Haem oxygenase-1 gene transfer protects retinal ganglion cells from ischaemia/reperfusion injury

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ABSTRACT

RGC (retinal ganglion cell) death following ischaemic insult is the major cause of a number of vision-threatening diseases, including glaucoma. The aim of the present study was to evaluate the role of HO-1 (haem oxygenase-1) in the retina against IR (ischaemia/reperfusion) injury. Adenovirus-mediated HO-1 gene transfer (Adv-HO-1) was carried out by injection into the vitreous body to induce HO-1 overexpression. At 3 weeks after transfection, levels of HO-1 expression, as measured by Western blot analysis, immunohistochemical staining and activity assay, were drastically upregulated. Transient retinal ischaemia was induced by raising the intraocular pressure to 150 mmHg for 60 min. Untreated IR caused a significant decrease in RGC numbers at 3 and 7 days after reperfusion (76.1 and 67.2 % of control eyes with sham IR respectively; \( P < 0.001 \)). Eyes pretreated with Adv-HO-1 had less RGC loss on day 3 and 7 following reperfusion compared with control eyes injected with Adv-GFP (adenovirus containing a gene for green fluorescent protein; 94.3 and 88.2 % respectively; \( P = 0.007 \) and 0.001). SnP (tin protoporphyrin), an HO-1 inhibitor, counteracted the effects of Adv-HO-1. In conclusion, these findings provide evidence that augmentation of HO-1 enzyme overexpression by intravitreal injection is able to protect RGCs against IR-induced damage.

INTRODUCTION

Retinal IR (ischaemia/reperfusion) injury, leading to RGC (retinal ganglion cell) death, is a common aetiology accounting for a number of ocular diseases, including central retinal artery occlusion, diabetic retinopathy and glaucoma [1,2]. Complex molecular pathways are activated following IR [3], including calcium entry, excitotoxicity, oxidative stress, induction of the apoptosis process and activation of multiple signalling pathways [4]. Such ischaemic episodes can cause particularly severe damage to the inner retina. Various strategies to reduce ischaemia-induced neuronal death are being widely investigated.

HO (haem oxygenase) is a key enzyme in haem catalysis [5], and three HO isoforms have been identified: HO-1, HO-2 and HO-3. HO-1, also known as HSP32 (heat-shock protein 32) [6], was first described in 1969 [7] and can be induced by stress, fever and various endogenous/exogenous chemicals [8,9]. HO catalyses haem into biliverdin, free iron and CO. Under the action of biliverdin reductase, biliverdin is subsequently converted into bilirubin [10]. Both biliverdin and bilirubin are potent

Key words: adenovirus, gene therapy, haem oxygenase, ischaemia/reperfusion, ocular disease, retinal ganglion cell.
Abbreviations: Adv-GFP, adenovirus containing the gene for green fluorescent protein; HEK-293 cells, human embryonic kidney cells; HO, haem oxygenase; Adv-HO-1, adenovirus containing the gene for HO-1; IOP, intraocular pressure; IR, ischaemia/reperfusion; IVI, intravitreal injection; RGC, retinal ganglion cell; SC, superior colliculi; siRNA, small interfering RNA; SnP, tin protoporphyrin.
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antioxidants [11]. CO serves as a second messenger to regulate vascular tone, and free iron stimulates ferritin formation, which decreases iron-mediated accumulation of free radicals as well as up-regulating cytoprotective genes [12–14]. Induction of HO-1 is neuroprotective against ischaemic stroke and oxidative-stress-mediated neuronal death [15,16]. On the other hand, cells with compromised HO-1 expression had a decrease in stress defence [17]. HO-1 alleviates tissue injury not only by acting as an antioxidant, but also by modulating inflammatory immune reactions, and activating the anti-apoptotic process and other signal transduction pathways [18–20].

