Published in final edited form as:

Neuroscience. 2015 April 16; 291: 250–259. doi:10.1016/j.neuroscience.2015.02.001.

Neuroprotective Activity of (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (4*R*) *in vitro* and *in vivo* in Rodent Models of Brain Ischemia

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Abstract

(1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol (4*R*) is a precursor to key flavor ingredients in leaves of *Nicotiana* species. The present study shows 4*R* decreased brain damage in rodent ischemic stroke models. The 4*R*-pretreated mice had lower infarct volume ($26.2 \pm 9.7 \text{ mm}^3$) than those in control groups (untreated: $63.4 \pm 4.2 \text{ mm}^3$, DMSO: $60.2 \pm 14.2 \text{ mm}^3$). The 4*R*-posttreated rats also had less infarct volume ($120 \pm 65 \text{ mm}^3$) than those in the rats of DMSO group ($291 \pm 95 \text{ mm}^3$). The results from *in vitro* experiments indicate that 4*R* decreased neuro2a cells (neuroblastoma cells) apoptosis induced by oxygen glucose deprivation (OGD), and improved the population spikes (PSs) recovery in rat acute hippocampal slices under OGD; a phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin, abolished the effect of 4*R* on PSs recovery. Furthermore, 4*R* also inhibited monocyte adhesion to bEND5 cells (murine brain-derived endothelial cells) and upregulation of intercellular adhesion molecule-1 (ICAM-1) induced by OGD/reoxygenation (OGD/R), and restored the p-Akt level to pre-OGD/R values in bEND5 cells.

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Disclosure/conflict of interest The authors declare no conflict of interest.

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In conclusion, the present study indicates that 4R has a protective effect in rodent ischemic stroke models. Inhibition of ICAM-1 expression and restoration of Akt phosphorylation are the possible mechanisms involved in cellular protection by 4R.

Keywords

4R cembranoid; oxygen-glucose deprivation; inflammation; intercellular adhesion molecule-1; ischemia; neuroprotection

1. INTRODUCTION

The neuronal death after ischemic stroke cannot be well controlled because of a lack of efficient therapeutic strategies. Therefore, there is an urgent need for developing effective treatments for stroke. Targeting post-ischemic inflammation and anti-apoptotic pathways may give a promising approach for therapy of ischemic stroke.

Post-ischemic inflammation plays an important role in neuronal injury and death during brain ischemia, and is considered as an important player in the secondary brain injury. Stroke patients with inflammatory problems exhibit poorer outcomes after ischemic stroke. The up-regulation of cellular adhesion molecules (CAMs), such as vascular cell adhesion molecule (VCAM-1), ICAM-1 and E-selectin, enhances recruitment of monocytes and other inflammatory cells into the brain tissue (Kalogeris et al., 1999; Khan et al., 1995; Marui et al., 1993; Weber et al., 1995). The recruitment of immune cells across the blood brain barrier (BBB) play an important role in the pathology of ischemic stroke and this cellular response is closely related to the BBB damage. Because adhesion of monocyte and lymphocytes to endothelial cells is the first step of vascular-neuronal inflammation, inhibition of adhesion and recruitment of inflammatory cells will have a beneficial effect on the outcome of ischemic stroke. Anti-inflammatory approaches have successfully decreased infarct size and improved neurological deficit in animal stroke models (Wang, 2005; Zhang et al., 2003). Particularly, ICAM-1 antibody (Enlimomab) was shown to reduce brain damage in the animal ischemic stroke model by inhibition of post-ischemic inflammation, but clinical use of this anti-ICAM-1 antibody failed due to host immune responses against the murine IgG (Furuya et al., 2001). In addition to inflammation, the anti-apoptotic Akt pathway is another target for stroke therapy, since Akt plays a critical protective role against ischemia/reperfusion injury. After phosphorylation by PI3K, the phosphorylated Akt (pAkt) inhibits a number of pro-apoptotic molecules such as GS3-K (Koh et al., 2006) and activates other anti-apoptotic pathways resulting in the inhibition of cell apoptosis. Beside its anti-apoptotic activity, pAkt was also shown to have some anti-inflammatory activities (Guha and Mackman, 2002; Luyendyk et al., 2008; Tyagi et al., 2010). Consequently, a therapeutic approach for brain ischemia logically would aim to suppress neuronal apoptosis by inhibition of inflammation and activation of the Akt signaling pathways.

