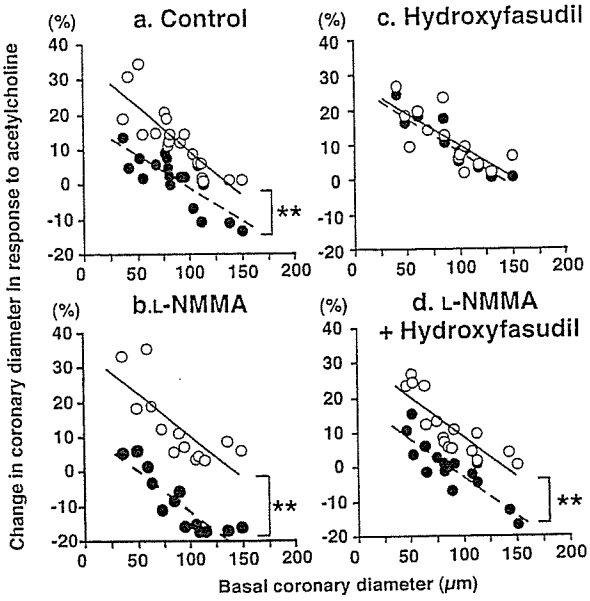

Figure 5. Coronary microvascular responses to acetylcholine before and after coronary ischemia/reperfusion (I/R) injury in dogs in vivo. Under control conditions, I/R significantly impaired coronary vasodilator response to acetylcholine (**a**), whereas hydroxyfasudil completely preserved the responses in the absence (**c**) or presence of L-NMMA (**d**) compared with that in the presence of L-NMMA alone (**b**). Number of vessels per animals used was 19/7 under control conditions (before I/R: $y = -0.3x + 35.9$, $r = 0.85$; after I/R: $y = -0.2x + 18.1$, $r = 0.80$), 13/4 for L-NMMA alone (before I/R: $y = -0.2x + 35.1$, $r = 0.76$; after I/R: $y = -0.2x + 12.2$, $r = 0.88$), 14/7 for hydroxyfasudil (before I/R: $y = -0.2x + 27.9$, $r = 0.73$; after I/R: $y = -0.2x + 27.4$, $r = 0.80$), and 16/7 for hydroxyfasudil plus L-NMMA (before I/R: $y = -0.2x + 31.8$, $r = 0.83$; after I/R: $y = -0.2x + 19.2$, $r = 0.86$). **$p < 0.01$. **Open circles** = before I/R; **solid circles** = after I/R.

variables at baseline did not significantly change after I/R compared with those before I/R (Table 1).

Effects of Rho-kinase inhibition on serotonin-induced coronary responses. Intracoronary administration of serotonin caused coronary vasoconstriction of small arteries and vasodilation of arterioles under control conditions (Figs. 3a and 3b, both $p < 0.01$ vs. basal coronary diameter). Intracoronary administration of L-NMMA enhanced the serotonin-induced vasoconstriction and abolished the serotonin-induced vasodilation of arterioles (Fig. 3b, $p < 0.01$ vs. serotonin, S). By contrast, hydroxyfasudil reversed the serotonin-induced vasoconstriction of small arteries to vasodilation while it further enhanced the serotonin-induced vasodilation of arterioles (Figs. 3a and 3b, both $p < 0.01$). The vasodilator effect of hydroxyfasudil on the coronary response to serotonin was significantly attenuated by L-NMMA in both-sized arteries (Figs. 3a and 3b, both $p < 0.01$). As a result, serotonin-induced increase in CBF (Fig. 3c) was significantly inhibited by L-NMMA ($p < 0.05$) and enhanced by hydroxyfasudil ($p < 0.05$), the effect of which was significantly attenuated by L-NMMA ($p < 0.01$).

Endothelium-dependent coronary vasodilation before and after I/R. Under control conditions (before I/R), intracoronary administration of acetylcholine caused a significant coronary vasodilation to a greater extent in arterioles than in small arteries (Figs. 4a, 4b, and 5a, $p < 0.01$). Coronary I/R significantly impaired the coronary vasodilation to acetylcholine in both sized arteries (both $p < 0.01$) and L-NMMA further reduced the vasodilation (Figs. 4a, 4b, and 5b both $p < 0.01$), whereas hydroxyfasudil completely preserved (small artery $p < 0.05$, arteriole $p < 0.01$) the acetylcholine-induced coronary vasodilator response after I/R (Figs. 4a and 4b). The vasoconstriction by L-NMMA was significantly attenuated by hydroxyfasudil in both sized arteries (both $p < 0.05$) with decrement of CBF (Figs. 4a to 4c). When the coronary vasodilator response to acetylcholine was expressed as a function of basal coronary diameter, hydroxyfasudil preserved the response after I/R injury at all sized coronary arteries either in the absence

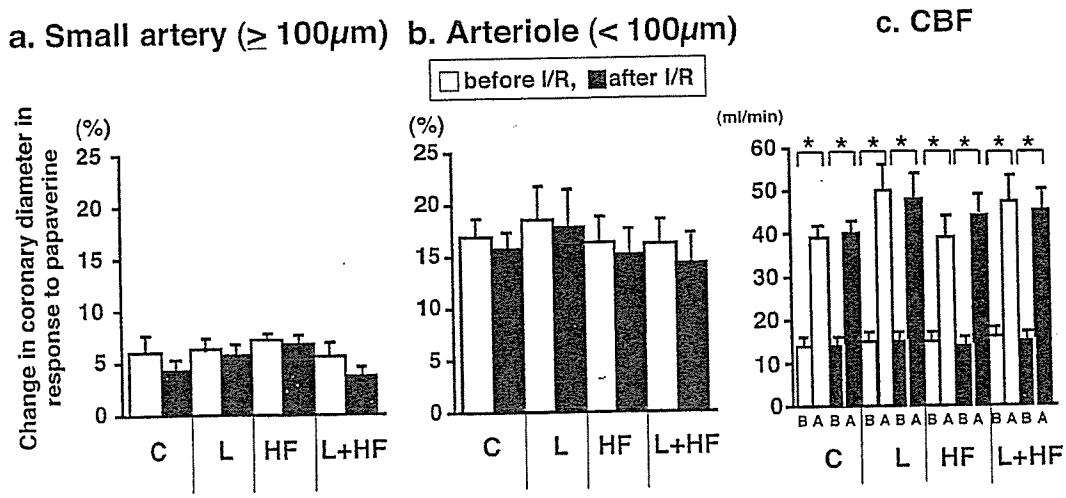

Figure 6. Endothelium-independent coronary vasodilation before and after coronary I/R injury in dogs in vivo. Coronary vasodilator response to papaverine was comparable under all conditions in both small arteries and arterioles. Number of vessels per animals used was 7/6 for control (mean diameter 120 ± 7 μm), 5/4 for L-NMMA (123 ± 8 μm), 6/4 for hydroxyfasudil (118 ± 8 μm), and 5/4 for hydroxyfasudil plus L-NMMA (125 ± 9 μm) in small arteries; and 12/6 for control (70 ± 6 μm), 8/4 for L-NMMA (69 ± 7 μm), 8/5 for hydroxyfasudil (68 ± 7 μm), and 11/6 for hydroxyfasudil plus L-NMMA (71 ± 5 μm) in arterioles. C = control; L = L-NMMA; HF = hydroxyfasudil. I/R = ischemia/reperfusion. B = before papaverine; A = after papaverine.

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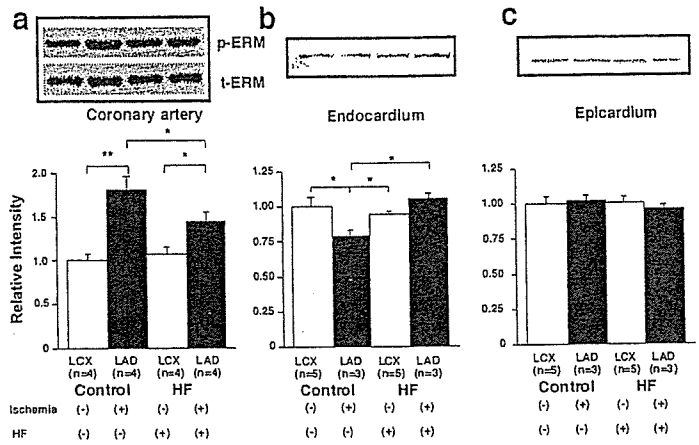

Figure 7. Western blotting showing the effects of hydroxyfasudil (HF) on Rho-kinase activity and on eNOS protein expression in the myocardium of LAD and LCX. (a) Rho-kinase activity in coronary artery; (b) expression of eNOS protein in endocardium; (c) expression of eNOS protein in epicardium. Rho-kinase activity was determined by the degree of ezrin-radixin-moesin phosphorylation (p-ERM/t-ERM). Rho-kinase activation in the ischemic LAD was completely inhibited by cotreatment with hydroxyfasudil. Expression of eNOS protein in the ischemic endocardium of LAD area was significantly decreased compared with the non-ischemic endocardium of LCX area, which was again improved by hydroxyfasudil. $^{*}p < 0.05$, $^{**}p < 0.01$.

(Figs. 4a, 4b, and 5c, df 2, 25, $p < 0.01$) or presence (Figs. 4a, 4b, and 5d, df 2, 24, $p < 0.01$) of L-NMMA compared with that in the presence of L-NMMA alone (Figs. 4a, 4b, and 5b).

Endothelium-independent coronary vasodilation. Coronary vasodilator response to papaverine was comparable under all conditions in both small arteries and arterioles (Figs. 6a and 6b). Similarly, the increase in CBF to papaverine (Fig. 6c) was also comparable under all conditions in both-sized arteries. Those coronary vasodilator responses were resistant to the blockade of NO synthesis with L-NMMA (Figs. 6a and 6b).

