Protective effect of beraprost sodium, a new chemically stable prostacyclin analogue, against the deterioration of baroreceptor reflex following transient global cerebral ischaemia in dogs

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- 1 A possible cerebroprotective effect of a chemically stable prostacyclin analogue, beraprost sodium, was investigated in a canine model of cerebral ischaemia. Cerebral ischaemia was produced by the combined occlusions of the left subclavian and the brachiocephalic arteries with preceding ligations of the intercostal arteries.
- 2 The decrease in baroreceptor reflex sensitivity (BRS), measured by phenylephrine-induced reflex bradycardia, following 5 min ischaemia was used to assess the cerebroprotective effect.
- 3 Beraprost ($1 \mu\text{g kg}^{-1} \text{min}^{-1}$ i.v., infused for 15 min just before ischaemia) completely prevented the decrease in BRS. Although the lower dose of beraprost ($0.1 \mu\text{g kg}^{-1} \text{min}^{-1}$ i.v.) failed to show such a protective effect, its inhibitory effect on ADP-induced platelet aggregation was as potent as that of the higher dose.
- 4 The extent of decrease in BRS was inversely correlated with the extent of the residual blood flow in the medulla oblongata during ischaemia. Since beraprost did not affect the extent of the residual blood flow during ischaemia, its cerebroprotective effect could not be ascribed to the reduction of the degree of ischaemia by increasing collateral blood flow to the brain.
- 5 Post-ischaemic reduction of the regional blood flow in the medulla and the cerebral cortex was completely prevented by the higher dose of beraprost.
- 6 The present study suggests that the cerebroprotective effect of beraprost may be independent of its anti-aggregatory and vasodilator effects. It is possible that the protection may be due to a prostacyclin-like cytoprotective effect through membrane stabilization.

Introduction

Prostacyclin (PGI_2) is a potent endogenous vasodilator and an inhibitor of platelet aggregation (Bunting *et al.*, 1976). Since the labile property of PGI_2 has limited its clinical use, a number of chemically stable analogues have been synthesized. Thus, beraprost sodium (Figure 1) is an orally active prostacyclin analogue which inhibits platelet aggregation and adhesion, disaggregates platelet clumps and causes vasodilatation (Akiba *et al.*, 1986; Umetsu *et al.*, 1986; Nishio *et al.*, 1988; Toda, 1988).

In addition to these effects on platelet and vascular smooth muscle, PGI_2 has been shown to possess a cytoprotective effect, which may be useful for preservation of platelets (Blackwell *et al.*, 1982) and protection of various tissues from ischaemia or hypoxia (Ogletree *et al.*, 1979; Araki & Lefer, 1980). Therefore, the present study was conducted to investigate whether or not beraprost has a cytoprotective effect in a canine model of transient global cerebral ischaemia. In this model, we have already demonstrated that cerebral ischaemia of longer than 5 min produced a marked decrease in barore-

ceptor reflex sensitivity (BRS) during the reperfusion period (Kurihara *et al.*, 1989a). Since the decrease in BRS is accompanied by a decreased cardiac response to the vagal afferent nerve stimulation but not to the vagal efferent nerve stimulation, this decrease in BRS seems to be due to the dysfunction of the central pathway of the baroreceptor reflex mechanism. Furthermore, we have shown that the decrease in BRS was inhibited by pretreatment with flunarizine, a Ca^{2+} entry blocker with a cerebroprotective effect (Kurihara *et al.*, 1989b). In the present study a protective effect of beraprost against the decrease in BRS following 5 min global cerebral ischaemia was investigated and compared with the effects on platelet aggregation as well as cerebral blood flow.

Methods

Animals

Mongrel dogs of either sex weighing 8 to 15 kg were anaesthetized with sodium pentobarbitone (32 mg kg^{-1} , i.v. followed by an infusion of $3.2 \text{ mg kg}^{-1} \text{ h}^{-1}$, i.v.). Under artificial ventilation with room air (a tidal volume of 20 ml kg^{-1} at a rate of 20 to 25 breaths min^{-1}) animals were immobilized with suxamethonium chloride (2 mg kg^{-1} , i.v. followed by an infusion of $1 \text{ mg kg}^{-1} \text{ h}^{-1}$, i.v.). Appropriate volumes of O_2 and CO_2 were provided to maintain arterial Po_2 and Pco_2 at about 100 mmHg and 35 mmHg, respectively. Rectal temperature was maintained at about 38°C .