Cembranoids are 14-carbon cembrane ring cyclic diterpenoids (Hann et al., 1998). Our group discovered that certain cembranoids are noncompetitive inhibitors of nicotinic acetylcholine receptors (nAChRs) (Eterovic et al., 1993; Ferchmin et al., 2001; Hann et al., 1998). As one of cembranoids, 4R (Fig. 1) has been reported to protect acute hippocampal

slices against the neurotoxicity of NMDA (Ferchmin et al., 2005) and of paraoxon, a toxic organophosphorous compound (Eterovic et al., 2011). In addition, specific analogues of 4R were found to protect the hippocampal slices against the neurotoxicity of diisopropylfluorophosphate, a surrogate of the chemical war nerve agent sarin (Eterovic et al., 2013). 4R neuroprotective activity was found to be mediated by activation of the PI3K/Akt antiapoptotic cascade (Ferchmin et al., 2005). In vivo experiments demonstrated that 4R ameliorated brain degeneration caused by diisopropylfluorophosphate when injected 24 hours after organophosphate poisoning (Ferchmin et al., 2014). For the first time, the present study demonstrated that 4R protected brain against ischemic stroke-induced injury in rodents.

2. Materials and Methods

2.1. Permanent middle cerebral artery occlusion (pMCAO) in mouse and transient MCAO (tMCAO) in rats

All animal procedures were approved by the Institutional Animal Care and Use Committees at University of Cincinnati and Universidad Central del Caribe and they complied with pertinent NIH guidelines for care and use of animals. The pMCAO in mice: CD1 female mice (body weight 28~32 g) supplied by Charles River (Wilmington, MA, USA) were kept under standardized light/dark (12 hours), temperature (22°C), and humidity (70%) conditions, with rodent chow and water available ad libitum. The MCAO was induced as described previously (Hao et al., 2008). A dose of 4R (6mg/kg, produced in Dr. Khalid El Sayed's lab) in 30 µl DMSO or equal volume of DMSO or phosphate buffer solution (PBS) was administered into mice by intraperitoneal (i.p.) injection 10 min before MCAO. At 24 hours after pMCAO, mice were euthanized by decapitation; brain coronal sections (1mm) were cut on mouse brain slicer. The tMCAO in rats: male Sprague-Dawley rats (250~300g) from UCC colony, kept under standardized light/dark (12 hours) and temperature (25°C) conditions, were anesthetized with isoflurane and N₂O, and subjected to transient MCAO (tMCAO) for 1 hour as described in (Ford et al., 2006). Briefly, a surgical monofilament nylon suture (Doccol) was inserted 18 to 20 mm from the bifurcation of the common carotid artery to occlude the MCA. After 1 hour of ischemia, the nylon suture was withdrawn to allow blood reperfusion. 6 mg/kg 4R in DMSO, or the same volume of DMSO, was administered by s.c. injection 1 hour after reperfusion. The brain was removed 24 hours after induction of ischemia and coronal brain sections (3 mm) were prepared. All brain sections were stained with 2,3,5-triphenyltetrazolium chloride (TTC) as described previously (Mdzinarishvili et al., 2005). After TTC staining, images were acquired by digital camera (Nikon), and areas of the infarct regions were quantified for each slice using Image J analysis software (NIH, Rockville, MD, USA). Infarct volumes in mice or rats were calculated as described previously (Hao et al., 2008).

2.2. Cell culture

The neuro-2a cell line (ATCC) and bEND5 endothelial cell line were grown in DMEM media (Mediatech Inc.) supplemented with 10% (v/v) FBS (fetal bovine serum) (Atlanta Bio Inc.), 1 mM sodium pyruvate, 4 mM L-glutamine, 1% (v/v) non-essential amino acids, 1%

(v/v) 100 IU/ml penicillin 100 mg/ml streptomycin (ATCC). The cell lines were maintained at 37°C, 5% CO₂ and 95% relative humidity.

