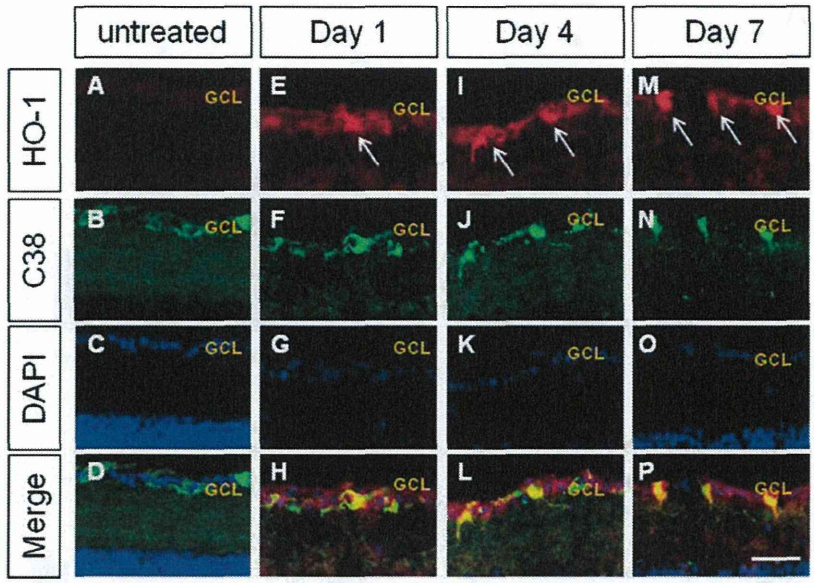

Fig. 2. Changes in HO-1 expression after NC. **A–D:** Normal retina. **E–H:** One day after NC. **I–L:** Four days after NC. **M–P:** Seven days after NC. HO-1 immunoreactivity was detected in the GCL 4 days and 7 days after NC. Arrows indicate HO-1 in the GCL. Scale bar = 50μm.

NC, immunostaining showed that HO-1-positive cells were present in the GCL (Fig. 2E,I,M). Double staining for HO-1 and C38 protein showed that the cells expressing HO-1 were all RGCs, although not all RGCs expressed HO-1 (Fig. 2H,L,P). We also found that, over time, the ratio of C38 protein-stained RGCs to DAPI-stained RGCs slowly decreased ($P < 0.05$; Fig. 3A). The proportion of HO-1 positive cells, however, rose significantly in the GCL after NC ($P < 0.05$; Fig. 3B). This tendency did not change near the optic nerve (500 μm) and far from it (1,000 μm).

CoPP Upregulated the Expression of HO-1 Protein

It is difficult to collect cells including protein because of their scarcity in the retina, especially after NC. We could not obtain enough protein in purified RGCs for immunoblot analysis, so we tried qPCR and immunoblot analysis in the retina. The levels of *Ho-1* mRNA in the retina did not increase after 1 or 2 days of CoPP administration (data not shown). However, 3 days of CoPP administration resulted in increased *Ho-1* mRNA expression. We therefore chose a CoPP treatment without NC lasting for 6 days. The levels of HO-1 protein in the retina also increased significantly 3 days after the injection of CoPP ($P < 0.05$; Fig. 4B). Additionally, immunofluorescence analysis showed that IP injection of CoPP induced expression of HO-1 in the GCL (Fig. 4C).

Effects of CoPP on Protection of RGCs From NC

Before NC, there was no significant difference in the density of FG-labeled RGCs in mice treated with vehicle (3,160 ± 435 cells/mm^2), CoPP (3,549 ± 475 cells/mm^2), or a combination of CoPP and SnPP (3,156 ± 658 cells/mm^2). Seven days after NC, the density of FG-labeled RGCs in the mice treated with CoPP was significantly higher than in those treated with vehicle (1,313 ± 137 cells/mm^2 and 868 ± 253 cells/mm^2, respectively, $P < 0.01$, $P < 0.05$), whereas the mice treated with a combination of CoPP and SnPP (808 ± 262 cells/mm^2) did not show a significant difference from those treated with vehicle (Fig. 5).

