Paraplegia **32** (1994) 524–530

Increased production of eicosanoids, TXA_2, PGI_2 and LTC_4 in experimental spinal cord injuries

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Arachidonate metabolites have many kinds of bioactivities. Thromboxane A_2 (TXA_2) stimulates platelet aggregation and vasoconstriction, whereas prostaglandin I_2 (PGI_2) antagonises its activities. Thromboxane B_2 (TXB_2) and 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) are determined in biological materials. Production of TXB_2, 6-keto-$PGF_{1\alpha}$ and leukotriene C_4 (LTC_4), which have potent vascular permeability, was measured by radioimmunoassay in experimental spinal cord injured animals. TXB_2 level in the rat spinal cord reached a peak concentration of 133.6 ± 3.8 pmol/g cord, and 6-keto-$PGF_{1\alpha}$ increased to 26.2 ± 11.7 pmol/g cord 5 minutes after the injury. There was good correlation between TXB_2 production and vascular damage as monitored by fluorescein uptake. When OKY-046 ((E)-3-[4-(1-imidazolylmetyl) phenyl]-2-propenoic acid), which selectively inhibits TXA_2 synthetase activity, was administered 10 minutes before injury, the increase in TXB_2 production was inhibited by more than 80%, but the degree of vascular damage was reduced by only 40%. In the guinea pig spinal cord, LTC_4 levels reached a peak concentration of 2.2 ± 0.4 pmol/g cord 10 minutes after compression, while that of TXB_2 reached 146.8 ± 6.2 pmol/g cord. The increased production of TXB_2 was correlated with the degree of compression injury while that of LTC_4 production did not. These findings suggest that vasoactive eicosanoids, TXA_2, PGI_2 and LTC_4, play important roles in secondary damage following spinal cord injury, although their roles may be different among species of animals.

Keywords: spinal cord injuries; eicosanoids; thromboxane A_2; prostaglandin I_2; leukotriene C_4; experimental spinal cord injury.

Introduction

In order to know the pathogenesis of spinal cord injury, pathological, physiological and biochemical approaches have been attempted in a wide range of studies. After Jonsson *et al*[1] found an increased production of prostaglandin E (PGE) in the injured spinal cord, a number of articles have been published on the relationship between the increased production of eicosanoids (PGE_2, PGF_2, TXB_2) and the pathogenesis of spinal cord injury. Among the vasoactive eicosanoids, thromboxane A_2 (TXA_2) and prostaglandin I_2 (PGI_2) are the most potent. The balance between TXA_2 and PGI_2 effects vascular homeostasis. Recently, attention has been focused on the relation between early vascular changes in the injured spinal cord and arachidonate metabolites, particularly TXA_2 and PGI_2. Hsu *et al*[2] have shown that a greater increase in TXB_2 compared to 6-keto-prostagland $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$) might cause vascular damage in experimental spinal cord injury.

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Materials and methods

Spinal cord injury model

One hundred and thirty-four adult Wistar rats (250–350 g body weight) and 186 Hartley guinea pigs (300–400 g body weight) were used in the experiments. All animals

were initially anaesthetised by an intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and a three-segment laminectomy was performed at the lower thoracic spine. The spinal cord was subjected to compression by different weights (5, 10, 20 and 40 g) with an area of 4 mm^2 placed on the spinal cord at T12 for 10 minutes. At various time intervals after the termination of compression, the animals were frozen in liquid nitrogen. The T12 cord segment was then removed and the dura mater was discarded. Sham-operated animals were used as controls. A polyethylene catheter had been previously inserted into the femoral vein for intravenous injection of OKY-046 ((E)-3-[4-(1-imidazolylmethyl) phenyl]-2-propenoic acid),[3] water soluble flavonoid (5-hexyloxy-3′, 4′-dihydoxy-6, 7-dimethoxy-flavone, 4′-disodium phosphate)[4] and physiological saline. This catheter was also used for the injection of a sodium fluorescein solution.