Measurement of blood pressure, heart rate and EEG

Arterial blood pressure was measured from the left femoral artery by means of a pressure transducer (Nihon Kohden, TP-200T, Japan) and heart rate was measured by a heart rate counter (Nihon Kohden, AT-600G) triggered by lead II ECG.

Figure 1 Chemical structure of beraprost sodium.

The cortical EEG was recorded by means of stainless steel electrodes screwed bilaterally into the parietal skull.

Measurement of baroreceptor reflex sensitivity (BRS)

Baroreceptor reflex was assessed by bolus injections of 4 to 5 doses of (–)-phenylephrine hydrochloride within the dose range of 0.3 to 10 $\mu\text{g kg}^{-1}$, i.v. Phenylephrine-induced increase in pulse interval (ms) was correlated with the increase in mean arterial blood pressure (mmHg) by the method of least squares. Then, the slope of the regression line (ms mmHg^{-1}) was used as a measure of BRS, because ischaemia did not displace the regression line, but altered the slope (Kurihara *et al.*, 1989a). BRS was measured before ischaemia (before drug infusion, see below) and during the periods of 60 to 90, 120 to 150, and 180 to 210 min of reperfusion.

Production of cerebral ischaemia

Thoracotomy was performed at the fifth intercostal space. Approximately 14 intercostal arteries descending along the thoracic aorta were permanently ligated to obstruct severely the collateral blood flow to the brain. Transient global cerebral ischaemia was produced by a 5 min occlusion with clamps of the left subclavian artery and the brachiocephalic artery as close as possible to the aortic arch. A sufficient reduction in cerebral blood flow in experiment 1 (see below) was suggested by the rapid flattening of the EEG within 1 min of ischaemia and confirmed in experiment 2 (see below) by continuous monitoring of cerebral blood flow by the thermocouple electrodes.

Experiment 1

Eighteen animals were divided into 3 equal groups. The first group received an infusion of beraprost (0.1 $\mu\text{g kg}^{-1} \text{min}^{-1}$, i.v.). Infusion was started at 15 min before ischaemia and was continued until the first measurement of BRS after ischaemia was completed. In the second group, an infusion of beraprost (1 $\mu\text{g kg}^{-1} \text{min}^{-1}$, i.v.) was started at 15 min before ischaemia, and was stopped at the start of ischaemia, because the hypotensive effect of this dose was marked (Table 1). The third group received an infusion of saline for 15 min just before ischaemia as the control group.

Measurement of platelet aggregation Platelet aggregation induced by adenosine diphosphate (ADP) (10 μM) was examined in animals which received beraprost. Arterial blood (2.5 to 3 ml) was withdrawn into a plastic syringe containing sodium citrate (about 0.1% final concentration) from the right saphenous artery. Blood sampling was done before infusion and at the period of 10 to 12 min of infusion. Platelet-rich plasma (PRP) was immediately obtained by centrifugation for 10 min at 1500 *g*. The residual sample was recentrifuged for 5 min at 1500 *g* to prepare platelet-poor plasma (PPP). Platelet aggregation was measured at 37°C by means of an aggre-

gometer (Sienco, DP-247E, U.S.A.); light transmission of PRP and PPP was regarded as 0 and 100%, respectively.

Experiment 2

Thirty animals were divided into three groups. The first ($n = 6$) and the second ($n = 12$) group received an infusion of beraprost (0.1 and 1 $\mu\text{g kg}^{-1} \text{min}^{-1}$, i.v., respectively). Infusion was started at 15 min before ischaemia and was stopped at the start of ischaemia. In the third group ($n = 12$), saline was infused instead of beraprost in the same way.

