Retrograde Perfusion of the Cerebral Vein with Antioxidant LY231617 Reduces Brain Damage in the Rat Focal Ischemia Model

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Abstract

Retrograde perfusion of the cerebral vein (RPCV) with antioxidant LY231617 was evaluated in the focal ischemia model in rats as a new therapeutic route to deliver cytoprotective agents more selectively and efficiently into ischemic brain tissue. Thirty-six Sprague-Dawley rats were divided into Groups A through D. Focal ischemia was induced for 3 hours in the rats, then all groups were treated differently for 2 hours and then sacrificed. Rats in Group A ($n = 10$) served as the control group and was left untreated. Rats in Group B ($n = 10$) received an intravenous infusion of LY231617 (10 mg/kg/hr). Rats in Group C ($n = 6$) received saline (86 μ l/min) through RPCV. Rats in Group D ($n = 10$) received LY231617 (10 mg/kg/hr) in saline (86 μ l/min) through RPCV. The regional cerebral blood flow (rCBF) was measured using [14 C]iodoantipyrine autoradiography, and phorbol 12,13-dibutyrate (PDBu) binding by *in vitro* [3 H]PDBu autoradiography. Ischemic brain damage was assessed quantitatively after staining with cresyl violet and Luxol fast blue. Rats in Group D showed significantly higher rCBF (41–400%, $p < 0.05$) in the ischemic cortical and subcortical areas, and a significant reduction (66%, $p < 0.01$) in the total volume of ischemic damage and reduction of PDBu binding ($p < 0.05$) in the lateral striatum of the ischemic hemisphere, as compared to the rats in Groups A–C. RPCV with antioxidant LY231617 achieves a more beneficial effect on focal ischemic tissue than regular systemic administration.

Key words: ischemic brain damage, phorbol-dibutyrate binding, retrograde perfusion, cerebral vein, antioxidant, cerebral blood flow

Introduction

Experimental studies of ischemic brain injury have demonstrated a direct relationship between excess production of free radicals^{3,15,16,21,36,37)} and the release of excitatory amino acids which are induced in a series of molecular events linked to ischemic neuronal cell death.^{27,30)} Experimental data have also indicated that various free radical scavengers or antioxidants significantly reduce damage induced by ischemia.^{1,2,7,9,13,17,19,22,32,45)} However, systemic

administration of free radical scavengers or antioxidants was only of benefit if started before or shortly after the onset of cerebral ischemia.^{1,2,7,9,13,17,19,22,32)} Therefore, the time window for effective systemic administration of these agents is too narrow.⁵⁾

We previously developed a method of retrograde perfusion of the cerebral vein (RPCV) for delivering a cytoprotective agent into ischemic tissue *via* a cerebral vein.^{14,42,43,46)} The cerebral veins have no valves, undergo minimal atherosclerotic changes,^{38,39)} and are provided with rich micro-collateral chan-

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Fig. 1 Chemical structure of antioxidant LY231617.

nels.⁴⁾ RPCV is a more selective and efficient delivery route into the ischemic tissue than regular systemic administration.^{42,43)} Our recent study showed that RPCV of the calcium channel blocker verapamil achieved a significant improvement in ischemic brain in the focal ischemia for 3 hours in rats.¹⁴⁾

The new antioxidant LY231617 (2,6-bis(1,1-dimethylethyl)-4-[(1-ethyl)amino]methylphenol hydrochloride, Fig. 1) inhibits iron-dependent lipid peroxidation and blocks a key enzyme activity, that of the arachidonic acid cascade.^{8,9)} In addition, this drug easily penetrates the blood-brain barrier and produces a vasodilating effect.⁹⁾

The present study compared the therapeutic efficacy of RPCV with systemic administration of LY231617 (10 mg/kg/hr) using the focal cerebral ischemia model in rats. The effectiveness of the treatment was evaluated by quantitative double-tracer autoradiography with [¹⁴C]iodoantipyrine (IAP) to measure regional cerebral blood flow (rCBF)³⁵⁾ and [³H]phorbol 12,13-dibutyrate (PDBu) to measure the activity of PDBu binding.^{28,44)} The volume of ischemic damaged tissue was also measured using a modified method of Osborne *et al.*²⁹⁾

