Pharmacologic amelioration of Severe Hypoglycemia-induced Neuronal Damage

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Abstract

Hypoglycemia is a common complication for insulin treated people with diabetes. Severe hypoglycemia, which occurs in the setting of excess or ill-timed insulin administration, has been shown to cause brain damage. Previous pre-clinical studies have shown that memantine (an N-methyl-D-aspartate receptor antagonist) and erythropoietin can be neuroprotective in other models of brain injury. We hypothesized that these agents might also be neuroprotective in reponse to severe hypoglycemia-induced brain damage. To test this hypothesis, 9-week old, awake, male Sprague-Dawley rats underwent hyperinsulinemic (0.2 U.kg^{-1}.min^{-1}) hypoglycemic clamps to induce severe hypoglycemia (blood glucose 10–15 mg/dl for 90 minutes). Animals were randomized into control (vehicle) or pharmacological treatments (memantine or erythropoietin). One week after severe hypoglycemia, neuronal damage was assessed by Fluoro-Jade B and hematoxylin and eosin staining of brain sections. Treatment with both memantine and erythropoietin significantly decreased severe hypoglycemia-induced neuronal damage in the cortex by 35% and 39%, respectively (both $p<0.05$ vs controls). These findings demonstrate that memantine and erythropoietin provide a protective effect against severe hypoglycemia-induced neuronal damage.

Keywords

hippocampus; cortex; insulin; memantine; erythropoietin

Introduction

Hypoglycemia is the major obstacle in achieving tight glycemic control in people with diabetes [1]. Intensive insulin therapy increases the risk of severe hypoglycemia [2]. By depriving the brain of glucose, severe hypoglycemia can cause brain damage leading to long-term impairments in learning and memory [3;4]. Episodes of severe hypoglycemia have been shown to cause significant cognitive damage in many [5–12] but not all [13–18] studies. Unlike retrospective clinical studies, carefully controlled prospective pre-clinical

studies have unambiguously demonstrated that acute severe hypoglycemia induces neuronal damage, especially to the vulnerable neurons in the cortex and hippocampus [3;19–25]. Deficits in learning and memory have been shown to be a direct consequence of this severe hypoglycemia-induced hippocampal neuronal damage [3;21;22;25].

Mechanistically, similarities and differences have been noted between neuronal injury induced by hypoglycemia and neuronal damage caused by other forms of acute neurologic insult [26]. Both ischemic and hypoglycemic neuronal damage are caused by an excitotoxic amino acid mediated increase in intracellular calcium, production of reactive oxygen species and apoptosis [27;28]. Agents that have been shown to be neuroprotective in animal models of brain ischemia warrant investigation to determine if they would be neuroprotective in severe hypoglycemia-induced brain injury. There are many optimistic reasons to think that neuroprotective agents would be particularly efficacious to prevent severe hypoglycemia-induced brain damage. Since cerebral blood flow is not impeded during hypoglycemia (as it is with a stroke), administered neuroprotective agents should have unrestricted ability to reach vulnerable brain areas. Neuroprotective agents used in pre-clinical hypoglycemia models may translate well into clinical trials given the unique nature of insulin-induced hypoglycemic brain damage which is unlike the heterogeneous types of stroke damage (i.e., lacunar, hemorrhagic, or embolic with or without reperfusion). Finally, the inherent delay in administering neuroprotective agents in the clinical setting of a stroke (due to transportation to a hospital or waiting for a brain imaging) could be minimized in a patient recovering from severe hypoglycemia if a neuroprotective agent could be administered on-site, simultaneously with a rescue intravenous bolus of glucose.

In this study, we tested memantine (an N-methyl-D-aspartate receptor antagonist) and erythropoietin as potential neuroprotective agents in a model of severe hypoglycemia. Previously, an older N-methyl-D-aspartate (NMDA) receptor antagonist MK-801 (dizocilpine), has been shown to be neuroprotective in response to ischemia [29] and severe hypoglycemia [30] but the neurobehavioral changes induced by this older NMDA agonist (ie, induction of cataplexy) limit its potential clinical utility. Memantine, a lower affinity NMDA receptor antagonist already in clinical use with limited side effects [31] has been shown to be neuroprotective in models of focal cerebral ischemia [29] and traumatic brain injury [32].

Erythropoietin, in addition to its role in the regulation of erythropoiesis, has neuroprotective, anti-inflammatory and neurotrophic properties and has been found to play a role in neurogenesis [33]. When recombinant erythropoietin was given intraperitoneally 24 hours prior to, and up to six hours after focal brain ischemia in rats, infarct volume was decreased by 50–75% [34]. Similarly, infusion of erythropoietin into the lateral ventricle of gerbils prevented ischemia-induced cell death in the hippocampus [35].