Measurement of cerebral blood flow The animals were placed in a prone position and the head fixed rigidly in a stereotaxic frame. The head was tilted down at an angle of about 30 degrees to help the manipulation of the brainstem. Craniotomy was performed at the parietal region about 1 cm frontal to the external meatus. The foramen magnum was enlarged by removal of the caudal edge of the occipital bone. Regional cerebral blood flow (rCBF) was continuously measured by a tissue flow monitor (Unique Medical, UMW-101, Japan) using the plate type thermocouple electrodes placed on the surface of the parietal portion of the left hemisphere and on the left fasciculus cuneatus in the dorsal medulla oblongata proximal to the obex. In some animals, platinum electrodes were further inserted contralaterally to the thermocouple electrodes to a depth of 1 to 2 mm, and rCBF was measured by the hydrogen clearance method before drug infusion and at the reperfusion period of 60, 120 and 180 min.

The degree of ischaemia was assessed by calculating the residual blood flow during ischaemia with the data obtained by thermocouple electrodes (Figure 2). Blood flow level at the start of drug infusion (15 min before ischaemia) was regarded as 100%, and zero flow was obtained by killing the animal with saturated KCl at the end of each experiment. Then, blood flow during ischaemia was plotted at 30 s interval, and the percentage ratio of the integrated residual blood flow (hatched area in Figure 2) to the total integrated flow (area of the square ABCD) was calculated as a measure of the residual blood flow during ischaemia.

Drugs used

Beraprost sodium was supplied by Toray Industries, Inc., Basic Research Laboratories, Japan. Stock solution of beraprost (10 mg ml^{-1} in saline) was stored at 3°C and diluted with saline just before use. (–)-Phenylephrine hydrochloride (Sigma Chemical Company, U.S.A.) and suxamethonium chloride (Tokyo Kasei, Japan) were also dissolved in saline.

Statistics

Values in different groups were analysed by the one-way analysis of variance followed by wholly-significant-different

Table 1 Effects of beraprost on arterial blood pressure and heart rate

Dose ($\mu\text{g kg}^{-1} \text{min}^{-1}$)	Blood pressure (mmHg)						Heart rate (beats min^{-1})	
	Before	Systolic Max. change*	Before	Mean Max. change*	Before	Diastolic Max. change*	Before	Max. change*
0.1	176 $\pm$ 9	–12 $\pm$ 4	149 $\pm$ 7	–13 $\pm$ 1	132 $\pm$ 8	–14 $\pm$ 2	152 $\pm$ 7	5 $\pm$ 2
1	163 $\pm$ 10	–59 $\pm$ 6**	136 $\pm$ 13	–67 $\pm$ 8*	124 $\pm$ 12	–71 $\pm$ 7**	143 $\pm$ 4	–1 $\pm$ 0.5
								11 $\pm$ 2
								–20 $\pm$ 3*

Data from 6 animals in each group are indicated as means $\pm$ s.e.mean.

* Maximum change during 15 min infusion of beraprost before ischaemia.

** $P < 0.01$, ** $P < 0.001$; significantly different from the corresponding value in the animals treated with the lower dose of beraprost.

Figure 2 Calculation of the residual blood flow during ischaemia. Blood flow was continuously monitored by means of thermocouple electrodes. Blood flow level at the start of drug infusion and that after killing the animal were regarded as 100% and 0%, respectively. Percentage ratio of the hatched area to the area of the square ABCD was calculated as a measure of the integrated residual blood flow during ischaemia.

method (Tukey's *t* test) or control-significant-difference method (Dunnett's *t* test). Differences between the values before and after ischemia were analysed by the paired Student's *t* test. The regression line was analysed by the analysis of covariance. Differences giving $P < 0.05$ were regarded as statistically significant.

Results

Experiment 1

Blood pressure and heart rate As shown in Table 1, beraprost produced a dose-dependent decrease in arterial blood pressure. There was no significant difference in the extent of decrease in systolic, mean and diastolic blood pressure. Change in heart rate induced by beraprost was biphasic. The lower dose of beraprost mainly produced a tachycardia, while the higher dose produced a marked and consistent bradycardia following tachycardia. Additionally, it was confirmed in the preliminary experiment that the effects of the higher dose of beraprost on blood pressure and heart rate disappeared within 30 min after cessation of infusion.

