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Simvastatin promotes heat shock protein 27 expression and Akt activation in the rat retina and protects axotomized retinal ganglion cells in vivo

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Heat shock proteins (Hsps) are stress proteins that mediate protein stabilization in various tissues and protect cells from environmental stress. Novel evidence suggests that overexpression of the small heat shock protein 27 (Hsp27) in neurons protects against neurotoxic stimuli and may act as an inhibitor of neurodegeneration. Overexpression of Hsps has been achieved by different means including pharmacological induction. Here, we show that intravitreal injection of the 3-Hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor simvastatin induces Hsp27 expression in axotomized retinal ganglion cells (RGCs) and enhances RGC survival 7 and 14 days after optic nerve (ON) axotomy by 90% and 19%, respectively. The flavonoid quercetin inhibited Hsp27 induction and abrogated simvastatin-mediated neuroprotection. Simvastatin increased Akt phosphorylation in vivo, indicating that the PI3K/Akt pathway contributes to central nervous system (CNS) protective effects achieved. We propose the use of statins as a feasible approach to reduce lesion-induced CNS neuronal degeneration in vivo.

Keywords: Apoptosis; Heat shock protein; HMG-CoA reductase inhibitors; Neurodegeneration; Neuroprotection; PI3K/Akt; Retina; Statins

Introduction

Axotomy of the optic nerve (ON) in adult rats results in degeneration and apoptotic death of more than 80% of the retinal ganglion cell (RGC) population within 14 days (Berkelaar et al., 1994; Garcia-Valenzuela et al., 1994; Isenmann et al., 1997; Schmeer et al., 2002). There is an ongoing search for factors that enhance or prolong the survival of injured neurons and prevent further neuronal degeneration. ON transection causes severe changes in RGC protein expression that relate to ongoing apoptosis. These include increased expression of c-Jun (Robinson, 1995), the pro-apoptotic protein Bax (Isenmann et al., 1999), and activation of death promoting caspase-3 (Kermer et al., 1999). Recently, Krueger-Naug et al. (2002) showed that ON injury also induces the small heat shock protein Hsp27 in three distinct layers of the rat visual system: sensory RGCs, glial cells of the optic tract, and astrocytes in the optic layer of the superior colliculus.

Heat shock proteins (Hsps) are ubiquitous and highly conserved proteins whose expression is upregulated in response to a wide variety of physiological dysbalances and environmental stress factors, and they allow cells to survive otherwise lethal insults (Parcellier et al., 2003). Most Hsps are chaperones involved in proper folding and/or elimination of misfolded proteins. Furthermore, they protect subcellular structures, particularly mitochondria, which are crucial in antioxidant and antiapoptotic cell protection, and they can directly interfere with apoptotic pathways (Garrido et al., 1999). Overexpression of Hsp27 has been shown to protect against hyperthermia, oxidative stress, staurosporines, ligation of Fas/Apo-1/CD95 death receptor, and cytotoxic drugs (Mehlen et al., 1996a,b; Garrido et al., 1997; Yu et al., 2001). Age-acquired resistance of adult sensory neurons against axotomy has been attributed to Hsp27 upregulation as opposed to death susceptibility of Hsp27 negative embryonic neurons (Lewis et al., 1999). Likewise, Hsp27 protects RGCs from ischemic injury (Yokoyama et al., 2001), possibly by Hsp27 contribution in HIF-1-mediated ischemic preconditioning (Whitlock et al., 2005a) and caspase-3 inactivation (Whitlock et al., 2005b). Antiapoptotic effects in lesioned neurons have been explained by ATF-3-dependent Hsp27 and Akt activation and inactivation of death promoting c-Jun (Nakagomi et al., 2003). In addition, Hsp27 prevents apoptosome formation and caspase activation (Garrido et al., 1999) and can

Abbreviations: FG, fluorogold; GCL, ganglion cell layer; HMG-CoA, 3-Hydroxy-3-methylglutaryl-CoA; Hsp27, heat shock protein 27; i.o., intraocular; ON, optic nerve; Q, quercetin; RGC, retinal ganglion cell; RRR, RGC rescue rate (cp. Materials and methods); WM, wortmannin.

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stabilize actin microfilaments (Lavoie et al., 1995). Hsp72 induction has also been shown to prevent apoptotic degeneration in a rat glaucoma model (Caprioli et al., 2003). Furthermore, neuroprotective effects of immunophilin ligands such as FK506 and its non-calcineurin inhibiting derivatives have been suggested to be mediated by rapid Hsp induction (Klettner and Herdegen, 2003). Overexpression of Hsps has been achieved by direct delivery of the recombinant protein (Yu et al., 2001) or by recombinant expression from adenoviral and herpes simplex virus vectors (Nakagomi et al., 2003; Vander-Heide, 2002; Wagstaff et al., 1999), by electroporation (Yokoyama et al., 2001), and by treatment with pharmacological agents including polyisoprenoids and statins (Ishii et al., 2003; Wang et al., 2003).