The goals of these studies are to determine whether these pharmacological agents, previously shown to be neuroprotective in pre-clinical models of ischemia, would be neuroprotective in response to brain damage induced by severe hypoglycemia.

Methods

Animals

Nine week old male Sprague-Dawley rats (Charles River Laboratories) were individually housed in a temperature and light controlled environment maintaining the animal's diurnal cycle (12hrs light, 12hrs dark), and with an ad lib diet. All studies were done in accordance with the Animal Studies Committee at the Washington University School of Medicine and the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Implantation of arterial and venous catheters

Anesthesia was induced with an intraperitoneal injection of ketamine 87 mg/kg and xylazine 2.6 mg/kg. A Micro-Renathane brand (Braintree Scientific, Boston, MA) catheter was inserted into the left common carotid artery and two catheters were implanted into the right jugular vein. To prevent clotting, catheters were filled with a 40% polyvinylpyrrolidone (Sigma, St. Louis, MO) heparin (1000 USP U/ml) solution (Baxter Healthcare Corporation, Deerfield, IL).

Severe Hypoglycemia Clamp

Clamp experiments were performed after the rats were allowed to recover for one week following implantation of arterial and venous catheters. After an overnight fast, vascular catheters were externalized and attached to infusion pumps. After a basal period to allow the rats to acclimate, insulin (Humulin R, Eli Lilly and Co., Indianapolis, IN) was infused intravenously (0.2 U.kg^{-1}.min^{-1}) into awake, unrestrained rats. Throughout the clamp, blood glucose was measured from the arterial cannula every fifteen minutes using Ascensia Contour blood glucose monitors (Bayer Healthcare, LLC, Mischawake, IN). Previous studies determined the nadir hypoglycemia necessary to reproducibly cause neuronal damage was a glucose level <15 mg/dl [24;25]. Once the blood glucose dropped to below 15 mg/dl, hypoglycemia was maintained at 10–15 mg/dl for ninety minutes by adjusting the rate of continuous intravenous glucose infusion (50% dextrose). After the ninety minutes of severe hypoglycemia, hypoglycemia was terminated with infusions of dextrose to restore euglycemia. Throughout this recovery period blood glucose readings were made every 15 minutes and the rates of glucose infusion were slowly decreased as the glucose-lowering effects of insulin waned.

Experimental groups

Memantine group—Based on previous neuroprotective studies [32;36;37], memantine hydrochloride (Sigma, St. Louis, MO) was dissolved in saline. Memantine (20mg/kg) (*n*=8) or saline vehicle (*n*=7) was injected intraperitoneally immediately following 90 minutes of severe hypoglycemia.

Erythropoietin group—Vehicle solution was made with 5.8mg of sodium citrate (Sigma, St. Louis, MO), 5.8mg of sodium chloride (Sigma, St. Louis, MO), 0.06mg of citric acid (Sigma, St. Louis, MO), and 2.5mg of albumin from human serum (Sigma, St. Louis, MO) dissolved in 1ml of water. Based on previous neuroprotective studies [38–43] animals were given 5000IU/kg (*n*=11) of Epoetin alfa (Amgen, Thousand Oaks, CA) or vehicle solution (*n*=10) on three occasions; intraperitoneally 24 hours prior to the hypoglycemic clamp, intravenously immediately following 90 minutes of severe hypoglycemia, and intraperitoneally 24 hours after the hypoglycemic clamp.

Histology—One week following the episode of severe hypoglycemia, rats were anesthetized with isoflurane (Butler Animal Health Supply, Dublin, OH) and then transcardially perfused with 100 ml of 0.01 M phosphate buffered saline (Sigma) and 100 ml of 4% paraformaldehyde (PFA) (Electron Microscopy Scientific). Perfused brains were left in 4% PFA overnight in 4 °C and then cryoprotected in 30% sucrose (Sigma, St. Louis, MO). Brains were then set in Tissue-Tek OCT Compound (Sakura Finetek, Torrance, CA) and stored at −20 °C. Using a Leica CM1850 cryostat (Leica Microsystems Inc., Bannockburn, IL), 6–10 coronal sections (10 and 20μm) taken 120 μm apart were collected starting 2.8m posterior to the bregma. Sections were stained with either Fluoro-Jade B (Chemicon International, Inc., CA) and Dapi (Sigma, St. Louis, MO) or hematoxylin and

eosin (H&E, Sigma, St. Louis, MO) and examined with an epifluorescent microscope (Zeiss) using fluorescein isothiocynate (FITC) (for Fluoro-Jade B).