Platelet aggregation Table 2 summarizes the *ex vivo* inhibitory effect of beraprost on ADP-induced platelet aggregation. Platelet aggregation was significantly inhibited by either dose of beraprost. There was no significant difference in the magni-

Table 2 Effect of beraprost on ADP-induced platelet aggregation

Dose ($\mu\text{g kg}^{-1} \text{ min}^{-1}$)	Platelet aggregation (%)		
	Before infusion	During* infusion	% inhibition
0.1	63.6 $\pm$ 4.3	6.1 $\pm$ 3.7*	59.6 $\pm$ 3.6
1.0	64.7 $\pm$ 2.6	18.1 $\pm$ 4.5*	72.7 $\pm$ 6.0

Data from 6 animals in each group are indicated as means $\pm$ s.e.mean.

* Platelet aggregation of blood obtained at 10 to 12 min of infusion.

* $P < 0.001$; significantly different from the value before infusion.

Figure 3 Baroreceptor reflex sensitivity following 5 min global cerebral ischaemia in animals treated with saline (open column), beraprost ($0.1 \mu\text{g kg}^{-1} \text{ min}^{-1}$ i.v.) (hatched column) and beraprost ($1 \mu\text{g kg}^{-1} \text{ min}^{-1}$ i.v.) (closed column). Each group consists of 6 animals. Data are indicated as percentage of the value before ischaemia. Vertical lines indicate s.e. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; significantly different from the value before ischaemia. † $P < 0.05$; †† $P < 0.01$; significantly different from the value in saline-treated animals.

tude of the anti-aggregatory effect of beraprost at either level of infusion.

BRS during the reperfusion period Absolute values of BRS before ischaemia (before drug infusion) in animals treated with beraprost (0.1 and $1 \mu\text{g kg}^{-1} \text{ min}^{-1}$, i.v.) and saline were 2.05 ± 0.28 , 2.28 ± 0.37 and $2.15 \pm 0.22 \text{ mmHg}^{-1}$ ($n = 6$ in each group), respectively. Figure 3 summarizes BRS during the reperfusion period as a percentage of the value before ischaemia.

In the animals treated with the lower dose of beraprost as well as saline, a significant decrease in BRS was observed during the reperfusion period of 60 to 210 min. There was no difference between these two groups in the extent of the decrease in BRS. In contrast, the decrease in BRS was not produced in the animals pretreated with the higher dose of beraprost. BRS in this group was significantly greater than that in saline-treated animals at any period of reperfusion. These results indicate that beraprost ($1 \mu\text{g kg}^{-1} \text{ min}^{-1}$, i.v.) completely protects the central baroreceptor reflex system from 5 min global cerebral ischaemia.

Experiment 2

Correlation between the residual blood flow in the medulla during ischaemia and the post-ischaemic decrease in BRS In the control animals treated with saline (Figure 4) BRS during the reperfusion period correlated linearly with the residual blood flow in the dorsal medulla; smaller residual blood flow during ischaemia produced a more marked decrease in BRS. The correlation in the animals pretreated with beraprost ($1 \mu\text{g kg}^{-1} \text{ min}^{-1}$, i.v.) was also linear. However, the slope of the regression line was significantly smaller than that in the control animals (Figure 4). Mean residual blood flow in the medulla of beraprost-treated and control animals was 39.9 ± 5.7 and $43.6 \pm 6.8\%$ ($n = 12$ in each group), respectively, and there was no significant difference between these values. Additionally, beraprost ($0.1 \mu\text{g kg}^{-1} \text{ min}^{-1}$, i.v.) did not affect the residual blood flow and the post-ischaemic decrease in BRS. These results indicate that prevention by beraprost of the decrease in BRS cannot be ascribed to the reduced degree of ischaemia.

Regional cerebral blood flow (rCBF) in the medulla and the cerebral cortex during reperfusion period Figure 5 summarizes the effects of pretreatment with beraprost on rCBF

Figure 4 Correlation between the residual blood flow in the dorsal medulla during ischaemia (abscissa scale) and baroreceptor reflex sensitivity during the reperfusion period of 60 to 210 min (ordinate scale) in animals pretreated with saline (○) and beraprost ($1 \mu\text{g kg}^{-1} \text{min}^{-1}$ i.v.) (●). Data from 12 animals in each group are plotted. Regression line for open and closed circles are indicated as dotted ($Y = 7.52 + 0.93X$, $n = 30$, $r = 0.775$, $P < 0.001$) and solid ($Y = 76.60 + 0.32X$, $n = 35$, $r = 0.395$, $P < 0.05$) lines, respectively. Difference in the slope of the lines was significant ($P < 0.005$ by analysis of covariance).

