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Inhibiting caspase-8 after injury reduces hypoxic–ischemic brain injury in the newborn rat

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Abstract

A broad spectrum caspase inhibitor reduces brain injury. Will a caspase-8 inhibitor provide protection? Seven-day-old rat pups had the right carotid artery ligated, then were subjected to 2.5 h of 8% oxygen. Caspase-8 activity in the right cortex was measured enzymatically. Caspase-8 activity was increased at 12 and 24 h after injury and IETD-CHO, (Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Pro-Ile-Glu-Thr-Asp-CHO, CHO is aldehyde) a cell permeable caspase-8 inhibitor, given by i.c.v. injection after the hypoxic period eliminated this increase with significant effect at 15 and 50 μg/pup (1.7 μmol/kg). Thirty pups were randomly assigned to receive 50 μg/pup of IETD-CHO or vehicle i.c.v. immediately after the hypoxic period. The loss of brain weight in the right hemisphere 22 days after injury was 29 ± 5% in the vehicle-treated animals and 12 ± 5% in the IETD-CHO-treated animals ($P < 0.05$). Inhibiting caspase-8 activity after hypoxic–ischemic brain injury reduces brain injury.

Keywords: Caspase; Neuroprotection; Apoptosis; Stroke

1. Introduction

Hypoxic–ischemic brain injury induces cell death in neurons by the mechanisms of apoptosis and necrosis (Northington et al., 2001b; Graham and Chen, 2001). Apoptotic mechanisms may be more important in fetal and neonatal animals, since that is an age when apoptosis is normally occurring in the brain (Hu et al., 2000). The initiator caspases in apoptotic cell death in the extrinsic and mitochondrial pathways are caspase-8 and caspase-9, respectively. We have shown that inhibition of caspase-9 in the newborn rat model is neuroprotective (Feng et al., 2003). Northington et al. (2001a,b) have shown that markers of the extrinsic, caspase-8-dependent pathway are activated by hypoxic ischemia in the newborn rat model. Genetically engineered mice, deficient in caspase-8, die during the embryonic period from abnormal heart and neural tube development and inadequate hematopoeitic cell development (Sakamaki et al., 2002; Wang and Lenardo, 2000). Does the inhibition of caspase-8 reduce hypoxic–ischemic brain injury in vivo? Caspase-8 is activated by tumor necrosis factor-α (TNF-α) or other fas receptor ligands, acting on the fas cell surface receptor. Activated caspase-8 can either activate caspase-3 directly or activate bid. Bid deactivates bcl-2 and by this mechanism releases mitochondrial cytochrome *c*. Cytochrome *c* activates caspase-9, which activates caspase-3 (Northington et al., 2001a,b; Benchoua et al., 2001; Felderhoff-Mueser et al., 2000). Cheng et al. (1998) showed that treatment after injury with a broad spectrum caspase inhibitor was neuroprotective in the neonatal rat hypoxic–ischemic brain injury model. Will a cell permeable, specific (Garcia-Calvo et al., 1998) caspase-8 inhibitor produce neuroprotection? In the present study, we measured the time course of activation of caspase-8 in the neonatal rat hypoxic–ischemic brain injury model (Rice et al., 1981), and demonstrated that it was possible to inhibit this increase of activity with a cell permeable caspase-8 inhibitor IETD-CHO (Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Pro-Ile-Glu-Thr-Asp-CHO, CHO is aldehyde, Calbiochem, San Diego, CA, USA) with i.c.v. administration. Lastly, we demonstrated that the use of this agent in a dose shown to inhibit caspase-8 activity reduces brain injury in this model.

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2. Materials and methods

2.1. Animal protocol

Our institutional committee on animal use approved this protocol. Rats were cared for in accordance with the National Institute of Health guidelines. Seven-day-old Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) weighing 12 to 17 g of either sex were anesthetized with isoflurane and had the right common carotid artery isolated from the nerve and vein, divided and ligated. The whole procedure took less than 7 min. The pups were returned to their dam for at least 3-h recovery after surgery. The pups were then placed in sealed jars in a 37 °C water bath and subjected to a warmed, humidified mix of 8% oxygen and 92% nitrogen delivered at 4 l/min for 2.5 h. The rats were then returned to their dams. Rats not subjected to hypoxic–ischemic injury (shams, six pups) and rats subjected to hypoxic–ischemic injury were anesthetized with Phenobarbital and killed by decapitation at 6 h (seven pups), 12 h (five pups), and 24 h (six pups) after hypoxic–ischemic injury. Brains were removed and the right and left cortex was analyzed for caspase-8 activity. Because caspase-8 activity was increased at 12 and 24 h after injury, we chose 24 h after injury to assay the dose response curve of IETD-CHO on caspase-8 activity. Rats were treated with IETD-CHO at doses of 5 (six pups), 15 (six pups), 50 μg/pup (six pups) in 1 μl of dimethylsulfoxide plus 4 μl of phosphate buffered saline immediately after the hypoxic period or an equal volume of vehicle (six pups) i.c.v. (Cheng et al., 1998).

