Neuroprotective effect of hydroxypropyl-β-cyclodextrin in hypoxia-ischemia

Jack R. Rivers, Simran D.S. Maggo and John C. Ashton

Neonatal cerebral ischemic injury is a common and debilitating pathology for which there is currently no known purely pharmacological treatments that are effective when delivered immediately after injury. Cyclodextrins are cyclic oligosaccharides that can remove cholesterol from cell membranes and thereby affect receptor function. Cyclodextrins have previously been shown to be neuroprotective *in vitro*. We showed that hydroxypropyl-β-cyclodextrin is neuroprotective in rats *in vivo* when delivered by intraperitoneal injection 30 min following hypoxia-ischemia, when assessed 15 days after surgery. A single dose of 1 g/kg hydroxypropyl-β-cyclodextrin reduced brain infarction size by 28.57% compared with control ($P<0.001$). We also report that the same compound reduces neuronal excitability in hippocampal slices and propose that hydroxypropyl-β-cyclodextrin is neuroprotective by reducing excitotoxicity in the delayed phase of brain damage. *NeuroReport* 23:134–138 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

NeuroReport 2012, **23**:134–138

Keywords: cyclodextrins, excitability, hydroxypropyl-β-cyclodextrin, hypoxia-ischemia, neuroprotection

Department of Pharmacology and Toxicology, Otago School of Medical Sciences, University of Otago, Dunedin, New Zealand

Correspondence to John C. Ashton, Department of Pharmacology and Toxicology, Otago School of Medical Sciences, University of Otago, PO Box 913, Dunedin 9054, New Zealand
Tel: +64 3 479 3040; fax: +64 3 479 9040; e-mail: john.ashton@otago.ac.nz

Received 31 October 2011 **accepted** 4 November 2011

Introduction

Neonatal and infant hypoxia is a major cause of cognitive disability [1]. It has been estimated that approximately half of hypoxic-ischemia-induced encephalopathy causes death, and a further 25% of cases result in loss of neurological function [2]. Although there are some partially effective treatments for improving the symptoms of neonatal hypoxia [3,4], only hypothermia has been shown to be neuroprotective in the acute stages of injury. There is a clear need for inexpensive and simple pharmacological interventions in acute treatment that can reduce neurological damage and improve patient prognosis.

Cyclodextrins are cyclic oligosaccharides with a lipophilic central domain and a hydrophilic surface. This makes cyclodextrins useful carrier molecules for lipophilic drugs [5]. Some cyclodextrins also remove cholesterol from cell membranes, affecting receptor function by altering lipid-raft structure and receptor stability in the membrane. Several classes of cyclodextrins protect cultured cortical neurons against oxygen–glucose deprivation, and against *N*-methyl-D-aspartic acid (NMDA) and glutamate-induced excitotoxicity, without affecting normal calcium signaling [6]. Methyl-β-cyclodextrin has also been found to protect hippocampal slices from the effects of anoxia [7]. One of the neuroprotective cyclodextrins investigated by Abulrob *et al.* [6], 2-hydroxypropyl-β-cyclodextrin (HP-CD), has also been extensively characterized in rats by Valenzano *et al.* [5]. The authors of that study found that intraperitoneal injection of 10 ml/kg 25% HP-CD did not cause any neurological impairment or other adverse effects.

We hypothesized that HP-CD could be neuroprotective in a rat model of neonatal hypoxia when administered after injury. HP-CD has been used as a vehicle for experimental lipophilic drugs [5] and also as a deodorizer. Other lipophilic drug vehicles such as ethanol [8] and dimethyl sulfoxide [9,10] have been shown to have neuroprotective properties. Therefore, to test whether any neuroprotective effect of HP-CD was due to a class property of lipophilic drug vehicles or was specific to cyclodextrins, we compared the effects of HP-CD with those of another commonly used lipophilic drug vehicle [11,12] [a combination of 5% dimethyl sulfoxide, 5% cremophor, and 5% ethanol in 0.01 M PBS (henceforth DCE)]. We also compared the results with rats that had received no treatment (naive controls). We hypothesized that HP-CD would reduce neuronal excitability, as has been described for other β-cyclodextrins [13], and tested this by measuring synaptic transmission in the CA1 region of isolated rat hippocampal slices.