Activation of Rho-kinase by ischemia-reperfusion causes down-regulation of eNOS protein expression. Rho-kinase activity after a 90-min period of ischemia was significantly greater in the ischemic LAD than in the nonischemic LCX in the control group (Fig. 7a, $p < 0.01$). This Rho-kinase activation was significantly suppressed by hydroxyfasudil in the ischemic LAD (Fig. 7a, $p < 0.01$). Expression of eNOS protein in the ischemic endocardium of the LAD area (as determined by Western blotting) was significantly decreased (79 ± 4%, $p < 0.05$) compared with the nonischemic endocardium of the LCX area (100 ± 7%), which was also improved by hydroxyfasudil (105 ± 6%) (Fig. 7b, $p < 0.05$). There was no significant difference in the eNOS expression in the epicardium between the LAD and LCX area (Fig. 7c).

Effect of Rho-kinase inhibition on I/R-induced myocardial infarct size. Ischemia-reperfusion injury caused myocardial infarct area that was approximately 50% of the left ventricular risk area, and intracoronary L-NMMA did not further increase the I/R-induced infarction size (Fig. 8a). Intracoronary pretreatment with hydroxyfasudil markedly reduced the infarct size ($p < 0.01$ vs. control), and this beneficial effect of hydroxyfasudil was significantly attenuated by L-NMMA (Fig. 8a $p < 0.01$). In the control group, there was an inverse relation between the infarct area and collateral blood flow measured by microsphere technique ($r = 0.93$, $p < 0.01$), and hydroxyfasudil significantly shifted the regression line downward as compared with the control group ($p < 0.01$), that is, smaller infarct size for a given collateral flow (Fig. 8b).

DISCUSSION

The major findings of the present in vivo study in the canine coronary microcirculation were that: 1) a specific Rho-kinase inhibitor hydroxyfasudil preserved the endothelium-dependent coronary vasodilator responses after coronary I/R injury, 2) hydroxyfasudil also reduced myocardial infarct size, and 3) NO may be involved in those cardiovascular protective effects of hydroxyfasudil. To the best of our knowledge, this is the first report that demonstrates the usefulness of a Rho-kinase inhibitor to prevent coronary I/R injury in vivo.

Validations of experimental model and methodology. On the basis of the previous reports (4,12,20), we chose the adequate dose of hydroxyfasudil, acetylcholine, papaverine, and L-NMMA to examine the effects of the Rho-kinase inhibition, endothelium-dependent and -independent vasodilator responses, and inhibition of NO synthesis on coronary vascular responses before and after coronary I/R, respectively. The methodologic validity of the present study has been confirmed previously (15). After 60 to 90 min of ischemia, ultrastructural damage of coronary endothelium was observed particularly in the subendocardium in the present study, a consistent finding to the previous study (21).

Hydroxyfasudil as a specific Rho-kinase inhibitor in the coronary microcirculation in vivo. Shimokawa et al. (11) have recently demonstrated that hydroxyfasudil is a specific Rho-kinase inhibitor that markedly inhibits coronary vasospastic responses in a porcine model; its inhibitory effect on Rho-kinase is 100 times greater than on protein kinase C and

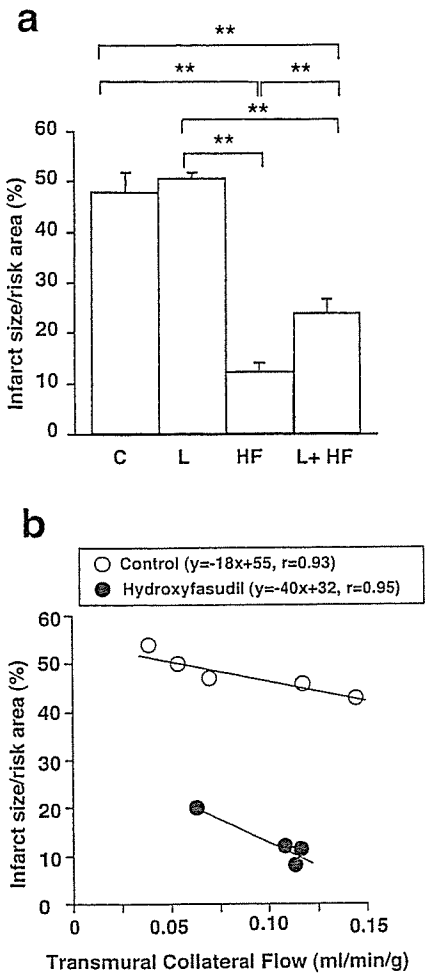

Figure 8. (a) Ischemia/reperfusion (I/R)-induced LV infarct size in dogs in vivo. Hydroxyfasudil significantly reduced the I/R-induced LV infarct size. The beneficial effect of hydroxyfasudil was partially attenuated by L-NMMA, while L-NMMA alone did not significantly increase the infarct size. Number of animals used was each 7 for C, HF, and L + HF, and 4 for L. C = control; L = L-NMMA; HF = hydroxyfasudil. $^{**}p < 0.01$. (b) Plot of infarct size expressed as a percentage of the risk area and regional collateral flow during ischemia. In the control group, there was an inverse relation between infarct area and collateral flow measured by microsphere ($r = 0.93$, $p < 0.01$), and hydroxyfasudil significantly shifted the regression line downward as compared with the control group ($p < 0.01$). Number of animals used was five for control conditions and four for hydroxyfasudil.

1,000 times greater on myosin light-chain kinase. Hydroxyfasudil has potent vasodilator effects on coronary arteries through inhibition of Rho-kinase-mediated phosphorylations of myosin light chains (11). In the present study, intracoronary hydroxyfasudil caused coronary microvascular vasodilation in a dose-dependent manner in vivo, and its vasodilator effect was greater in arterioles than in small arteries (Fig. 2). Hydroxyfasudil suppressed the serotonin-induced vasoconstriction of small arteries, whereas it enhanced the serotonin-induced vasodilation of arterioles in vivo (Fig. 3). This finding is in accordance with the hypothesis that the calcium sensitization of vascular smooth-muscle cells mediated by Rho-kinase plays a key role in the molecular mechanisms of coronary hyperconstriction (12). Furthermore, in the present study, intracoronary L-NMMA significantly attenuated serotonin-induced coronary vasodilator responses, which were improved by hydroxyfasudil, indicating an involvement of NO-mediated mechanism in the beneficial effects of the Rho-kinase inhibitor. Lamping et al. (22) demonstrated that coronary vascular response to serotonin is determined by a balance between 5-HT_1 receptor-mediated dilatation of coronary arterioles and 5-HT_2 receptor-mediated vasoconstriction of small coronary arteries. Inhibition of NO synthase enhances coronary vasoconstriction to serotonin in both-sized arteries. Our present results are in agreement with those of Lamping et al. The beneficial vasodilator effect of hydroxyfasudil on coronary vascular response to serotonin is mediated by its action on both vascular smooth muscle and the endothelium as shown in Figure 3. Thus, it is possible that the beneficial effect of Rho-kinase blockade with hydroxyfasudil is mediated by its action on both vascular smooth muscle and the endothelium (22). Serotonin released by aggregating platelets has been implicated for coronary vasospasm in the presence of damaged vascular endothelium (5,23).

Beneficial effects of a Rho-kinase inhibitor on coronary I/R injury. In the present study, hydroxyfasudil exerted beneficial effects on I/R-induced endothelial injury in the canine coronary microcirculation in vivo through the NO-dependent mechanism (Figs. 4 and 5). This dose of hydroxyfasudil (100 μg/kg) selectively inhibits Rho-kinase activity and effectively prevents serotonin-induced coronary hyperconstriction. Recent studies have demonstrated that cGMP-dependent protein kinase inhibits RhoA phosphorylation by inhibiting the membrane binding of RhoA, in which the NO-mediated mechanism may inhibit the RhoA/Rho-kinase pathway (24–26). It was previously demonstrated that statins attenuate I/R injury of the heart and the brain in rats and mice, demonstrating the Rho-mediated and NO-dependent protective effect of statins (27,28). Hydroxyfasudil also inhibits the production of superoxide anions in neutrophils (29) and various chemoattractant-induced migration of those cells (14) in a canine model of cerebral ischemia. Furthermore, treatment with hydroxyfasudil in human saphenous vein endothelial cells reversed the hypoxia-induced decrease in eNOS activity as examined by the citrulline conversion assay and 4,5-diaminofluorescein diacetate fluorescence method (13). In the present study, I/R increased Rho-kinase activity, and hydroxyfasudil significantly inhibited the Rho-kinase activation. These findings suggest that NO is involved in the protective effect of hydroxyfasudil with an increase in eNOS activity and a decrease in Rho-kinase activity during reperfusion injury.

In the present study, the vasodilator effects of hydroxyfasudil were significantly attenuated by L-NMMA (Figs. 3 and 4). The eNOS expression was decreased in the ischemic area of the endocardium compared with that of the epicardium under control conditions, which was improved by hydroxyfasudil (Fig. 7). We have previously demonstrated that endocardial arteriolar dilation during reactive hyperemia is more sensitive to L-NMMA than epicardial arte-

Table 1. Hemodynamics During Myocardial Ischemia-Reperfusion Injury in Dogs

		Before I/R			After I/R		
	n	Baseline	ACh	Papaverine	Baseline	ACh	Papaverine
MBP (mm Hg)							
Control	7	91 ± 4	90 ± 6	92 ± 5	89 ± 4	89 ± 5	92 ± 6
L-NMMA	4	88 ± 8	86 ± 5	91 ± 7	86 ± 4	86 ± 4	85 ± 4
Hydroxyfasudil	7	92 ± 9	92 ± 8	93 ± 8	93 ± 6	90 ± 6	91 ± 7
L-NMMA + hydroxyfasudil	7	89 ± 6	89 ± 5	89 ± 5	91 ± 8	87 ± 10	89 ± 9
Heart rate (beats/min)							
Control	7	151 ± 5	156 ± 3	155 ± 3	155 ± 5	153 ± 5	152 ± 5
L-NMMA	4	147 ± 7	149 ± 8	149 ± 8	145 ± 11	146 ± 11	145 ± 11
Hydroxyfasudil	7	152 ± 7	151 ± 8	148 ± 8	148 ± 7	149 ± 7	150 ± 7
L-NMMA + hydroxyfasudil	7	151 ± 6	152 ± 6	151 ± 6	154 ± 6	151 ± 6	153 ± 7

Results are expressed as mean ± SEM.
ACh = acetylcholine; I/R = ischemia/reperfusion; MBP = mean blood pressure.

riolar dilation (30). These findings indicate that the perfusion of the endocardium is more dependent on NO than that of the epicardium and that endothelial damage after I/R in arterioles may be greater in the endocardium than in the epicardium.