DISCUSSION

It is well known that axonal damage induces significant RGC death, but a small number of RGCs are nevertheless able to survive 7 days after NC. To search for new treatments for retinal diseases, we tried to identify patterns of change in the gene expression of these axonal-damage-resistant RGCs. A microarray assessment of sorted post-NC RGCs revealed that the expression of HO-1, in particular, increased significantly. Immunofluorescence analysis also revealed that a significantly higher proportion of HO-1-positive cells was present in the RGCs 1, 4, and 7 days after NC and that the ratio of HO-1-positive cells in the RGCs gradually increased during that time. Finally, an examination of retinas pretreated with CoPP, which is a nonsubstrate HO-1 inducer, revealed a higher density of surviving RGCs after NC, whereas retinas pretreated with both CoPP and SnPP showed no difference in RGC survival from the untreated group. These data strongly suggest that molecules expressed in surviving RGCs, especially HO-1, contribute to the survival of RGCs through a neuroprotective effect.

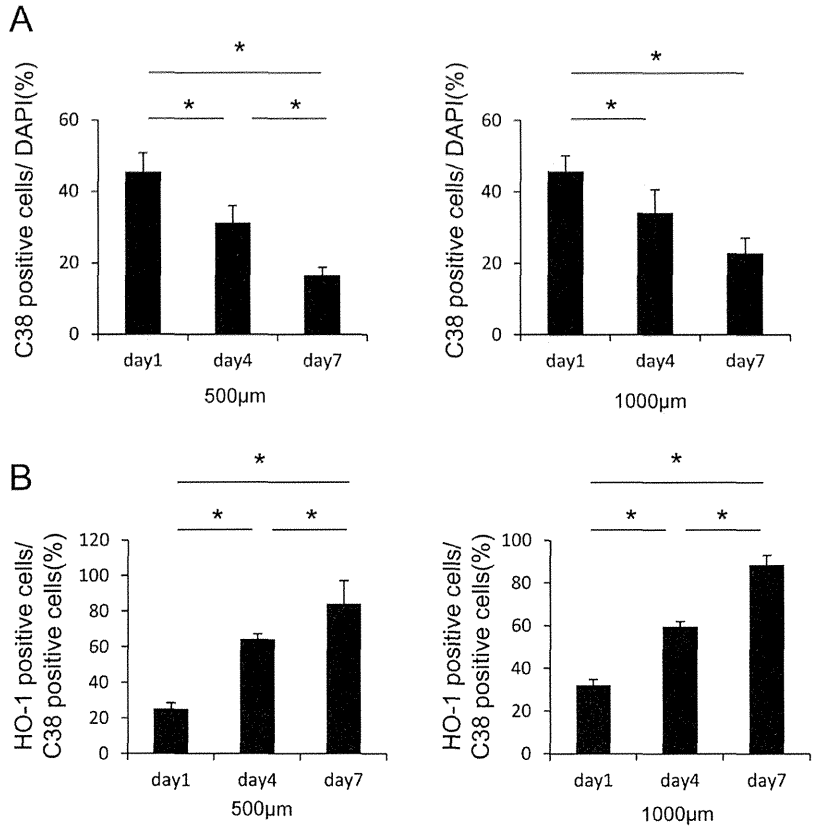

Fig. 3. Quantitative analysis of HO-1 positive cell numbers in the GCL after NC. The left and right graphs represent measurements in areas 500 μm and 1,000 μm from the center of the optic nerve, respectively. **A:** The ratio of C38 protein-stained RGCs to DAPI-stained RGCs (n = 4 in each group). **B:** The proportion of HO-1 positive cells to C38 protein-stained RGCs (n = 4 in each group). $*P < 0.05$.

RGCs represent only a small fraction of the total retinal cell population, so it is easy to overlook responses specific to them. To overcome this obstacle, we used FACS, a technique capable of identifying RGC-specific changes that has recently been introduced (Fischer et al., 2004). FACS functions by isolating RGCs from other cells and examining gene expression in them exclusively. Our experience indicates that a combination of sorting by retrograde fluorescent labeling and cell size is most useful in isolating the RGCs and allows us to obtain the purest samples. Moreover, in our previous research, cell sorting very clearly revealed markers specifically expressed by RGCs (Himori et al., 2013). The ratio of $4Di\text{-}10ASP^{+}$ RGCs to 4′-6-diamino-2-phenylindole $(DAPI)^{+}$ cells rose from only 0.2% to 96.4% with sorting (as shown in Fig. 1). This high level of purity gave us exceptionally large sample sizes for both the microarray and the qPCR analyses and allowed us to perform very effective statistical analyses of biological variations and obtain highly reliable results. The possibility remains, however, that our analysis was affected by the exclusion of small RGCs. Therefore, further experiments may be required to provide more precise results.