2.3. *In vitro* endothelial inflammatory model

After incubation with 50 µg/ml of TNF-α for 4 hours, the bEND5 cells were treated with 4 µM, 8 µM, 16 µM of 4R or the same volume of DMSO respectively for 24 hours. The bEND5 cells were then lysated and the protein was extracted for western blotting for detection of ICAM-1 and p-Akt expressions with appropriate antibodies. β-Actin antibody (Cell Signaling Technology, Inc.), ICAM-1 and p-Akt antibodies (Santa Cruz Biotechnology, Inc.).

2.4. Oxygen-glucose deprivation (OGD)

Hypoxia was induced by placing cells in a sealed chamber (Billups-Rothenberg Inc.) at 37°C, and introducing a flush with 95% N₂/5% CO₂ gas until the complete removal of O₂. Aglycemia was performed by using RPMI 1640 medium without D-glucose. In experiments related to ischemia without reperfusion, the cells were kept under OGD condition for 6 h. For experiments using OGD and reperfusion (OGD/R) conditions, the cells were kept in OGD for 6 h followed by normal growth condition and normoxic atmosphere for an additional 4 h.

2.5. Ischemia model in acute hippocampal slices

Acute rat hippocampal slices were prepared and the population spikes (PSs) measured as described previously (Ferchmin et al., 2005). Briefly, for each individual experiment, about 30 slices from the hippocampi of two rats were distributed equally among the three lanes of the chamber, superfused with artificial cerebrospinal fluid (ACSF) (Ferchmin et al., 2005) saturated with 95% O₂ and 5% CO₂. Following the acquisition of the initial PS, the slices were exposed for 10 minutes to OGD, which consisted in changing the gaseous phase to 95% N₂ and 5% CO₂ and removing glucose from ACSF. After 10 min of OGD, the gaseous phase was switched back to normal and the slices were washed for 30 minutes with regular ACSF. Then, the slices were superfused for 1 hour to ACSF containing one of the following: 1. 0.1% v/v DMSO; 2. 10µM 4R in 0.1% DMSO; 3. 10nM wortmannin plus 10µM 4R, where wortmannin was applied five minutes before 4R; or 4. 10nM wortmannin. The area of the initial PS was compared with the final PS elicited by the same stimulus recorded from the same position in the hippocampal slice. The results are expressed as the %Recovery of PS (100 * final PS/initial PS).

2.6. Apoptosis measurement

The neuro-2a cells were seeded in 6-well plates and divided into two groups after 80% confluence. The control group was kept in normal condition. The other groups were maintained in OGD conditions. The cells were harvested and centrifuged after 6 h OGD. Apoptosis assay was performed using Annexin-V/propidium iodide (PI) cell apoptosis kit (BD biosciences, Inc.) according to the manufacturer's instructions. Briefly, the pellets were suspended in 200 µl binding buffer, and then 5 µl of Annexin-V-PE was added to cell suspension and incubated at room temperature for 10 min in the dark. After washing with

binding buffer twice, 5 μ l of PI solution was added into suspension and incubated for 5 min. Finally, another 300 μ l of binding buffer was added to the mixture, and was analyzed by flow cytometry (Becton Dickinson). At least 10,000 cells were analyzed in each sample.

2.7. Monocyte adhesion assay

The different groups of bEND5 cells (4 μ M, 8 μ M, 16 μ M of 4R treatment or DMSO control groups) were subjected to OGD/R as described early. For the adhesion assay, U937 cells (a monocyte cell line, from ATCC) were labeled with 2',7'-Bis- (2-Carboxyethyl)-5- (And-6)-carboxyfluorescein (BCECF-AM, 5 mg/ml) for 30 minutes at 37°C. After washed with PBS the U937 cells were resuspended in serum-free media. The bEND5 cells then were co-cultured with BCECF-AM-labeled U937 cells (10^6 cells/well) for 30 minutes at 37°C. Non-adhering U937 cells were removed, and the bEND5 cells were washed with phosphate-buffered saline (PBS). Then cells were lysed in 0.1% Triton X-100 in 0.1M Tris-HCl (pH7.4). Fluorescence (F) was measured with a microplate fluorescence reader using excitation at 492 nm and emission at 535 nm. The monocyte adhesion was calculated as: Adhesion (%) = $100 \times F_{\text{sample}} / F_{\text{total}}$ (fluorescence intensity of 10^6 cells).