Materials and methods

All surgical procedures and animal care practices were carried out in accordance with the University of Otago Animal Ethics Committee guidelines on the use of laboratory animals. Unless stated otherwise, all chemicals used were acquired from Sigma-Aldrich (St Louis, Missouri, USA); all surgical materials used were acquired from Southern Medical Products Ltd (Dunedin, New Zealand), and all statistical analyses were carried out on Minitab15 Statistical Software (Minitab Ltd, Coventry, UK).

0959-4965 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/WNR.0b013e32834ee47c

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Hypoxia-ischemia surgery

We used the Variable Hypoxia-Ischemia model (Variable hypoxic-ischemia), which we have described previously [14]. Briefly, 23 male P26 Wistar rats (70–85 g) were anesthetized using 2.5% halothane in 100% oxygen. A unilateral carotid artery ligation was performed. The left common carotid artery was exposed and then bluntly dissected away from the internal jugular vein and the vagus nerve. The artery was then permanently ligated using two 5-0 silk surgical sutures. The incision was then closed with surgical clips, and the animals were left to recover for a period of 2–3 h. After recovery, the animals were placed in an airtight chamber in which the air temperature was maintained at 32.5 ± 0.5°C. A constant flow of a humidified (85–100% relative humidity) hypoxic gas of 8% oxygen/92% nitrogen was perfused through the chamber. Each rat was removed from the hypoxia chamber immediately after it experienced a tonic clonic seizure as described in Rivers *et al.* [14].

Treatments

Thirty minutes after removal from the chamber, the rats were randomly allocated into three groups: one received no treatment (naive group) and the other two groups were administered either the DCE vehicle containing 5% (v/v) dimethyl sulfoxide (VWR laboratory supplies, Poole, England; Cat# 103234L), 5% (v/v) cremophor EL (Sigma-Aldrich; Cat# C5135-500G), 5% (v/v) ethanol, and 85% (v/v) 0.01 M PBS at 4 ml/kg, or 25% (w/v) HP-CD (Sigma-Aldrich; Cat# H107-100G) in PBS at 4 ml/kg (providing a dose of 1 g/kg).

Tissue processing and infarction assessment

Fifteen days after Variable hypoxic-ischemia, the animals were killed, and the brains were quickly removed and placed in chilled 20 mM PBS. The brains were then soaked in 10% phosphate-buffered formalin for 48 h at 4°C. This was followed by a 48 h emersion in 30% sucrose PBS solution at 4°C. The brains were then embedded in ProLabo optimum cutting temperature compound (VWR International) and frozen and stored at −18°C. The embedded brains were then sectioned transversely using a cryotome, and a section was taken every 1 mm starting from the rostral end of the striatum and stained using 0.1% (w/v) toludine/saline solution (Fig. 1). Each transverse section was then photographed using a Dinolite camera (AnMo Electronics Corp., New Taipei City, Taiwan), and the area of infarction was delineated manually using Image J software (National Institutes of Health, Bethesda, Maryland, USA) by a person blinded to the treatment groups. The infarction was then approximated by subtraction of the healthy area of the ipsilateral side from the area of the contralateral side [14]. This method of infarction assessment accounts for the effects of edema.