Microscopy and Cell Counting—H&E staining was used to identify the different brain regions and identify neuronal cell damage as noted by pyknotic cells. For quantification purposes, the number of Fluoro-Jade B positive cells per high power field was assessed by an observer blinded to the treatment conditions. Using two sections per animal, the data is expressed as the average number of Fluoro-Jade B positive cells per brain region of interest.

Statistical Analysis—All data is expressed as mean ± SEM. Statistical significance was determined by a two-tailed student t-test, $p < 0.05$.

Results

Memantine

In the memantine and control groups, there was no difference in blood glucose values at baseline (MEM 66 ± 4 mg/dl vs. CONT 63 ± 3 mg/dl, p=NS), during ninety minutes of severe hypoglycemia (MEM 11 ± 0.5 mg/dl vs. CONT 11 ± 0.2 mg/dl, p=NS), or during recovery (MEM 177 ± 22 mg/dl vs. CONT 187 ± 31 mg/dl, p=NS) (Fig 1A). Treatment with memantine decreased hypoglycemia-induced neuronal damage in the cortex by 35% (MEM 838 ± 137 vs. CONT 1282 ± 121 cells, p=0.02)(Fig 1B, 2). Although memantine treatment also decreased the amount of neuronal damage in the hippocampus (MEM 827 ± 155 vs. CONT 1075 ± 213 cells, p=NS), these results were not statistically significant (Fig 1B).

Erythropoietin

There was no difference in glucose levels at baseline (EPO 61 ± 4 mg/dl vs. CONT 65 ± 5 mg/dl, p=NS), during severe hypoglycemia (EPO 12 ± 0.4 mg/dl vs. CONT 11 ± 0.2 mg/dl, p=NS), or during recovery (EPO 194 ± 23 mg/dl vs. 167 ± 31 mg/dl, p=NS) (Fig 1C). In the cortex, animals treated with erythropoietin had a 39% decrease in the amount of hypoglycemia-induced neuronal damage (EPO 1350 ± 241 vs. CONT 2202 ± 211 cells, p=0.01)(Fig 1D, 2). Although erythropoietin also attenuated damage in the hippocampus, results were not statistically significant (EPO 1655 ± 209 vs. CONT 1982 ± 126 cells, p=NS) (Fig 1D).

Discussion

In this study we tested whether or not the administration of pharmacologic agents shown to be neuroprotective in other models of acute neuronal injury would be similarly neuroprotective against the brain cell damage caused by severe hypoglycemia. We demonstrate that memantine and erythropoietin protect against severe hypoglycemia-induced cortical brain damage.

In this study a hyperinsulinemic hypoglycemic clamp technique was used to precisely match for duration (90 minutes) and nadir of hypoglycemia (10–15 mg/dl). Our lab [24;25] and others [3;19–23] have previously shown that this degree of hypoglycemia consistently and reproducibly causes neuronal brain damage in the cortex and hippocampus. We also noted that there was equal damage in both the left and right hemispheres (data not shown) in response to global severe hypoglycemia indicating that cannulation of the left common carotid artery did not appear to have any significant detrimental effects.

Although anesthesia is often used in severe hypoglycemia experiments; to create a more real-world model of hypoglycemia, our experiments were performed in awake, freely mobile

rats. We were thus able to circumvent the confounding effects of anesthesia including, 1) reduced brain glucose uptake which may increase brain damage, 2) abrogated seizure activity which is often associated with severe hypoglycemia, 3) induced hypothermia, 4) impaired increase blood pressure (Cushing) response to hypoglycemia, and 5) neurotoxicity [44–48].

During the ninety minutes of severe hypoglycemia, animals went into a coma (as evidenced by the fact that they loss their righting reflex) and exhibited characteristic tonic-clonic seizure-like behavior noted by characteristic brief (5–10 seconds) neck extensions, tonic stretching, uncontrolled limb movements, and spontaneous spinning [24;25]. In the absence of electroencephalogram (EEG) monitoring, the effect of subclinical seizures (i.e. seizures not associated with noticeable motor activity) on brain damage could not be assessed. Also, since potassium was not measured, we could not rule out the possibility that the effects of insulin induced hypoglycemia on brain damage were mediated, at least in part, by insulin induced hypokalemia.

Rather than employing a pre-treatment protocol, memantine were administered following the episode of hypoglycemia, at the time of glucose administration. This post-hypoglycemia administration of memantine was designed to mimic real-world conditions where severe hypoglycemia would be terminated in the Emergency Room with the infusion of glucose and a potential neuroprotective agent. Since severe hypoglycemia causes neuronal damage via excitotoxicity [26], memantine's efficacy in this study is likely attributed to its mechanism of action as a low-affinity, uncompetitive NMDA blocker that blocks receptor in situations of excessive or pathologic activity [31] [29;32].

