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Alpha-2 agonist attenuates ischemic injury in spinal cord neurons

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ARTICLE INFO

Article history:
Received 6 May 2014
Received in revised form
4 December 2014
Accepted 17 December 2014
Available online 23 December 2014

Keywords:
Aortic surgery
Ischemia–reperfusion injury
Paraplegia
Dexmedetomidine

ABSTRACT

Background: Paraplegia secondary to spinal cord ischemia–reperfusion injury remains a devastating complication of thoracoabdominal aortic intervention. The complex interactions between injured neurons and activated leukocytes have limited the understanding of neuron-specific injury. We hypothesize that spinal cord neuron cell cultures subjected to oxygen-glucose deprivation (OGD) would simulate ischemia–reperfusion injury, which could be attenuated by specific alpha-2a agonism in an Akt-dependent fashion.

Materials and methods: Spinal cords from perinatal mice were harvested, and neurons cultured *in vitro* for 7–10 d. Cells were pretreated with 1 μM dexmedetomidine (Dex) and subjected to OGD in an anoxic chamber. Viability was determined by MTT assay. Deoxyuridine-triphosphate nick-end labeling staining and lactate dehydrogenase (LDH) assay were used for apoptosis and necrosis identification, respectively. Western blot was used for protein analysis.

Results: Vehicle control cells were only 59% viable after 1 h of OGD. Pretreatment with Dex significantly preserves neuronal viability with 88% viable ($P < 0.05$). Dex significantly decreased apoptotic cells compared with that of vehicle control cells by 50% ($P < 0.05$). Necrosis was not significantly different between treatment groups. Mechanistically, Dex treatment significantly increased phosphorylated Akt ($P < 0.05$), but protective effects of Dex were eliminated by an alpha-2a antagonist or Akt inhibitor ($P < 0.05$).

Conclusions: Using a novel spinal cord neuron cell culture, OGD mimics neuronal metabolic derangement responsible for paraplegia after aortic surgery. Dex preserves neuronal viability and decreases apoptosis in an Akt-dependent fashion. Dex demonstrates clinical promise for reducing the risk of paraplegia after high-risk aortic surgery.

1. Introduction

Paraplegia remains a devastating complication in thoracoabdominal aortic intervention resulting in significant burdens on the patients, their families, and the medical system. Despite advances in surgical practice, paraplegia continues to be an obstacle in complex thoracoabdominal aortic surgery [1]. Spinal cord injury still occurs in up to 20% of

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0022-4804/$ – see front matter
http://dx.doi.org/10.1016/j.jss.2014.12.033

high-risk patients [2]. Ischemia to the spinal cord leads to complex metabolic derangement and inflammatory responses that further contribute to neuronal degeneration, known as ischemia–reperfusion (IR) injury [3].

IR injury has been an area of intensive investigation with alpha-2 agonists being widely studied. Alpha-2 agonists have been shown to attenuate IR injury in cerebral [4–7], cardiac [8], lung [9], and renal [10] models. Dexmedetomidine (Dex), a highly selective alpha-2a agonist, has been shown in *in vivo* murine models to improve neuronal viability and provide functional attenuation of spinal cord IR injury [11]. Dex is commonly used in the intensive care unit as a sedative, so translation into a neuroprotective agent would not be challenging. However, the mechanism by which Dex provides neuroprotection remains to be elucidated, especially within specific populations of cells within the spinal cord.

IR produces cytokine release [12] and induces apoptotic events, which initiate a cascade of processes that lead to cell injury and death. Promoting the phosphatidylinositol 3-kinase (PI3K-Akt) survival pathway reduces apoptotic cell death [13]. The Akt pathway is a prosurvival pathway, which has been implicated as having a role in the protection by Dex [8,14–16].

Our previously published murine model showed the protective effects of Dex in spinal cord IR injury [11]; however, the neurons could not be separated from the other cellular milieu for specific investigation. We hypothesize that isolated spinal cord neurons subjected to oxygen-glucose deprivation (OGD) would simulate IR injury seen in aortic surgery and that Dex preserves neuronal viability through an Akt-dependent mechanism.

