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PHOTOBIOMODULATION PARTIALLY RESCUES VISUAL CORTICAL NEURONS FROM CYANIDE-INDUCED APOPTOSIS

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Abstract—Near-infrared light via light-emitting diode treatment has documented therapeutic effects on neurons functionally inactivated by tetrodotoxin or methanol intoxication. Light-emitting diode pretreatment also reduced potassium cyanide-induced cell death, but the mode of death via the apoptotic or necrotic pathway was unclear. The current study tested our hypothesis that light-emitting diode rescues neurons from apoptotic cell death. Primary neuronal cultures from postnatal rat visual cortex were pretreated with light-emitting diode for 10 min at a total energy density of 30 J/cm^2 before exposing to potassium cyanide for 28 h. With 100 or 300 μM potassium cyanide, neurons died mainly via the apoptotic pathway, as confirmed by electron microscopy, Hoechst 33258, single-stranded DNA, Bax, and active caspase-3. In the presence of caspase inhibitor I, the percentage of apoptotic cells in 300 μM potassium cyanide was significantly decreased. Light-emitting diode pretreatment reduced apoptosis from 36% to 17.9% (100 μM potassium cyanide) and from 58.9% to 39.6% (300 μM potassium cyanide), representing a 50.3% and 32.8% reduction, respectively. Light-emitting diode pretreatment significantly decreased the expression of caspase-3 elicited by potassium cyanide. It also reversed the potassium cyanide-induced increased expression of Bax and decreased expression of Bcl-2 to control levels. Moreover, light-emitting diode decreased the intensity of 5-(and -6) chloromethy-2′, 7-dichlorodihydrofluorescein diacetate acetyl ester, a marker of reactive oxygen species, in neurons exposed to 300 μM potassium cyanide. These results indicate that light-emitting diode pretreatment partially protects neurons against cyanide-induced caspase-mediated apoptosis, most likely by decreasing reactive oxygen species production, down-regulating pro-apoptotic proteins and activating anti-apoptotic proteins, as well as increasing energy metabolism in neurons as reported previously. © 2006 Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: CM-H_2DCFDA, 5-(and -6) chloromethy-2′, 7-dichlorodihydrofluorescein diacetate acetyl ester; DAB, 3,3′-diaminobenzidine; HRP, horseradish peroxidase; KCN, potassium cyanide; LED, light-emitting diode; NIR, near-infrared; PBS, phosphate-buffered saline; ROS, reactive oxygen species; RT, room temperature; ssDNA, single-stranded DNA.

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Low-energy laser irradiation in the far red to near-infrared (NIR) range is found to modulate various biological processes (Karu, 1999) by increasing mitochondrial respiration or ATP synthesis (Passarella et al., 1984; Morimoto et al., 1994; Karu et al., 1995; Yu et al., 1997a; Wilden and Karthein, 1998), facilitating wound healing (Conlan et al., 1996; Yu et al., 1997b; Sommer et al., 2001; Whelan et al., 2001) and promoting cell survival (Shefer et al., 2002). Possible mechanisms involve an acceleration of electron transfer in the respiratory chain and activation of photoacceptors, such as cytochrome oxidase, thus pointing to a particular role for mitochondria. Mitochondria are sensitive to irradiation with monochromatic NIR, 50% of which is reportedly absorbed in the liver by mitochondrial chromophores, such as cytochrome oxidase (Beauvoit et al., 1994).

Direct benefit of photobiomodulation on cytochrome oxidase activity was tested and confirmed in primary neurons functionally inactivated by tetrodotoxin (TTX) (Wong-Riley et al., 2001, 2005) or poisoned by potassium cyanide (KCN) (Wong-Riley et al., 2005), an irreversible inhibitor of cytochrome oxidase. Photobiomodulation also has therapeutic benefit on rat retinal neurons poisoned by methanol-induced formate (Eells et al., 2003), a reversible inhibitor of cytochrome oxidase. Thus, NIR via light-emitting diode (LED) treatment appears to compete with inhibitors of cytochrome oxidase in activating this enzyme, resulting in increased enzyme activity, increased ATP production, and increased metabolic activity of neurons (Wong-Riley et al., 2005). The ability of LED pretreatment to reduce the number of cell deaths caused by KCN or azide, another inhibitor of cytochrome oxidase, is particularly intriguing (Wong-Riley et al., 2005) and deserves further investigation.