Extraction and purification of arachidonate metabolites from the spinal cord

The spinal cord was homogenised in 10 volumes of cold 80% ethanol using a Polytron homogenizer (Kinematica, Luzern, Switzerland). After centrifugation at 9500 × g for 20 minutes at −20 °C, the supernatant fluid was evaporated *in vacuo*. The residue was resuspended in 1 ml buffer A (10 mM sodium phosphate buffer, ph 7.3, containing 0.1 M NaCl, ImM $MgCl_2$ and 0.1% NaN_3) and centrifuged at 1500 × g for 10 minutes. The supernatant fluid was subjected to radioimmunoassay for TXB_2 and 6-keto-$PGF_{1\alpha}$. Recovery in the extraction procedure was about 67% for TXB_2 and 75% for 6-keto-$PGF_{1\alpha}$, using ^3H-labelled compounds as the internal standards. In the case of LTC_4, 0.2 M acetic acid buffer (pH 5.4) was added to the 80% ethanol extract mentioned above to obtain a 15% ethanol solution (pH 5.4), and this solution was loaded on a SEP · PAK C_{18} column (Waters, Milford, USA) and LTC_4 was eluted with 80% ethanol. The recovery rate of LTC_4 and 50% in this two-step purification process using ^3H-labelled compounds as the internal standard. High performance liquid chromatography (HPLC) was used for further purification of some samples as follows. HPLC using a TSK-GEL column (6.0 × 150 mm) (Toso, Japan) was performed to purify LTC_4 (at 280 nm) and TXB_2 (at 192 nm). LTC_4 was eluted with acetonitrile:methanol:water:acetic acid = 270:100:400:0.8 by volume and adjusted to pH 5.7 with ammonium hydroxide, and TXB_2 was eluted with acetonitrile:water: acetic acid = 33:67:0.1 by volume, at a flow rate of 1.0 ml/min.

Radioimmunoassay (RIA)

For radioimmunoassay of TXB_2, 6 keto-$PGF_{1\alpha}$ and LTC_4, [5, 6, 8, 9, 11, 12, 14, 15(n)-^3H] TXB_2, 6-keto [5, 8, 9, 11, 12, 14, 15(n)-^3H] $PGF_{1\alpha}$ and [5, 6, 8, 9, 11, 12, 14, 15(n)-^3H] LTC_4 were purchased from Amersham International (Buckinghamshire, England). The reaction mixture was incubated at 37 °C for 60 minutes, followed by overnight incubation at 4 °C. The immunoprecipitate was collected by adding polyethylene glycol 6000 and bovine γ-globulin, and radioactivity was counted in a liquid scintillation counter.

Evaluation of vascular damage

OKY-046 (500 μg/kg body weight) or the vehicle, and sodium fluorescein dissolved in physiological saline (40 mg/kg body weight) were sequentially administered to rats via the catheter 10 minutes before compression. The animals were immediately frozen in liquid nitrogen 60 minutes after the termination of compression, and the T12 cord segment was removed. The fluorescein in the extract was measured using a spectrophotofluorometer at an excitation wavelength of 440 nm and an emission wavelength of 518 nm, and the readings were converted into μg of fluorescein employing a standard curve.

Effect of indomethacin and flavonoid on the production of LTC_4 and TXB_2

Indomethacin (Signa, St Louis, USA) was injected intraperitoneally (10 mg/kg body weight) 30 minutes before injury induction with a 20 g weight in guinea pig. Flavonoid (200 mg/kg body weight) was injected intravenously 15 minutes before injury.

The concentrations of LTC_4 and TXB_2 were determined by radioimmunoassay 10 minutes after compression.

Error bars in figures represent standard error.

Results

Figure 1 demonstrates TXB_2 and 6-keto-$PGF_{1\alpha}$ concentration at the tissue level in the rat spinal cord following a compression injury induced by placing 20 g of weights for 10 minutes. The TXB_2 level markedly increased immediately and reached a peak value of 133.6 ± 13.8 pmol/g cord 5 minutes after the termination of compression. It then decreased but remained above the control level for up to 1 hour after compression. In contrast, only a slight increase of 6-keto-$PGF_{1\alpha}$ production (20–30 pmol/g cord) was observed from 5 to 30 minutes after compression was terminated. In the sham-operated rats, TXB_2 and 6-keto-$PGF_{1\alpha}$ levels remained at less than 1 pmol/g cord. The degree of vascular damage determined by fluorescein uptake and TXB_2 production increased as the compression of the spinal cord was increased (Fig 2). 6-keto-$PGF_{1\alpha}$ production, however, remained almost unchanged.