To assess the neuroprotective effect of IETD-CHO, pups were randomized to treatment with vehicle ($n = 16$) or 50 μg/pup IETD-CHO ($n = 14$) immediately after the hypoxia. The dose was chosen from the dose response curve. Pups were returned to their dams and allowed to recover and grow for 22 days. Rectal temperature was taken with a 36-gauge flexible thermocouple (Omega Engineering, Stamford, CT) in a sub-set of these pups (four from the vehicle group and four treated with 50 μg/pup of IETD-CHO) at 0, 1, 2, and 6 h after dosing.

Rat pups were anesthetized with pentobarbital and decapitated 22 days after hypoxic exposure. The brains were removed, scored and weighed by an observer blind to the code. Brains were scored normal, mild, moderate or severe by the method of Palmer et al. (1990). "Normal" meant no reduction in the size of the right hemisphere, "mild" meant visible reduction in right hemisphere size, "moderate" meant large reduction in hemisphere size with a visible infarct in the right parietal area, and "severe" meant near total destruction of the hemisphere (Palmer et al., 1990). After removing the cerebellum and brain stem, the brain was divided into two hemispheres and weighed by an observer blinded to the experimental group of the pups. Results are presented as a percent loss of right hemispheric weight relative to the left [(left − right)/left × 100]. The loss of hemispheric weight can be used to measure brain damage in this model if enough time after injury has elapsed to allow resorption of the dead brain tissue (Trescher et al., 1997). After removal, the brains were stored in 10% buffered formalin. Sections were then embedded with paraffin. Five-micron coronal sections were cut in the parietal region aiming for the equivalent of Bregma −4.3 to −4.5 mm (Kruger et al., 1995) in the adult rat and then stained with hemotoxylin and eosin. Cerebral cortex was scored by an observer blind to the treatment group of the animal from 0 to 5 by the method of Cataltepe et al. (1995), where "0" is normal, "1" is 1–5% of neurons damaged, "2" is 6 to 25% of neurons damaged, "3" is 26–50% of neurons damaged, "4" is 51–75% of neurons damaged, "5" is >75% of neurons damaged. Damaged neurons for scores of 1–3 usually were shrunken cells with picnotic nuclei and eosinophillic cytoplasm replacing most of the health neurons in patchy areas of the cortex. Damaged neurons for scores of 4 and 5 usually showed loss of the cortex with the tissue partially replaced by a small amount of inflammatory cells and connective tissue.

2.2. Caspase-8 and 9 assays

Caspase-8 activity was assayed as previously described by Selzner et al. (2000) In brief, pups were anesthetized with 50 mg/kg of phenobarbital. The cortex in both lesioned and unlesioned hemispheres was separately dissected and homogenized in four volumes of buffer (100 mM NaCl, 50 mM HEPES, 10 mM DL-threo-1,4-Dimercapto-2,3-butanediol, 1 mM EDTA, 10% glycerol, 0.1% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulonate CHAPS, pH 7.4). Injury effects cortex, basal ganglion and hippocampus, but the volume of effected cortex is much larger than that of basal ganglion or hippocampus (Almli et al., 2000). Caspase-8 activity was measured using assay kits (Calbiochem), following the manufacturer's instructions. Supernatant was mixed with buffer containing the recognition sequence for the caspase attached to *p*-nitroanilide and the results measured colorimetrically. Since the cortex from the whole hemisphere and not just the area of injury was assayed, the increase in activity seen with injury as a percent of baseline is less than that reported by some investigators (Northington et al., 2001a,b).

In order to demonstrate the specificity of IETD-CHO for caspase-8, relative to caspase-9, caspase-9 activity was assayed as previously described (Feng et al., 2003) in shams, and in pups treated with 50 μg/pup i.c.v. of IETD-CHO or vehicle immediately after hypoxia and assayed for caspase-9 activity 24 h after hypoxia. Tissue was processed as for caspase-8. Caspase-9 activity was measured using assay kits (Alexus Biochemicals, San Diego, CA, USA), following the manufacturer's instructions. Supernatant was mixed with reaction buffers containing the recognition sequence for caspase-9 attached to *p*-nitroanilide and the results measured colorimetrically.