Hippocampal slices

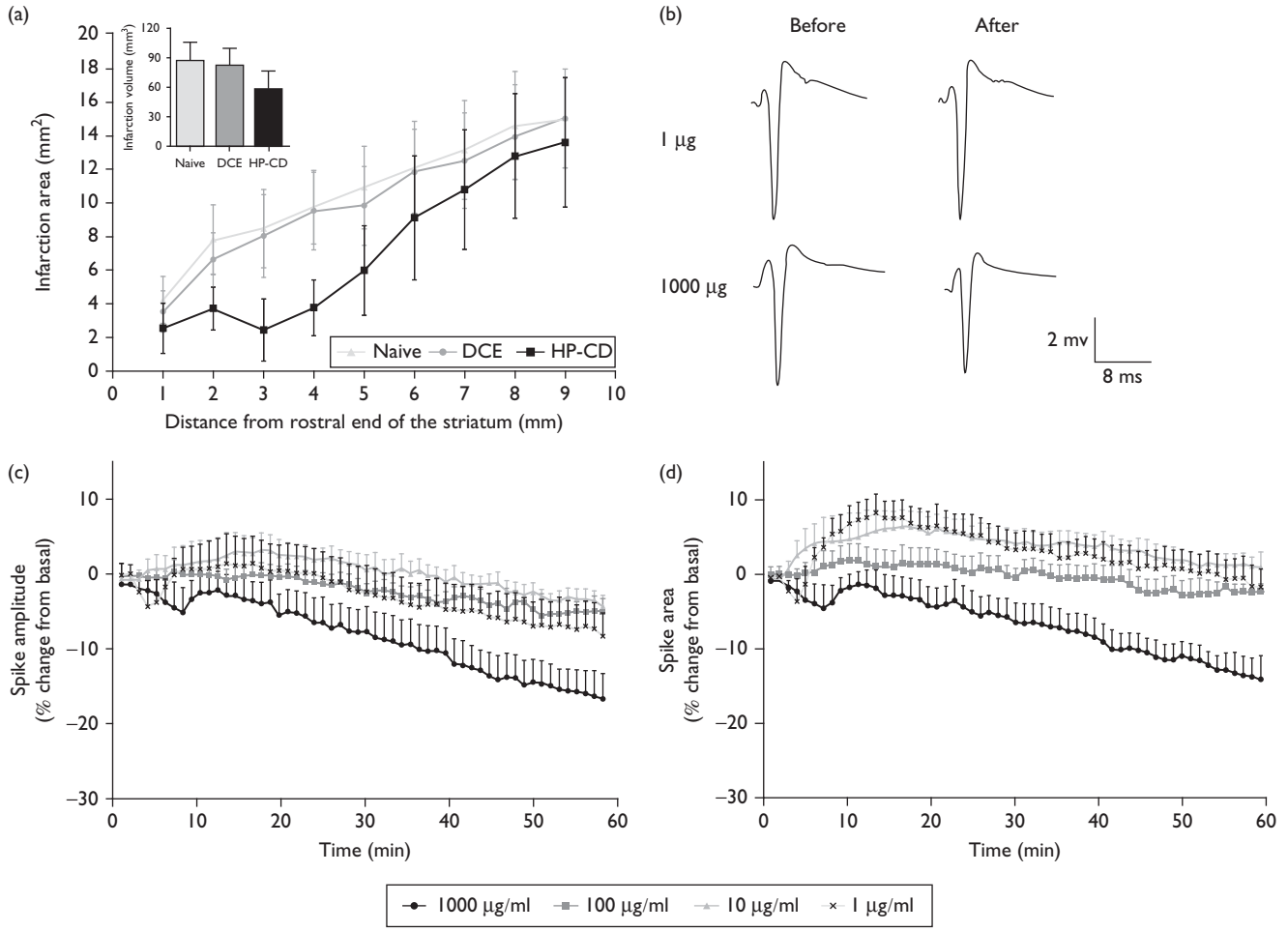
Hippocampal slices (400 µm transverse sections) were obtained from adult Wistar rats (300–350 g) following established procedures [15,16]. Briefly, brains were rapidly removed after decapitation and placed in ice-cold artificial cerebrospinal fluid consisting of 124 mM NaCl, 3.2 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 10 mM glucose (21–23°C, saturated with 95% O_2/5% CO_2). Hippocampi were dissected out and 400-µM-thick slices were obtained using a Stoelting tissue slicer (Stoelting, Wood Dale, Illinois, USA). Slices were maintained in an in-vitro brain slice-holding chamber at room temperature for at least 90 min before recording observations. Slices were fully submerged in a brain slice-holding chamber (KSI instruments, Christchurch, New Zealand) and constantly perfused with artificial cerebrospinal fluid maintained at a temperature of 32–33°C. For each slice, before commencement of baseline recording, test stimuli were adjusted such that the level of evoked response was at or near 75% of the maximal orthodromic population spike (PS) amplitude. Evoked responses were then monitored for 30 min before the addition of drug/vehicle to the perfusion medium. Slices not exhibiting stable baseline potentials for at least 20 min before drug/vehicle administration were discarded from the study. HP-CD was washed into the chamber for a period of 1 h, with continuous recording throughout. For each drug/vehicle paradigm, the time-course of specific effects was assessed online following analog-to-digital conversion of waveforms and storage using LabChart Analytical Software (developed by A.D. Instruments, Dunedin, New Zealand). PS area (area under the curve) and amplitude, which reflect the number of neurons firing in response to a given stimulus and synchronicity of firing, respectively, were assessed every 5.7 s by measuring the difference in mV between the negative point of the PS and the two positive points of inflection on either side. Data were expressed as a percentage change from baseline values, with mean and error bars shown for four to six hippocampi.