Cyanide toxicity mediated through the inhibition of cytochrome oxidase causes histotoxic action (Mills et al., 1996; Lee et al., 1998; Bhattacharya and Lakshmana Rao, 2001) and compromises cellular energy status, resulting in cell death (Wong-Riley et al., 2005). However, cyanide can produce neuronal deaths either by apoptosis or necrosis, depending on its concentration (Li et al., 2002; Prabhakaran et al., 2004) or differential susceptibility of brain areas (Mills et al., 1999). Cyanide exposure reportedly produces primarily apoptosis in cortical neurons but necrosis in mesencephalic cells (Prabhakaran et al., 2002). Our previous studies on primary visual cortical neurons indicated that cyanide at different concentrations caused varying proportions of cell deaths

and that pretreatment with LED was effective in rescuing many neurons from dying (Wong-Riley et al., 2005). However, the mode of cell death was unclear.

The goal of the present study was to test our hypothesis that LED protects neurons from apoptotic cell death induced by 100–300 μM KCN. Various indicators of apoptosis were used to determine if apoptotic indices would be reduced by LED and to test for possible mechanisms of LED rescue. Moreover, the possibility that apoptosis occurred via a caspase-mediated pathway was investigated. Primary cultures of postnatal rat visual cortical neurons were used as our model.

EXPERIMENTAL PROCEDURES

All experiments were carried out in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Medical College of Wisconsin regulations. All efforts were made to minimize the number of animals and their suffering.

Primary neuronal cultures

Sprague–Dawley rats (1-day-old) were anesthetized with CO_2, and brains were removed, placed in balanced salt solution (Invitrogen, Carlsbad, CA, USA) and dissected. Meninges and surface blood vessels were stripped, visual cortices were minced into 1 mm^3 pieces and digested with 0.75% trypsin at 37 °C for 15 min. Cells were mechanically dissociated, and plated on poly-L-lysine-(Sigma, St. Louis, MO, USA) coated coverslips at a density of 5×10^5 cells/ml. The medium was changed to Neurobasal/B27 supplement (Invitrogen) and the cultures were maintained at 37 °C with 5% CO_2 in a humidified incubator. Cytosine arabinoside (Ara-C) (Sigma) was added on the second day of plating neurons to inhibit the replication of non-neuronal cells. Neuronal cultures were maintained by replacing half of the medium every five days. In this culture system, 95% of the cell population were neurons (Zhang and Wong-Riley, 1999).

Culture treatments

Experiments were carried out on 7–10 day old cultures of visual cortical neurons, with or without 670 nm LED pretreatment for 10 min at a power intensity of 50 mW/cm^2, giving a total energy density of 30 J/cm^2. An LED array (25 cm×10 cm) with a peak wavelength at 670 nm (Quantum Devices, Inc. Barnaveld, WI, USA) was placed beneath the covered culture dish of 60 mm diameter, with the room light turned off and irradiated accordingly. Cells were subdivided into eight groups: 1) cells pretreated with LED and then exposed to 100 μM KCN for 28 h; 2) cells exposed to 100 μM KCN for 28 h only; 3) cells pretreated with LED and then exposed to 300 μM KCN for 28 h; 4) cells exposed to 300 μM KCN for 28 h only; 5) normal cells treated with LED for 10 min and assayed 24 h later; 6) cells exposed to 1 mM KCN for 28 h; 7) cells exposed to caspase inhibitor I (at 1 μM, 3 μM, 5 μM, 7 μM, and 10 μM concentrations; Calbiochem, San Diego, CA, USA) in the medium for 30 min before exposure to 300 μM KCN for 28 h; and 8) normal cells without exposure to KCN or treatment with LED as normal controls.