The mechanism of erythropoietin-induced neuroprotection in the cortex of rats in this study is likely multifactorial and may be related to its inhibition of proinflammatory cytokines [49], inhibition of nitric oxide synthesis [35], stimulation of anti-apoptotic proteins [43;50] or protection against reactive oxygen species and free radicals [51]. Since studies have shown erythropoietin to be neuroprotective when given both before and after several models of brain injury [35;38–43;49;51]; we chose to administer erythropoietin both prior to and following severe hypoglycemia in order to increase the likelihood of observing an effect. It remains to be determined if erythropoietin would be similarly neuroprotective if given only after an episode of severe hypoglycemia.

Both memantine and erythropoietin significantly protected against cortical brain damage, but the trend towards neuroprotection against hippocampal brain damage did not reach significance suggesting that there may be a regional susceptibility to hypoglycemia induced brain damage and/or regional responsiveness to neuroprotective agents. In previous studies, the cortex has consistently been shown to be more vulnerable than the hippocampus to glucose deprivation [24;25;52–54]. It is therefore speculated that differential susceptibilities to damage and differential responses to neuroprotective agents may be due to regional variations in antioxidant defenses [53–55], mitochondria bioenergetics [27;52], and/or distribution of NMDA receptors [56].

In summary, the administration of memantine and erythropoietin significantly decreased severe hypoglycemia-induced cortical neuronal damage by 35% and 39% respectively in our model (p<0.05 vs controls). Further studies are needed to determine the mechanisms by which neuronal damage is caused by severe hypoglycemia and identify the pathways by which neuroprotective pharmacological agents act to ameliorate damage. It is concluded that memantine and erythropoietin have the potential to be clinically efficacious agents that confer neuroprotection in response to severe hypoglycemia induced brain damage.

Acknowledgments

## Abbreviation

NMDA   N-methyl-D-aspartate

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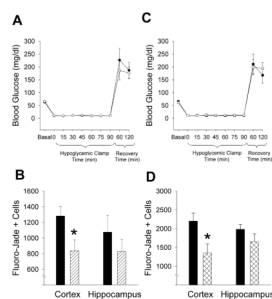
Figure 1. Memantine and Erythropoietin attenuate cortical brain damage after 90 minutes of severe hypoglycemia

(A) During a hyperinsulinemic severe hypoglycemic (10–15 mg/dl) clamp, blood glucose was not significantly different between vehicle-treated (n=7; ◆) and memantine-treated (n=8; ◇) rats before, during or after 90 minutes of severe hypoglycemia. (B) Neuronal damage resulting from severe hypoglycemia was quantified using Fluoro-Jade B staining in vehicle treated (black bar) and memantine-treated (lined bar) rats. Treatment with memantine significantly reduced the number of degenerating neurons compared to vehicle-treated animals in the cortex (*p=0.02) but not in the hippocampus (p=NS). (C) During a hyperinsulinemic severe hypoglycemic (10–15 mg/dl) clamp, blood glucose was not significantly different between vehicle-treated (n=10; ■) and erythropoietin-treated (n=11; □) rats before, during or after 90 minutes of severe hypoglycemia. (D) Neuronal damage resulting from severe hypoglycemia was quantified using Fluoro-Jade B staining in vehicle-treated (black bar) and erythropoietin-treated (cross-hatched bar) rats. Treatment with erythropoietin significantly reduced the number of degenerating neurons compared to vehicle-treated animals in the cortex (*p=0.01) but not in the hippocampus (p=NS).
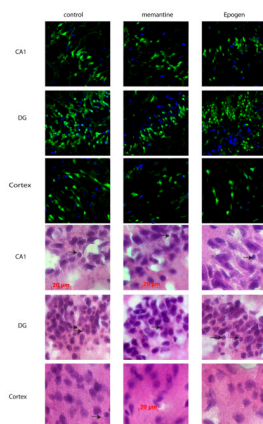
Figure 2. Neuronal damage as assessed by hematoxylin and eosinophilic staining and by Fluoro-Jade B staining

In the top 9 photomicrographs Fluoro-Jade B-positive (green) and Dapi (blue) staining are shown and in the bottom 9 photomicrographs Hematoxylin and eosinophilic staining are shown from representative sections in the hippocampus [CA 1 and dentate gyrus (DG)] and ectorhinal cortex superior to the rhinal fissure viewed at X 400 magnification in control, memantine-treated, and erythropoietin (Epogen) treated animals. Brain cells damaged by severe hypoglycemia are characterized by condensed shrunken morphology and pyknotic nuclei with eosinophilic staining (arrows). Scale bar indicates 20 microns.