OKY-046, a selective TXA_2 synthetase inhibitor, showed the following effects on

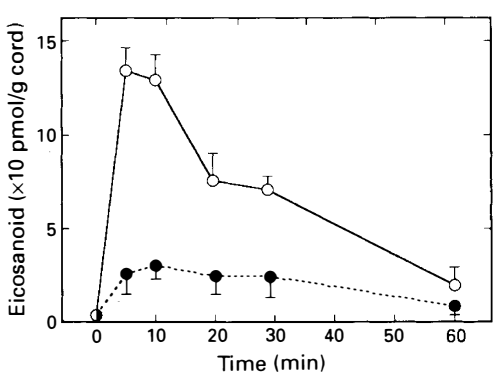

Figure 1 TXB_2 (○) and 6-keto-$PGF_{1\alpha}$ (●) levels in the rat injured spinal cord ($n = 4$ in each measurement) at various time intervals after the termination of compression. Compression injury was induced by placing 20 g of weight on spinal cord for 10 minutes.

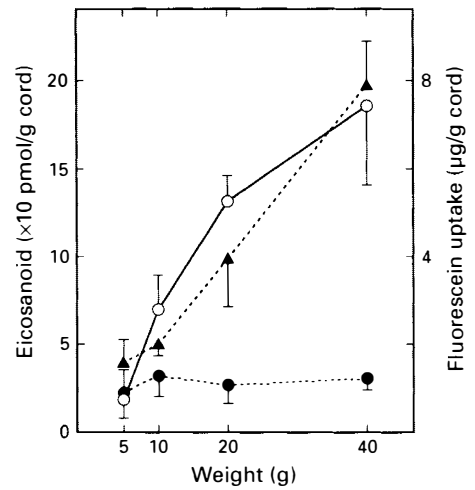

Figure 2 TXB_2 level and correlation between TXB_2 level and fluorescein uptake in the injured rat spinal cord induced by various degrees of compression ($n = 4$ in each measurement). The concentrations of TXB_2 (○) and 6-keto-$PGF_{1\alpha}$ (●) in the spinal cord were measured by radioimmunoassay 5 minutes after the termination of compression. The TXB_2 levels (○) were compared with fluorescein uptake (△) in the spinal cord.

TXB_2 production and vascular damage in the injured rat spinal cord. When OKY-046 (500 μg/kg body weight) was injected intravenously 10 minutes prior to 20 g compression, TXB_2 production was reduced to 20%. The inhibitory effect of OKY-046 on TXB_2 biosynthesis decreased as the interval between induction of spinal cord injury and its administration elapsed (Fig 3). Figure 4 presents the effects of OKY-046 (500 μg/kg body weight) on vascular damage in the injured spinal cord. Maximum inhibition of 40% was observed with 40 g compression.

As TXA_2 has no physiological activity directly leading to vascular damage, the involvement of other chemical mediators was indicated. The production of LTC_4, which strongly promotes vascular permeability, was thus evaluated using a guinea pig spinal cord injury model because production of LTC_4 could not be detected in the rat injured spinal cord.

Production of LTC_4 after compression of the spinal cord in guinea pigs is presented in Figure 5. After compression of the spinal

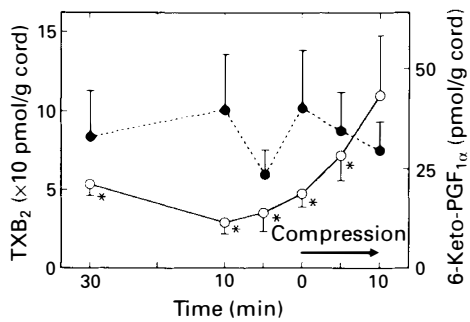

Figure 3 The production of TXB_2 and 6-keto-$PGF_{1\alpha}$ in the rat injured spinal cord ($n = 4$ in each measurement) treated with intravenous injection of OKY-046 at various time intervals before and after the start of compression. The concentrations of TXB_2 (○) and 6-keto-$PGF_{1\alpha}$ (●) in the spinal cord were measured by radioimmunoassay 5 minutes after the termination of compression. $^*p < 0.01$ compared with the control value of TXB_2 (133.6 ± 3.8 pmol/g cord) without OKY-046 treatment.

cord in guinea pigs with a 20 g weight for 10 minutes, LTC_4 level reached a peak concentration of 2.2 ± 0.4 pmol/g cord 10 minutes after the end of compression and then gradually decreased to undetectable levels 60 minutes later. TXB_2 level reached a peak value 5 minutes after compression, then gradually decreased, but remained at half of the peak level even 60 minutes after compression. TXB_2 production was correlated with the degree of compression, although LTC_4 production was not. In sham-operated guinea pigs, LTC_4 production was not detected, and TXB_2 production increased slightly (Fig 6).

