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Growth hormone reverses excitotoxic damage induced by kainic acid in the green iguana neuroretina

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ABSTRACT

It is known that growth hormone (GH) is expressed in extrapituitary tissues, including the nervous system and ocular tissues, where it is involved in autocrine/paracrine actions related to cell survival and anti-apoptosis in several vertebrates. Little is known, however, in reptiles, so we analyzed the expression and distribution of GH in the eye of green iguana and its potential neuroprotective role in retinas that were damaged by the intraocular administration of kainic acid (KA). It was found, by Western blotting, that GH-immunoreactivity (GH-IR) was expressed as two isoforms (15 and 26 kDa, under reducing conditions) in cornea, vitreous, retina, crystalline, iris and sclera, in varying proportions. Also, two bands for the growth hormone receptor (GHR)-IR were observed (70 and 44 kDa, respectively) in the same tissues. By immunofluorescence, GH-IR was found in neurons present in several layers of the neuroretina (inner nuclear [INL], outer nuclear [ONL] and ganglion cell [GCL] layers) as determined by its co-existence with NeuN, but not in glial cells. In addition, GH and GHR co-expression was found in the same cells, suggesting paracrine/autocrine interactions. KA administration induced retinal excitotoxic damage, as determined by a significant reduction of the cell density and an increase in the appearance of apoptotic cells in the INL and GCL. In response to KA injury, both endogenous GH and Insulin-like Growth Factor I (IGF-I) expression were increased by 70 ± 1.8% and 33.3 ± 16%, respectively. The addition of exogenous GH significantly prevented the retinal damage produced by the loss of cytoarchitecture and cell density in the GCL (from 4.9 ± 0.79 in the control, to 1.45 ± 0.2 with KA, to 6.35 ± 0.49 cell/mm^2 with KA + GH) and in the INL (19.12 ± 1.6, 10.05 ± 1.9, 21.0 ± 0.8 cell/mm^2, respectively) generated by the long-term effect of 1 mM KA intraocular administration. The co-incubation with a specific anti-GH antibody, however, blocked the protective effect of GH in GCL (1.4 ± 0.23 cell/mm^2) and INL (11.35 ± 1.06), respectively. Furthermore, added GH induced an increase of 90 ± 14% in the retinal IGF-I concentration and the anti-GH antibody also blocked this effect. These results indicate that GH and GHR are expressed in the iguana eye and may be able to exert, either directly of mediated by IGF-I, a protective mechanism in neuroretinas that suffered damage by the administration of kainic acid.

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1. Introduction

Synthesis of growth hormone (GH), or its mRNA expression, have been described in the retina of chicken (Baudet et al., 2003), rat (Harvey et al., 2006), human (Harvey et al., 2009) and, recently, in the green iguana (Harvey et al., 2016). It has been proposed that GH promotes cell survival from apoptotic waves during normal development of the retina in embryonic chick (Sanders et al., 2011), while in humans the retinal ganglion cell survival was correlated with the expression of GH (Sanders et al., 2009). In addition, it is known that persistent neurogenesis in the retina associates with increased GH levels in teleosts (Otteson et al., 2002). In reptiles, however, little is known about the distribution of GH in ocular tissues and its role on the retina. This becomes relevant since some diurnal reptiles (ectotherms), including the green iguana, are exposed to high light intensities that could eventually cause retinal damage, as has been evidenced in other models, showing that exposure to UV light induces cellular death in the retina (Atlasz et al., 2011; Oguni et al., 1996). Scarce studies of retinal damage in reptiles have been developed; however, in avian and mammalian models the intraocular injection of kainic acid (KA), an agonist of kainate type glutamate receptors, has been employed to induce retinal damage (Han et al., 2005; Morgan and Ingham, 1981), which results from high intracellular calcium accumulation, mitochondrial dysfunction and reactive oxygen species generation

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(Zhang and Zhu, 2011), similar to that reported for UV damage (Atlasz et al., 2011).

It is also known that GH has anti-apoptotic effects that promote neural protection after exposure to diverse insults such as hypoxia/ischemia, brain trauma or kainate-induced injury (Alba-Betancourt et al., 2013; Arce et al., 2013; Devesa et al., 2013), and that it is able to stimulate neurogenesis (Arámburo et al., 2014).

In this work, we studied GH expression in diverse iguana ocular tissues as well as in several layers of the retina, and assessed the possible survival role of GH in retinal neural cells exposed to excitotoxic damage through the development of a KA-induced lesion model, to determine if it could participate in mechanisms involved in neuroprotection in reptiles.

2. Material and methods

2.1. Animals

One-year-old green iguanas (*Iguana iguana*), were kindly donated by "Las Brisas" Environmental Management Unit (EMU), from Acapulco, Guerrero, México. Animals were kept in rooms of $3 \times 3 \times 2$ m under controlled conditions (at 30–35 °C with a relative humidity of 60–70%, and in a 12-h dark/12-h light photoperiod); they were fed daily with alfalfa pellets and water *ad libitum*. Animals, which were injected on eyes, were previously anesthetized intramuscularly with a xylazine (1 mg/kg) and ketamine (30 mg/kg) cocktail. Intraocular injection was performed with ultra-fine syringes (gauge needle 31G) introducing the needle into the posterior ocular chamber at a 45° angle. Animals recovered in individual cages at 38 °C and, if needed in the experimental design, were killed by decapitation following a protocol approved by the Institute's Bioethics Committee (Instituto de Neurobiología, UNAM). Eyes were rapidly enucleated and fixed in Bouin–Hollande solution (Romeis, 1989) or frozen immediately at −80 °C until use.