2.3. Statistics

The statistical comparisons were made using χ^2 for categorical variables, and Mann–Whitney or Kruskal–Wallis for ordinal variables and analysis of variance (ANOVA) for continuous variables using Newman–Keul's test for individual comparisons.

3. Results

The time course of elevation of caspase-8 activity is shown in Fig. 1. There was a statistically significant increase in caspase-8 activity from 201 ± 15 fmol/mg protein/min (mean ± S.E.M.) in the shams ($n = 6$) to 295 ± 15 fmol/mg protein/min at 12 h ($n = 5$, $P < 0.01$ vs. shams), and to 242 ± 10 fmol/mg protein/min at 24 h ($n = 6$, $P < 0.05$ vs. shams) after the hypoxic period. There was no statistical significant increase in caspase-8 activity at 6 h (194 ± 5 fmol/mg protein/min, $n = 7$).

In Fig. 2, the dose response curve from 5 to 50 μg/pup for i.c.v. administration of IETD-CHO immediately after the hypoxic period with caspase-8 activity measured 24 h after injury is shown. Significant reductions in caspase-8 activity were seen for doses of 15 and 50 μg/pup (50 μg/pup is approximately 3.3 mg/kg or 1.7 μmol/kg since the pups weight approximately 15 g).

Caspase-9 activity in the right cortex was 52.6 ± 4.7 pmol/mg protein in the shams ($n = 19$), and increased to 100.1 ± 10.7 pmol/mg protein in the vehicle-treated animals 24 h after hypoxia ($n = 12$, $P < 0.05$ vs. shams). Caspase-9 activity in pups treated with 50 μg/pup of IETD-CHO given immediately after the hypoxia by i.c.v. injection and measured 24 h after hypoxia was 72.8 ± 3.5 pmol/mg protein ($n = 13$, $P < 0.05$ vs. shams and $P < 0.05$ vs. vehicle).

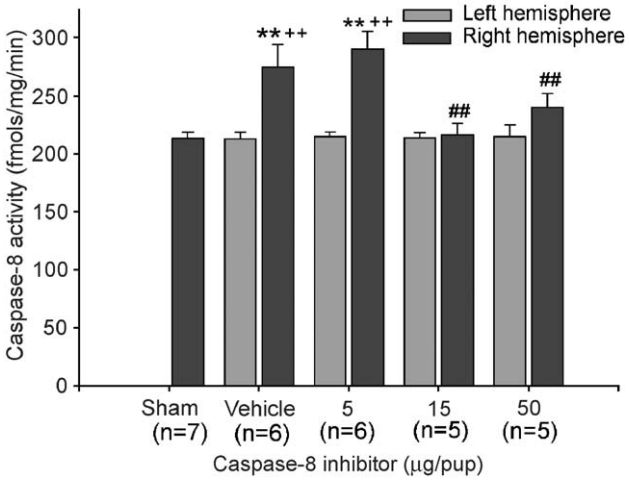

Fig. 2. The change in caspase-8 activity with inhibitor dose. Doses were given i.c.v. immediately after the hypoxic period. Doses of 15 and 50 μg/pup reduced caspase-8 activity relative to the vehicle group. $^{**}P < 0.01$ vs. sham, $^{++}P < 0.01$ vs. left contralateral side, $^{\#\#}P < 0.01$ vs. right side vehicle group.

We have shown that LEHD-CHO (Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Leu-Glu-His-Asp-CHO [MW = 2036.5], Calbiochem) a cell permeable caspase-9 inhibitor, is neuroprotective in this model (Feng et al., 2003). We have not previously tested LEHD-CHO's specificity relative to caspase-8 under the conditions where neuroprotection was demonstrated. Caspase-8 activity in the right cortex was 208 ± 8.6 fmol/mg protein/min in the shams, $n = 12$, and increased to 263 ± 24 fmol/mg protein/min at 24 h after hypoxia in the vehicle-treated animals ($n = 7$, $P < 0.05$ vs. shams). Caspase-8 activity in pups treated with 50 μg/pup of LEHD-CHO given immediately after the hypoxia by i.c.v. injection and measured 24 h after

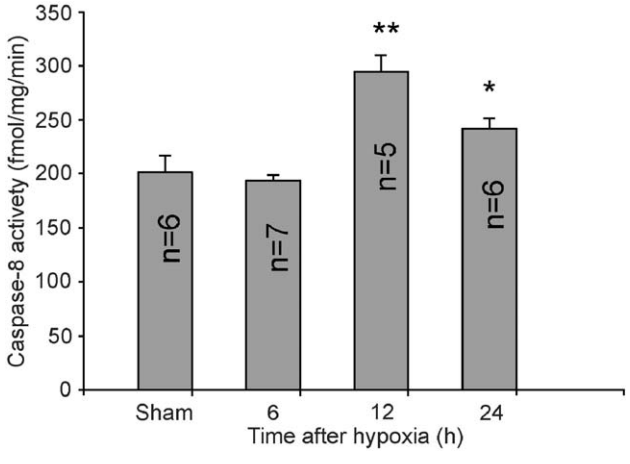

Fig. 1. The change in caspase-8 activity in the right cortex with time after injury. Caspase-8 activity was significantly increased at 12 and 24 h after injury. Data are presented as mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. sham.