Statistical analysis

Infarction distribution for the two treatment groups was assessed for statistical difference using general linear model (GLM) two-factor analysis of variance with repeated measures. This technique compared whether the infarction areas were different between sections in relation to the distance from the rostral end of the striatum, and in relation to treatment groups, and also assessed whether there was an interaction between treatment and section distance from the rostral end of the striatum. The variances were assessed and found to be equal using the Bartlett test for the GLM. All data were assessed for normality using the Anderson–Darling test. Hippocampal slice data were analyzed using linear regression and analysis of covariance to test for significant

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

Fig. 1



(a) Mean infarction areas and SEM for sequential sections 1 mm apart starting at the rostral end of the striatum, comparing control rats ($n=8$) with DCE-treated rats ($n=8$) and HP-CD-treated (1 g/ml) rats ($n=7$). Inset shows total infarct volumes for the three groups; (b) representative stimulated population spikes from the CA1 region in isolated hippocampi, showing the reduction in spike amplitude and after 1 h of treatment with 1 mg/ml HB-CD; (c) percentage changes in spike amplitudes in isolated hippocampi over 1 h of treatment with 1, 10, 100, or 1000 µg/ml of HP-CD data points are the means of changes from baseline taken from four to six rats, plotted as a mean of 11 measurements for each minute. Error bars are SEM; (d) population spike areas in isolated hippocampi over 1 h of treatment. Conventions are as for C above. DCE, a combination of 5% dimethyl sulfoxide, 5% cremophor, and 5% ethanol in 0.01 M PBS; HP-CD, 2-hydroxypropyl-β-cyclodextrin.

differences in the slope of PS area or amplitude against time.

Results

Two-factor analysis of variance with repeated measures using a GLM showed that HP-CD treatment significantly reduced infarctions in the brains of rats when assessed 15 days after injury (Fig. 1a). Both treatment ($F = 14.64$, $P < 0.001$) and distance of section along the brain ($F = 32.57$, $P < 0.001$) were highly significant factors. Bonferroni simultaneous tests showed that HP-CD-treated rats had significantly smaller infarctions compared with both control rats ($t = 4.343$, $P < 0.001$) and DSE-treated rats ($t = 5.074$, $P < 0.001$). Mean infarcts in the three treatment groups were 78.2, 81.7, and 56.7 mm^2 for control, DSE-treated, and HP-CD-treated rats, respectively (Fig. 1b). This represents a 21.5 and 25 mm^2 reduction in mean infarct volume in HP-CD-treated rats compared with control and DSE-treated rats (i.e. 28.5 and 30.6% reduction in infarcts). The reduction in infarction areas in HP-CD-treated rats was greatest in the striatum and adjacent cortex and was least in the hippocampus and adjacent cortex. Ventricular enlargement was also more pronounced in the striatal sections of control and DCE-treated rats.

