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Upregulation of IGF-I in the goldfish retinal ganglion cells during the early stage of optic nerve regeneration

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Abstract

Goldfish retinal ganglion cells (RGCs) can regrow their axons after optic nerve injury. However, the reason why goldfish RGCs can regenerate after nerve injury is largely unknown at the molecular level. To investigate regenerative properties of goldfish RGCs, we divided the RGC regeneration process into two components: (1) RGC survival, and (2) axonal elongation processes. To characterize the RGC survival signaling pathway after optic nerve injury, we investigated cell survival/death signals such as Bcl-2 family members in the goldfish retina. Amounts of phospho-Akt (p-Akt) and phospho-Bad (p-Bad) in the goldfish retina rapidly increased four- to five-fold at the protein level by 3–5 days after nerve injury. Subsequently, Bcl-2 levels increased 1.7-fold, accompanied by a slight reduction in caspase-3 activity 10–20 days after injury. Furthermore, level of insulin-like growth factor-I (IGF-I), which activates the phosphatidyl inositol-3-kinase (PI3K)/Akt system, increased 2–3 days earlier than that of p-Akt in the goldfish retina. The cellular localization of these molecular changes was limited to RGCs. IGF-I treatment significantly induced phosphorylation of Akt, and strikingly induced neurite outgrowth in the goldfish retina *in vitro*. On the contrary, addition of the PI3K inhibitor wortmannin, and IGF-I antibody inhibited Akt phosphorylation and neurite outgrowth in an explant culture. Thus, we demonstrated, for the first time, the signal cascade for early upregulation of IGF-I, leading to RGC survival and axonal regeneration in adult goldfish retinas through PI3K/Akt system after optic nerve injury. The present data strongly indicate that IGF-I is one of the most important molecules for controlling regeneration of RGCs after optic nerve injury.

Keywords: Goldfish; Retina; Retinal ganglion cells; IGF-I; p-Akt; Survival; Optic nerve regeneration

1. Introduction

Axonal injury of adult mammalian CNS neurons generally leads to retrograde neuronal degeneration and cell death. In parallel paper, we reported caspase-3 dependent apoptosis and downregulation of insulin-like growth factor-I in rat retinal ganglion cells (RGCs) after optic nerve (ON) injury. On the other hand, RGCs of lower vertebrates such as fish can successfully regenerate their axons after ON injury (Arora and Sperry, 1957). There have been many studies on morphological and behavioral changes involved in fish ON regeneration (Grafstein, 1975; Springer and Agranoff, 1977; Benowitz et al., 1981; Kato et al., 1999, 2007; Rodger et al., 2005). Furthermore, biochemical and genetic approaches for identifying molecules involved in ON regeneration in fish have recently accumulated (Schwalb et al., 1996; Liu et al., 2002; Matsukawa et al., 2004a; Fischer et al., 2004; Sugitani et al., 2006). However, there are few reports clarifying the reason why fish can regenerate optic axons after injury. In the present study, we examined goldfish RGCs as counterparts of rat RGCs with respect to regenerative properties after ON injury. The process of goldfish ON regeneration might be divided into two

Abbreviations: GCL, ganglion cell layer; IGF-I, insulin-like growth factor-I; INL, inner nuclear layer; ON, optic nerve; PI3K, phosphatidyl inositol-3-kinase; RGC, retinal ganglion cell

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mechanisms: (1) RGC survival, and (2) axonal elongation. We first measured enzymatic activities and protein levels of cell survival/death-related molecules such as members of the Bcl-2 family, which are major pro-apoptotic and anti-apoptotic proteins or the phosphatidyl inositol-3-kinase (PI3K)/Akt system which plays a central role in cell survival (Yamaguchi and Wang, 2001). The present study clearly showed early upregulation of phospho-Akt (p-Akt) and subsequent upregulation of Bcl-2 activity with a concomitant downregulation of caspase-3 activity in the goldfish RGCs after ON injury. Furthermore, we observed an earlier upregulation of insulin-like growth factor-I (IGF-I) than that of p-Akt in the goldfish RGCs after nerve injury. Addition of IGF-I to the culture medium dose- and wortmannin-dependently induced activation of Akt and axonal elongation in adult goldfish RGCs. Thus, the present data demonstrated, for the first time, a signaling pathway for the initial upregulation of IGF-I leading to optic nerve regeneration via the PI3K/Akt system in the goldfish RGCs, after nerve injury.