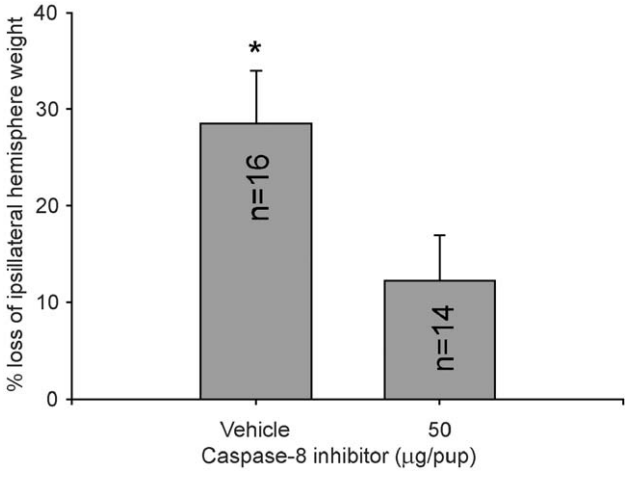

Fig. 3. Caspase-8 inhibition is neuroprotective. Fifty microgram per pup of the caspase-8 inhibitor IETD-CHO given i.c.v. reduces loss of ipsilateral hemisphere weight at 22 days after injury. $^{*}P < 0.05$ vehicle vs. caspase-8 inhibitor.

hypoxia was 276 ± 11 fmol/mg protein/min ($n = 8$, $P < 0.05$ vs. shams and $P =$ ns vs. vehicle).

The effect of 50 μg/pup of the caspase-8 inhibitor given immediately after hypoxia on the reduction in right hemispheric weight is shown in Fig. 3. Right hemisphere weight 22 days after injury was reduced by 28.5 ± 5.4% in the 16 vehicle-treated animals, and by 12.2 ± 4.8% in the 14 IETD-CHO-treated animals ($P < 0.05$). Left hemisphere weight is unchanged by hypoxic ischemia (vehicle 480 ± 7 mg, IETD-CHO 501 ± 12 mg, $P =$ ns).

Brain score by blinded observer 22 days after injury in the vehicle-treated pups was normal in 3/16 (19%), mild in 4/16 (25%), moderate in 4/16 (25%), and severe in 5/16 (31%). Brain score in the pups treated with 50 μg/pup of IETD-CHO immediately after hypoxia was normal in 7/14 (50%), mild in 5/14 (36%), moderate in 1/14 (7%), and severe in 1/14 (7%), $P < 0.05$. Relative to the vehicle group, the treatment group has less moderate and severe injuries and more normal and mild brain injuries ($P < 0.05$).

Histologic grading of the cortex 22 days after injury by blinded observer was 2 [0, 3.5] in the vehicle group (median [25%tile, 75%tile]), and 0 [0, 0] in the IETD-CHO group ($P < 0.05$), with the IETD-CHO group showing significantly better histologic outcome (Fig. 4).

There was no difference in rectal temperature between the vehicle and the IETD-CHO groups (data not shown). Body weight of the IETD-CHO-treated group was not significantly different from the control group before injury or at 4, 7, 11, 14, or 22 days after injury. Body weights increased significantly with time in both groups.