In the present study, hydroxyfasudil exerted cardiovascular protective effects on coronary I/R injury, as did preconditioning (31,32). However, the mechanism by which hydroxyfasudil and preconditioning protect coronary I/R injury appears to be different. Endogenous NO does not alter the infarct size after I/R and is not involved in the protective mechanism of preconditioning in pigs or rabbits (33,34). It has been suggested that preconditioning preserves myocardial creatine phosphate and intracellular pH (35). Furthermore, ischemic preconditioning increases adenosine production and activates protein kinase C, which also enhances adenosine production during I/R injury.

In the present study, hydroxyfasudil significantly reduced myocardial infarct size with increment of coronary collateral blood flow, at least in part, thorough the NO-mediated mechanism (Fig. 8). Shimokawa et al. (11) demonstrated that hydroxyfasudil inhibits both MLC mono- and diphosphorylations. Satoh et al. (14) showed that hydroxyfasudil also protects the brain from ischemic injury through inhibition of superoxide production and neutrophil infiltration. Mohri et al. (36) demonstrated that fasudil suppresses coronary microvascular spasm in patients with microvascular angina. Wolfrum et al. (37) recently demonstrated that inhibiting Rho-kinase has cardioprotective effects to reduce infarct size by activating phosphatidylinositol 3-kinase/protein kinase Akt/eNOS pathways. All these mechanisms may be involved in the beneficial effects of hydroxyfasudil on the I/R-induced myocardial injury.

Hydroxyfasudil increases blood supply to the ischemic region of the myocardium and prevents I/R-induced myocardial injury. Furthermore, it has been recently demonstrated that an estrogen receptor modulator, raloxifene, also reduces I/R-induced myocardial infarct size, whereas an inhibitor of NO synthesis (L-NAME) or a blocker of calcium-activated K^+ channels (charybdotoxin) partly attenuates the effect of raloxifene (19). These results suggest that cardioprotective effects of those inhibitors may be mediated in part by the compensatory effects of NO and endothelium-derived hyperpolarizing factor (20). Several studies using NO synthase inhibitors (38,39) or eNOS-deficient mice (40) demonstrated an increase in infarct size after I/R. The effect of NO synthesis inhibition on the infarct size might be species- and dose-dependent.

Clinical implications and conclusions. The present study has demonstrated for the first time that hydroxyfasudil, a specific Rho-kinase inhibitor, has NO-dependent cardiovascular protective effects on coronary I/R injury in vivo. Rho-kinase inhibitor has also an antianginal effect in a canine model of angina (41), patients with effort angina (42), and those with vasospastic angina (43). Moreover, it has been recently reported that hydroxyfasudil may be effective for the treatment of pulmonary hypertension (44). Indeed, Rho-kinase inhibitors may be useful for the treatment of a wide range of cardiovascular diseases (10). The present study suggests that Rho-kinase inhibitors may also be useful for the treatment of coronary I/R injury in humans.

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Bilateral induction of the S-100A9 gene in response to spreading depression is modulated by the cyclooxygenase-2 activity

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Abstract

Cyclooxygenase-2 (COX-2) was reported to be induced in the infarcted human brain. Spreading depression (SD) is thought to play a role in this induction. In this study, we correlated the expression of SD-associated genes with COX-2 production in brains after SD. Rats were divided into 3 groups: rats that did not undergo SD (group I saline controls, $n=7$), rats that underwent unilateral SD as a result of KCl application (group II, $n=9$), and rats that were pretreated with the selective COX-2 inhibitor, JTE-522 3 h before the induction of SD (group III, $n=7$). The expression of the SD-associated genes, S-100A9, and mitogen-activated proteinkinase phosphatase (cpg21) was analyzed 2 h later using a cDNA array. In group II, COX-2 and cpg21 mRNA expression, as determined by RT-PCR, were significantly upregulated in the hemisphere undergoing SD. While the expression of S-100A9 mRNA was bilaterally upregulated in these animals, this expression was significantly reduced in group III, and was accompanied by reduced bilateral production of PGE_2. Thus, the bilateral induction of expression of the S-100A9 gene in response to SD was associated with COX-2 activation.

Keywords: Spreading depression; S-100A9; cpg21; Cyclooxygenase-2; JTE-522; Rat

1. Introduction

Spreading depression (SD), characterized by reversible depression of cortical electrical activity in the brain that spreads like a wave, can be induced by a variety of electrical, chemical, and mechanical stimuli in the normal brain [1]. While preconditioning by the repeated induction of SD was shown to induce tolerance to subsequent ischemia [2], the induction of repetitive SDs in the ischemic cortex within a few hours after ischemia was found to contribute to the expansion of the infarcted areas in rat and cat models [3–6]. Thus, SD appears to have dual effects on the brain.

The activity of cyclooxygenase-2 (COX-2), the rate-limiting enzyme in prostaglandin synthesis, was upregulated by SD [7] and focal brain ischemia [8] in the cortex of primates and nonprimates [9,10]. Koistinaho and Chan [11] reported that SD directly induced COX-2 expression in focal brain ischemia by stimulating the *N*-methyl-D-aspartate (NMDA) receptor and activating phospholipase A2. Nogawa et al. [12] suggested that COX-2, induced at the infarct border, might be involved in delaying neuronal death, though they did not assess COX-2 expression in the contralateral hemisphere. On the other hand, COX-2 expression was shown to be globally induced in the infarcted human brain, and delayed COX-2 induction in the hemisphere contralateral to the ischemia was speculated to play a role in promoting the remodeling of neural networks [13]. SD is thought to be involved in the induction of COX-2 in brain areas great distances from the ischemic

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regions [14], however, the mechanisms of COX-2 expression in remote brain areas from affected site have remained to be clarified.

To help clarify these mechanisms, we examined the alteration of gene expression in the whole brain under eliciting SDs after administration of a selective COX-2 inhibitor.

2. Materials and methods

2.1. SD model and brain preparation

All animal procedures were approved by our Institutional Animal Research Committee and were performed in accordance with the standards published by the National Research Council.

Male, Sprague–Dawley rats (240–350 g, $n=23$) were randomly divided into 3 groups. Rats in group I (control, $n=7$) were not subjected to SD, while rats in groups II ($n=9$) and III ($n=7$) were subjected to SD, with the rats in group III also receiving a selective COX-2 inhibitor JTE-522 (4-[4-cyclohexyl-2-methyloxazol-5-yl]-2-fluorobenzenesulfonamide; Central Pharmaceutical Research Institute of Japan Tobacco Inc., Osaka, Japan) 3 h prior to SD elicitation. JTE-522, which was suspended in a 0.5% carboxy methylcellulose solution and administered orally at a dose of 10 ml/kg, was reported to selectively inhibit COX-2 without affecting COX-1 [15]. All animals were anesthetized with chloral hydrate (400 mg/kg body weight i.p.) prior to the induction of SD.

SD was evoked by applying 3.3 mol/L KCl to the cortex. The anesthetized animals were mounted on a stereotaxic instrument in the prone position, with their head restrained using teeth and ear-bars. After the frontoparietal cranium was exposed by a midsagittal incision, two small burr holes were made in the right parietal skull bone and the dura carefully excised; the rostral burr hole was used to apply potassium chloride (KCl) while the more caudal hole was used to take direct current (DC) potential recordings. These two burr holes were made 7-mm apart (Fig. 1A). The DC potential was monitored with an amplifier (Iso-DAM8, World Precision Instruments, Sarasota, FL, USA) that was connected to microelectrodes (TM33B10, World Precision Instruments, Sarasota, FL, USA) that were inserted into the cortex to a depth of 1 mm. In the rats in group I, physiological saline, instead of KCl, was applied to the cortex through the rostral burr hole. Rectal temperature of all treated rats was monitored and their body temperature maintained around 37 °C with the aid of heating pads. An arterial catheter placed into the right femoral artery was used to continuously monitor heart rate and arterial pressure.

Two hours after KCl or saline application, brain tissues were perfused with cold saline and the animals were sacrificed by exsanguination under chloral hydrate anesthesia. The brains were then cut into 3 coronal sections as shown in Fig. 1. The section between slices 1 and 2 was frozen in isopentane-dry ice and stored at −80 °C for biochemical analyses.