Intense light stimulation and hypoxia/ischaemia can stimulate HO-1 expression in the retina [21–23]. This up-regulation is considered to serve as a cellular defence mechanism against oxidative damage and is rendered ineffective if HO-1 is blocked by siRNA (small interfering RNA) [21]. Hegazy et al. [24] delivered HO-1 genes into the SC (superior colliculi) of rats to enhance HO-1 expression, where they found approx. 15 % less RGC loss at 1–3 weeks after ischaemia. Although SC administration of viral vector selectively transfects RGCs, causing less ocular damage and possibly providing better RGC protection than intraocular injection [25], the invasive approach is not a feasible clinical approach for humans. Additionally, only the number of living RGCs was given by Hegazy et al. [24]. Hence the present study was undertaken to examine the effects of HO-1 overexpression in the RGC against IR injury by IVI (intravitreal injection) of an adeno virus-mediated gene [Adv-HO-1 (adenovirus containing the gene for HO-1)], as well as investigation of related biomolecular features of HO-1.

**MATERIALS AND METHODS**

**Animals**

Experiments were conducted on female Wistar rats of 8–10 weeks of age (body weight, 200–250 g), obtained from the Taiwan National Laboratory Animal Center, Taipei, Taiwan. The care and handling of animals were in accordance with the International Guiding Principles for Animal Research adopted by the Laboratory Animal Center, National Taiwan University. Rats were kept in a cyclic light environment with a 12 h light/dark cycle. Experiments were carried out under general anaesthesia with an intraperitoneal injection of a mixture of ketamine (40 mg/kg of body weight; Pfizer) and xylazine (8 mg/kg of body weight; Sigma).

**Preparation of Adv-HO-1**

Recombinant adenovirus was constructed using a method described previously [26]. Human HO-1 cDNA containing the entire coding sequence was subcloned into the adenovirus shuttle plasmid vector pAd-CMV, which contains a cytomegalovirus promoter and a polyadenylation signal from bovine growth hormone. To generate the Adv-GFP (adenovirus containing the gene for green fluorescent protein), a shuttle vector containing the human phosphoglycerate kinase gene promoter was used. The recombinant adenovirus was generated by homologous recombination and amplified in HEK-293 cells (human embryonic kidney cells) as described previously [26]. Large-scale production of viral vectors were purified by CsCl ultracentrifugation and stored in 10 mmol/l Tris/HCl (pH 7.4), 1 mmol/l MgCl₂ and 10 % (v/v) glycerol at −70 °C until use. Virus titres were determined by a plaque assay on a HEK-293 cell monolayer. For transfection, the left eye of a rat was injected with 2 μl of Adv-HO-1 [1.0 × 10⁸ pfu (plaque-forming units)/μl] into the vitreous cavity with a glass micropipette, while the right eye of the same rat received an equivalent volume of Adv-GFP as the control. At 3 weeks after transfection, the retina were isolated for analysis.

**Induction of retinal IR**

At 3 weeks after Adv-HO-1 gene transfection, anaesthetized rats were placed on a stereotaxic frame. A 30-gauge needle connected to a saline bottle was introduced into the anterior chamber of the eye, and IR was induced by elevation of the bottle to a height which produced an IOP (intraocular pressure) of 150 mmHg for 60 min. After this time had elapsed, the needle was withdrawn and the corneal puncture site was sealed with glue. Retinal ischaemia was confirmed by whitening of the iris and loss of the red reflex. In eyes subjected to a sham IR, the procedure used was the same as above, except that the saline bottle was not elevated.

**Measurement of HO-1 expression by Western blotting**

Animals were killed with an overdose of anaesthetics. After enucleation and removal of the anterior segment of the eyeball, the entire resected retina was homogenized for 30 min on ice in buffer containing 50 mmol/l Tris/HCl (pH 8.0), 120 mmol/l NaCl, 1 % (v/v) Nonidet P40 and a mixture of proteinase inhibitors (Roche Diagnostics). The protein concentration was determined using the Bradford assay (BioRad Laboratories). Retinal protein (30 μg per sample) was separated by SDS/PAGE on 15 % (w/v) acrylamide gels and electroblotted on to a nitrocellulose membrane. The membrane was blocked with 5 % (w/v) non-fat milk powder in TBS (Tris-buffered saline) for 1 h and then incubated with a mouse monoclonal anti-HO-1 antibody (1:500; Stressgen Bioreagents) overnight at 4 °C. After three washes with TBS/0.1 % Tween 20 (BioRad Laboratories), a secondary peroxidase-labelled antibody was applied for 1 h at room temperature (25 °C). The membrane blots were developed using chemiluminescent reagents (PerkinElmer Life Sciences).
Picturcs were scanned and quantified further with a densitometer (GelPro 3.1; Media Cybernetics). The density of HO-1 staining was compared with that of constitutively expressed β-actin.