2.8. Statistical analysis

The data were statistically analyzed using Sigma-Plot software (Systat Software, Inc.). Statistical analysis was performed by ANOVA, followed by post-tests (Dunett's test or Newman-Keuls) for comparison of experimental groups vs. control conditions. $p < 0.05$ indicates statistical significance.

3. RESULTS

3.1. 4R treatment reduces infarction size in brain ischemic stroke in mouse and rats

Cerebral blood flow (CBF) was monitored using Laser-Doppler flowmetry as described previously (Mdzinarishvili et al., 2005). All groups of animals had comparable reductions in CBF to 25% or less of baseline after induction of pMCAO. There are no significant changes in CBF among the 4R treated and control groups (Fig. 2A). When mice were treated with 4R at a dose of 6mg/kg of body weight 10 min before pMCAO, the infarct sizes were significantly reduced compared with those in the mice of the control groups. 4R treatment reduced the extent of brain damage as reflected in a reduced infarct volume of 26.2 ± 9.7 mm³, while the infarct volume was 63.4 ± 4.2 mm³ in animals that received no treatment (PBS). There was no significant effect of vehicle, as indicated by a corresponding volume of 60.2 ± 14.2 mm³ in the DMSO group. The neuroprotective effect of 4R was highly significant (Fig. 2B, $p < 0.01$).

We also evaluated the post-treatment effect of 4R on infarction size in the rat tMCAO. A dose of 6mg/kg of 4R significantly decreased the infarct size in tMCAO when injected at 1 hour after reperfusion. The infarct volume in DMSO treated rats was 291 ± 95 mm³ and 120 ± 65 mm³ in 4R treated rats (Fig. 2E, $p < 0.01$). Also, the 4R treatment decreased the body weight loss during the first 24 hours after tMCAO in rats, which was $12.6\% \pm 3.7\%$ compared with the body weight loss of $19.52\% \pm 1.35\%$ in DMSO group (Fig. 2D).

3.2. 4R ameliorated the OGD-induced damage in rat acute hippocampal slice

Furthermore, to address the mechanism of 4R's action we have performed experiments with rat acute hippocampal slices subjected to OGD. A 10-minute exposure to OGD caused a significant reduction of PS to $18.9 \pm 2.6\%$ of the initial value. However, when OGD was followed by incubation with $10 \mu\text{M}$ 4R, the final PS was increased to $52.1 \pm 3.9\%$ of the initial value. 10 nM wortmannin, a PI3 kinase inhibitor, completely blocked the effect of 4R on the recovery of PS, and wortmannin itself did not have any effect on PS recovery (Fig. 3).

3.3. Effect of 4R on the expression of ICAM-1 in bEND5 cells after OGD

We first identified the concentration of 4R required for an optimal response in decreasing the basal expression of ICAM-1 in bEND5 cells. In the presence of 4R at $4 \mu\text{M}$, $8 \mu\text{M}$, and $16 \mu\text{M}$, the expression of ICAM-1 was significantly inhibited in bEND5 cells as evaluated by western blotting. This inhibitory effect was dose dependent, since ICAM-1 expression decreased to $68.0\% \pm 6.5$, $30.0\% \pm 5.5$, $17.3\% \pm 8.9$ as compared with controls (100%) (Fig. 4A, 4B). The ability of 4R in suppression of ICAM-1 expression was then investigated by the experiments in an inflammatory state, for which we added 50 ng/ml of $\text{TNF-}\alpha$ to the bEND5 cells. We observed that $\text{TNF-}\alpha$ caused an up-regulation of ICAM-1 to $151.3\% \pm 25.8$ of control in DMSO group and this up-regulation of ICAM-1 was reverted to basal levels with value of $91.4\% \pm 19.2$ of control after incubation with $8 \mu\text{M}$ 4R (Fig. 4D, 4E). To determine the potential of 4R in decreasing the expression on ICAM-1 in bEND5 cells in ischemia-like condition, OGD/R condition, we exposed the bEND5 cells to OGD/R. The results showed that ICAM-1 level was increased to $146.8\% \pm 11.1$ after OGD/R in the cells with normal media, and to $193.4\% \pm 43.9$ in cells submitted to OGD/R plus DMSO. The expression of ICAM-1 significantly decreased to basal levels with value of $98.4\% \pm 18.4$ after $8 \mu\text{M}$ of 4R treatment (Fig. 5A and 5B).