The authenticity of LTC_4 detected by RIA in the injured guinea pig spinal cord was confirmed by an immunochromatographic analysis (Fig 7). The extract from a guinea pig spinal cord subjected to compression was purified in a SEP-PAK C_{18} column and analysed by HPLC coupled with radioimmunoassay. Column A represents the retention time for the authentic eicosanoids. As observed in crude samples, increased production of LTC_4 and TXB_2 was demonstrated in the purified samples in column C. Slight increase of TXB_2 production and no increase of LTC4 were indicated in column B in the sham-operated animals.

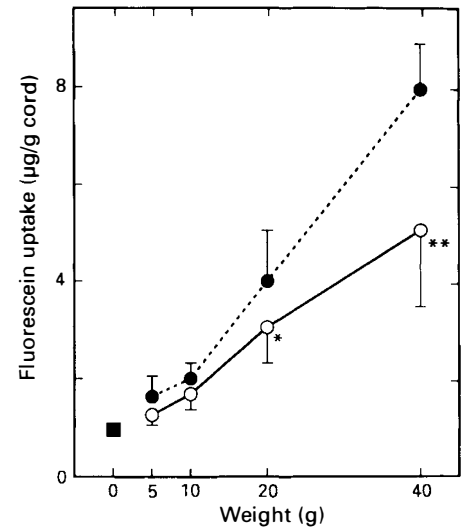

Figure 4 Effects of OKY-046 in the injured and sham-operated (■) rat spinal cord on vascular damage determined by uptake of fluorescein. OKY-046 (○) or vehicle (●), and fluorescein solution were administered sequentially 10 minutes before compression using various weights. $^*p < 0.05$, $^{**}p < 0.01$ compared with vehicle value.

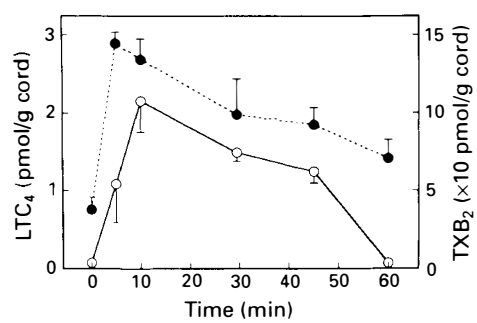

Figure 5 LTC_4 (○) and TXB_2 (●) levels in the injured spinal cord of guinea pigs ($n = 9$ in each measurement) at various time intervals after the termination of compression. Compression injury was induced by placing 20 g of weight on spinal cord for 10 minutes.

LTC_4 and TXB_2 production in the injured spinal cord in the presence of flavonoid, a specific 5-lipoxygenase inhibitor, and of indomethacin, a cyclooxygenase inhibitor, are shown in Figure 8. Flavonoid (200 mg/kg body), when administered intravenously 15 minutes before compression,

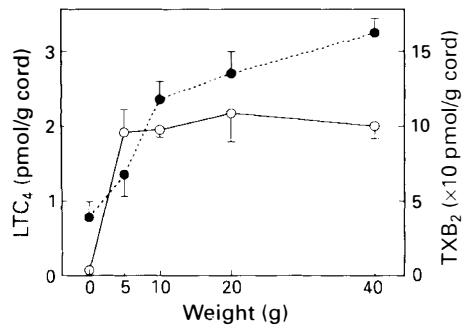

Figure 6 LTC_4 (○) and TXB_2 (●) levels in the injured spinal cord of guinea pigs following various degrees of compression ($n = 9$ in each measurement). The materials were taken 10 minutes after the termination of compression.