**Measurement of HO protein activity**

The activity of the HO enzyme was detected using the method described by Abraham et al. [27], which measures the generation of bilirubin. Eight retinas were pooled and homogenized on ice in a 10 mmol/l Tris/HCl lysis buffer (pH 7.4) containing 0.5% Triton X-100 and protease inhibitors. Homogenates (100 μl) were mixed with 0.8 mmol/l NADPH, 0.8 mmol/l glucose 6-phosphate, 1.0 unit of glucose-6-phosphate dehydrogenase, 1 mmol/l MgCl₂ and 10 ml of purified rat liver containing biliverdin reductase at 4 °C. The reaction was initiated by the addition of haemin (0.25 mmol/l). The reaction mixture was then incubated at 37 °C for 15 min in the dark. At the end of the incubation period, any insoluble material was analysed. A molar absorption coefficient of 40 litre · mmol⁻¹ · cm⁻¹ at λ₆₄₅–₅₃₀ was used to determine the amount of bilirubin formed. Controls included samples without the NADPH-generating system.

**Measurement of HO-1 expression by immunohistochemistry**

For the immunohistochemical analysis, enucleated eyes were first fixed in 4% (w/v) paraformaldehyde in 0.1 mol/l PBS for 1 h at 4 °C. Whole eyeballs were then cryopreserved in a 30% (w/v) sucrose solution overnight, and frozen in OCT (optimal cutting temperature) embedding medium over liquid nitrogen; sagittal and frozen in OCT (optimal cutting temperature)–cryopreserved in a 30% (w/v) sucrose solution overnight, and the bilirubin concentration of the supernatants was analysed. A molar absorption coefficient of 40 litre · mmol⁻¹ · cm⁻¹ at λ₆₄₅–₅₃₀ was used to determine the amount of bilirubin formed. Controls included samples without the NADPH-generating system. In some rats, SnP (tin protoporphyrin; FrontierScientific) was used to block HO-1 activity. SnP (50 μg · kg⁻¹ · kg⁻¹ of body weight) was dissolved in equal amounts of PBS and 0.1 mol/l NaOH and then administered intraperitoneally 24 h prior to the experiments [28,29].

**RGC labelling and counting**

Experimental animals were divided into five groups: sham + Adv-GFP (n = 6); sham + Adv-HO-1 (n = 6); IR + Adv-GFP (n = 6); IR + Adv-HO-1 (n = 6); and IR + Adv-HO-1 + SnP (n = 4).

RGCs were retrogradely labelled with dye 1 week before ischaemia. The scalp of a rat was incised, and two small holes were drilled on the skull bilaterally 6 mm posterior to the bregma and 1.5 mm lateral to the midline. A total of 2 μl of 5% (v/v) Fluoro Gold (Sigma) was injected with a micropipette 3.8, 4.0 and 4.2 mm below the skull. The micropipette was held in each position for 3 min to prevent dye reflux. After the procedure, antibiotic ointment was applied to the wound and the skin was sutured. Rats were killed by an overdose of chlorohydrate (Kanto Chemical) at 3 and 7 days after reperfusion, and the 12:00 position of each rat’s eye was first marked with a suture to facilitate orientation. Retinas were isolated after removal of the anterior segment and vitreous. Following incubation in 4% (w/v) paraformaldehyde for 1 h, the retina was placed flat on slides and divided equally into four quadrants. Each retinal quadrant was divided into central, middle and peripheral locations (approx. 1, 2 and 3 mm from the optic disc respectively) for counting. In each location, the numbers of RGCs in six microscopic fields of 430 μm² each along the medial line were counted. These 72 fields used for counting RGCs corresponded to approx. 13.3% of each retina, and the mean RGC density was expressed as the total number of counted RGCs divided by the total counted area of each retina.