Our previous work showed that expression of *Ho-1* had increased 1 day after NC (Himori et al., 2013). Four days after NC, *Ho-1* continued to have a high level of expression. Seven days after NC, moreover, expression of *Ho-1* had increased still further. This study included a microarray analysis of the purified RGCs, which clearly showed high HO-1 expression in the surviving RGCs 7 days after NC. Approximately 7 days after NC, even though almost all the RGCs were disrupted by the apoptotic reaction, a fraction of cells survived. Although factors other than HO-1 might rise after injection of CoPP, we focused on HO-1 because many researchers have suggested that HO-1 has a strong cellular protective effect in RGCs and in Müller cells (Arai-Gaun et al., 2004; Sun et al., 2010). Increased levels of HO-1 protein have been observed in RGCs exposed to hydrostatic pressure in vitro and in mouse retinas exposed to acute elevated IOP (Liu et al., 2007). Hypothesizing that the neuroprotective factor after NC

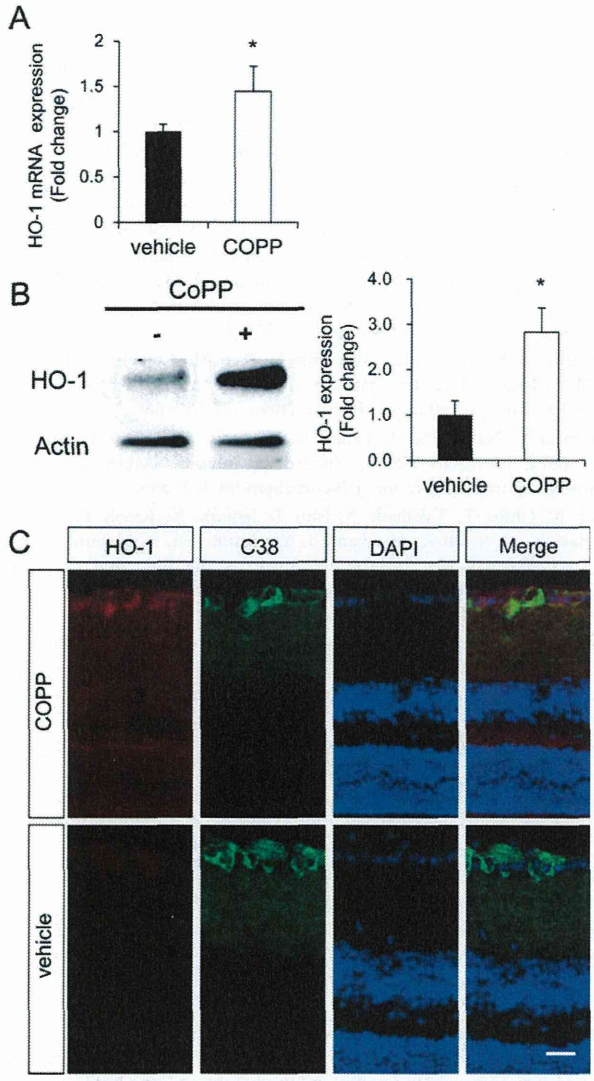

Fig. 4. **A**: The left graph shows qPCR data on *Ho-1* mRNA expression changes. **B:** Representative immunoblotting data, with antibodies for HO-1 and β-actin (top and bottom), on retinal HO-1 induction in mice pretreated with either vehicle or CoPP. The right graph shows the band intensities of HO-1 relative to β-actin, measured with densitometry (n = 3–4 in each group). $*P < 0.05$. **C:** Immunofluorescence images showing the localization pattern of HO-1 protein. HO-1 was detected only in the CoPP-pretreated retina without NC. Scale bar = 20μm.