Materials and Methods

The focal ischemia model was prepared in 36 Sprague-Dawley rats. Details of the surgical preparation and monitoring of physiological parameters have been previously published.^{14,42,43)} In brief, anesthesia was induced through inhalation of 2.0–2.5% halothane for 3–5 minutes and maintained by spontaneous respiration of 0.5–1.0% halothane in room air through a mask. Polyethylene catheters were placed in the femoral artery for the monitoring of blood pressure, blood gases, hematocrit, blood glucose concentration, and radioisotope content, and in the femoral vein for administration of radioisotopic tracers and drugs. A small craniectomy was made in the left subtemporal region with a dental drill. After electrocoagulation of all len-

ticulostriate branches of the middle cerebral artery (MCA) under a microscope, the left MCA was occluded by a Zen clip (Ohwa Tsusho Ltd., Tokyo) proximal to the lateral striate arteries, using a modified method of Tamura *et al.*^{14,41)} The MCA remained occluded by the clip until decapitation. After occlusion of the left MCA, a small craniectomy was performed at the left inferior posterior part of the squamosal bone for the cannulation of the inferior cerebral vein in rats used for RPCV. A tapered PE-10 polyethylene catheter was cannulated backwards into the inferior cerebral vein. After completion of the surgical procedure, all rats were immobilized using loose-fitting plaster casts, and halothane was discontinued to remove the effect of general anesthesia. All rats recovered completely from general anesthesia at least 1 hour before decapitation. Lidocaine hydrochloride was used for local anesthesia of the surgical wounds during the experiments.

Three hours after occlusion of the MCA, the rats were divided into four groups. Rats in Group A (n = 10) served as the control group and received no agents. Rats in Group B (n = 10) received an intravenous infusion of LY231617 (10 mg/kg/hr) (Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind., U.S.A.) in saline (86 μ l/min) into the femoral vein. Rats in Group C (n = 6) received saline (86 μ l/min) through RPCV, and rats in Group D (n = 10) received LY231617 (10 mg/kg/hr) in saline (86 μ l/min) through RPCV, both through the inferior cerebral vein. The saline was delivered using an infusion pump over a period of 2 hours. During the 2 hours of RPCV, the infusion pressure of LY231617 solution was kept constant at 150 mmHg.³⁹⁾ The body temperature was maintained at $37 \pm 0.5^\circ\text{C}$ with a heating lamp. Systemic blood pressure and blood gases were checked regularly and maintained within physiological ranges. Hematocrit and blood glucose concentration were determined intermittently using arterial blood samples.

Four hours and 59 minutes after occlusion of the left MCA, all rats received an injection in the femoral vein of [¹⁴C]IAP (30 μ Ci) (DuPont Canada Inc., Mississauga, Ontario, Canada; specific activity 50 mCi/mmol) in normal saline (1 ml) over a period of 1 minute. A 20 μ l arterial blood sample was drawn every 5 seconds during injection of the tracer to determine the ¹⁴C radioactivity in the arterial plasma. The animals were decapitated 1 minute after the start of [¹⁴C]IAP infusion. The brains were quickly removed and frozen in liquid Freon-12 (Histo Freeze, Fisher Scientific Co., Pittsburgh, Pa., U.S.A.). The frozen

brains were sliced into 20 μm thick sections by a cryostat (-22°C). The sections were mounted on a microscopic slide and rapidly dried on a hot plate for autoradiography. Alternate sections were used for [^{14}C]IAP and [^3H]PDBu autoradiography experiments.

The brain sections for [^{14}C]IAP autoradiography were exposed to Kodak SB-5 films (Rochester, N.Y., U.S.A.) for 1 week with Carbon-14 Standards (American Radiolabeled Chemicals Inc., St. Louis, Mo., U.S.A.). Densitometric measurements of the autoradiograms were performed with a digital image analyzer (The Image Calculator; McGill University, Montreal, Canada). Mean values for the ^{14}C tissue radioactivities were obtained by measuring the optical density on autoradiograms of the same locus on three consecutive brain slices as designated by the rat brain atlas. rCBF was calculated using the operational equation of Sakurada *et al.*³⁵⁾ A tissue-blood partition coefficient of 0.8 was used.