**Statistical analysis**

All values are means ± S.E.M. ANOVA was carried out for multiple group comparisons, followed by Duncan’s test of significance. Significant differences were accepted at P < 0.05.

**RESULTS**

**Up-regulation of HO-1 expression by Adv-HO-1 transfection**

Transgene expression of the HO-1 protein in the retina after IVI of the Adv-HO-1 was confirmed by Western blot analysis, where the HO-1 enzyme presented as a 32 kDa protein band. Figure 1(A) shows the time course of HO-1 protein expression. The level of HO-1 protein in the retinas of Adv-HO-1-treated eyes increased drastically at 3 weeks following transfection. Up-regulation of the HO-1 enzyme continued for 6 weeks after virus delivery. A quantitative analysis of Figure 1(A) is shown in Figure 1(B). Compared with Adv-GFP-treated control eyes, an 8–9 times increase in HO-1 protein in the retina was observed after Adv-HO-1 transfection for 3 and 6 weeks (P < 0.001 and P = 0.002 respectively). In accordance with the findings of the Western blot analysis, the activity of the HO-1 enzyme in the retina 3 weeks after Adv-HO-1 was significantly enhanced.
Figure 1 Western blot analysis of HO-1 protein induction in retina of rats at 3 and 6 weeks after injection of Adv-HO-1.

(A) Transfection of Adv-HO-1-induced HO-1 overexpression in retina after 3 weeks of IVI injection of Adv-HO-1. The up-regulation of HO-1 protein was sustained 6 weeks after administration of Adv-HO-1. (B) Mean absorbance (IOD) of immunoreactive bands in (A). The HO-1/β-actin ratio was determined. GFP, Adv-GFP. Two independent experiments were carried out (n = 3) at each time point. **P < 0.01 and ***P < 0.001 compared with the control (Adv-GFP) group.

Figure 2 Levels of HO protein expression determined using an activity assay

Retinas were isolated for measurement 3 weeks after transfection of Adv-HO-1. Changes in the experimental groups were compared with control transfection with Adv-GFP (GFP). Two independent experiments were carried out (n = 3) at each time point. *P < 0.05 and **P < 0.01.

(Figure 2). Although the HO-1 protein expression, as examined by Western blot, increased by 8–9 times over the Adv-GFP group, the HO-1 activity in eyes subjected to Adv-HO-1 for 3 weeks had an approx. 3.5 times increase (P < 0.001). HO-1 activity was blocked by SnP, a competitive inhibitor of HO-1 (P = 0.03).

Figure 3 HO-1 immunohistochemistry with and without administration of Adv-HO-1

(A) Control eyes transfected with Adv-GFP (GFP) revealed very little HO-1 immunoreactivity. (B) The intensity of HO-1 immunostaining increased 3 weeks after transfection with Adv-HO-1. The enhanced signal was found along the inner limiting membrane, in the ganglion cell layer (GCL), in some cell processes spanning the entire inner plexiform layer (IPL) (arrow) and in the inner nuclear layer (INL). ONL, outer nuclear layer.

We also used an immunohistochemical method to localize the HO-1 distribution in normal retina and in retina 3 weeks after Adv-HO-1 administration (Figure 3). In the normal retina, only faint HO-1 immunoreactivity was seen in the inner retina. Following Adv-HO-1 transfection, HO-1 expression was markedly stronger and was distributed in the inner limiting membrane, ganglion cell layer, the process of inner plexiform layer and inner nuclear layer (n = 3).

