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NEUROPROTECTION OF ADULT RAT DORSAL ROOT GANGLION NEURONS BY COMBINED HYPOTHERMIA AND ALKALINIZATION AGAINST PROLONGED ISCHEMIA

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Abstract—Ischemia and ischemia-induced secondary events, such as acidosis and excessive activation of receptors by amino acids, trigger neuron death. The isolation and dissociation of dorsal root ganglion (DRG) involves time during which the neurons are ischemic due to being densely packed within the intact DRG and surrounded by a connective tissue coat. Thus, the longer the time between killing the host animal and when the DRG are dissociated, the longer the neurons are ischemic and exposed to ischemia-induced secondary causes of neuron death. It is well established that hypothermia and alkalinization each separately protect neurons from ischemia and ischemia-induced secondary causes of neuron death, but there are no data on the neuroprotection provided by simultaneous hypothermia and alkalinization. The present experiments were designed to determine the combination of hypothermic and alkaline conditions that yield the largest number of viable neurons dissociated from intact DRG maintained ischemic for up to 4 h. Hypothermia (20 °C>15 °C>37 °C) and alkalinization (pH 9.3>pH 8.3>pH 7.4) increased the yield of viable neurons compared with the yield from DRG maintained under physiological conditions. Hypothermia and alkalinization combined (20 °C/pH 9.3) provided the greatest neuroprotection with a yield of viable neurons after 1 h of ischemia 2.5-fold larger than that from DRG maintained under physiological conditions (37 °C/pH 7.6). Over 4 h of ischemia, the yield of viable neurons from DRG maintained under both hypothermic/alkaline and physiological conditions decreased in a linear manner, but those at 20 °C/pH 9.3 had a 4.5-fold greater yield of viable neurons than those at 37 °C/pH 7.6. Thus, combined hypothermia and alkalinization provide significantly greater protection against ischemia and ischemia-induced secondary causes of neuron death than either alone. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: anoxia, neurotoxicity, neuron death.

CNS trauma triggers insults, such as ischemia, lipid peroxidation, loss of calcium homeostasis by neurons, and acidosis, all of which exacerbate neuronal death leading to neurological deficits. To minimize the loss of neurological function following CNS trauma requires minimizing the number of neurons killed by ischemia and its secondary insults. Although various methods provide neuroprotection against secondary insults of trauma, improved methods are required to reduce further neuron death.

Electrophysiological and other studies on adult rat dorsal root ganglion (DRG) neurons indicate that small changes in the cellular environment (predominantly increases in temperature and decreases in pH) result in dramatic changes in membrane properties that damage and kill neurons (Adachi et al., 1998; Maginot et al., 1998). Other triggers of neuron death are excitotoxins, such as glutamate (Adachi et al., 1998) and aspartate (Follis et al., 1994, 2000; Cho et al., 2000), extracellular acidification (Sucher et al., 1997), trauma-induced inflammation (Schnell et al., 1999a,b), membrane peroxidation (Inci et al., 2001), and excessive NMDA receptor activation leading to massive calcium influx and disruption of calcium homeostasis (Cebers et al., 1998; Leist and Nicotera, 1998; Wang et al., 2002).

Systemic hypothermia provides protection against ischemia to adult rat CNS neurons (Wagner et al., 2002; Debillon et al., 2003). Clinically, whole body hypothermia provides neuroprotection, but it is limited, probably due to requiring interactions with an additional environmental condition. However, systemic hypothermia is dangerous due to its triggering cardiac arrest, and causing permanent damage to, and failure of, the liver and kidneys (Biberthaler et al., 2001b).

Regional hypothermia significantly reduces the clinical problems resulting from systemic hypothermia. Clinically, patients who undergo regional, rather than systemic hypothermia during cerebral ischemia, suffer less significant neurological deficits (Aebert et al., 2001).

Animal model experiments have shown that regional hypothermia provides protection against prolonged ischemia to cerebral and spinal neurons. However, in these experiments the extent of hypothermia providing optimal neuroprotection varied from mild (33–35 °C; Mori et al., 1998, 2001) to moderate (30–32 °C; Kwun and Vacanti, 1995; Mori et al., 1998; Yamamoto et al., 1999) to severe (17–29 °C; Lucas et al., 1994; Mori et al., 1998; Sueda et al., 2002) and to extreme (4–8 °C; Herold et al., 1994; Biberthaler et al., 2001a).

In addition to hypothermia, alkalinization provides neuroprotection against ischemia (Ishikawa and Marsala, 1999). Alkalinization to pH 8.2 protects adult rat CNS neurons against ischemic effects of infarct (Dietrich et al., 1993), and mouse neocortical neurons in primary culture from azide-induced chemical anoxia (Jorgensen et al., 1999).

A number of experiments have demonstrated that neuroprotection by one mechanism is enhanced when com-

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Abbreviations: CNS, central nervous system; DMEM, Delbecco's Modified Eagle's Medium; DRG, dorsal root ganglion; EAA, excitatory amino acid; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide.

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bined with another. The neuroprotection provided by regional hypothermia against 45 min of ischemia is increased by the simultaneous infusion with adenosine (Herold et al., 1994; Lucas et al., 1994; Parrino et al., 1999; Ross et al., 2000). Similarly, regional hypothermia together with the simultaneous infusion of a NMDA receptor antagonist enhances the neuroprotection provided by hypothermia (Lucas et al., 1994). There are no data on the neuroprotection provided by simultaneous hypothermia and alkalinization.