PDBu binding activity was determined by the *in vitro* receptor binding method using [^3H]PDBu autoradiography of Worley *et al.*⁴⁴⁾ and Onodera *et al.*²⁸⁾ in Groups A, B, and D. The slices were divided into two groups: one for [^3H]PDBu autoradiography and the other for the verification of [^{14}C]IAP washout. All slices were mounted on gelatin-coated microscopic slides and soaked twice in ice cold buffer (50 mM Tris HCl/pH 7.7, 100 mM NaCl, 1 mM CaCl_2) for 5 minutes to reduce the [^{14}C]IAP activity to less than 1%. The slices for [^3H]PDBu autoradiography were incubated in 25°C buffer with 2.5 nM [^3H]PDBu (DuPont Canada Inc.; specific activity 20 Ci/mmol) for 1 hour, washed twice in ice cold buffer for 2 minutes, and then briefly rinsed in ice cold distilled water. Preliminary experiments verified that specific [^3H]PDBu binding reached a plateau during these incubation periods. Non-specific labeling was assessed by adding 1 μM PDBu to the incubation solution. All specimens were imme-

diately dried under a stream of cold air and exposed to Hyperfilm- ^3H (Amersham, Arlington Heights, Ill., U.S.A.) for 1 week with Tritium Standards (American Radiolabeled Chemicals Inc.). Densitometric measurements of the autoradiograms were performed in the same manner as for [^{14}C]IAP autoradiography. Optical densities of [^3H]PDBu binding were measured and converted to fmol/mg tissue based on the values for the Tritium Standards.

Measurement of the volume of ischemic brain used frozen coronal tissue sections of 20 μm thickness obtained from the areas adjacent to those used for the autoradiographic studies. The most posterior coronal section was taken 3.2 mm anterior to the occipital pole, and six more sections were taken at 1.28 mm intervals toward the anterior side. These sections were then fixed in 10% formaldehyde solution for over 24 hours and stained by a combined method of cresyl violet and Luxol fast blue.^{14,29)} The unstained ischemic areas were manually outlined on the digitized images of these sections with a digital image analyzer (The Image Calculator). Any indistinct border of an ischemic area was verified under light microscopic examination for neuronal ischemic damage.²⁹⁾ The total volume of early ischemic brain damage was determined by the integration of the seven areas with the distance between neighboring sections.

All data were expressed as mean $\pm$ SD. The statistical analysis of all data was performed using a one-way analysis of variance, followed by Tukey's intergroup comparison test. A p-value of <0.05 was considered significant.

Results

There were no significant differences in blood pressure, blood gases, body temperature, hematocrit, and blood glucose concentrations during

Table 1 Physiological variables during experiments

	Group A (n = 10)	Group B (n = 10)	Group C (n = 6)	Group D (n = 10)
MABP (mmHg)	122 $\pm$ 6	124 $\pm$ 8	118 $\pm$ 6	120 $\pm$ 10
PaCO ₂ (torr)	40.7 $\pm$ 4.1	42.1 $\pm$ 4.7	41.2 $\pm$ 5.3	42.3 $\pm$ 3.9
PaO ₂ (torr)	100 $\pm$ 7	107 $\pm$ 12	109 $\pm$ 10	105 $\pm$ 13
pH	7.41 $\pm$ 0.05	7.41 $\pm$ 0.03	7.39 $\pm$ 0.02	7.40 $\pm$ 0.03
Ht (%)	46.4 $\pm$ 0.9	45.6 $\pm$ 1.6	45.5 $\pm$ 1.7	45.6 $\pm$ 1.7
Temp ($^{\circ}\text{C}$)	37.0 $\pm$ 0.2	37.1 $\pm$ 0.3	37.1 $\pm$ 0.1	37.0 $\pm$ 0.4
BG (mg/dl)	188 $\pm$ 35	166 $\pm$ 37	173 $\pm$ 28	177 $\pm$ 19

All values are mean $\pm$ SD. BG: plasma glucose level, Ht: hematocrit, MABP: mean arterial blood pressure, Temp: body temperature.

Table 2 Changes of rCBF in rats

Structure	Group A (n = 10)		Group B (n = 10)		Group C (n = 6)		Group D (n = 10)	
	Left	Right	Left	Right	Left	Right	Left	Right
Frontal cortex	71 ± 17	163 ± 27	82 ± 20	163 ± 23	65 ± 19	168 ± 27	100 ± 22 [‡]	171 ± 21
Sensorimotor cortex	18 ± 16	139 ± 22	20 ± 18	140 ± 13	18 ± 15	161 ± 21	60 ± 21 ^{##}	150 ± 23
Anterior parietal cortex	15 ± 18	133 ± 23	10 ± 18	139 ± 15	18 ± 12	163 ± 20	47 ± 18 [#]	148 ± 19
Posterior parietal cortex	19 ± 24	136 ± 20	5 ± 6	136 ± 16	20 ± 12	160 ± 16	51 ± 18 [#]	141 ± 20
Lateral caudate	13 ± 17	140 ± 24	42 ± 42	141 ± 17	20 ± 24	134 ± 28	57 ± 29 [*]	134 ± 19
Medial caudate	54 ± 27	133 ± 27	75 ± 38	132 ± 19	58 ± 11	128 ± 31	109 ± 20 [‡]	132 ± 19
Posterolateral portion of caudo-putamen	13 ± 13	123 ± 18	34 ± 29	127 ± 16	18 ± 16	123 ± 25	65 ± 32 [‡]	120 ± 20