The statins simvastatin and lovastatin, which act as HMG-CoA reductase inhibitors, specifically stimulate Hsp27 accumulation in osteoblasts in vitro (Wang et al., 2003). Statin therapy has pleiotropic effects, including protection against acute and chronic neurodegeneration occurring in ischemic stroke, in Alzheimer's disease, and in inflammatory CNS pathologies (Amarenco et al., 2004; Fassbender et al., 2001; Youssef et al., 2002). Likewise, atorvastatin and simvastatin, when administered early after stroke, reduced neurological deficits in a rat model of middle cerebral artery occlusion (Chen et al., 2003). These benefits appear to be mediated by complex mechanisms including endothelial eNOS induction, inhibition of metalloproteinases, local antioxidant, and antiinflammatory effects and, potentially, by direct neuroprotection via induction of phosphatidylinositol 3-kinase-Akt (PI3K/Akt) and Ras/Erk pathway (Urbich et al., 2002).

As reported by Krueger-Naug et al. (2002), minimal naive Hsp27 expression is substantially increased after RGC injury, and there was a positive correlation between surviving RGCs and augmented Hsp27 expression. The aim of this study was to investigate if exogenous induction of Hsp27 using pharmacological HMG-CoA reductase inhibitors can further improve the survival of injured RGCs in vivo and to examine the putative role of Hsps and the PI3K/Akt pathway in statin-mediated effects.

Materials and methods

Animal guidelines

All experiments were performed in accordance with the European Convention for Animal Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

Optic nerve transection and fluorescent labeling

Adult female Sprague–Dawley rats (200–250 g, Charles River, Germany; 4–6 animals per experimental group) were used. Rats were anesthetized by an intraperitoneal injection of chloral hydrate (7% in PBS; 420 mg/kg body weight). The cornea was anesthetized with oxybuprocaine-hydrochloride (4 mg/ml). The conjunctiva was incised temporally and the ON was exposed between the external ocular muscles and transected at a distance of approximately 2 mm from the eye bulb while preserving retinal blood supply. For retrograde RGC labeling, a small piece of gel foam soaked in a 5% aqueous solution of the fluorescent dye fluorogold (FG; Fluorochrome Inc., Denver, CO, USA) was placed at the ON stump immediately after axotomy. Operated rats were monitored until they were awake and moved spontaneously. Animals with persistent retinal ischemia (verified fundoscopically) were excluded. RGC densities were evaluated 3, 7, and 14 days after injury.

Intraorbital transection of the ON induces RGC death starting from days 4 to 5 after axotomy (Peinado-Ramon et al., 1996). The density of FG-labeled RGCs 3 days after axotomy is not affected by apoptotic RGC death (Isenmann et al., 2003) and was taken to define physiological RGC numbers.

Intravitreal injection and drug administration

For intraocular injections, animals were anesthetized shortly with a gas mixture of Enflurane, N_2O, and oxygen. Liquid volume in the vitreous was considered to be approximately 80 μl in average. Two microliters of simvastatin freshly activated under physiological pH (final concentration in the eye: 30 μM; dissolved in 50% ethanol; Calbiochem, San Diego, CA, USA), the PI3K inhibitor wortmannin (WM; 0.1 mM; dissolved in 15% dimethylsulfoxide; Sigma, Taufkirchen, Germany), and solvent solution at corresponding pH were injected into the vitreous body posterior to the ora serrata by means of a 34-gauge Hamilton syringe (Hamilton, Reno, Nevada, USA). WM was administered twice, 24 h before simvastatin administration, and together with simvastatin. Solvent treatment with DMSO and/or ethanol did not influence protein expression analysis or cell survival. The effect of simvastatin on RGCs survival was evaluated on days 7 and 14 after ON transection. For evaluation on day 7, the drug was administered 24, 48, and 72 h after ON transection. For evaluation on day 14, two different protocols were used: (i), the drug was administered 24, 48, 72, and 96 h after ON transection; (ii) the drug was administered 2, 4, 7, and 10 days after ON transection in order to evaluate if delayed, but prolonged treatment would also be effective in rescuing injured neurons. To elucidate the role of Hsp expression in the neuroprotective effects of simvastatin, quercetin at 4 mg/kg (dissolved in 0.1% DMSO, Sigma) was administered intraperitoneally to a group of animals 24 h prior to simvastatin injection and in combination with it 24, 48, and 72 h after ON transection. The effect on protein expression and cell survival was evaluated 48 h and 7 days after ON transection, respectively.

Evaluation of RGC densities

Rats were killed by an overdose of chloral hydrate 3, 7, or 14 days after ON transection. FG labeled retinae of axotomized and simvastatin- or control-injected animals with or without concomitant quercetin treatment were dissected, fixed in 4% paraformaldehyde (PFA) in PBS, and flat-mounted (Isenmann et al., 1998). Retinae were examined under epifluorescence using a 4,6-diaminido-2 phenylindole (DAPI) filter (365/397 nm) for FG fluorescence (Axiophot, Zeiss, Germany). RGC densities were determined by counting tracer-labeled RGCs in 12 grid-defined areas of 62,500 μm each (three areas per retinal quadrant at 1/6, 3/6, and 5/6 of the retinal radius; Isenmann et al., 1998; Schmeer et al., 2002). Cell counting was performed by two independent investigators according to a double-blind protocol. In addition to RGCs, there were also endothelial cells of retinal vessels and microglial cells labeled with FG. These were discriminated by stringent morphological criteria, as detailed and illustrated previously (Isenmann et al., 1998; Schmeer et al., 2002; Straten et al., 2002), and excluded from counting. Briefly, vascular endothelial