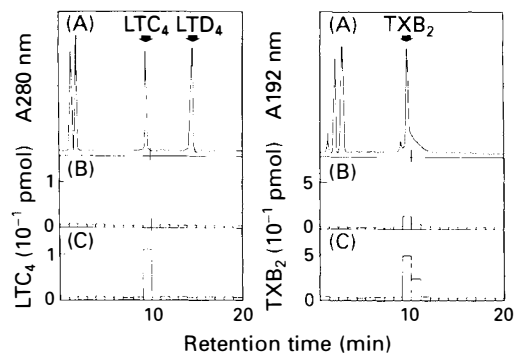

Figure 7 Immunochromatographic analysis of LTC_4 and TXB_2 in the spinal cord extracts of guinea pig. Authentic LTC_4, LTD_4, TXB_2 (A) and the samples passed through SEP-PAK C_{18} from the sham-operated spinal cord (B) and from the injured spinal cord (C) were separated by HPLC. Each fraction of the spinal cord was analysed by radioimmunoassay for LTC_4 and TXB_2. The injury was induced by 20 g weight placement for 10 minutes followed by 10 minutes recovery.

inhibited LTC_4 production to approximately *50%, but did* not inhibit TXB_2 production. Indomethacin (10 mg/kg body), injected intraperitoneally 30 minutes before compression, inhibited TXB_2 production by more than 90%. Although there was no statistically significant difference, LTC_4 production tended to increase in the presence of indomethacin.

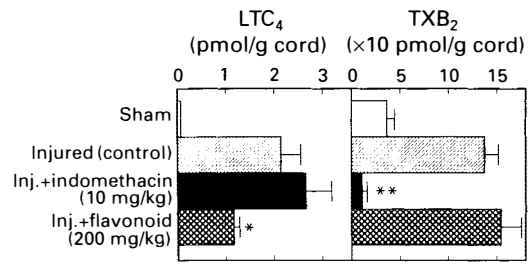

Figure 8 Effects of intraperitoneal injection of indomethacin 30 minutes before injury and intravenous injection flavonoid 15 minutes before injury on the production of LTC_4 and TXB_4 in guinea pig spinal cord ($n = 9$ in each measurement). Injury was induced by placing 20 g of weight for 10 minutes and the samples were taken 10 minutes after the termination of compression. $^*p < 0.05$, $^{**}p < 0.01$ compared with control value.

Discussion

Arachidonic acid is metabolized by cyclooxygenase to produce prostaglandins and by lipoxygenase to produce leukotrienes. These biologically active metabolites of arachidonic acid (eicosanoids) have been implicated as important pathophysiological factors in a variety of acute traumatic insults to the central nervous system, including brain ischaemia and concussive head trauma, as well as spinal cord injury. Once the spinal cord is injured, post-traumatic necrosis subsequent to the initial physical insult keeps progressing. This is considered to be a form of ischaemic necrosis in which vascular damage plays a major role. Especially in experimental spinal cord injury, morphological observations demonstrated endothelial damage with platelet adherence, intravascular platelet aggregation, microvascular occlusion, dissemination of emboli and continued vasogenic oedema. Following these vascular changes, ischaemic necrosis in the parenchyma of the spinal cord advances, resulting in degeneration of the necrosis tissue. Demopoulos *et al*[5] suggested that endothelial damage, platelet aggregation and microcirculatory stasis in the spinal cord lesion might be due to a TXA_2-PGI_2 imbalance that would favour thrombosis. This was confirmed by Hsu *et al*[2] and Demediuk & Faden[6] in experimental spinal

cord injury caused by the weight drop method. They reported that the TXB_2 level increased more than the 6-keto-$PGF_{1\alpha}$ level in the injured spinal cord and that the extent of this discrepant increase was correlated with the degree of injury. Hsu *et al*[7] demonstrated that the extent of vascular injury, determined by extravasation of ^{125}I-labelled human serum albumin, was related to the TXB_2/6-keto-$PGF_{1\alpha}$ ratio.