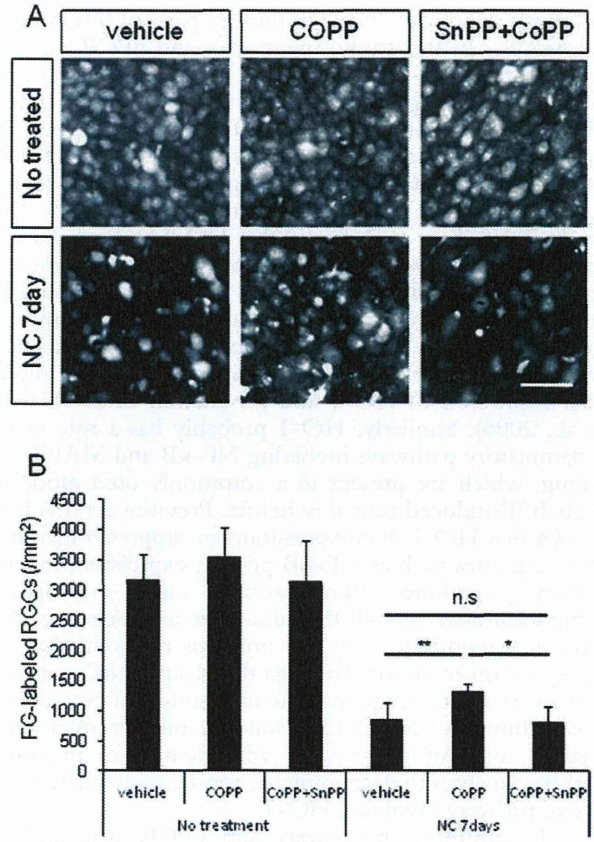

Fig. 5. Effect of HO-1 on RGC protection after NC. **A:** Representative photographs of RGCs in flat-mounted retinas. **B:** Quantitative data on the density of RGCs after NC (n = 4–7 in each group). $*P < 0.05$, $**P < 0.01$. Scale bar = 50μm.

was stronger in these surviving RGCs than in the apoptotic cells, we performed the microarray analysis, which revealed the high expression of HO-1. Additionally, experimental studies showed that free-radical scavengers effectively prevented glaucomatous tissue injury, including glutamate- and IOP-induced RGC death (Inomata et al., 2006; Munemasa et al., 2009). Oxidative stress is one of the most common stress signals inducing HO-1 expression, and it has been suggested that it plays a role in signaling RGC death in glaucoma (Tezel, 2006; Guo et al., 2011).

HO-1 belongs to the heat shock family of proteins, a group that can function as antioxidants, antiapoptotics, cytoprotectors, or anti-inflammatory agents in different pathologic conditions. The induction of HO-1 is regulated at the level of transcription by HSF1, AP-1, NFκβ, and Nrf2. Nrf2, in particular, has an important role in the expression of HO-1 (Naidu et al., 2009; Koriyama et al., 2010). Nrf2 normally resides in the cytoplasm bound by its cytosolic inhibitor, Keap1, which targets it for proteosomal degradation (Itoh et al., 1997, 1999). Our previous work suggested that the large quantity of ROS induced by NC disrupts the association of Nrf2 and Keap1, which leads to nuclear translocation of Nrf2 and the transcriptional activation of cytoprotective genes (Himori et al., 2013). We believe that mitochondrial dysfunction can induce the generation of ROS and that HO-1 is a key part of the antioxidant enzymes that form in response. Additionally, members of the Bcl2 gene family, including Bcl2, BclxL, and Bax, play an important role in regulating RGC death in glaucoma (Nickells et al., 2008). Cell

apoptosis stands out as one of the key parts of this biological process. In this study, microarray and qPCR analyses revealed that HO-1 is upregulated in the RGCs after NC. Although this might reflect only the nature of NC injury, pharmacological induction of HO-1 with CoPP has also been shown, in a previous study, to contribute to the reduction of RGC death and to play a beneficial role in retinal protection after ischemia–reperfusion injury (Sun et al., 2010). Sun et al. found that HO-1 was related to a reduction in the recruitment of macrophage infiltration in the retina through the suppression of monocyte chemoattractant protein. A different study showed that a combination of astrocytes and microglia could prevent excessive inflammatory responses in the brain by regulating microglial expression of HO-1 and production of ROS (Min et al., 2006). Similarly, HO-1 probably has a role in the inflammatory pathways involving NF-κB and MAPK signaling, which are present in a commonly used model of high-IOP-induced retinal ischemia. Previous reports have shown that HO-1 overexpression can suppress inflammatory responses such as NF-κB protein expression, inflammatory cytokine upregulation, and macrophage infiltration after high-IOP-induced retinal ischemia. We have also reported, from our previous research, that the upregulation of antioxidants via the Keap1–Nrf2 pathway was a very important part of the mechanism of cytoprotection (Himori et al., 2013). Building on our microarray analysis, our future research will include an ingenuity pathway analysis to determine the most closely related oxidative pathway involving HO-1.

In summary, microarray and qPCR data analyses indicated that RGC gene responses were closely linked to optic nerve injury in our mouse NC model. Neuroprotective strategies have been proposed and are being investigated as new goals for glaucoma therapy. Effective neuroprotection, aimed at salvaging functional RGCs and their axons before they are irreversibly damaged, requires early intervention and targeting of upstream events. Identification of the early clinical molecular events in RGCs would add to our understanding of the nature of glaucomatous injury and provide potential targets for neuroprotective strategies. This study demonstrates that inducing the overexpression of HO-1 may have promise as a neuroprotective treatment for glaucoma, and in particular for NTG, which is the most prevalent type of glaucoma in Asian countries.

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