In hippocampal slices, HP-CD treatment caused a time-dependent decrease in neuronal excitability (Fig. 1c) in terms of both stimulated PS amplitudes (Fig. 1d) and areas under the curve (Fig. 1e). Linear regression

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

analysis with analysis of covariance showed that in both cases the linear regression slopes for all series were significantly nonzero ($P < 0.001$) and were significantly different between concentrations ($P < 0.001$). For both outcome measures, the slope for the 1-mg/ml-treated slices was greatest, such that after 1 h of treatment PS amplitude and area had both decreased to less than 85% of baseline measurements. PS area represents the total recruitment of synapses, and for this outcome measure a dose response was particularly clear, with 100 μg/ml HP-CD causing an intermediate level of inhibition of neuronal excitability between 1 mg/ml and the lower doses.

Discussion

Present treatment for hypoxic-ischemia is focused on managing symptoms in long-term care [3,4]. There are very few treatments available for the acute stages of hypoxic-ischemia; the most successful has been induced hypothermia [17]. Pharmacological intervention with neuroprotectants has been most successful in preclinical research, when the drug is given before the induction of neural injury [18]. However, this does not model clinical reality, in which the first warning of cerebral injury is often the event itself. Drugs that are neuroprotective when given before injury are not necessarily protective when given soon after injury [18]. In addition, drugs that appear neuroprotective when brain infarctions are assessed at 1–3 days following treatment are not necessarily neuroprotective when infarctions are measured weeks later. Therefore, the finding that HP-CD provides substantial neuroprotection up to 15 days after treatment that follows 30 min after Variable hypoxic-ischemia suggests a promising strategy for treatment of neonatal hypoxia.

Our finding that HP-CD reduced neuronal excitability in hippocampal slices is consistent with the results reported by Frank *et al.* [13] for other β-cyclodextrins, in particular methyl-β-cyclodextrin. Frank *et al.* [13] also reported that methyl-β-cyclodextrin treatment caused disruption of NMDA and AMPA receptor-dependent glutamate transmission, which they suggested is because of the ability of cyclodextrins to sequester cholesterol from postsynaptic densities. We propose that this may also explain the neuroprotective properties of HP-CD. The results of the experiments carried out by Abulrob *et al.* [6] and by Rufini *et al.* [7] suggest that by altering the distribution of synaptic ion channels, including NMDA receptor subunits, delayed excitotoxicity may be ameliorated, thereby resulting in reduced long-term damage.

The absence of any effect of an acute dose of HP-CD on performance in standard behavioral and motor function tests [5] suggests that the mechanism has a specific effect immediately following injury, but with minimal or no effects on normal neuronal function. In addition, HP-CD is used as a deodorizing agent for which, to our knowledge, no incidents of toxicity have been reported.

Conclusion

Systemic delivery of hydroxypropyl-β-cyclodextrin 30 min after Variable hypoxic-ischemia caused a significant reduction in brain injury in rats. Protection was robust up to at least 15 days after treatment and was most pronounced in the striatum, a key brain area in motor function. Hydroxypropyl-β-cyclodextrin has proven to have low toxicity, and our experiments only provide evidence for activity at high doses (particularly at 100 μg/ml and above *in vitro* and at 1 g/kg *in vivo*), making it a promising candidate for cyclodextrin-based treatment for cerebral ischemic injury.

Acknowledgements

This research was supported by the Marsden Fund of New Zealand.

Conflicts of interest

There are no conflicts of interest.