2.2. Gene analyses

cDNA array analyses were used to search for SD-associated genes that were modulated by the administration of JTE-522. These analyses were conducted using Motorola CodeLink Bioarrays (Motorola Life Science, IL, USA), each of which contained 10,060 elements. Poly $(A)^+$ RNA extracted from the right cortices of 3 animals in each of the 3 groups (animal no 1~no 9) was pooled together (the total

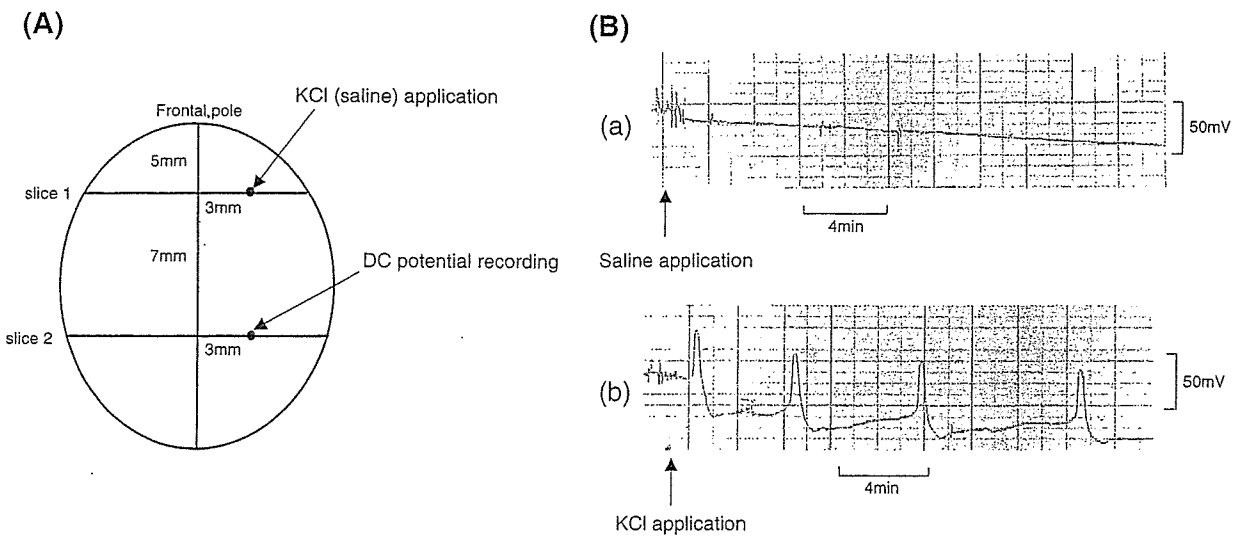

Fig. 1. Stereotaxic parameters and DC potential recordings from rats in groups I and II. (A) The brain was stereotaxically divided, on ice, into 3 coronal sections. (B) DC potentials were not detected after saline application to rats in group I (a). In the rats in group II, a total of four DC potentials were detected after KCl application (b). Tracings represent the data from a single rat in each group.

Table 1
Oligonucleotide primers used in RT-PCR

Gene	Sequences (Forward primer) (Reverse primer)	Cycling number (*N*) PCR product size
COX-2	CCCAGCACTTCACTCATCAGTTTTTCAAGA (F563)	32
	TTCCACCAGCAGGGCGGGATACAGTTCCAT (R1459)	926 bp
cpg21	GAGTATATCAAGCAGAGGAGGAGCGTGGTC (1045F)	32
	TTCCCTGAAGTGACAGAGGACAGAGACAGA (1761R)	746 bp
S-100A9	AGCGCAGCATAAGCACCATCATCAATGTTT (60F)	32
	ATTATTTCCCAGCCCCAGAACCAAGGTCAT (431R)	401 bp
GAPDH	ACCACAGTCCATGCCATCAC (586F)	23
	TCCACCACCCTGTTGCTGTA (1018R)	439 bp

COX-2, cyclooxygenase 2; cpg21, mitogen-activated protein kinase; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

amounted to about 30 μg) and was used to synthesize complementary DNA. The specific protocol involved the use of a Motorola CodeLink™ microarray and is described in detail elsewhere [16,17].

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

RT-PCR was used to examine the expression of COX-2 and SD-associated genes. Twenty samples were obtained from bilateral cortices in 10 animals (animal no10~no19). Primers for selected genes were obtained from Proligo (Kyoto, Japan). RT-PCR analysis was performed using KOD DNA polymerase (Toyobo, Osaka) as previously described [18]. The sequence of the primer pairs for each of the target genes and their cycling number (*N*) are described in Table 1. The basic cycling parameters were as follows: 3 min at 96 °C, followed by N cycles at 94 °C, 15 s; 55 °C, 2 s; 68 °C, 1 min. Cycling numbers were determined empirically using semi-quantitative PCR amplification. The amplification products were visualized by electrophoresis using a 2% ethidium bromide-stained agarose gel, and digitized using a DC290 with Kodak™ 1D 3.5.3 software. The digitized values of each gene were normalized with those of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.4. Measurement of prostaglandin concentration by radioimmunoassay

Tissue concentrations of prostaglandin E_2 (PGE_2) in both hemispheres of all animals ($n=23$) were determined using radioimmunoassay kits (PerkinElmer Life Sciences, Inc., MA, USA). Values were normalized for protein content.

2.5. Data analysis

A one-way analysis of variance (ANOVA) followed by a Fisher's post-hoc test was used to assess the differences in hemispheric gene and PGE_2 expression between the 3 groups. A Student's *t* test was used to analyze differences between the hemispheres in each group. A two-tailed *p*-value of <0.05 was considered to be significant. Data are expressed as the mean±standard deviation.

3. Results

The physiological parameters of all test animals did not change significantly during the experimental period (Table 2). No episodes of SD were observed in the control group (I; Fig. 1). The mean number of SDs evoked by KCl application in group II (4.7±2.4) was not significantly different from that seen in group III (3.6±1.6).

3.1. cDNA array analysis

The expression signals for S-100A9 and mitogen-activated protein kinase phosphatase (cpg21) in group II increased more than 2.5-fold compared to their expression in the rats in groups I and III (2.8-fold vs. group I, 2.8-fold vs. group III for S-100A9; 6.0-fold vs. group I and 2.7-fold vs. group III for cpg21). In group II, 35 elements were found to be at least 2.5-fold different than in the rats in group I,

Table 2
Physiological measurements

Group	*N*	Weight (mg)	SD	MAP (mm Hg)	Temp. (°C)	pH	Po_2 (mm Hg)	Pco_2 (mm Hg)
I	7	302±30	0	66.4±6.5	37.2±0.6	7.30±0.02	108±15	43±4
				66.1±6.4	37.2±0.5	7.26±0.02	108±15	42±4
II	9	301±33	4.7±2.4	65.3±10.7	37.4±0.6	7.30±0.04	97±19	44±5
				68.1±12.1	37.0±0.9	7.29±0.05	108±20	42±5
III	7	305±25	3.6±1.6	74.0±9.9	37.2±0.6	7.31±0.02	104±12	44±2
				68.1±15.4	37.2±0.4	7.30±0.04	109±13	43±5

Values are the mean±S.D.
Upper rows of each column indicate baseline measurements, while the lower rows indicate values obtained 2 h after KCl or saline application.

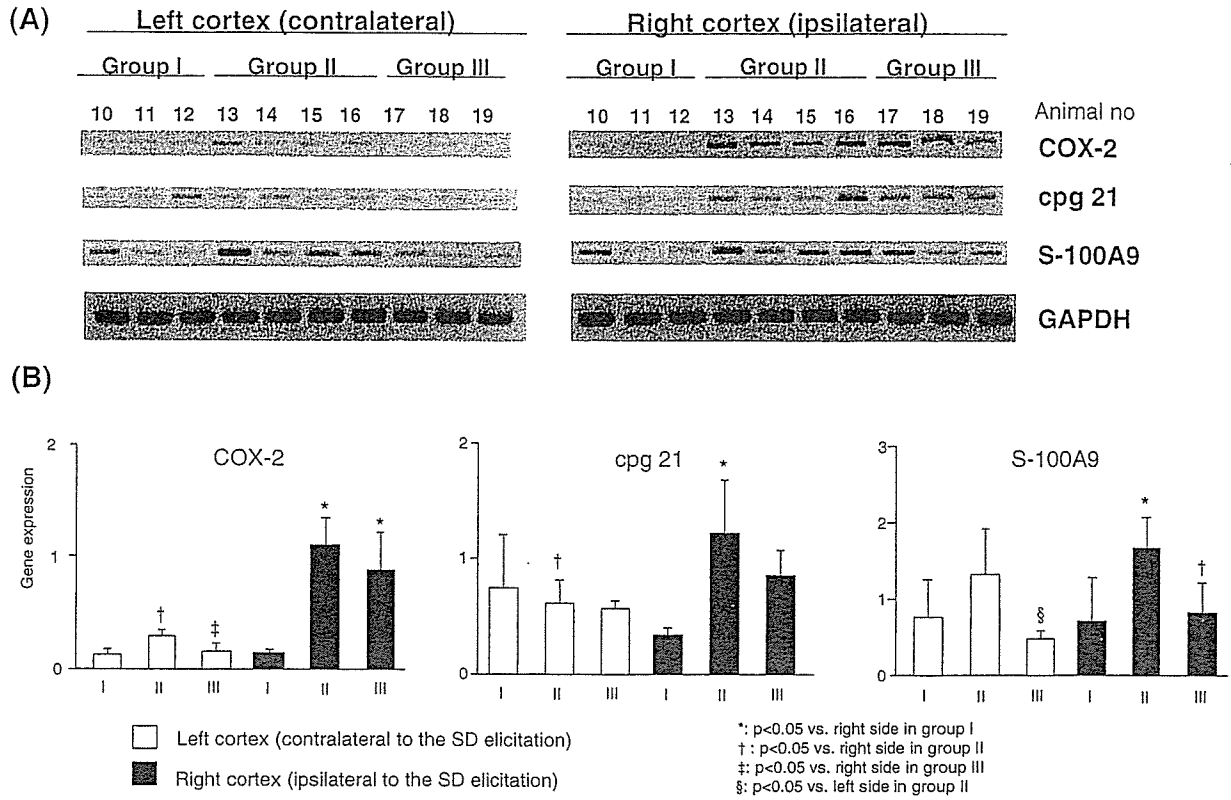

Fig. 2. RT-PCR analysis of COX-2, cpg21, S-100A9, and GAPDH mRNA in each group. (A) Autoradiograms of COX-2, cpg21, S-100A9, and GAPDH mRNA in each group. (B) Densitometric analysis showing the expression of the genes of interest normalized to GAPDH mRNA. Note that S-100A9 expression, which was upregulated in both cortices of rats in group II, was downregulated by the administration of JTE-522 (group III). The expression of COX-2 and cpg21 was prominent in the cortices undergoing SD in the rats in group II. Expression of GAPDH mRNA was equivalent between groups.

and 8 elements were found to be at least 2.5-fold different than in the rats in group III.