The present experiments on spinal cord injury induced by compression provided the same results. Production of TXB_2 and 6-keto-$PGF_{1\alpha}$ increased shortly after the termination of compression, but not during the period of spinal cord compression. The increase of TXB_2 level was greater than that of 6-keto-$PGF_{1\alpha}$ level 5 minutes after the termination of compression (Fig 1). Both the extent of post-traumatic vascular damage, determined by fluorescein uptake, and the increase of TXB_2 production were correlated with the degree of injury (Fig 2). These findings suggest that TXA_2 plays an important role in post-traumatic vascular injury. When a selective TXA_2 synthetase inhibitor, OKY-046, was injected intravenously 10 minutes prior to the injury, the increase of TXB_2 production was inhibited to 20%, but the degree of vascular damage was reduced by only 40%. In addition, when OKY-046 was injected after the start of compression, the inhibitory effect was reduced as the interval between injury and injection was prolonged. From a therapeutic standpoint, this finding suggests the difficulty in drug therapy for neurological recovery following spinal cord injury.

LTC_4 is a slow-reacting substance of anaphylaxis (SRS-A) which plays an important role in anaphylaxis and inflammation. Black & Hoff[8] successfully induced cerebral oedema by injecting leukotrienes (LTB_4, C_4 and D_4) into the rat brain. In addition, they found that BW755C which is an inhibitor of both cyclooxygenase and lipoxygenase, prevented the cerebral oedema caused by arachidonic acid infusion, whereas indomethacin which is a cyclooxygenase inhibitor did not have such an effect. Kiwak *et al*[9] investigated the productions of LTC_4 and LTD_4 in gerbil brain contusion model and suggested that leukotrienes are involved in the development of the cerebral oedema observed after contusion.

There are, however, few reports concerning increased production of peptide leukotrienes (LTC_4, LTD_4 and LTE_4) in the injured spinal cord. Faden *et al*[10] indicated, using rat spinal cord injury models, that BW755C administration inhibited TXB_2 production and also showed an effect on the recovery of neurological function; nevertheless, they failed to demonstrate the production of leukotrienes. There have been reports that LTB_4 production was found to be increased in spinal cord injury rabbit[11] and rat models.[12] Saunders *et al*[13] were the only investigators who reported the increased production of peptide leukotrienes in cat spinal cord injury models; however, their measurements were made only by RIA. Yokota *et al*[14] reported the immunological determination of eicosanoids in biological materials by using a bulk of endogenous inhibitors in the crude extract. They showed that it was necessary to purify the crude extract for the accurate detection of eicosanoids in biological materials. In the present experiments, we could detect the eicosanoids' values in purified samples using HPLC. Similar values were obtained in the crude extract as well as in the purified samples taking into account the recovery rate of purification.

In order to know a possible role for the LTC_4 in the pathogenesis of spinal cord injury, we evaluated the production of LTC_4, which strongly promotes vascular permeability, using guinea pig spinal cord injury models because production of LTC_4 could not be detected in the rat injured spinal cord.[6,12] Following compression, TXB_2 production also increased in proportion to the severity of spinal cord injury, whereas the increased production of LTC_4 remained almost consistent and was not related to the severity of the injury. Since the production of LTC_4 increased even with a mild degree of spinal cord injury, it is presumed that LTC_4 production is closely correlated to the development of oedema in the injured spinal cord.

Flavonoid, a specific inhibitor of 5-lipoxygenase, showed only about a 50% inhibition of increased LTC_4 production.

On the other hand, administration of indomethacin, a specific inhibitor of cyclooxygenase, inhibited more than 90% of TXB_2 production, and LTC_4 production tended to increased, although no statistically significant difference was observed (Fig 8). Dempsy *et al*[15] reported that the use of substrate in the lipoxygenase system was likely to be increased owing to the inhibitory effect of indomethacin on cyclooxygenase.

In conclusion, our findings demonstrated that the increased production of TXA_2 and LTC_4 in the injured spinal cord played important roles in the progress of damage secondary to spinal cord injury. In order to decrease this secondary damage, we suggest that it is important to inhibit TXA_2 and LTC_4 production using selective inhibitors for each, and that more potent inhibitors should be developed. It seems possible to elucidate the relationship between secondary injuries in the injured spinal cord and eicosanoids by virtue of performing histopathological estimation and also by further investigating the role of other chemical mediators or free radicals on pathogenesis of spinal cord injury.

Acknowledgements

We wish to thank Professor Shozo Yamamoto for his kind suggestions and the generous gifts of anti-eicosanoids antibodies and flavonoid. We also thank Ono Pharmaceutical Co Ltd for the gifts of authentic eicosanoids and OKY-046. This study was partly supported by grants from the Ministry of Education, Japan.

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