3.4. Effect of 4R on phosphorylation of Akt in bEND5 cells

In addition to decreasing ICAM-1 expression, 4R was also able to increase the Akt phosphorylation in the same cell samples of bEND5 cells, both in normoxic condition and in OGD/R condition (Fig. 4A, 4C). This effect was dose-dependent, being 112%, 122 % and 140% of control with $4 \mu\text{M}$, $8 \mu\text{M}$ and $16 \mu\text{M}$ 4R treatment, respectively (Fig. 4C). In OGD/R, p-Akt level was 70% of control in OGD/R group and 50% of control in OGD/R +DMSO group, while $8 \mu\text{M}$ 4R increased p-Akt level in OGD/R cells near to the normal level, which was 105% of control (Fig. 5C-5D).

3.5. 4R reduces monocyte adhesion to bEND5 cells induced by OGD/R

These experiments demonstrated that the effects of 4R seen in changes of ICAM-1 at the protein level were translated into the effects at the functional level, because the enhanced level of ICAM-1 would increase the percentage of monocytes adhesion to endothelial cells (Fischer et al., 2005). When bEND5 cells were subjected to OGD/R, the percentage of U937 cells adhesion to bEND5 cells was increased to $22.4\% \pm 0.8$ compared to that in normoxic control bEND5 cells with 17.7 ± 0.3 adherent monocytes. 4R treatments reduced the adherent U937 cells in a dose dependent manner. The percentage of the adherent U937 cells was

15.8%±0.3, 14.1%±1.1, and 13.2%±2.3 in 4µM, 8µM and 16 µM 4R treatments, respectively (Fig. 6A). The vehicle DMSO did not have any effect on the adhesion of U937 cells to bEND5 cells indicated by 23.9%±0.8 of adherent U937 cell to the bEND5 cells under OGD/R condition (Fig.6A).

3.6. 4R mediated decrease in apoptosis of neuro-2a cells is associated with Akt phosphorylation after OGD

Using neuronal cell line, neuro-2a, we have tested the capability of 4R in induction of the Akt phosphorylation. 4R increased Akt phosphorylation under both normoxic environment and OGD condition. The value of p-AKT expression for DMSO under normoxic environment was 113.6%±11.3 of control, and it was 210.0%±36.1, 72.32%±7.38, 81.3%±5.8, 164.6%±9.5 in 4R under normoxic environment, OGD alone, OGD+DMSO, OGD+4R condition respectively (Fig. 7A). Since Akt is involved in pro-survival pathway, we quantified cell apoptosis using the Annexin V and PI staining kit measured by flow cytometry. The percentage of apoptotic neuro-2a cells induced by OGD was 38% ± 11, and was 42.4 %± 4.9 in OGD+DMSO condition, and was 16% ±2.6 in cells treated with 8µM of 4R (Fig. 7B, 7C).

4. DISCUSSION

In the present study, we demonstrated that 6mg/kg of 4R decreased brain damage after transient and permanent cerebral ischemia in rodents. Although the optimal neuroprotective dose of 4R *in vivo* is not known, 6 mg/kg 4R did not alter exploratory activity in naïve rats but abolished behavioral sensitization to nicotine (Ferchmin et al., 2001). *In vitro* experiments suggest that even at lower doses, 4R could be effective (Ferchmin et al., 2005, Eterovic et al., 2013). On the basis of these *in vivo* and *in vitro* results, 6 mg/kg was used in this work.