3.2. SD-associated genes that were modulated by JTE-522

There were no significant differences in the expression of cpg21, S-100A9, and COX-2 in the right vs. left cortex of rats in group I. However, mRNA expression of all of these genes was significantly upregulated in the right cortex of rats in group II that had undergone SD, compared to controls (Fig. 2). The expression of both COX-2 and cpg21 mRNA in the right hemisphere in group II rats was also significantly upregulated compared to their left hemisphere. On the other hand, the expression of S-100A9 mRNA in the left cortex of these rats was similar to that seen in their contralateral hemisphere. The expression of S-100A9 mRNA in both hemispheres of the rats in group III was significantly reduced compared to that seen in corresponding sides of the brain in group II rats.

Table 3
Concentration of PGE_2 in brain samples

Group	Right cortex (ipsilateral)	Left cortex (contralateral)
I	22.8±12.3	36.3±16.9
II	17.8±12.6	29.5±13.8
III	8.0±4.9***	18.4±6.9***

pg/TP (total protein) mg.
* $p<0.05$ vs. right cortex in group I.
** $p<0.05$ vs. left cortex in group III.
*** $p<0.05$ vs. left cortex in group I.

3.3. PGE_2 synthesis

Mean tissue levels of PGE_2 in both hemispheres of group III rats were significantly lower than those seen in corresponding regions of the brains of control, group I rats (Table 3). The PGE_2 concentration in the right cortex of group III rats was significantly reduced compared to levels in their contralateral hemisphere.

4. Discussion

In this study, we examined the effects of the selective COX-2 inhibitor, JTE-522, on gene expression in brains that underwent SD. We confirmed the findings of Choudhuri et al. [19] that COX-2 was only upregulated in the hemisphere that underwent SD. On the other hand, PGE_2 production levels in the rats of group II were similar to those seen in group I; levels in the right cortex of the rats in group III were significantly lower than those seen in the contralateral cortex. These findings were likely

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attributable to either suppression of protein synthesis during the repetitive SDs [20], or the rapid kinetics of the COX enzyme [21,22].

cpg21, which shows 92% homology with dual specificity phosphatase 5 in humans, dephosphorylates and inactivates phosphorylated extracellular signal regulated kinase 1 (ERK1). Transient phosphorylation of ERK1/2 in a MAP kinase/ERK kinases (MEK)-dependent manner was shown to occur following SD, with phosphorylated ERK levels returning to control levels 45 min later [23]. Because JTE-522 did not suppress the expression of the cpg21 gene as shown by RT-PCR in the present study, upregulation of the cpg21 gene probably occurs concomitantly with phosphorylation of ERK1/2 after SD, and is not associated with COX-2 activation.

S-100A9 belongs to the S-100 family of calcium-binding proteins. The S-100 protein, which was first isolated from the brain by Moore in 1965 [24], exists in 3 dimeric forms [25] i.e., an alpha–alpha form known as S-100A(0), an alpha–beta form known as S-100A, and a beta–beta form known as S-100B [26]. Enhanced synthesis of S-100B by reactive astrocytes within the peri-infarct area was shown to participate in the inflammatory response that delayed infarct expansion after permanent focal ischemia in rats [27]. S-100A8 and S-100A9, which are produced by activated neutrophils and monocytes, are translocated to the cell membrane where they form a heterodimer that co-localizes with cytoskeletal proteins [28,29]. Postler et al. [30] demonstrated that microglial cells in the peri-infarct area expressed S-100A8 and S-100A9 in the early phase of human cerebral ischemia, though the role that they played in this process is not fully understood.

In the present study, we found that the expression of the S-100A9 gene was upregulated not only in the cortex that underwent SD but also in the contralateral cortex. The S-100A8/A9 complex was reported to specifically bind to polyunsaturated fatty acids in a calcium-dependent manner [31,32], and it has been implicated in the modulation of the activity of arachidonic acid-metabolizing enzymes [33]. Neuronal activity during SD that is mediated by the NMDA receptor could help propagate astrocytic calcium waves into the brain areas contralateral to the affected side [34–36]. Since S-100A9 gene expression and PGE_2 production were bilaterally downregulated in the hemispheres of rats administered JTE-522, the expression of the S-100A9 gene is likely affected not only by calcium mobilization but also by increased COX-2 activity during SD. Thus, expression of the COX-2 gene in the cortices undergoing SD could be modulated by the S100A9 gene, though it remains unclear whether the S100A9 gene could modulate contralateral COX-2 gene expression.

In conclusion, the bilateral induction of expression of the S-100A9 gene in response to SD may be modulated by prostaglandin synthesis, even though there was no upregulation of the COX-2 gene in the cortex contralateral to that which underwent SD.

Acknowledgments

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研究のスポット

「フレンチパラドックス」と核内受容体PPARとの新しい接点

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「フレンチパラドックス」という言葉は，中等度の赤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ことに由来している．赤ワインには，レスベラトロールと呼ばれるフィトアレキシン（抗菌性物質）が含まれており，このレスベラトロールの抗酸化活性が，「フレンチパラドックス」に関与していると考えられてきた．

筆者らは，プロスタグランジン産生の律速酵素である誘導型シクロオキシゲナーゼ（COX-2）の発現制御について，種々の疾患における役割を視野に入れて10年以上研究を続けている．その共同研究の中で，レスベラトロールが①COX-2の酵素活性や発現誘導を阻害すること，②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③PPARα ノックアウトマウスを用いた脳虚血モデル実験において，レスベラトロールの経口投与がPPARα を介して脳保護効果をもつことを見いだした[1]．これらの知見から，「フレンチパラドックス」の新しい分子作用機構としてシクロオキシゲナーゼ（COX）経路とともにPPARが中心的な役割を演じていると仮説を立て，現在研究を進めている．一方，レスベラトロールが寿命延長効果をもつカロリー制限模倣物質であること[2]，PPARα の新しい内因性リガンド候補として見いだされたオレイルエタノールアミドがPPARα 活性化によって食欲をコントロールすること[3]が，筆者らの報告とほぼ同時期に報告され，PPAR活性化→カロリー制限→長寿効果がつながっていく可能性が出てきた．本稿では，その背景，経緯，展望について紹介したい．なお，レスベラトロールに関する最近の動向については総説[4,5]を参照していただきたい．

シクロオキシゲナーゼ経路と核内受容体との関係

COXはプロスタグランジン（PG）産生の律速酵素で，アラキドン酸を基質にしてPGH_2 を生成する反応を触媒する．非ステロイド性抗炎症薬の作用はCOX活性の阻害によるPG産生抑制を介している．COXには構成型発現を示すCOX-1と誘導型COX-2の2種類のアイソザイムが存在する．COX-2の発現は炎症性刺激で迅速に誘導され，かつ抗炎症性ステロイド剤である合成グルココルチコイド・デキサメタゾン（DEX）によって抑制されることから，炎症反応への関与が想定されてい

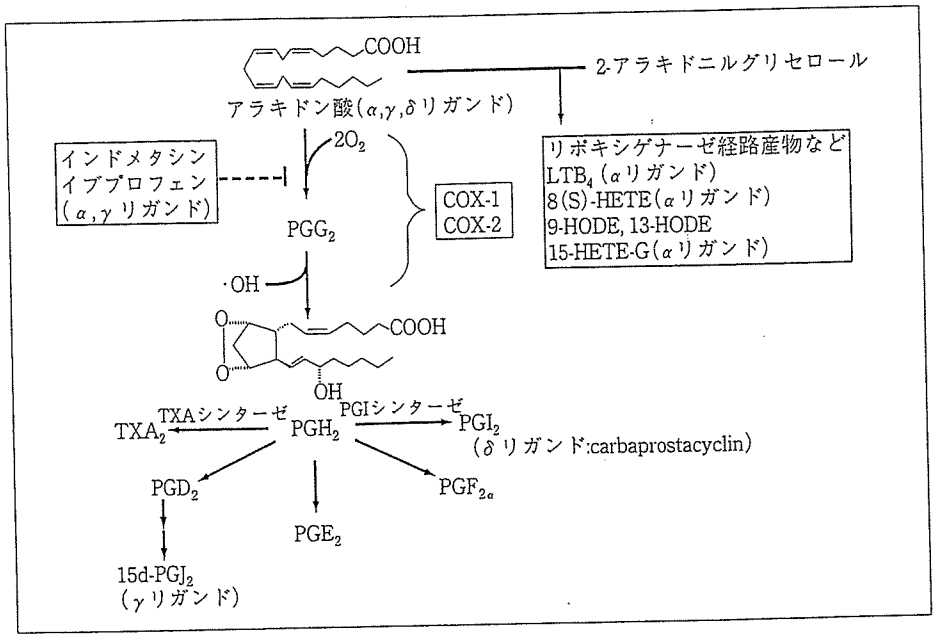

図1■シクロオキシゲナーゼ経路とPPARリガンド
プロスタグランジン産生系などアラキドン酸代謝物，およびその阻害剤の中に，PPARのリガンドとして働くものがいくつか報告されている．ただし，これらはPPARに対する結合能で検出され，高濃度でのみ働くものも多い．したがって，これらが実際に生体内でPPARを介して機能しているかどうかはさらに検討が必要である．

る．実際，最近開発されたCOX-2選択的阻害剤はCOX-1活性阻害に由来する副作用が少ない抗炎症薬として注目されている．さらに，COX-2ノックアウトマウスの解析や最近の臨床，疫学調査を含む研究などから，COX-2が発癌，アルツハイマー病，循環器系疾患にも関与し，様々な役割を担っていることが次第に明らかになってきた[6]．

