregulation of COX-2 mRNA expression was observed at 2 h (expression ratio was 7.4), but disappeared by 24 h in the ischemic temporal cortex, where cell injury was apparent by incorporation of dUTP. In the ischemic parietal cortex, where flow-metabolism uncoupling was observed, COX-2 mRNA was persistently induced even at 24 h after ischemia (expression ratio was 4.7), and few damaged cells could be detected by incorporation of dUTP as well as in each region from the hemisphere contralateral to the clot injection. Intense COX-2 immunoreactivity was found in discrete neurons in the ischemic parietal

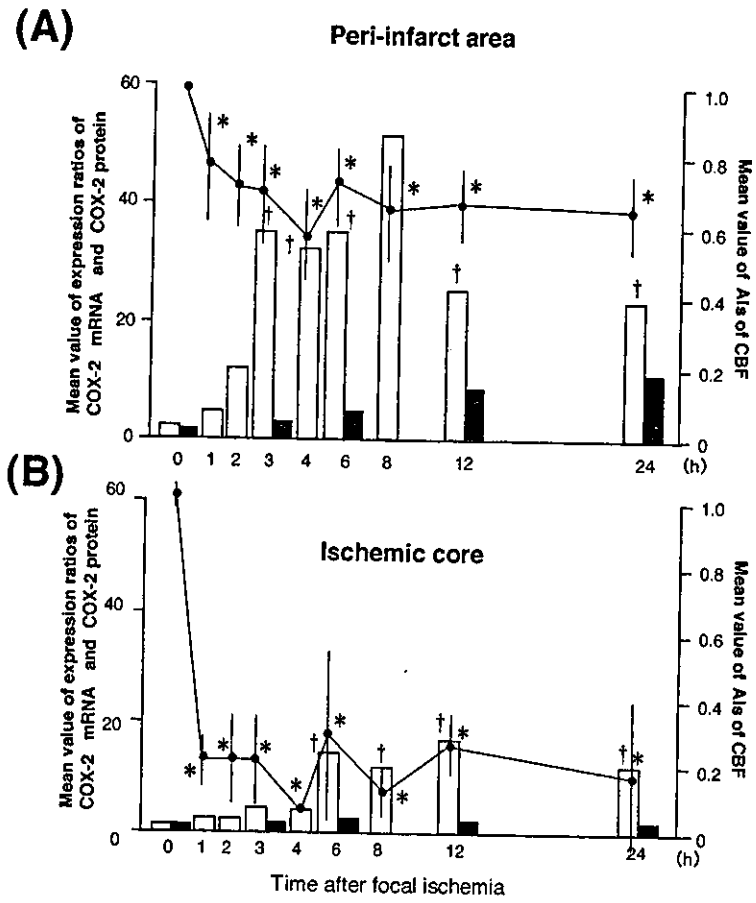

Fig. 1. Temporal profile of COX-2 expression associated with changes in CBF during 24 h of ischemia

Lines indicate the mean asymmetry index (AI) values of CBF. The open and solid columns correspond to the mean expression ratios of COX-2 mRNA and COX-2 protein, respectively. Figures A and B show the time course of COX-2 expression in the peri-infarct areas and ischemic core, respectively. A one-way ANOVA and post-hoc Fisher's tests were used to assess the differences in AIs and expression ratios of COX-2 mRNA between the different ischemic time points. CBF in the peri-infarct areas and ischemic core were significantly reduced compared to controls immediately after arterial occlusion (*: $p<0.05$). The mean CBFs in the ischemic core and peri-infarct areas were 0.19 ± 0.07 (mean ± SD) and 0.67 ± 0.06, respectively. The time course of COX-2 expression in the peri-infarct areas was different from that in the ischemic core. Thus, the expression ratios of COX-2 mRNA increased significantly after 3 h of ischemia (†: $p<0.05$), with COX-2 protein increasing with time in the peri-infarct areas. On the other hand, significant increases in COX-2 mRNA were found 6 h after ischemia (†: $p<0.05$), and increases in COX-2 protein were not observed during the ischemic period in the ischemic core.

cortex, although no significant increases in COX-2 protein level were shown either in the ischemic temporal or parietal cortices.