Administration of Adv-HO-1 promoted RGC survival against IR injury

Figure 4 shows the density of RGCs following the ischaemic event. On day 3 following reperfusion after ischaemia, the surviving RGCs decreased to 76.1 ± 6.7% of control in eyes receiving Adv-GFP (n = 6; P < 0.001; Figure 4F). The number of RGCs at 3 days following reperfusion had only fallen to 94.3 ± 4.6% of control in eyes pretreated with Adv-HO-1 for 3 weeks (n = 6, P = 0.007). SnP administration led to a decrease in the number of RGCs protected by Adv-HO-1 under retinal IR (77.1 ± 4.7%; n = 4, P = 0.04).

By 7 days after reperfusion, the surviving RGCs continued to decrease to 67.2 ± 2.6% of control in eyes...
Figure 4  Surviving RGCs at 3 and 7 days after retinal ischaemia

(A–E) Representative pictures of labelled RGCs at 3 days after retinal ischaemia. (F) The mean density of RGCs after 3 and 7 days of ischaemia in the different groups. *P < 0.05, **P < 0.01 and ***P < 0.001. GFP, Adv-GFP.

subjected to Adv-GFP (n = 6, P < 0.001, Figure 4F), whereas the density of RGCs 7 days after reperfusion had only fallen to 88.2 ± 3.5 % of control in eyes pretreated with Adv-HO-1 for 3 weeks (n = 4, P = 0.001). Administration of SnP caused a lower density of RGCs protected by Adv-HO-1 under retinal IR (75.3 ± 0.8 %; n = 4, P = 0.01).

DISCUSSION

In the present study, we found that Adv-HO-1 gene transfer by IVI efficiently stimulated HO-1 expression in the retina, as demonstrated by Western blot analysis, immunohistochemical staining and activity assay. In addition, the up-regulation of HO-1 protein by IVI conferred comparable protection for injured RGCs against ischaemia to SC administration.

Animal models of retinal IR that simulate various human neuroretinal diseases, especially glaucoma, have been developed in an attempt to investigate feasible treatments [30,31]. Glaucoma is a group of distinctive optic neuropathies characterized by typical optic disc changes and visual field progression. Apart from a high IOP-related mechanical mechanism, other critical mechanisms, such as growth factor deprivation, excitotoxicity and oxidative stress, lead to ischaemia-induced neuronal death [4].

HO-1 expression induced by stress has been documented in different cells and organs [19,32,33]. By binding to the regulatory regions, these inducers promote transcription of the HO-1 gene [9]. The HO system serves as an important endogenous cytoprotective system based on the following characters [21,33]: (i) HO-1 is a potent antioxidant; (ii) anti-inflammatory, antiapoptotic and antiproliferative actions by HO-1 and its reaction end products (CO and biliverdin) protect tissues against IR injury and prolong graft survival; and (iii) HO-1 is a ‘therapeutic funnel’ because other beneficial molecules (e.g. prostaglandins and heat-shock proteins) carry out their functions through HO-1 [19].

Other common agents that have been used to augment HO-1 production include heavy metals (cobalt and aluminium), haem compounds, organic solvents and others [9,22]. Up-regulation of HO-1 not only offers protection for the liver and kidney against ischaemic injuries [34,35], but its neuroprotective property has also been demonstrated in the brain and retina [15,22,24,36]. As HO-1 possesses such a spectrum of pleotropic cytoprotective effects, delivering it as a therapeutic gene to enhance
expression has been attempted previously. Abraham et al. [27] first reported the delivery of the HO-1 gene into rabbit eyes by injection of recombinant adenoviral construct of human HO-1 cDNA. Since then, approaches with HO-1 gene transfection have been conducted in a variety of organs, such as the cardiovascular system, liver and skeletal muscle [37–39]. The long-sustained site-specific supply of appropriate molecules to deficient cells is a major advantage of using viral vectors to deliver specific genes. Moreover, stimulation is less abrupt and more stable than single high-dose administration of chemical agents.