グルココルチコイドや活性型ビタミンDなどは生理活性をもつ脂溶性物質であり，これらをリガンドとする核内受容体群は，その構造上の相同性によってファミリーを形成している．PPARはこの核内受容体ファミリーに属するリガンド依存性転写因子である[6]．PPARには現在3種類のサブタイプ$\alpha, \gamma, \delta(\beta)$が知られている．PPAR$\alpha$は主に肝臓，腎臓に，PPAR$\gamma$は主に脂肪細胞，マクロファージに，PPAR$\delta$は様々な組織に発現している．PPAR$\alpha$に対する合成リガンド・フィブレート系誘導体は高脂血症治療薬として，PPARγに対する合成リガンド・チアゾリジン系誘導体はインスリン抵抗性改善薬として知られており，PPARは生活習慣病改善薬の標的分子として現在注目を集めている．PPARγの内因性リガンドは未だ不明であるが，その候補としてPGD_2の代謝産物15-deoxy-$\Delta^{12,14}$ PGJ_2(15d-PGJ_2)が報告された．また，アラキドン酸をはじめとする脂肪酸やその代謝産物，非ステロイド性抗炎症薬などもPPARのリガンドとして働く可能性が指摘され，食生活とも密接に関わる核内受容体であることがわかってきた．図1にCOX経路およびリポキシゲナーゼ経路でPPARのリガンドとして働く可能性があるものを示す．注目すべき点として，PPARが関与していると考えられる生体内での役割は，前述したCOX-2が関与する役割と重複している．

細胞の種類によって異なるCOX-2の発現調節

循環器系においては，プロスタサイクリン(PGI_2)とそれに拮抗する作用をもつトロンボキサン(TXA_2)の産生のバランスがホメオスターシスに重要であり，そのバランスの破綻は動脈硬化症をはじめとする様々な病態と関連している．PGI_2は主に血管内皮細胞で，一方TXA_2は血小板や活性化マクロファージで産生されるが，血小板以外の細胞ではCOX-2の発現が種々の刺激により誘導され，それぞれのPG類産生に寄与している．筆者らはその視点から，PGI_2を産生する培養血管内皮細胞とTXA_2, PGE_2を産生するマクロファージ系U937細胞でのCOX-2発現の相違に注目して研究を進めてきた．その結果，①COX-2遺伝子のプロモーター領域に存在する転写因子結合配列のうち，cAMP応答エレメント(CRE)，NF-IL6およびNF-κB結合配列がCOX-2の転写調節に関与していること，②これらのシスエレメントに，種々の転写因子が作用すること，③これら転写因子は細胞種，刺激剤，時間などによって異なった組み合わせで作用することを明らかにしてきた(図2)．

注目すべき点として，COX-2の発現に対するDEX作用の相違がある．DEXによるCOX-2発現抑制は上記に示したU937細胞など，種々の細胞で報告されているが，血管内皮細胞においてCOX-2の発現はDEXでほとんど抑制されない．この血管内皮細胞におけるDEX非

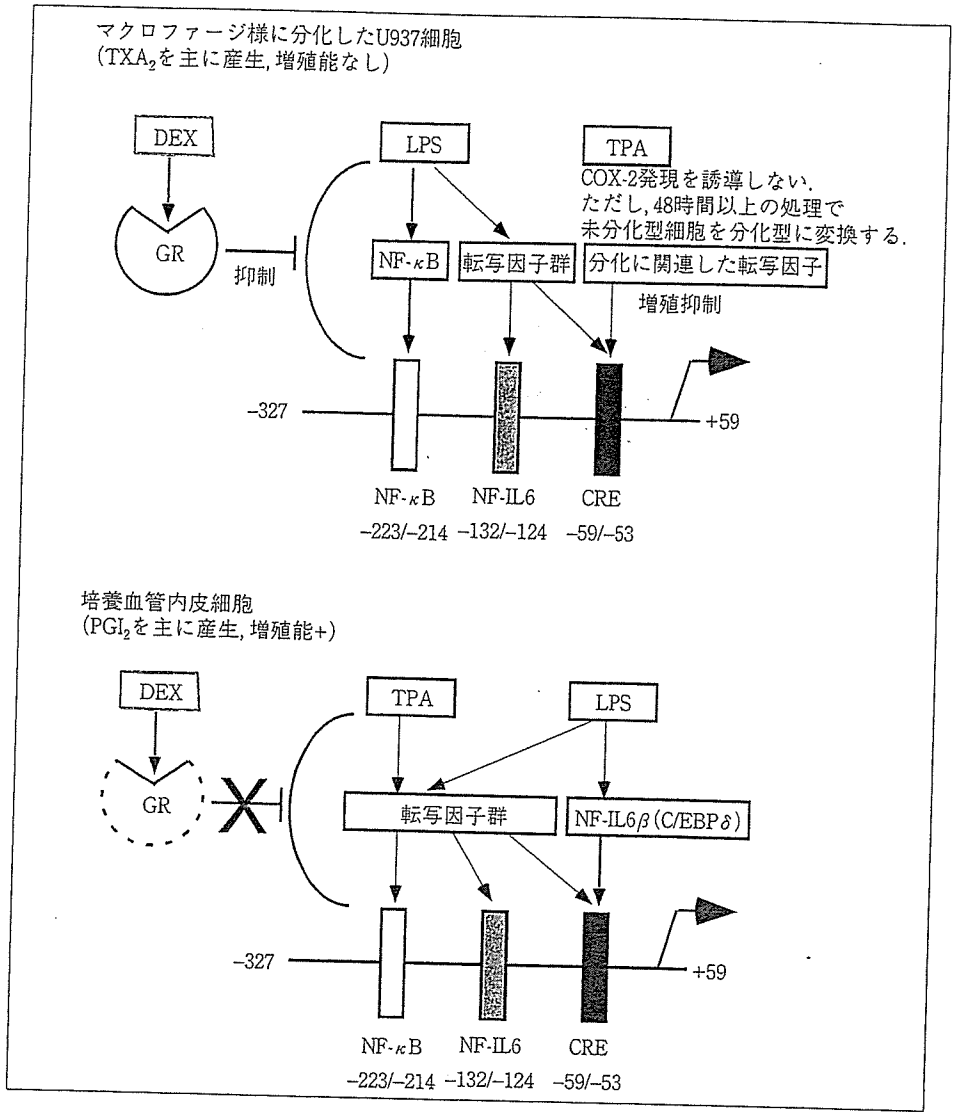

図2■マクロファージ系細胞と血管内皮細胞でのCOX-2遺伝子転写調節の比較

TPA：12-*O*-tetradecanoylphorbol 13-acetate（発がんプロモーターの一種）

感受性について検討した結果，ウシ動脈由来血管内皮細胞（BAEC）ではU937細胞に比べると，グルココルチコイド受容体（GR）の発現が非常に低いことがわかった．そこで，GR発現ベクターをBAECに導入したところ，DEXの濃度および導入するGR発現ベクター量に依存してCOX-2プロモーター活性が抑制された．したがって，BAECにおけるGRの低い発現レベルがDEX非感受性に関与していると結論した[8]．さらにDEXは，転写レベル以外に，転写後の段階でも働き，COX-2遺伝子の3′非翻訳領域がGRを介して転写後調節に関与していることが示唆された[9]．一方で筆者らは，血管内皮細胞において，静脈血程度の弱い流れ刺激（シェアストレス）でCOX-2が誘導され，PGI_2産生に関与することを明らかにした[10]．これらの知見から，生体の血管内皮細胞では流れ刺激によってCOX-2が構成的に発現し，PGI_2産生に関与していると考えられる．最近，COX-2選択的阻害薬投与によって心筋梗塞や脳卒中の危険度が増加することが指摘されているが，COX-2を介するPGI_2産生が関与している可能性が示唆される．

マクロファージにおけるPPARγによるCOX-2発現のフィードバック制御[11]

筆者らは，DEXの場合と同様，PPARγ内因性リガンド候補15d-PGJ_2によってCOX-2の発現がU937細胞では抑制されるが，血管内皮細胞では抑制されないことを見いだした．さらに，血管内皮細胞ではPPARγの発現がほとんど認められないこと，血管内皮細胞にPPARγ発現ベクターを導入すると，15d-PGJ_2によるCOX-2プロモーター活性が抑制されることを見いだした．一方，U937細胞ではPPARγ mRNAの顕著な発現が観察されるが，LPS(リポ多糖)刺激で迅速に抑制された．このPPARγの発現とは対照的に，GRはLPS刺激で誘導さ

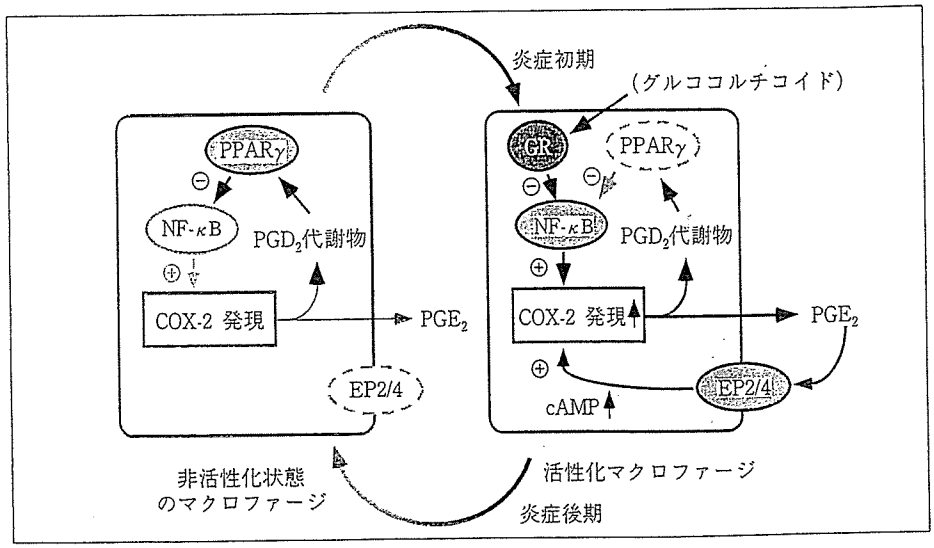

図3■PPARγによるCOX-2の発現制御

れた．U937細胞において，100 nM DEXではCOX-2の発現が90%以上抑制されるのに対し，10 μM 15d-PGJ$_2$では60%程度の抑制しか観察されない．このDEXと15d-PGJ$_2$のCOX-2発現抑制効果の相違は，核内受容体GRとPPARγの発現変動が関与していると考えられ，GRとPPARγはマクロファージにおけるCOX-2の発現，ひいてはPG産生に関して異なった役割をもつと考えられる．