Figure 5 Regional cerebral blood flow (rCBF) in (a) the dorsal medulla and (b) the cerebral cortex during the reperfusion period after 5 min global cerebral ischaemia. Open, hatched and closed columns indicate the mean values in animals pretreated with saline, beraprost ($0.1 \mu\text{g kg}^{-1} \text{min}^{-1}$ i.v.) and beraprost ($1 \mu\text{g kg}^{-1} \text{min}^{-1}$ i.v.), respectively ($n = 6$ for each group). Data are given as percentage of the value before ischaemia. Vertical lines indicate s.e. * $P < 0.05$; ** $P < 0.01$; significantly different from the value before ischaemia. †† $P < 0.01$; significantly different from the value in saline-treated animals.

during the reperfusion period of 60 to 180 min; rCBF values are expressed as a percentage of the value before ischaemia (before drug infusion). Absolute pre-drug rCBF values in the medulla of animals treated with beraprost (0.1 and $1 \mu\text{g kg}^{-1} \text{min}^{-1}$ i.v.) and saline were 32.5 ± 6.6 , 26.0 ± 3.6 and $25.3 \pm 3.5 \text{ ml min}^{-1} 100 \text{ g}^{-1}$ ($n = 6$ in each group), respectively. Those in the cerebral cortex were 39.3 ± 5.6 , 41.0 ± 2.3 and $42.3 \pm 2.8 \text{ ml min}^{-1} 100 \text{ g}^{-1}$ ($n = 6$ in each group), respectively. There was no significant difference in these pre-drug values among 3 groups.

In the animals treated with the lower dose of beraprost as well as saline, significant reduction of rCBF was observed in both the medulla and the cerebral cortex at any period of reperfusion. There was no difference in the extent of hypoperfusion between these two groups. Furthermore, the extent of hypoperfusion in the medulla was the same as that in the cerebral cortex. In contrast, a significant reduction of rCBF was not produced in the animals treated with the higher dose of beraprost. In this group, rCBF was significantly higher than that in saline-treated animals at any period of reperfusion. These results indicate that the higher dose of beraprost completely prevents the development of post-ischaemic hypoperfusion syndrome in the medulla as well as in the cerebral cortex.

Discussion

The present study demonstrates a protective effect of beraprost ($1 \mu\text{g kg}^{-1} \text{min}^{-1}$ i.v.) against the deterioration of baroreceptor reflex by 5 min global cerebral ischaemia. The result is consistent with the cerebral protection by PGI_2 and its analogue against ischaemia (Pluta, 1985; 1986; Masuda *et al.*, 1988; Terawaki *et al.*, 1988) or hypoxia/anoxia (Masuda *et al.*, 1986a,b). The aim of the present study was to determine the possible mechanism of the cerebroprotective effect of beraprost in a canine model of cerebral ischaemia.

It has been shown that platelet activation may be enhanced in stroke patients (Shah *et al.*, 1985; Joseph *et al.*, 1988) and experimental models of cerebral ischaemia (Dougherty *et al.*, 1979; Petito, 1979). The activation of platelets involves aggregation resulting in circulatory disturbance by the formation of microthrombi (Obrenovitch & Hallenbeck, 1985), and the release of biologically active substances which may affect vascular smooth muscle tone (Hamberg *et al.*, 1975) or increase vascular permeability to produce brain oedema (Turčáni *et al.*, 1988). Therefore, inhibition of platelet activation may be favourable for prevention of post-ischaemic circulatory and functional disorders in the brain. Although the potent anti-aggregatory effect of PGI_2 and its analogues may be promising in this regard, the present study did not find a close correlation between anti-aggregatory effect of beraprost and its protective effect against the decrease in BRS; only the higher dose produced protection in spite of the similar inhibition of ADP-induced platelet aggregation by the two doses used. The results with the lower dose of beraprost are similar to the previous reports that post-ischaemic infusion of PGI_2 did not improve the circulatory or functional disorders in the brain in spite of the inhibition of platelet aggregation or normalization of ischaemic activation of platelets (Hallenbeck & Furlow, 1979; Van den Kerckhoff *et al.*, 1983), and suggest that the contribution of the anti-aggregatory effect of beraprost to the protection of the central baroreflex system may be little, if any, at least in a canine model of transient global cerebral ischaemia.