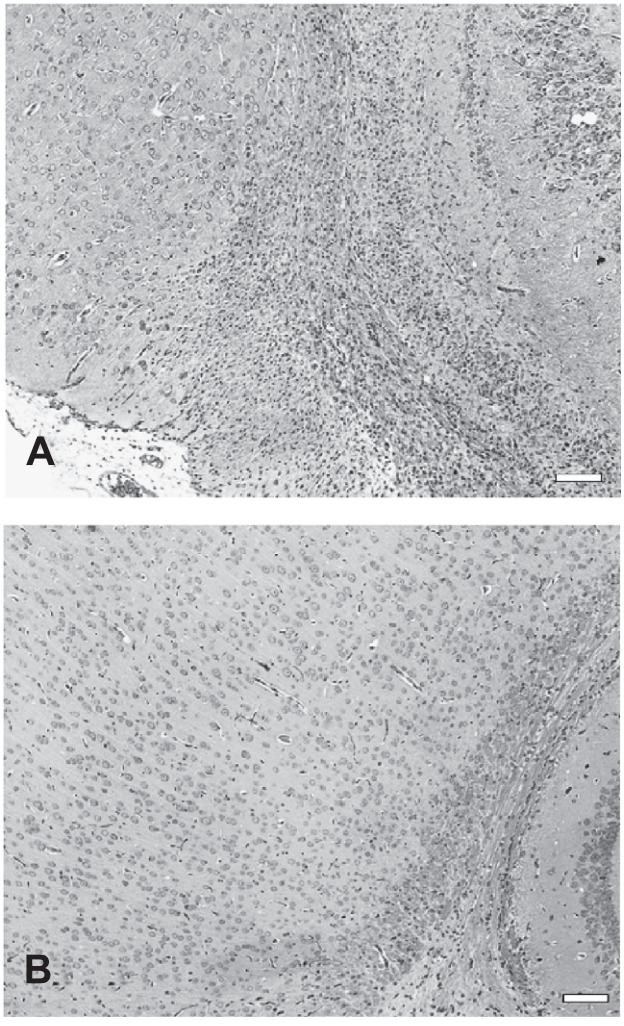

Fig. 4. Hemotoxylin and eosin stain of right (ipsilateral) cortex. The bar is 0.1 mm. "A" is from a vehicle-treated pup showing an infarct with loss of cortical tissue and infiltration of inflammatory cells. "B" is a view of the corresponding region from a pup treated with 50 μg/pup of IETD-CHO immediately after the hypoxic period, showing normal histology. The histopathological score, determined by an observer blind to the treatment group of the pups, was significantly better for the IETD-CHO-treated pups than that of the vehicle-treated pups ($P < 0.05$).

4. Discussion

IETD-CHO in vitro inhibits caspase-8 at 100-fold lower concentration than it inhibits caspase-9, 3, 7, 4 or 5 (Garcia-Calvo et al., 1998). In the conditions produced in this experiment, IETD-CHO produced inhibition of caspase-8 back to levels seen in the sham animals. Caspase-9 activity was reduced to half the increase in activity seen in the vehicle-treated pups over that seen is shams. Thus, IETD-CHO shows some specificity for caspase-8, but still shows some caspase-9 inhibitory activity. Whether IETD-CHO inhibits caspase-9 activity directly, or indirectly by inhibiting caspase-8 and thereby reducing bid linked caspase-9 activation is unclear from this experiment.

The caspase-9 inhibitor LEHD-CHO, which we have shown to be neuroprotective in the newborn rat model (Feng et al., 2003), does seem to be quite specific for caspase-9 over caspase-8. Caspase-8 is the major initiator caspase of the extrinsic, receptor dependent, apoptotic pathway (Graham and Chen, 2001), whereas, caspase-9 is the major initiator caspase of the intrinsic or mitochondrial pathway. Caspase-8 is activated by fas receptor ligands such as TNF-α. Fas receptors are up-regulated (Northington et al., 2001a,b) and TNF-α levels increased by hypoxic ischemia in the brain of the newborn rat (Silverstein et al., 1997). Fas activation activates caspase-8 which can then activate caspase-3 directly or caspase-8 can activate bid, which deactivates bcl-2 and thereby releases cytochrome *c* and activates Caspase-9 (Graham and Chen, 2001) which activates caspase-3. Caspase-9 is activated relatively late after injury, peaking at 24 h after injury, at about the same time that caspase-3 peaks (Northington et al., 2001b; Wang et al., 2001; Feng et al., 2003). Caspase-8 is activated somewhat earlier than caspase-9.

Giving one dose of caspase-8 inhibitor immediately after hypoxic ischemia continued to reduce caspase-8 activity 24 h after injury. Caspase-8 inhibition reduces brain injury, consistent with the hypothesis that caspase-8 and the extrinsic apoptotic pathway are important in hypoxic–ische-

mic injury in the newborn rat model. The degree of neuroprotection seen in the cortex is similar to that seen with a caspase-9 inhibitor (Feng et al., 2003) or with broad spectrum caspase inhibitors (Cheng et al., 1998), using the same newborn rat model with different outcome measures. Because of subtle differences between labs and over time, this is not the same as a direct comparison between these two agents. In conclusion, inhibiting caspase-8 activity in the brain with IETD-CHO is neuroprotective. Caspase-8 is important in the pathophysiology of hypoxic–ischemic brain injury in the newborn rat.

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