つまり，LPS刺激によりCOX-2が誘導され，多量のPGが産生された状態（これは活性化マクロファージの状態と想定される）において，GRの発現上昇によってグルココルチコイドに対するCOX-2の発現抑制感受性が上がることが示唆される．GRとは対照的に，PPARγはLPS刺激で抑制されるため15d-PGJ$_2$に対する感受性は低下する．一方，活性化マクロファージになる前の非活性化状態では，15d-PGJ$_2$がCOX-2の発現を抑制していると考えられる．さらに，U937細胞では15d-PGJ$_2$の前駆体となるPGD$_2$がCOX-2に依存して産生されること，PGD$_2$産生に関わるPGD合成酵素が発現していることを見いだしたので，マクロファージで合成されたPGD$_2$代謝産物がPPARγのリガンドとして作用することによってCOX-2発現は負のフィードバック制御を受けることが示唆された（図3）．

一方，活性化状態になりPPARγによるフィードバック制御から開放されたCOX-2の発現はPGE$_2$の産生を促し，それがマクロファージ系細胞で発現する細胞膜型受容体EP2あるいはEP4に働き，細胞内cAMP濃度を上昇させ，COX-2の発現を増強すると予想される．この予想に関連して，COX-2と同様，炎症性刺激で誘導され，DEXで抑制される性質を有し，COX-2の下流でPGE$_2$産生に関わる膜結合型PGE合成酵素が発見された．

以上，時間により異なる種類のPG産生が核内受容体と細胞膜型受容体へ異なる情報を伝達することで，マクロファージにおけるダイナミックなPG産生を可能にしていると考えられる．ただ，この制御機構についての理解は未だ不完全で，相反する知見も報告されている．しかし，少なくともCOX-2とPPARγが逆の発現パターンを示すことが多数報告されており，この制御機構の検証を今後も進めていきたいと考えている．

レスベラトロール：COX-2阻害剤かつPPARαおよびγの選択的デュアルアゴニスト

発癌とPGとの関わりについては以下のような報告がある．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%低いこと，②種々の化学発癌物質を用いたマウスでの発癌実験において，アスピリンなど非ステロイド性抗炎症薬は有意に癌の発生を抑制すること，③ヒト家族性大腸腺腫症モデルマウスにおいて，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ことなどである．一方，レスベラトロールはマウス皮膚癌モデルで発癌抑制効果を示す[12]．

このように，癌の研究者がCOX-2に興味をもつようになってきた．筆者らはコーネル大学Dannenberg博士との共同研究の結果，レスベラトロールが癌細胞においてCOX-2の活性および発現を抑制することを見いだした[13]．そこで，種々の細胞でレスベラトロールの効果

をさらに検討した結果，COX-2の発現の様相が細胞の種類によって異なっていることを見いだした（未発表）．したがって，DEXや15d-PGJ_2と同様に，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着想を得た．

そこで，核内受容体の専門家である故梅園和彦博士（京都大学ウイルス研究所），脳卒中の専門家である名村尚武博士（当時・国立循環器病センター，現Morehouse医学校神経科学研究所）と共同研究を行ない，①レスベラトロールは核内受容体群のうち，PPARαおよびPPARγを選択的に活性化すること，②その活性化は血管内皮細胞およびニューロンで認められること，③レスベラトロールおよびPPARαリガンドを3日間経口投与後に24時間脳虚血にすると，脳梗塞の体積が対照と比べて有意に減少し，脳保護効果が認められること，④その脳保護効果はPPARαノックアウトマウスでは認められないことを明らかにした．これらの結果から，レスベラトロールによるPPARαの活性化は，「フレンチパラドックス」を説明する新しい作用機構であり，PPARαが脳卒中に対する薬剤の新しい分子標的になることが示唆された[1]．

現在，PPARの活性化は脳保護効果以外にも生活習慣病に関連する種々の病態を改善することが報告されており，レスベラトロールの効果も広く生活習慣病予防に関与すると考えられる．しかし，その分子作用機構については不明な部分が多いので，現在，PPAR活性化の下流で働く分子群の同定とともに検討を進めている．

展望：カロリー制限，寿命延長効果，核内受容体，COX経路との関係

以前より，カロリー制限は，ラットで実験的に老化を遅らせ，寿命を延長させることが知られている．そこでカロリー制限の機構を解明し，その効果のみを模倣する薬剤（カロリー制限模倣剤）の開発が行なわれている．上記の筆者らの報告と同じ2003年に，レスベラトロールがそのような作用をもち，酵母の寿命をのばすことが報告された[2]．その効果はNAD^+依存性脱アセチル化酵素Sirtuinファミリーの活性化に由来すると報告されたが，Sirtuinはヒストンを介して様々な転写調節に関与しており，核内受容体とも相互作用していると考えられる．

さらに同じ2003年に，オレイルエタノールアミド（OEA）が核内受容体PPARαの活性化を介して食欲と体重をコントロールすることが報告された[3]．アラキドン酸のエタノールアミドであるアナンダミドはG蛋白質共役型受容体であるカンナビノイド受容体を介して，多幸感など，マリファナ様の作用を発揮することが知られている．そこで，オレイン酸のエタノールアミドであるOEAについてもカンナビノイド受容体に作用することが予想されたが，そのような作用は見いだされなかった．そしてOEAはPPARαに高い親和性をもち（EC_{50}＝10 nM），オレイン酸に対する親和性の1,000倍と報告された．一方，OEAはPPARδに対しても親和性をもつが（EC_{50}＝1.1 μM），PPARγには親和性をもたない．さらに，①OEAはPPARαの活性化を介して，脂質代謝に関わる蛋白質の転写を高め，摂食刺激に関与するNO合成酵素の誘導を抑制すること，②自由に摂食させているマウスの小腸を時間差で調べると，餌を食べる夜間はOEA濃度が低く，逆に満腹で休んでいる日中はOEA濃度が高いことが報告され，OEAがPPARαの活性化を介して食欲の調節をしていることが提唱された．

筆者らもその追試実験を行なったが，筆者らの系ではOEAのPPARα活性化能は見いだされていない．その理由として，①PPARに対する反応性に細胞特異的な機構が働いている可能性や，②報告された実験系では安定な遺伝子導入系を用いており，通常使われている一過性の遺伝子導入系とは異なっている可能性などが考えられる．魅惑的な仮説であるので，今後さらに検証していく必要がある．

一方，前述のカンナビノイド受容体の内因性リガンドとしては，アナンダミドよりも2-アラキドニルグリセロール（2-AG）のほうが有力な候補である．すなわち，2-AGの15-リポキシゲナーゼによる代謝産物である15-HETE-G (15-hydroxyeicosatetraenoic acid glyceryl ester) がPPARαリガンドとして働くことが報告された．これらの知見も含めて，さらに検討していく必要がある．

以上述べてきた事実は，筆者らが報告し，提唱しているPPARを介するレスベラトロールの生活習慣病予防と直接関連していると考えられる．図4にその概略を示す．最近，DNAチップによる解析で，カロリー制限を行なったネズミと核内受容体PPARαのアゴニストを投与したネズミではよく似た遺伝子の発現パターンを示し，PPARαが寿命延長に関与する可能性が報告されたが[14]，筆者らも同様の結果を得ている（投稿準備中）．しかし一方で，レスベラトロールで活性化されるSirtuinはPPARγの活性化を抑制することで白色脂肪細胞において脂肪代謝を活性化するという報告もある[15]．この結果は，レスベラトロールがPPARγも同時に活性化するという筆者らの結果とは一致しない．一方，共同研究の

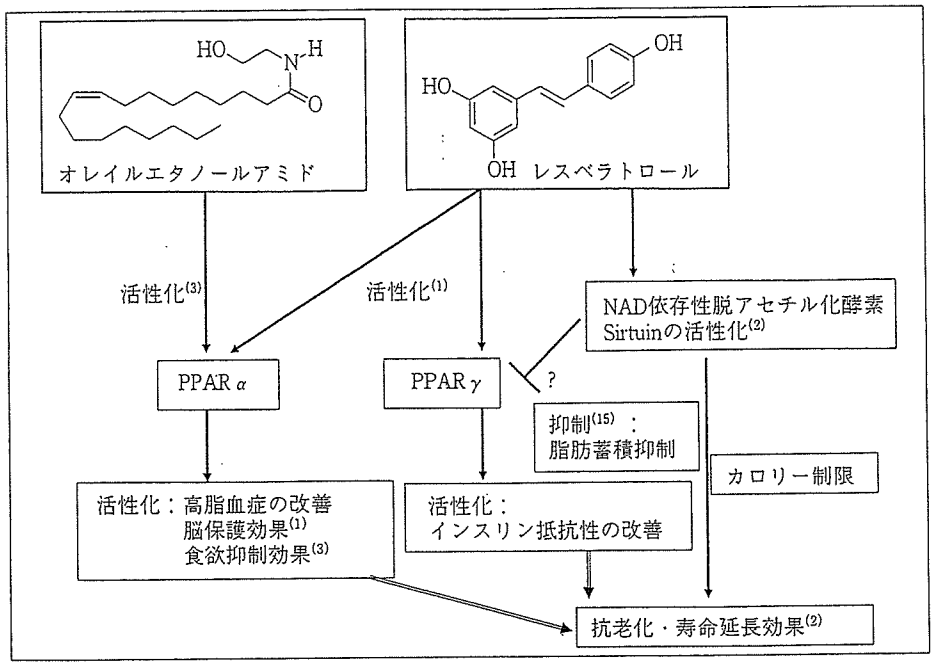

図4■レスベラトロールの作用機構

結果，γ-Mangostin や Chrysin などが COX-2 の発現を抑制することもわかってきた．したがって，レスベラトロールにとどまらず，食品中に含まれるその他のポリフェノール類と核内受容体や COX 経路との相互作用は，寿命延長，生活習慣病予防の視点から，今後さらに研究が進んでいくと考えられる．

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PPARsの基礎研究の進歩

PPARsの内因性リガンド

井上裕康

Endogenous ligands for PPARs

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Abstract

Peroxisome proliferator-activated receptors (PPARs), a family of three nuclear receptors/transcription factors, are widely recognized as molecular targets for drugs against lifestyle-related disease. In spite of intensive search for natural ligands, no truly endogenous ligand has been identified as yet. Rather, these results have lead to the suggestion that PPAR may act as various lipid sensors. Namely, the ligand binding modes of PPARs would be similar to those of odorant receptors or substrate-binding modes of drug-metabolizing enzyme P450 family. In this brief review, free fatty acids, lipid mediators in arachidonate cascade and polyphenolic compounds such as resveratrol will be discussed as natural ligands for PPARs.