EXPERIMENTAL PROCEDURES

Owing to their large size and numerous neurons, cervical and lumbar DRGs from adult rats were used for the present experiments. To allow comparison of the extent of neuroprotection provided by the different combinations of low and high temperature and pH it was essential to compare the yields of viable neurons only from paired DRG from the same spinal cord level that contain the same number of neurons. For each experiment, one DRG from each pair was maintained under one set of conditions while its twin was maintained under another set of conditions. This provided the relative differences between the yields of viable neurons from DRG under the different conditions. Except for the time the DRG were under the different conditions, they were treated identically.

Adult rat DRG were removed at room temperature (20 °C), and maintained as pairs in DMEM+F15 (50/50; Sigma Chemical, St. Louis, USA) culture medium without serum. After isolating all the required DRG, but without removing their surrounding connective tissue capsule, the DRG pairs were placed for 1–4 h in medium at different combinations of pH (adjusted to pH 7.4, 8.3 and 9.6 with HEPES buffer), and temperature (37 °C, 20 °C, and 15 °C). Stable temperatures were maintained by placing the DRG in an ambient air incubator at 37 °C, at room temperature (20 °C), or on a 15 °C stage.

After the specified time under their respective conditions all the DRG were placed in medium at 20 °C and pH 7.2, their connective tissue capsules removed using irredectomy scissors and the DRG cut into small pieces. The pieces were placed in medium containing the enzymes collagenase P (5 mg/ml), neutral Dispase II (8 mg/ml), and DNase (0.3 mg/ml; Roche Diagnostics, Indianapolis, IN, USA). The dishes were placed in an O_2/CO_2 incubator at 37 °C for 1.5 h and the pieces of DRG gently triturated five times every 15 min through a 1-ml plastic pipette tip with an inside diameter just larger than the pieces of DRG. Once the neurons were completely dissociated, the enzyme-containing medium was removed by four changes with fresh medium at 20 °C/pH 7.4.

Falcon tissue culture dishes were treated for 1 h with poly-L-lysine (1 mg/ml; Sigma Chemical), followed by 1 h with laminin (Sigma Chemical; 5 mg/ml). Fresh medium (DMEM+F-12 culture medium) was added to the dishes and the dissociated neurons plated using a siliconized micropipette with a tip of about 100 μm attached to a mouth sucker. Viable neurons attached to the substrate immediately. Heat-inactivated fetal bovine serum (Sigma) was added to the medium after 15 min to a final concentration of 10%. Addition of serum before plating the neurons prevented the neurons from adhering to the substrate. The dishes were placed in an O_2/CO_2 incubator (95/5%) at 37 °C, in a saturated water environment. The cultures were maintained without any neurotrophic or other factors. After 18 h, the number of viable neurons was counted. The 18-h time point was chosen because it was a convenient time the following day to count the yield of viable neurons after plating the dissociated neurons from that day's experiment.

In a second set of experiments, we compared the yields of viable neurons from intact DRG maintained ischemic up to 4 h at 37 °C/pH 7.4 vs. 20 °C/pH 9.3. For these experiments, one DRG was dissociated immediately and its twin dissociated after the specified time. The data were normalized against the starting number of neurons for each pair, which represented the largest possible yield of neurons from each DRG of that pair.

Assay of neuron viability

The yield of viable neurons was assessed using the Trypan Blue dye exclusion test. Trypan Blue was added to the cultures to a final concentration of 0.4% for 20 min, and the viable neurons that exclude dye counted.

Data analysis

The data were analyzed using two-way mixed-design ANOVA.

Animal studies

These studies were carried out in accordance with NIH Guide for the Care Use of laboratory Animals, and with the IACUC approval. All efforts were made to minimize the number of animals used, and their suffering.

RESULTS

Histological methods have yielded estimates of the number of neurons within intact adult rat adult rat DRGs C-4, 5, and 6 at 6500 (Pover et al., 1993; Coggeshall and Lekan, 1996) to 6825–7508 (Mille-Hamard et al., 1999). In the current experiments the maximum yield of viable dissociated neurons was determined when C-4 DRG were dissociated immediately after being isolated from the adult rat. These DRG yielded a mean of 4324 viable neurons±812 S.D. (N=4). This represented yields of viable neurons that were 61–67% of the total number of neurons estimated to be in these ganglia.

Intact DRG were maintained for 1 h in culture medium at different combinations of temperature (37 °C, 20 °C and 15 °C) and pH (7.4, 8.3 and 9.3) to determine the extent of neuroprotection provided by hypothermia and alkalinization alone, or in combination, against ischemia and trauma-induced secondary triggers of neuron death. The DRG were dissociated, all neurons from a single DRG plated in a single culture dish, and the dishes placed in an O_2/CO_2 incubator at 37 °C. After 18 h, the number of viable neurons in each dish was counted, and the data compared with determine the relative yields of viable neurons from each condition. Thus, all DRG received exactly the same treatment except for the time they were under the different combinations of temperature and pH.

Preliminary data indicated that hypothermia and alkalinization (20 °C/pH 9.3) provided the greatest degree of neuroprotection and thus the highest yield of viable neurons. Therefore, data from all the experiments were normalized against the yield under the most neuroprotective condition, viz. that provided by 20 °C/pH 9.3.

For DRG maintained at 37 °C, alkalinization from pH 7.6–8.3, and to 9.3 increased the yield of viable neurons from 40.7±1.9% to 47.5±1.5% (N=3), and to 53.0±4.0% (N=3). These changes represent increases of 14.3%

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