Since beraprost produces PGI_2 -like vasodilatation both *in vitro* and *in vivo* (Akiba *et al.*, 1986; Toda, 1988), it is possible that such a vasodilator effect may provide a beneficial effect by increasing cerebral blood flow during ischaemia and reperfusion. In the present study, however, cerebral blood flows in the medulla and the cerebral cortex were rather reduced by

$19.3 \pm 4.3\%$ and $11.6 \pm 3.5\%$ ($n = 12$), respectively, during the infusion of the higher dose of beraprost due probably to a marked decrease in blood pressure. This decrease in cerebral blood flow seemed not to affect cerebral function, because the EEG pattern did not change during infusion. Therefore, it is unlikely that secondary metabolic or functional depression due to a decrease in cerebral blood flow might contribute to the protective effect of beraprost. In this regard, Masuda *et al.* (1986b) also suggested that the protective effect of PGI₂ against cerebral hypoxia in mice was not due to a general depression of the central nervous system.

Furthermore, the residual blood flow in the medulla during ischaemia was not affected by the pretreatment with beraprost, indicating that the protective effect of beraprost cannot be due to the reduction of the extent of ischaemia by increasing collateral blood flow into the brain. These results are consistent with the result that OP-2507, a stable PGI₂ derivative, inhibited the oedema formation after occlusion of the feline middle cerebral artery without affecting cerebral blood flow (Terawaki *et al.*, 1988).

During the reperfusion period, a secondary hypoperfusion state is generally observed. In the present study, however, such a hypoperfusion was not observed in the animals pretreated with the higher dose of beraprost during the reperfusion period of 60 to 180 min. During this period of reperfusion, any direct cardiovascular effect of beraprost can be regarded as negligible, because the preliminary experiment confirmed that the cardiovascular effect as well as anti-aggregatory effect of beraprost disappeared within 30 min after cessation of infusion. Therefore, prevention of the post-ischaemic hypoperfusion by the higher dose of beraprost cannot be ascribed to the direct cardiovascular effect of beraprost, but may be due to a cytoprotective effect on the endothelium, perivascular glial cells, blood constituents or vascular smooth muscle which are suggested to be responsible for the development of post-ischaemic hypoperfusion (Fischer, 1973). However, it is

controversial whether such an inhibition of the post-ischaemic hypoperfusion may primarily contribute to the protection of central baroreflex system, because many investigators have failed to find a causative role of post-ischaemic hypoperfusion in the metabolic, functional or histological damage in several models of transient cerebral ischaemia (Welsh, 1985). Also in a canine model used in the present study, we have demonstrated that the effect of drugs on the post-ischaemic hypoperfusion could be separated from that on the post-ischaemic decrease in BRS (Kurihara *et al.*, 1989b). For example, flunarizine could prevent the decrease in BRS without improving post-ischaemic hypoperfusion, while improvement of cerebral circulation by papaverine did not provide protection of BRS. Furthermore, Kochanek *et al.* (1988) showed in a canine model of multifocal cerebral ischaemia that post-ischaemic administration of PGI₂, indomethacin and heparin produced a sustained improvement of the cerebral circulation, whilst enhancement of the recovery of somatosensory evoked potential was transient. These results suggest that protection of the central baroreflex system by beraprost may be independent of its beneficial effect on post-ischaemic cerebral blood flow.

In conclusion, the present study suggests that the protective effect of beraprost against the decrease in BRS by 5 min global ischaemia may be independent of its anti-aggregatory and vasodilator effects. As has been suggested for the protective effects of PGI₂ or its analogues in myocardial ischaemia (Ogletree *et al.*, 1979; Schrör *et al.*, 1981), hepatic hypoxia (Araki & Lefer, 1980) or hemorrhagic shock (Bitterman *et al.*, 1988), the most likely mechanism of the protection may be a direct cytoprotective effect through membrane stabilization and prevention of a cytotoxic enzyme leakage.

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