Key words: cyclooxygenase, PPAR, oleylethanolamide, resveratrol, polyphenol

はじめに

PPARは生活習慣病に対する薬剤標的として注目を集めている．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$\alpha, \gamma, \delta(\beta)$が知られており，それぞれ異なった生理作用に寄与している[1]．PPARのリガンド結合部位については結晶構造が決定され，ほかの核内受容体に比べ，リガンド結合ポケットが大きいと報告された[2]．この特徴が様々な脂溶性の物質をリガンドとして結合するPPARの性質に関与しており，一般的なホルモンと受容体の関係とは異なっていると考えられる．したがって内因性リガンド候補は幾つか報告されているが，'真の'内因性リガンドであるかどうかは明らかではない．考え方を変えると，'真の'内因性リガンドは単一には存在せず，薬物代謝系酵素であるP450の基質に対する結合様式や嗅覚受容体のリガンドに対する結合のように，様々な脂溶性物質を受容して，その情報を生体内に伝えている可能性も残されている．

一方，3つのPPARサブタイプのリガンド結合は異なっており，その相違がPPARの生体内での発現分布の相違とともにそれぞれの生理作用に寄与している．更にリガンド存在下でPPARγは不安定化するのに対し，PPARαは逆に安定化するとの報告があり，共通のリガンドに対しても異なるPPARは異なる働きをもつ可能性がある．

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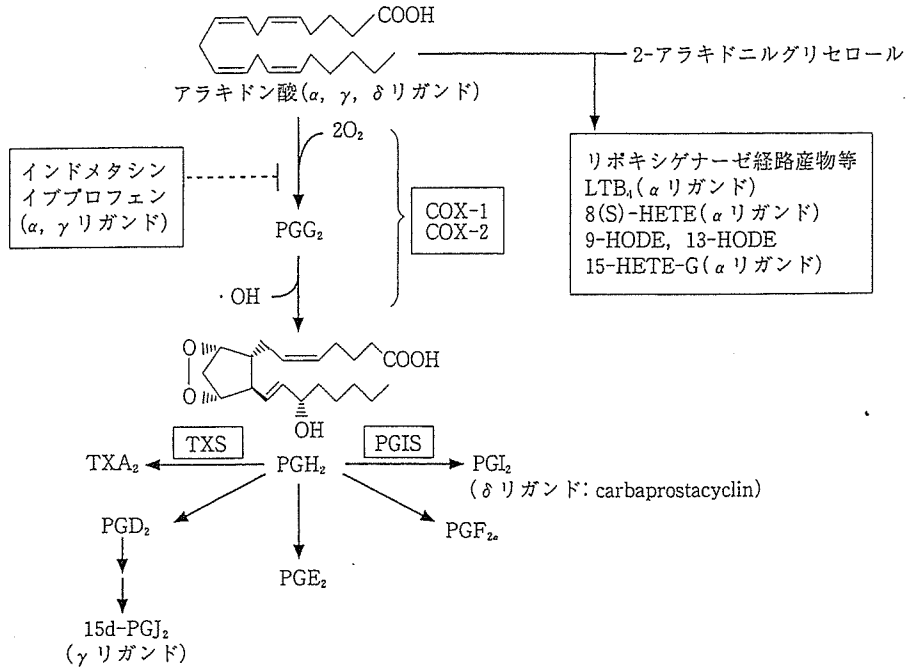

図1 シクロオキシゲナーゼ経路とPPARリガンド

プロスタグランジン産生系などアラキドン酸代謝物，およびその阻害剤の中に，PPARのリガンドとして働くものが幾つか報告されている．ただし，これらはPPARに対する結合能で検出され，高濃度でのみ働くものも多い．したがって，これらが実際に生体内でPPARを介して機能しているかどうかは更に検討が必要である．

1. 脂 肪 酸

必須脂肪酸であるリノール酸，リノレン酸，アラキドン酸などの多価不飽和脂肪酸はいずれのPPARに対しても生理的なリガンド候補となっている．パルミチン酸やステアリン酸など飽和脂肪酸や一価不飽和脂肪酸であるオレイン酸はPPARαにより親和性が高い．これは生理的には肝臓での飽和脂肪酸の代謝にPPARαが主に関与することを示唆している．構造上ではPPARαの結合ポケットがPPARγに比較して疎水性が高いことが報告されており，その性状が水酸化された遊離脂肪酸はPPARαよりPPARγに対して親和性が高いことを説明できるかもしれない．この知見に関連して，酸化LDLの構成成分である種々の物質が主にPPARγのリガンドになることが報告されている．これらの脂肪酸に対するPPARの親和性はnMオーダーを示す合成リガンド類に対する親和性に比べると低い．しかし細胞内で脂肪酸を基質とするアシルCoA合成酵素のKm値は20 μMであり，この値は脂肪酸のPPARに対する親和性と同程度である．また多種類の脂肪酸を組み合わせた細胞内の脂肪酸濃度は十分に高く，PPARが血中脂肪酸のセンサーとして働くとする考え方もある．一方，遊離脂肪酸は血液中ではアルブミンなどに結合しており，多量に細胞に入り込むことは考えにくい．したがって遊離脂肪酸が細胞内でどのような機構で輸送されPPARのリガンドとして働くのか，今後の検討を要する．

2. プロスタグランジン(PG)とロイコトリエン類

図1にCOX経路および，リポキシゲナーゼ(LOX)経路でPPARのリガンドとして働く可能性があるものを示す．PGD_2の代謝物である特殊なPG(15-deoxy-$\Delta^{12,14}$ PGJ_2以下，略して15d-PGJ_2)はPPARγの内因性リガンド候補として

て働くか?'という疑問とともに今後の課題である.

3. オレイルエタノールアミドと15-HETE-G

オレイルエタノールアミド(OEA)は核受容体PPARαの活性化を介して食欲と体重をコントロールすることが報告された[6]. 既にアラキドン酸のエタノールアミドであるアナンダミドはG蛋白質共役型受容体であるカンナビノイド受容体を介して, 多幸感など, マリファナ様の作用を発揮することが知られている. そこでオレイン酸のエタノールアミドであるOEAについてもカンナビノイド受容体に作用することが予想されたが, そのような作用は見いだされなかった. そしてOEAはPPARαに高い親和性をもち(EC_{50}=10 nM), その親和性はオレイン酸に対する親和性の1,000倍と報告された. 一方, OEAはPPARδに対しても親和性をもつが(EC_{50}=1.1 μM), PPARγには親和性をもたない. 更に, ①OEAはPPARαの活性化を介して, 脂質代謝にかかわる蛋白質の転写を高め, 摂食刺激に関与するNO合成酵素の誘導を抑制すること, ②自由に摂食させているマウスの小腸を時間差で調べると, えさを食べる夜間はOEA濃度が低く, 逆に満腹で休んでいる日中はOEA濃度が高いことが報告され, OEAがPPARαの活性化を介して食欲の調節をしていることが提唱された. 著者らもその追試実験を行ったが, 著者らの系ではOEAのPPARα活性化能は見いだされていない. その理由として, PPARに対する反応性において細胞特異的な機構が働いている可能性や報告された実験系では安定な遺伝子導入系を用いており, 通常使われている一過性の遺伝子導入系とは異なっている可能性などが考えられる. 魅惑的な仮説であるので, 今後更に検証していく必要がある.

一方, 前述のカンナビノイド受容体の内因性リガンドとしては, アナンダミドよりも2-アラキドニルグリセロール(2-AG)の方が有力な候補である. そのなかで, 2-AGが15リポキシゲナーゼによって代謝された産物15-HETE-G (15-hydroxyeicosatetraenoic acid glyceryl ester)がPPARαリガンドとして働くことが報告された[7]. これらの知見も更に検討していく必要がある.

4. 植物ポリフェノール類

レスベラトロールは赤ワインに含まれる抗酸化作用をも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. 著者らは最近, ①レスベラトロールは核内受容体群のうち, PPARαおよびPPARγを選択的に活性化すること, ②その活性化は血管内皮細胞およびニューロンで認められること, ③レスベラトロールおよびPPARαリガンドを3日間経口投与後に, 24時間脳虚血にすると, 脳梗塞の体積がコントロールに比べ有意に減少し, 脳保護効果が認められること, ④その脳保護効果はPPARαノックアウトマウスでは認められないことを明らかにした. これらの結果から, レスベラトロールによるPPARαの活性化は, '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[8].

一方, 以前よりカロリー制限は, ラットで実験的に老化を遅らせ, 寿命を延長させることが知られている. そこでカロリー制限の機構を解明し, その効果のみを模倣する薬剤(カロリー制限模倣剤)の開発が行われている. そのスクリーニングの結果, 興味深いことにレスベラトロールが見いだされ, 酵母の寿命を延ばすことが報告された[9]. その効果はNAD+依存性脱アセチル化酵素Sirtuinファミリーの活性化に由来すると報告されたが, Sirtuinはヒストンを介して様々な転写調節に関与しており, 核内受容体とも相互作用していると考えられる.

これらの結果と先に記したOEAの報告を考慮すると, PPARの活性化は寿命延長効果とも直接関連していると予想される(図3). 最近, DNAチップによる解析で, カロリー制限を行ったネズミと核内受容体PPARαのアゴニスト