References

1 Vannucci RC, Connor JR, Mauger DT, Palmer C, Smith MB, Towfighi J, *et al.* Rat model of perinatal hypoxic-ischemic brain damage. *J Neurosci Res* 1999; **55**:158–163.
2 Bass JL, Corwin M, Gozal D, Moore C, Nishida H, Parker S, *et al.* The effect of chronic or intermittent hypoxia on cognition in childhood: a review of the evidence. *Pediatrics* 2004; **114**:805–816.
3 Bhat MA, Charoo BA, Bhat JI, Ahmad SM, Ali SW, Mufti MUH. Magnesium sulfate in severe perinatal asphyxia: a randomized, placebo-controlled trial. *Pediatrics* 2009; **123**:E764–E769.
4 Vannucci RC, Vannucci SJ. Glucose, acidosis, and perinatal hypoxic-ischemic brain damage. *Ment Retard Dev Disabil Res Rev* 1997; **3**: 69–75.
5 Valenzano KJ, Tafesse L, Lee G, Harrison JE, Boulet JM, Gottshall SL, *et al.* Pharmacological and pharmacokinetic characterization of the cannabinoid receptor 2 agonist, GW405833, utilizing rodent models of acute and chronic pain, anxiety, ataxia and catalepsy. *Neuropharmacol* 2005; **48**: 658–672.
6 Abulrob A, Tauskela JS, Mealing G, Brunette E, Faid K, Stanimirovic D. Protection by cholesterol-extracting cyclodextrins: a role for N-methyl-D-aspartate receptor redistribution. *J Neurochem* 2005; **92**: 1477–1486.
7 Rufini S, Grossi D, Luly P, Tancredi V, Frank C, D'Arcangelo G. Cholesterol depletion inhibits electrophysiological changes induced by anoxia in CA1 region of rat hippocampal slices. *Brain Res* 2009; **1298**:178–185.
8 Kelly DF, Lee SM, Pinanong PA, Hovda DA. Paradoxical effects of acute ethanolism in experimental brain injury. *J Neurosurg* 1997; **86**:876–882.
9 Di Giorgio AM, Hou YJ, Zhao XR, Zhang B, Lyeth BG, Russell MJ. Dimethyl sulfoxide provides neuroprotection in a traumatic brain injury model. *Restor Neurol Neurosci* 2008; **26**:501–507.
10 Greiner C, Schmidinger A, Hulsmann S, Moskopp D, Wolfer J, Kohling R, *et al.* Acute protective effect of nimodipine and dimethyl sulfoxide against hypoxic and ischemic damage in brain slices. *Brain Res* 2000; **887**: 316–322.
11 Alvares LD, de Oliveira LF, Camboim C, Diehl F, Genro BP, Lanziotti VB, *et al.* Amnestic effect of intrahippocampal AM251, a CB1-selective blocker, in the inhibitory avoidance, but not in the open field habituation task, in rats. *Neurobiol Learn Mem* 2005; **83**:119–124.
12 Rahn EJ, Zvonok AM, Thakur GA, Khanolkar AD, Makriyannis A, Hohmann AG. Selective activation of cannabinoid CB2 receptors suppresses neuropathic nociception induced by treatment with the chemotherapeutic agent paclitaxel in rats. *J Pharmacol Exp Ther* 2008; **327**:584–591.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.

13 Frank C, Rufini S, Tancredi V, Forcina R, Grossi D, D'Arcangelo G. Cholesterol depletion inhibits synaptic transmission and synaptic plasticity in rat hippocampus. *Exp Neurol* 2008; **212**:407–414.
14 Rivers JR, Sutherland BA, Ashton JC. Characterization of a rat hypoxia-ischemia model where duration of hypoxia is determined by seizure activity. *J Neurosci Methods* 2011; **197**:92–96.
15 Mockett BG, Guévremont D, Wutte M, Hulme SR, Williams JM, Abraham WC. Calcium/calmodulin-dependent protein KINASE II Mediates Group I metabotropic glutamate receptor-dependent protein synthesis and long-term depression in rat hippocampus. *J Neurosci* 2011; **31**:7380–7391.
16 Sari P, Kerr DS. Domoic acid-induced hippocampal CA1 hyperexcitability independent of region CA3 activity. *Epilepsy Res* 2001; **47 (1–2):** 65–76.
17 Hoehn T, Hansmann G, Buhrer C, Simbruner G, Gunn AJ, Yager J, *et al*. Therapeutic hypothermia in neonates. Review of current clinical data, ILCOR recommendations and suggestions for implementation in neonatal intensive care units. *Resuscitation* 2008; **78**:7–12.
18 Wahlgren NG, Ahmed N. Neuroprotection in cerebral ischaemia: facts and fancies – the need for new approaches. *Cerebrovasc Dis* 2004; **17**: 153–166.

Copyright © Lippincott Williams & Wilkins. Unauthorized reproduction of this article is prohibited.