2.2. Experimental design

Initially, the histological morphology of the iguana's retina was determined using fixed tissues, as described below. Subsequently, experiments were conducted to appraise the kainic acid doses capable to exert a moderate damage to the retina. To do this, three groups were formed and the animals were injected in the left eye with 5 µl of either 0.1, 1.0 or 10 mM kainic acid (KA) solutions, respectively. The right eyes were used as controls and were injected with 5 µl of vehicle (PBS). After 24 h, iguanas were killed and eyes were collected for histological procedures or biochemical assays.

To evaluate the GH effect upon retinas damaged by excitotoxic lesions, iguanas were injected with 1 mM KA (5 µl) in the left eye, while the right eyes (used as controls) were injected with PBS (5 µl), and after 4 h received the intraocular treatments with either recombinant chicken GH (rcGH) and/or polyclonal antibody directed against chicken GH (Arámburo et al., 1989), dissolved in PBS and 0.1 µg/µl of bovine serum albumin (BSA). Treatments consisted of: (a) rcGH (100 ng); (b) rcGH preabsorbed with polyclonal anti-cGH antibody 1:1000; (c) anti-rcGH antibody diluted 1:1000; and (d) vehicle (PBS-BSA). Treatments (3 doses) were administered every 24 h. Then, 24 h after the last dose, the animals were decapitated and eyes were collected and processed for histological or biochemical procedures.

2.3. Histology

Eyes were fixed in Bouin–Hollande solution (Romeis, 1989) for 72 h and later were dehydrated with a graded series of alcohol solutions (60%, 70%, 80%, 96%, and 100% ethanol [2×] for 1 h at each step, and subsequently in ethanol-xylol 1:1, and xylol [2×], for 2 h at each). Dehydrated tissue was immersed in paraffin two times (1.5 h each) and cut into 5-µm thin slices with a microtome (LEICA RM2135) and mounted in charged slides. Paraffin was washed out from tissues with Citrisolve (Fisher), and they were rehydrated with a reverse series of alcohol solutions (absolute ethanol [2×], 96% ethanol [2×], 70% ethanol and 50% ethanol, for 5 min each). Then, histological sections were incubated with lugol for 2 min [2×] in order to eliminate the mercury content in the fixing solution, and finally with 5% $Na_2S_2O_3$ for 2 min to eliminate the iodine. The retina morphology was analyzed employing the classic Harris hematoxylin-eosine staining (Junqueira et al., 1998).

2.4. Fluorescence Immunohistochemistry

To identify if GH was synthesized by retinal neurons or by glial cells, confocal analysis was carried out following the protocol standardized by Alba-Betancourt et al. (2011). Briefly, histological sections were obtained as described in Section 2.3 and were equilibrated in Tris-Buffered Saline (TBS, pH 7.6) for 10 min. To retrieve epitopes, slides were incubated in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0) for 30 min at 80 °C. Tissues were washed with TBS 10 min [3×] and non-specific binding sites were blocked for 2 h with 5% dry fat-free milk (Bio-Rad) in TBS. Retinal auto-fluorescence was eliminated by incubating preparations for 30 min at room temperature with Sudan black (0.1% in 70% alcohol). After washing 3 times the preparations with TTBS (TBS containing 1% Triton X-100), slides were incubated overnight with heterologous rabbit polyclonal antibody directed against chicken GH (1:200) and either mouse anti-human neural nuclear antigen (NeuN, 1:200, Chemicon International Inc., CA, USA), mouse anti-rat glial fibrillary acid protein (GFAP, 1:100, Covance Inc., Princeton, NJ, USA) or mouse anti-rat growth hormone receptor (GHR, 1:250, Santa Cruz Biotechnology Inc., Dallas, TX, USA) monoclonal antibodies. Sections were washed with TTBS [3×] and incubated with the secondary polyclonal antibodies rabbit anti-mouse-FITC (1:200, Zymed Laboratories, San Francisco, CA, USA), goat anti-rabbit-Cy3 (1:5000, Zymed Laboratories) or rabbit anti-goat-Cy3 (1:5000, Zymed Laboratories), for 2 h at room temperature. Tissues were counterstained with 5 ng/ml 4′,6′-diamidino-2-phenylindole (DAPI, Invitrogen) in TBS for 30 min and then washed 3 times with TBS. Preparations were mounted with Vectashield mounting media (Vector Laboratories, Burlingam, CA, USA) and analyzed using a Carl Zeiss LSM 780 confocal microscope with laser excitation wavelengths of 488 nm (FITC), 561 nm (Cy3) and 350 nm (DAPI).

2.5. TUNEL assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed to identify cells undergoing apoptosis. Preparations were obtained as described above and washed 3 times with PBS, treated with proteinase K (2 µg/ml; 10 mM Tris–HCl, pH 7.4) at 37 °C for 10 min and then washed with PBS [3×]. Auto-fluorescence was eliminated with Sudan black followed by three washes with PBS. Cell death was assayed using the *In situ* Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals, Mannheim, Germany) incubating tissues with 50 µl of reaction mixture at 37 °C for 1.5 h in a humid chamber following the manufacturer's indications. After washing three times with PBS, samples were counterstained with DAPI in PBS for 30 min and washed again. Slides were mounted and analyzed as described above.

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