All values are mean ± SD (ml/100 g/min). * $p < 0.05$, significant difference from Group A. [‡] $p < 0.05$, significant difference from Groups A and C. [#] $p < 0.05$, ^{##} $p < 0.01$, significant difference from Groups A–C.

Fig. 2 Autoradiograms of [¹⁴C]IAP in the coronal section of the sensorimotor cortex and in the region of the caudate nucleus. Group D rats (D) showed a marked improvement of rCBF compared to rats in Groups A (A), B (B), and C (C).

transvenous retrograde perfusion of the brain and administration of LY231617 between the groups of rats (Table 1).

The rCBF in Groups B and C showed no significant difference compared to Group A (Table 2). In Group D, rCBF increased significantly in the ischemic cerebral cortices (frontal cortex: 41%, $p < 0.05$; sensorimotor cortex: 233%, $p < 0.01$; anterior parietal cortex: 213%, $p < 0.05$; posterior parietal cortex: 168%, $p < 0.05$) and in the ischemic subcortical areas (caudate nucleus: 220%, $p < 0.05$; posterolateral portion of caudo-putamen: 400%, $p < 0.05$) compared to the corresponding areas of Group A (Table 2). Figure 2 shows representative

autoradiograms for all groups.

[³H]PDBu binding in the ischemic area of Groups A and B was 12–24% higher than the homologous area of the non-ischemic hemisphere (Table 3). In Group D, [³H]PDBu binding in the ischemic area was similar to the homologous area of the non-ischemic hemisphere (Table 3). [³H]PDBu binding in the posterolateral portion of the caudo-putamen of the ischemic hemisphere of Group D was reduced significantly (34–39%, $p < 0.05$) compared to Groups A and B. [³H]PDBu in the remainder of the ischemic hemisphere of Group D was moderately reduced (16–35%), but not significantly, compared to Groups A and B (Table 3). Figure 3 shows representative autoradiograms for all groups.

The volume of ischemic brain damage in Groups B and C showed no significant difference compared to Group A (Table 4). Group D rats showed a reduction of 66% in the total volume of ischemic damaged tissue ($p < 0.01$) compared to Group A (Table 4). The area of ischemic damaged tissue was reduced significantly at the sensorimotor level (69%, $p < 0.05$) and the parietal level (59%, $p < 0.05$) compared to Group A (Table 4).

Discussion

The inferior cerebral vein in rats, which collects venous blood from the centroparietal temporal regions,⁴⁾ can be used as a retrograde route to deliver cytoprotective agents to ischemic brain tissue. The cerebral vein can tolerate up to 150 mmHg retrograde perfusion pressure, without any change in blood-brain barrier permeability.⁴³⁾ Our previous study demonstrated that RPCV, with the calcium channel blocker verapamil, resulted in a significant increase of rCBF in the ischemic cerebral cortices

Table 3 Concentration of [³H]PDBu binding in rats

Structure	Group A (n = 10)		Group B (n = 10)		Group D (n = 10)	
	Left	Right	Left	Right	Left	Right
Sensorimotor cortex	843 ± 263	751 ± 265	927 ± 252	782 ± 241	709 ± 150	722 ± 293
Anterior parietal cortex	1078 ± 471	956 ± 427	970 ± 217	797 ± 156	704 ± 265	723 ± 218
Lateral caudate	925 ± 296	780 ± 263	911 ± 217	733 ± 182	700 ± 184	741 ± 267
Posterolateral portion of caudo-putamen	1178 ± 559	1027 ± 441	1079 ± 247	875 ± 173	713 ± 227†	717 ± 297

All values are mean ± SD (fmol/mg). †p < 0.05, significant difference from Groups A and B.

Fig. 3 Autoradiograms of [³H]PDBu binding in the coronal section of sensorimotor cortex and caudate nucleus region. Rats in Group D (C) showed a significant reduction (34–39%) of PDBu binding in the ischemic lateral caudate nucleus compared to rats in Groups A (A) and B (B).