Hegazy and co-workers [24] successfully injected HO-1 cDNA into the SC of rats and found that RGC loss was decreased by approx. 15 % after ischaemic injury. The overall rescue rate for RGC against ischaemia in the present study is approx. 18 %. Although our present study shares some similarities with the work by Hegazy et al. [24], some major differences exist. First, and most importantly, is the mode of delivery. The advantages of SC administration of virus vector include selective infection of RGCs as well as no IVI-induced ocular damage and inflammation. One study administered adenovirus containing interleukin-4 and interleukin-10 into the SC and reported a higher rate of RGC survival compared with IVI [25]. Our present study did not show any lesser protection of RGC by IVI than SC administration of Adv-HO-1. Virus vector delivered by IVI resulted in a predominant transfection of Müller cells [40], a class of retinal macroglial cells. Apart from their structural support for the retina, they are also essential for maintenance of homoeostasis, secretion of neurotrophin factors and recycling of neurotransmitters [41]. These cells span the whole thickness of the retina and ensheathe all kinds of retinal neurons. That the border surface of the internal limiting membrane is composed of the basal end-feet of Müller cells, leading to better absorption of viral particles, might explain the preferential viral infection of Müller cells. Another possible reason is that the efficiency of vector internalization might differ between neurons and glia [40]. The expression of HO-1 in retina occurs mainly in Müller cells, and inhibition of HO-1 in Müller cells by siRNA resulted in more severe retinal destruction [21]. The increased reaction of Müller cells after ischaemia is associated with the repair of the retina [42]. Further evidence for the supportive functions of glial cells for neurons is from a study by Min et al. [36]. These authors showed that, in addition to the directly protective role, astrocytes also act indirectly in co-operation with microglial cells to prevent excessive brain inflammation by the induction of HO-1. Consequently, despite the fact that IVI of the HO-1 gene did not exclusively infect RGCs, the benefit to RGCs was not inferior to SC administration of the HO-1 gene. Both the direct and indirect interactions among RGC and other non-RGC neurons or glial cells are vital for this protection. Therefore the mode of delivery adopted in the present study is a highly feasible strategy for gene therapy in humans.

Secondly, the experimental designs were not identical. The experiment in the study by Hegazy et al. [24] was based on reports that RGCs transfected with adenovirus were observed at 7 and 14 days in mice [43]; HO-1 mRNA in RGCs was elevated 3–5 days after microinjection and the up-regulation gradually decreased at 21 days [44]. However, in our preliminary study, there was no functional protection of RGCs by HO-1 protein, 2 weeks after transfection, implying different efficiency of the virus vectors used in the present study and by Hegazy et al. [24]. This accounts for the different time spans of the ischaemic experiments in the two studies. The levels of retinal damage depend on the severity (the pressure) and the duration of ischaemic challenge, which contributes to the different survival rates of RGC after reperfusion in these two studies. In the present study, a larger retinal area was included for counting. We believe this approach is able to reduce the error from counting only a small percentage of RGCs. Regarding the methods of dye delivery, Hegazy et al. [24] injected Fluoro Gold and Adv-HO-1 at the same time (4 days prior to ischaemia). It usually takes at least 3 days for retrograde transport to move the dye to the RGC cell body. The timing of dye injection (4 compared with 7 days) should have no influence on the results as the difference is small. However, whether tracer and virus vector administered simultaneously would interact with each other is unknown. Thirdly, in the present study, we measured both HO-1 protein level and its activity following Adv-HO-1 administration, not only its protein or mRNA expression level.

HO-1 overexpression following ischaemia is believed to be an endogenous means of self-protection [22]. However, it also has been found that the level of HO-1 induction under pathological states, such as in atherosclerotic lesions, is presumed to be not enough to conquer oxidative challenge [45]. Boosting HO-1 expression in vascular cells by gene transfection successfully attenuates the formation of atherosclerosis [45]. The limitation of the present study is that we did not analyse whether the increased HO-1 expression generated by adenoviral transfection is accentuating the natural defence mechanism or is an additional effect. This issue needs to be addressed in further studies.

In conclusion, our present findings suggest that the modulation of HO-1 protein expression by IVI of an adenovirus vector offers protection of RGCs against an IR challenge. The present study provides a therapeutic approach to extend neuronal survival.

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