First, we found that treatment with 4R before pMCAO in mice reduced infarct volume by 56.5 % compared to that in the DMSO group (Fig. 2B and 2C). This protection by 4R was not due to the effect of 4R on the cerebral blood flow, because there are no observed differences in cerebral blood flow among animals in different groups (Fig. 2A). The results using pre-treatment with 4R are highly relevant to the prevention of ischemic stroke, but are not feasible for the treatment of stroke. Therefore, we also performed experiments to evaluate post-treatment effect of 4R using the tMCAO rat model, since it is more relevant to clinical therapeutic application. We observed that post-treatment by 4R was able to reduce the infarct size in rats by 58.8% (Fig. 2E and 2F), and this protection was associated with less body weight loss after stroke (Fig. 2D), which indicates less severity of stroke (Petullo et al., 1999). Notably, some studies suggested that DMSO had a protective effect after cerebral ischemia, presumably due to its radical scavenger effect (Bardutzky et al., 2005; Nagel et al., 2007). In order to exclude any vehicle effect we included two cohorts of control animals (comparing PBS vs. DMSO treated animals) in our studies in mouse. Our results showed that there was no statistical difference in the infarct volume (Fig. 2B) between these two control groups. The difference between our results and reports mentioned above on DMSO effect in ischemic stroke may be due to different doses and administration time and

routes. The pharmacokinetics study indicated that the brain contained the lowest concentration of DMSO in all the tissue studied, and that there was little DMSO remaining in plasma or any tissue at 8 h after i.v. injection of DMSO in mouse (Kaye et al., 1983). Based on the known pharmacokinetic characteristics of DMSO, we assume that there would be little DMSO retained in the brain after i.p. or s.c. injection, as in our experiments.

The effect of 4R on infarction size is most likely due to a central effect, since pharmacokinetics studies have demonstrated that 4R crosses the blood-brain barrier and accumulates in the brain (Ferchmin et al. 2013). In addition, the studies with the acute hippocampal slices reported here also indicate a direct effect of 4R on the hippocampal pyramidal neurons since we observed that PSs were reduced after OGD, and 4R was able to ameliorate this damage (Fig. 3). As an indicator of neuronal function, the reduction of PSs induced by OGD indicates either neuronal death or dysfunction. The improved recovery of PSs by 4R gives indirect evidence of greater cell survival or enhanced functional integrity. Notably, 4R-mediated neuroprotection was inhibited by wortmannin, an inhibitor of PI3-kinase, indicating the involvement of PI3K/Akt pathway in the neuroprotective effect. Consistent with this result, we found that 4R was able to increase phosphorylation of Akt in a neuronal cell line, neuro-2a cell, in both normoxic cells and OGD conditions. Furthermore, the Akt phosphorylation was associated with less cell apoptosis induced by OGD (Fig. 7). Our data of 4R-mediated neuroprotection agree with the previous study (Ferchmin et al., 2005), where 4R protected acute hippocampal slices against NMDA-induced excitotoxicity by affecting the activity of the PI3K/Akt antiapoptotic cascade (Ferchmin et al., 2005). These data give some insight into the mechanism of 4R-mediated neuroprotection in the ischemic stroke models.

Over decades, the Akt pathway has proven to be an anti-apoptotic pathway, which is involved in the cell survival and growth. Akt plays a critical protective role in various organs affected by ischemia/reperfusion injury including brain (Akbar et al., 2005; Brazil et al., 2002; Feng et al., 2006; Fukunaga and Kawano, 2003). As a survival kinase against brain ischemia/reperfusion injury, Akt is activated by PI3K through phosphorylation to produce its active form, p-Akt. The p-Akt phosphorylates a number of apoptosis-regulatory molecules such as BAD, caspase 3 and 9, GSK-3 β , I κ B kinase, cAMP-responsive element binding protein (CREB), forkheads, proline-rich Akt substrate (PRAS). Apoptotic functions of BAD, caspase 3 and 9, GSK-3 β , forkheads, PRAS are inhibited, while anti-apoptotic functions of CREB, NF- κ B are activated after phosphorylated by p-Akt (Chan, 2004; Harada et al., 2004). All of the studies on Akt have shown that up-regulation of Akt has a protective effect on ischemic organs. In addition, increased level of Akt phosphorylation is associated with inhibitory effect on inflammation (Song et al. 2013; Yan et al.; Zhu et al., 2003). From a therapeutic perspective, the up-regulation of Akt could potentially be used alone or in combination with other therapeutic strategies to treat brain ischemic injury.