Table 4 Changes in area and volume of ischemic damage in the ischemic hemisphere

Distance from occipital lobe	Group A (n = 10)	Group B (n = 10)	Group C (n = 6)	Group D (n = 10)
9.56 mm (frontal cortex, mm ²)	15.6 ± 7.8	9.8 ± 5.2	12.8 ± 4.2	4.9 ± 2.8*
8.28 mm (sensorimotor cortex, mm ²)	23.2 ± 8.5	15.8 ± 7.7	19.2 ± 6.7	7.2 ± 3.2‡
7.00 mm (anterior parietal cortex, mm ²)	18.3 ± 6.1	18.1 ± 8.3	17.0 ± 5.1	7.8 ± 2.7†
5.72 mm (posterior parietal cortex, mm ²)	13.4 ± 3.3	15.0 ± 6.4	12.6 ± 3.0	5.3 ± 3.1#
Total damaged volume (mm ³)	106.1 ± 26.3	99.7 ± 35.6	104.0 ± 24.2	36.6 ± 9.2##

All values are mean ± SD. *p < 0.01, significant difference from Group A. †p < 0.01, significant difference from Groups A and B. ‡p < 0.05, significant difference from Groups A and C. #p < 0.05, ##p < 0.01, significant difference from Groups A–C.

and subcortical areas, with a reduction of ischemic damaged tissue in these areas.¹⁴⁾ We also obtained similar results using a bypass between an extracerebral artery and a cerebral vein for retrograde verapamil infusion into the focal ischemic tissue without any increase of the superior sinus pressure in rats.⁴⁶⁾ The present study showed that RPCV with LY231617 achieved a significant improvement in both rCBF and ischemic damaged tissue in the rat focal ischemia model. These findings strongly suggested that RPCV enhanced the therapeutic effect of the cytoprotective agents although direct comparison of the effect on cerebral ischemia between the

cytoprotective agents cannot be made.

Oxygen radicals cause brain edema^{3,19,45)} and pronounced vascular effects, including increased vascular permeability and endothelial injury.^{16,18,36,37)} Furthermore, oxygen free radicals present endothelium-dependent relaxation by endothelium-derived relaxing factor, which has recently been identified as either nitric oxide or a closely related compound.^{11,23,24,26,33,34)} Lipid peroxidation and the arachidonic acid cascade also cause aberrant vascular reactions.^{16,36,37)} These harmful effects to the cerebral vessels result in decreased CBF and brain edema. Beneficial effects of free radical scavengers

on CBF have been demonstrated in ischemia models.^{7,13,22,25)} The favorable effect of antioxidants on CBF is achieved by direct action upon endothelium and smooth muscle, and also by a reduction of brain edema.^{1,19,45)} Evidence from several experiments indicates that the phorbol ester receptor is identical to protein kinase C (PKC).^{6,26,28,44)} The activity of PKC can be assessed by binding of the phorbol ester receptor ligand [³H]PDBu.⁴⁴⁾ Activation of PKC causes contraction of the arterial smooth muscle,^{20,40)} which also affects CBF. In the early phase of biochemical events occurring during cerebral ischemia, PKC is activated by diacylglycerol and is important in the postischemic modulation of neuronal cell death. Excitatory amino acids activate the phosphatidylinositol cycle through the activation of phospholipase C resulting in generation of diacylglycerol which activates PKC,¹²⁾ and also activate the arachidonic acid cascade system.¹⁰⁾ Pellegrini-Giampietro *et al.* have reported that exogenously generated free radicals increase the release of excitatory amino acids from rat hippocampal slices,^{30,31)} and that various free radical scavengers reduced *in vitro* ischemia-induced excitatory amino acids release.³¹⁾

Our study revealed that PDBu binding in the ischemic area of untreated rats was increased by 12–19%, as compared to the homologous area in the non-ischemic hemisphere. RPCV with LY231617 reduced the PDBu binding in the ischemic hemisphere significantly compared to the ischemic hemisphere in untreated and systemically treated rats. In addition, the PDBu binding values in the ischemic lateral caudate nucleus revealed no significant difference from the non-ischemic hemispheres of all rats. LY231617 may restrain the vicious circle between free radicals and excitatory amino acids as mentioned above. A recent study has also indicated that LY231617 is effective in reducing damage in the hippocampal CA1 subfield in rats subjected to 30 minutes of four-vessel occlusion with reperfusion.⁹⁾

Our present results indicate that RPCV delivery of the antioxidant LY231617 provides significant reduction of tissue damage in the 3-hour focal ischemia rat model, which is not achieved through regular intravenous administration of LY231617.

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