2. Experimental procedures

2.1. Animals and surgery

The common goldfish (*Carrassius auratus*; length, 6–7 cm tip-to-tip) was used throughout this study. The experiments were carried out in accordance with Committee on Animal Experimentation of the Kanazawa University. Goldfish were anesthetized in ice-cold water. The ONs were crushed using tweezers 1 mm away from the eyeball for 10 s, to carefully avoid vessels. After this treatment, goldfish were kept in appropriate conditions until the indicated time points after ON crush.

2.2. Cell counts of RGCs

To evaluate survival of goldfish RGCs after ON crush, we used a retrograde neurotracer, fluorogold. The ON was treated with small particles of fluorogold (10 mg/ml) at the crushed site for 1–2 days. The number of RGCs in the flat-mounted goldfish retina was counted with a fluorescent microscope. More than three retinal preparations were analyzed for each sample at indicated time points after ON crush. Values were expressed as means ± S.E.M. of RGCs before and after ON crush (flat-mounted retinas).

2.3. Caspase-3 activity in the retina

Retinal samples at indicated time points following ON injury were homogenized with 50 mM Tris–HCl buffer (pH 7.5) containing 1 mM EDTA and 1 mM dithiothreitol, and then centrifuged at 1800 × *g* at 4 °C for 1 h. The assay method was the same as that in parallel paper (Homma et al., 2007).

2.4. Western blotting of cell survival and death signal molecules

Goldfish retinal extracts were prepared at the indicated times after ON injury. Retinas were immersed in Tris–HCl buffer (pH 7.4) containing a protease inhibitor cocktail (Sigma), and were then sonicated and centrifuged at 1000 × *g* for 10 min, as described previously (Koriyama et al., 2003). The electrophoresis and blotting were the same as that in parallel paper (Homma et al., 2007).

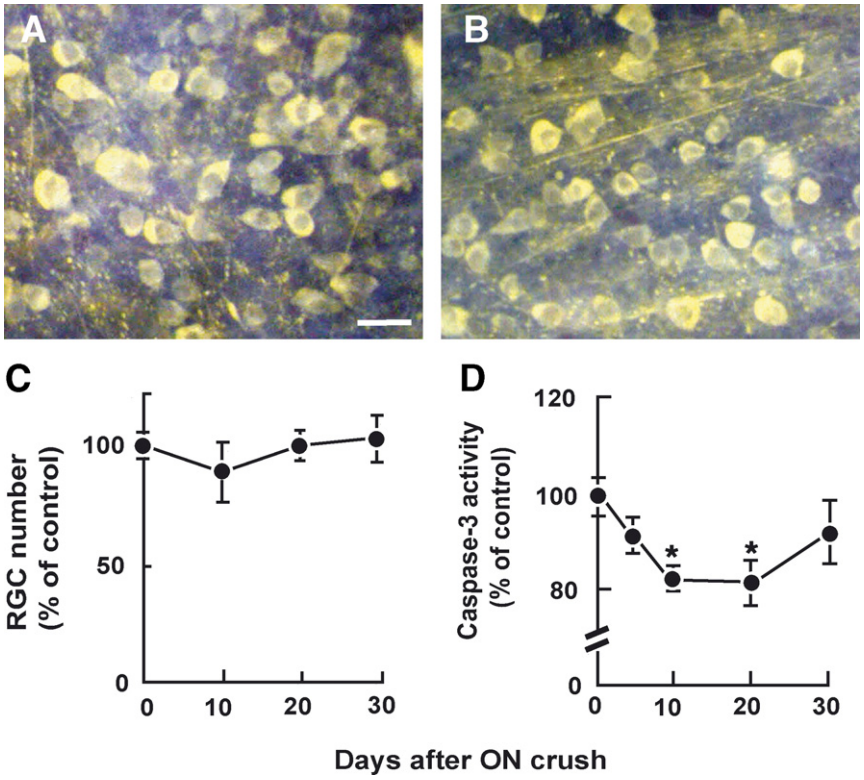

Fig. 1. Cell survival of goldfish RGCs after optic nerve crush. (A–C) In goldfish retinas, the number of ganglion cells retrogradely labeled with fluorogold was assessed before and after ON crush. Cell number did not change during the 30-day of treatment (B) compared to control retinas (A). Scale bar = 20 μm. A graphical analysis of these results was shown in (C). The experiment was repeated three times ($n = 3$). (D) Caspase-3 activity in the goldfish retina after ON crush. Caspase-3 activity was significantly decreased by 20% of that of the control value 10–20 days after nerve crush. Each value indicates the means ± S.E.M. ($n = 3$). *$P < 0.01$ compared to controls (untreated).