To elucidate if other mechanisms are involved in 4R-mediated neuroprotection, we have performed studies to investigate the effects of 4R in the brain endothelial cell line bEND5. The bEND5 cells mimic the vascular portion of the BBB *in vitro*, where ICAM-1 and other endothelial factors can be up-regulated to induce post-ischemic inflammation. In the present study we focused on ICAM-1, because normally ICAM-1 is expressed at low levels on brain

endothelium and perivascular astrocytes, but its expression rises dramatically in pathological conditions like ischemic stroke (Vemuganti et al., 2004). Therefore, inhibition of ICAM-1 should protect neurons from inflammatory injury and death in cerebral ischemic stroke. Although anti-ICAM-1 antibody failed for stroke treatment (Furuya et al., 2001), the therapeutic strategy targeting ICAM-1 is still valuable for ischemic stroke. The inhibitory effects of 4R on ICAM-1 expression (Fig. 4B, 4E; Fig. 5B) and monocyte adhesion (Fig. 6) indicate that 4R could protect neurons against stroke by inhibiting the post-ischemic inflammation. These results are consistent with the anti-inflammatory properties of 4R due to inhibition of prostaglandin synthesis (Olsson et al., 1993). Anti-inflammatory effect of 4R may also be due to enhancement or maintenance of Akt phosphorylation in brain endothelial cells under ischemic condition (Fig. 5C, 5D), because phosphorylation of Akt could inhibit inflammation by suppression of ICAM-1 and other inflammatory factors (Song et al., 2013; Yan et al., 2013; Zhu et al., 2003). These data provide further evidence in the mechanism of 4R protection in ischemic stroke. Notably, 4R may have a relatively longer therapeutic window by targeting post-ischemic inflammation, which usually lasts from hours, days to weeks after ischemic stroke. The long therapeutic window afforded by 4R indicates its high significance for clinical application, because it offers more opportunities for treatment.

5. CONCLUSION

Our study suggests that 4R possesses neuroprotective capability in *in vitro* and *in vivo* models of cerebral ischemia. 4R protection was mediated by inhibition of ICAM-1 expression and up-regulation of Akt phosphorylation. Although the exact mechanism of action of 4R is still not fully understood, this study reveals a possible new drug that can protect the neurons directly against ischemia by Akt activation and decrease the secondary brain damage via inhibition of neuroinflammation. This is highly significant for treatment of ischemic stroke, in which multiple pathological mechanisms are involved, because a multifunctional drug could have superior therapeutic effect, and possibly reduce unwanted effects in comparison to conventional therapy with monospecific drugs or polypharmaceutical combinations of different agents. Thus the results of the present study suggest that 4R could become a promising lead structure for the development of novel drugs in treating ischemic stroke. Of course, further studies are needed to evaluate the effect of 4R on behavior outcomes after ischemic stroke and elucidate the mechanism of action of 4R protection *in vivo*, e.g. using Akt inhibitors or other pharmacological approaches to confirm if activation of Akt signaling pathway or other molecules are involved in the 4R-mediated neuroprotection.

ACKNOWLEDGMENTS

This work was supported by NIH grants 5U54RR022762-05 (AHM), SNRP U54 NS039408-10 (AHM), 8G12MD007583-26 (PAF) and 5U01NS063555 (PAF) and The James L. Winkle College of Pharmacy new faculty start-up funding (JH). The authors also acknowledge the generous support by Fundación Segarra of Puerto Rico (VAE). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We would like to thank Dr. Zhenfeng Xu's contribution to the manuscript. Sadly, Dr. Zhenfeng Xu recently passed away.

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Highlights

- Treatment with 4R reduces infarct volume in rodent ischemic stroke models.
- 4R decreases apoptosis of neuroblastoma cells induced by OGD.
- 4R improves the population spikes recovery in rat acute hippocampal slices in OGD.
- 4R inhibits ICAM-1 expression in murine brain-derived endothelial cells.
- 4R restores Akt phosphorylation in murine brain-derived endothelial cells in OGD.

Figure 1.
Structure of (1*S*,2*E*,4*R*,6*R*,*-7E*,11*E*)-2,7,11-cembratriene-4,6-diol (4*R*)

Figure 2.