3. 3 Focal brain ischemia in rats

Table 1. Prostaglandin production (pg/mg total protein) in the ischemic hemisphere

Prostaglandin	Duration of ischemia	Peri-infarct area	Ischemic core
PG E_2	0 hours	60.8 ± 16.6	21.4 ± 11.4
	3 hours	156.6 ± 70.1	54.4 ± 22.3
	24 hours	2,609.0 ± 2,522.0 *†	414.6 ± 226.3 *†
Prostacyclin	0 hours	122.3 ± 47.6	47.6 ± 23.0
metabolite	3 hours	200.8 ± 59.7	93.4 ± 43.5
(6-keto-PG $F_{1\alpha}$)	24 hours	1,143.0 ± 623.7 *†	341.6 ± 84.5 *†

* $p < 0.05$ vs. 0 h (control) ; † : $p < 0.05$ vs. 3 h ischemia by ANOVA
The values were the mean ± SD.

Significant reductions in CBF in the peri-infarct areas and ischemic core were demonstrated in animals at each ischemic time point compared to the controls (Fig. 1). The expression ratios of COX-2 mRNA increased significantly between 3 and 24 h of ischemia in the peri-infarct areas compared to the controls. In the ischemic core, significant increases in COX-2 mRNA were seen following 6 h of ischemia, which remained through 24 h. The peak value of the expression ratio of COX-2 protein in the peri-infarct area was 10.7 at 24 h of ischemia, while the peak expression ratio in the ischemic core was 2.0 at 6 h of ischemia. COX-2 immunoreactive neurons were found predominantly in the peri-infarct area. Elevations in the immunohistochemical staining of discrete neuronal populations were also observed in the ischemic core. Although no significant increases in PGE_2 and prostacyclin levels were observed in the peri-infarct and ischemic core areas following 3 h of ischemia, significant increases in prostaglandin levels were found in the ischemic hemisphere following 24 h of ischemia. In particular, PGE_2 levels in the peri-infarct area increased significantly (Table 1).

4. Discussion

The CBF pattern obtained in the SD model of primates differed from those obtained in other studies using rat- and cat-SD models[13,14]. The focal hyperemia was not followed by prolonged hypoperfusion. The changes in CBF during SD phenomenon in primates also differed from those in patients with migraine[15]. In biochemical analysis for brain tissues, COX-2 was induced in the cortices where SD was recorded, being in accord with prvious observations in rodent models[5].

We observed COX-2 expression during focal brain ischemia in a primate thromboembolic stroke model. In the ischemic core, in which a signficnat decrease in CBF were accompanied by reduced CMRglc, upregurated COX-2 mRNA at 2 h-ischemia but decreased by 24 h. Disappearance of COX-2 at 24 h-ischemia was parallel to a housekeeping GAPDH-mRNA reduction, indicating that ischemic injury was already apparent at 24-ischemia in the temporal cortex and the basal ganglia. In the peri-infarct area, on the contrary, induced expression of COX-2 mRNA was still found at 24-h ischemia in the parietal cortex with a mild CBF reduction and maintained CMRglc.

In the focal ischemia in rats, the time course of COX-2 expression in the ischemic core was different from that seen in the peri-infarct area. The upregulation of COX-2 mRNA in the peri-infarct area persisted for at least 24 h after ischemia, as did the production of COX-2 protein, which lead

to significant increases in prostacyclin as well as PGE_2 levels following 24 hours of ischemia, though significant increases in COX-2 mRNA persisted during the 24 h of ischemia, though significant increases in COX-2 protein were not observed. This latter finding may be attributable to the severe ischemic injury that was caused by reduced CBF, which likely affected protein synthesis[2]. In spite of these effects on COX-2 protein, significant increases were seen in the concentration of prostaglandins in the ischemic core 24 hours after ischemia. Local increases in neuronal COX-2 expression in the ischemic core, as determined by immunohistochemical analysis, could have accounted for this increase in prostaglandin concentration.

The induction of neuronal COX-2 is important for the regulation of prostaglandin signaling in post-ischemic regions, and the magnitude of COX-2 activity and prostaglandin production is determined by the degree and duration of CBF reduction. Before novel therapeutic options for stroke patients can be developed, further clarification of the effects of COX-2 during and after ischemia will be required.