Electron microscopy

Cultures were fixed at 4 °C in a solution of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB), pH 7.2 and 4% sucrose for 1 h. After washing in phosphate-buffered saline (PBS), they were postfixed with 1% osmium tetroxide for 30 min, dehydrated in an ascending series of alcohol, and embedded in Durcupan (Sigma). Ultrathin sections were cut and collected on 200-mesh grids (Electron Microscopy Sciences, Hatfield, PA, USA). Grids were post-stained with 2% uranyl acetate at 37 °C for 30 min and 2.5% Reynold's lead citrate at room temperature (RT) for 25 min and examined with a JEOL 100CX transmission electron microscope.

Nuclear (DNA) staining

To quantify and assess nuclear morphology, cortical neurons were fixed with 2% paraformaldehyde in PBS (pH 7.4) followed by cold methanol at RT. After washing with PBS, cultures were stained for 15 min with 1 μg/ml of the fluorescent DNA-binding dye Hoechst 33258 (Sigma) to reveal nuclear condensation or aggregation, as described previously (Zhang et al., 2000). Hoechst-stained cells were visualized and photographed using the BA450 filter under the fluorescent microscope (Nikon). Five hundred to 1000 cells in five to 10 separate fields of each coverslip in each group were counted, and counts were made under the same treatment condition and repeated at least three times.

Single-stranded DNA (ssDNA) immunostaining

Immunolabeling of ssDNA was performed as described by Frankfurt and colleagues (Frankfurt et al., 1996; Frankfurt and Krishan, 2001a,b) with slight modifications. Cells were washed in PBS, pH 7.4 at 4 °C and re-fixed in methanol/PBS (4:1, vol/vol) at −20 °C. Coverslips were heated to 75 °C for 20 min in an oven and cooled for 4 min at 4 °C with formamide. After blocking with 3% nonfat dry milk at 37 °C for 1 h, cells were incubated with anti-ssDNA monoclonal antibodies MAB 3299 (Chemicon, Temecula, CA, USA) at 1:200 dilution for 40 min. This was followed by secondary goat-anti-mouse antibodies conjugated to horseradish peroxidase (HRP) (Bio-Rad, Hercules, CA, USA) at 1:100 dilution for 30 min at RT. The labeling was visualized by the 3,3′-diaminobenzidine (DAB) (Sigma) reaction. Finally, cells were stained with hematoxylin (Sigma) at RT for 30 s and differentiated in 1% hydrochloric acid in 70% alcohol for 10 s. Nuclear labeling of ssDNA-positive cells and normal cells were counted (~500–1000 cells per group), and counts were made in at least five separate fields per treatment condition. The experiment was repeated at least three times.

Immunocytochemistry for active caspase-3, Bax, and Bcl-2

Neurons were washed with PBS and fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 30 min on ice. After blocking non-specific binding with 2% H_2O_2 for 30 min, and 1% bovine serum albumin with 4% normal goat serum (NGS) for 1 h, cells were incubated with polyclonal antibodies against active caspase-3 (Chemicon) at 1:200 dilution; monoclonal antibodies against Bax or Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1:200 dilution (for both) overnight at 4 °C. This was followed by goat-anti-rabbit (Bio-Rad) or goat-anti-mouse secondary antibodies conjugated to HRP at 1:100 dilution for 2 h at RT. The labeling of cytoplasm was visualized by using the DAB reaction.

Optical densitometry analysis

To analyze quantitative changes in active caspase-3, Bax, and Bcl-2 immunoreactivity following different treatments, optical densities of reaction product were measured by means of a Zonax MPM03 photometer (Zeiss, Thornwood, NY, USA) attached to a Zeiss compound microscope. Measurements were used with a 25× objective and a spot size of 2 μm diameter directed at the centers of cytoplasm of individual neurons. Possible variations in coverslip and slide thickness were negated by adjusting a blank region of each coverslip/slide to zero. For each experiment of