4R treatment reduces infarction size after MCAO in mice and rats. A. Quantification of blood flow in mice after pMCAO untreated or treated either with DMSO or 6 mg/Kg of 4R (Mean±SD; n=6 per group; n.s.: not statistically significant). B. Quantification of brain infarct volume in mice identified by TTC staining (Mean±SD; untreated n=7; DMSO n=6; 4R n=6; ** p<0.01). C. Representative images of brain slices after pMCAO in mice stained by TTC. White area corresponds to the infarct area. The damaged area in the control groups involved cortical, striatal and hippocampal areas. The cortex area is preserved and the residual affected area in 4R treated mice was typically confined to striatum. D. Measurement of body weight of rats 24 hours after tMCAO. Body weights are expressed as percentage of reduction of body weight 24 hours after the surgery (Mean±SD; DMSO n=6 and 4R n=4, **p<0.01). E. 4R decreased the infarct area in rats when administered 1 hour after reperfusion. Statistic analysis of the brain damage expressed as % of healthy brain hemisphere (Mean±SD, DMSO n=5, 4R n=5, **p<0.01). F. The representative images of rat brain slices after MCAO stained by TTC.

Figure 3.

A. 4R protects the rat hippocampal slice function from OGD-induced damage. The area of synaptically elicited PSs recorded in stratum pyramidale is reported as the % recovery of the PS obtained after treatment compared to the PS obtained before treatment. Statistical analysis was done by one-way ANOVA followed by the Dunn's test: OGD+DMSO (n=56), OGD+10 μ M 4R+10nM wortmannin (n=7), OGD+10nM wortmannin (n=14), OGD +10 μ M 4R (n=35). Mean $\pm$ SEM, *p<0.05 (OGD+DMSO versus OGD+10 μ M 4R), # p<0.05 (OGD+10 μ M 4R versus OGD+10 μ M 4R+10nM wortmannin) B. Representative recordings of PSs before and after OGD. The PS is the area (ms x mv) of the negative peak, which represents the sum of the axon potentials.

Figure 4.

Effect of 4R on expression of ICAM-1 and pAkt expression in normoxic condition and *in vitro* inflammatory condition. A. Representative Western blot assay showing the effect of different 4R concentrations in ICAM-1 and pAkt expression using bEND5 in normoxic condition. B. Quantification of ICAM-1 expression. C. Quantification of pAkt expression. D. Representative western blotting image showing the effect of 4R in ICAM-1 expression in bEND5 after induction of inflammation by 50 ng/ml of TNF- α . E. Quantification of ICAM-1. Mean $\pm$ SD, n=6-10, **p<0.05.

Figure 5.

Effect of 4R on expression of ICAM-1 and pAkt in bEND5 cells under OGD. A. Representative image of ICAM-1 expression by Western blot assay. B. Quantification of ICAM-1 expression in bEND5 cells, $n=8$ in all treatments $**p<0.05$. C. Representative image of p-Akt expression in bEND5 cells by Western blot assay. D. Quantification of p-Akt expression in bEND5 cells. Mean $\pm$ SD, $n=6-8$, $**p<0.05$.

Figure 6.

Effect of 4R on monocyte adhesion to bEND5 cells subject to OGD/R condition. A. 4R reduces fraction of U937 cell adhesion to bEND5 cells under OGD/R condition (Mean±SD, n=6, **p<0.05). B. Representative images of U937 cell adhesion to bEND5 cells. The adherent U937 cells were labeled as green color, and the bEND5 cells were stained with DAPI shown as blue color. Image a: normal bEND cells without OGD/R. Image b: bEND5 cells in OGD/R incubated with DMSO. Image c: bEND5 cells in OGD/R incubated with 4μM of 4R. Image d: bEND5 cells in OGD/R incubated with 8μM of 4R. Image e: bEND5 cells in OGD/R incubated with 16 μM of 4R.

Figure 7. Effect of 4R on pAkt expression and apoptosis in neuro-2a cells under normoxic and OGD conditions

A. 4R induces pAkt after OGD, (Mean±SD, n=6, **p<0.05). The expression of p-Akt was detected by ELISA. B. A representative result of flow cytometry quantifying propidium iodide positive cells. C. Quantification of the effect of 4R on cell apoptosis indicated by propidium iodide. (Mean±SD, n=6, **p<0.05).