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急性期局所脳虚血病態時の脳循環代謝に対するプロスタグランジン制御

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我々は，急性期病態モデルとして，局所脳虚血モデルと spreading depression (SD) モデルを用いて，脳循環代謝変化と cyclooxygenase-2 (COX-2) 発現につき調べた．サル SD モデルにおいて，SD に伴う脳血流量の変化は一過性の脳血流上昇であり，持続的な血流低下を伴わなかった．SD 誘発サルの脳神経細胞には，COX-2 蛋白が発現していた．サル塞栓性脳梗塞モデルにおいて，虚血 24 時間までは主に脳神経細胞に COX-2 が発現し，特に脳糖代謝率が保たれている梗塞周囲領域での発現が増強していた．局所脳虚血ラット実験より，虚血周辺部と中心部では，COX-2 (mRNA，蛋白) 発現の時間経過と発現量，およびプロスタグランジン生成量は異なっていた．COX-2 蛋白は，神経細胞に発現していた．本研究により，急性期脳虚血病態と脳組織での COX-2 誘導およびプロスタグランジン産生は深く関連していることが明らかとなった．

キーワード：局所脳虚血，cyclooxygenase-2，サル，spreading depression

トピックス

赤ワインに含まれるポリフェノール・レスベラトロールに関する最近の話題

レスベラトロールは赤ワインに含まれる抗酸化作用を持つフィトアレキシン(抗菌性物質)である．レスベラトロールは中等度のワイン消費が心血管病，脳卒中，痴呆の危険度と負の相関を示す，いわゆる「フレンチパラドックス」に関与する物質と考えられてきた．我々は最近，レスベラトロールが核内受容体PPAR(peroxisome proliferators activated receptor)αとPPARγを選択的に活性化すること，さらにPPARα活性化が脳保護効果をもたらすことを見いだした[1]．これらの知見は「フレンチパラドックス」を説明する新しい作用機構を提供すると考えている．一方で，レスベラトロールは寿命延長効果を持つカロリー制限模倣物質であること[2]，オレイルエタノールアミドがPPARαの新しい内因性リガンドであり，その活性化によって食欲をコントロールすること[3]が報告されている．そこでこれらの知見を含めて，今後の展望とともに紹介したい．

PPARはビタミンD受容体やグルココルチコイド受容体と同様，核内受容体ファミリーに属し，現在3種類のサブタイプα，γ，δ(β)が知られている．PPARはレチノイドX受容体(RXR)とヘテロ二量体を形成し，PPAR応答エレメントを介して，様々な遺伝子の転写調節に関与している．肝臓で主に発現しているPPARαは種々の脂肪酸により活性化されるので，血中遊離脂肪酸のセンサーとして働くという考え方がある．また，PPARαアゴニストには抗高脂血症治療薬fibratesが知られている．一方，PPARγは脂肪細胞やマクロファージで主に発現し，PPARγアゴニストとしてインスリン抵抗性改善薬rosiglitazoneなどのチアゾリジン誘導体，プロスタグランジン(PG)D_2の代謝産物15-deoxy-$\Delta^{12,14}$ PGJ_2などが知られている．PPARδは広く種々の細胞で発現し，その内因性リガンドとしてはプロスタサイクリンが候補となっているが，PPARはいずれも脂肪酸をリガンドとしている．最近，PPARδは脂肪燃焼の制御因子として，PPARγは脂肪貯蔵の制御因子として働くことが提唱されている．以上，PPARの3種類のサブタイプはそれぞれ異なった場所で異なった機能を発揮すると考えられるが，いずれも生活習慣病の標的分子として世界的に注目を集めている分子群である[4]．

我々はアスピリンなど非ステロイド性抗炎症薬の標的であるPG産生の律速酵素・誘導型シクロオキシゲナーゼ(COX-2)に関する研究を進めている．そしてグルココルチコイド受容体やPPARγなどの核内受容体がCOX-2の細胞特異的発現調節に関与することを報告した[5][6]．一方，表1に示すように発がんとPG，レスベラトロールの関わりについてはいくつかの報告がある．共同研究の結果，レスベラトロールががん細胞において，COX-2の活性および発現を抑制することを見いだした[10]．そこで種々の細胞でレスベラトロールの効果を検討した結果，COX-2の発現について細胞の種類によって異なっており，レスベラトロールがある種の核内受容体リガンドとして作用しているのではないかいう新しい

表1．発がんとプロスタグランジン，レスベラトロールの関わり．

1. 疫学的調査の結果，リウマチ患者などでアスピリンを長期間常用している患者は大腸がんによる死亡率が40～50％低い[7]．
2. 種々の化学発がん物質を用いたマウスでの発がん実験において，アスピリンなど非ステロイド性抗炎症薬(NSAIDs)は有意にがんの発生を抑制する．
3. ヒト家族性大腸腺腫症のモデルとなるマウスでの実験の結果，COX-2の活性を抑制すると，腺腫の大きさと数が有意に減少する[8]．
4. 赤ワインに含まれるレスベラトロールはマウス皮膚がんモデルで発がん抑制効果を示す[9]．