2. Methods

2.1. Materials

Dex, atipamezole, and LY294002 were purchased from Tocris (Ellisville, MO). The dose of Dex was determined by prior dose response curves. All antibodies including pAkt, Akt, and secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA).

2.2. Animals

All experiments were approved by the Animal Care and Use Committee at the University of Colorado at Denver Health Sciences Center, and conformed to the Guide for the Care and Use of Laboratory Animals (US National Institutes of Health publication No. 85-23, National Academy Press, Washington DC, revised 1996). Postnatal day 2–3 old C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) were used for all experiments. Each litter was considered $n = 1$ (all pups from 1 litter are considered as one group, and then a second litter would be a second n).

2.3. Cell culture

Primary spinal cord neuron cultures were obtained from 2–3-day-old mice. Briefly, mice were euthanized with isoflurane then decapitated. The vertebral column was dissected out, and the spinal cord was removed *en bloc* via injection of phosphate-buffered saline (PBS, pH 7.4) through the spinal canal. Spinal cord tissue was minced and then digested in Hibernate-A (Invitrogen, Carlsbad, CA) with Papain (Worthington, Lakewood, NJ). Neurons were isolated using an OptiPrep (Sigma–Aldrich, St Louis, MO) density gradient adapted from Brewer [17] and plated on Poly-D-Lysine (Sigma–Aldrich) coated plates at 300,000 cells per well on a 24-well plate in Neurobasal-A (Invitrogen), B27 (Invitrogen), Glutamax (Invitrogen), and Penicillin/Streptomycin (Gibco, New York, NY). On *in vitro* day three, AraC (Sigma–Aldrich) was added to prevent astrocyte replication. Cell cultures were maintained in a humidified atmosphere containing 5% CO_2 at 37°C and underwent half media change every 3 d. The cultures were >90% neurons as seen by morphology on light microscope and confirmed with microtubule-associated protein 2 (MAP2) positive staining. Cultures of neurons from perinatal mice are considered mature on *in vitro* day 7–10, and thus were used for experimentation after *in vitro* day 7.

2.4. Immunofluorescence

On *in vitro* day 8, the cells were fixed with 4% paraformaldehyde on their culture slides, then blocked with 5% normal goat serum in 0.1% Triton X-100 (Sigma-Aldrich, St Louis, MO). Cells were incubated with primary antibody MAP2 (Cell Signaling Technology) at 1:50 overnight at 4°C, followed by secondary antibody Anti-Rabbit IgG Alexa Fluor 488 Conjugate (Cell Signaling Technology) at 1:1000 for 1 h. Prolong Gold Anti-Fade Reagent with 4′, 6-diamidino-2-phenylindole (Cell Signaling Technology) was added to slides and coverslips applied. Images were acquired using Intelligent Imaging Innovations Slidebook (Denver, CO) software.

2.5. Oxygen-glucose deprivation

On the day of the experiment, the experimental medium Dulbecco Modified Eagle Medium without glucose (Gibco) was placed in the Ruskin Bug Box Plus humidified airtight hypoxic chamber for 2 h. The Ruskin Bug Box Plus was used as per manufacturer's protocol to maintain an environment of 95% N_2/5%CO_2 at 37°C and verified with Anaerobic Indicator (Oxoid Ltd, Basingstoke, Hants, United Kingdom). The maintenance culture medium was removed, cells washed with PBS, and experimental hypoxic medium was added to the cell culture wells. OGD was induced by placing the plates in the hypoxic chamber. Dex at 1 μM or vehicle control (VC) was added 30 min before OGD and added to the OGD media during anoxia (Fig. 1). The concentration of Dex used was determined by a literature review. Antagonists and/or inhibitors were added before Dex at a concentration of 10 μM.

2.6. Viability studies

Cell viability was determined by MTT Cell Proliferation Kit (Roche, Indianapolis, IN) according to the procedure provided by the manufacturer. Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well at a concentration of 0.5 mg/mL and incubated for 4 h at 37°C,