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Original research article

A biochemical basis for induction of retina regeneration by antioxidants

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ABSTRACT

The use of antioxidants in tissue regeneration has been studied, but their mechanism of action is not well understood. Here, we analyze the role of the antioxidant N-acetylcysteine (NAC) in retina regeneration. Embryonic chicks are able to regenerate their retina after its complete removal from retinal stem/progenitor cells present in the ciliary margin (CM) of the eye only if a source of exogenous factors, such as FGF2, is present. This study shows that NAC modifies the redox status of the CM, initiates self-renewal of the stem/progenitor cells, and induces regeneration in the absence of FGF2. NAC works as an antioxidant by scavenging free radicals either independently or through the synthesis of glutathione (GSH), and/or by reducing oxidized proteins through a thiol disulfide exchange activity. We dissected the mechanism used by NAC to induce regeneration through the use of inhibitors of GSH synthesis and the use of other antioxidants with different biochemical structures and modes of action, and found that NAC induces regeneration through its thiol disulfide exchange activity. Thus, our results provide, for the first time, a biochemical basis for induction of retina regeneration. Furthermore, NAC induction was independent of FGF receptor signaling, but dependent on the MAPK (pErk1/2) pathway.

1. Introduction

Regeneration of damaged tissue would be the ultimate cure for many degenerative diseases. While some simple organisms and lower vertebrates are able to regenerate lost structures, or even an entirely new organism in the case of hydra and planarians, higher vertebrates do not have the innate ability to regenerate most tissues. Unfortunately, with increased organismal complexity, regenerative potential has been lost (Bely and Nyberg, 2010).

Stem cells are a common source for replenishing cells after injury or cell death in regenerating organisms. Specifically in the eye, stem cells present in the ciliary margin (CM) of several organisms are induced to proliferate and differentiate to replace damaged tissue (Fischer et al., 2013). In addition, stem cells must perform self-renewal to promote the maintenance of the stem cell niche, otherwise, exhaustion of these cells would hinder healing processes (Jopling et al., 2011). Understanding the mechanism of stem cell activation and maintenance will help contribute to the induction of the lost regenerative potential in higher vertebrates including humans.

Redox status, the balance of reactive oxygen species (ROS) and cellular antioxidants, is one of the main regulators of stem cell self-renewal (Sart et al., 2015). A hypoxic niche and a low level of intracellular ROS is important for the maintenance of stem cells (Lonergan et al., 2007) because, as the levels of ROS increase, mainly due to shifts in metabolism, ROS can act as important second messengers enhancing cell differentiation (Sart et al., 2015). This differentiation is accompanied with changes in expression of redox sensitive stemness factors such as, Sox2, Oct4, Nanog, Klf4, Tra-160, and an increased number of mature mitochondria (Ji et al., 2010; Lonergan et al., 2007).

NAC is a well-documented antioxidant that controls the redox status of cells through scavenging free radicals and/or reducing oxidized proteins and lipids (Zafarullah et al., 2003). NAC is able to reduce free radicals directly (scavenging activity) or by serving as a precursor for cysteine, which is necessary for the synthesis of glutathione (GSH) (Cotgreave, 1997; Laragione et al., 2003). Additionally, NAC has the ability to reduce cellular proteins through its thiol-disulfide exchange activity (Laragione et al., 2003). Specifically, NAC has been reported to directly interact with target proteins that contain cysteine residues or thiol groups such as Raf-1, MEK, and ERK (Kim et al., 2001).

NAC has been shown to play a role in regeneration in several model systems (Drowley et al., 2010; Uzun et al., 2009; Welin et al., 2009; Xiong et al., 2012; Yamada et al., 2013), however, the specific

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inductive mechanism is not clear. Here, we analyze the properties of NAC to determine how it induces retina regeneration in the embryonic chick. Retina regeneration is normally induced in the embryonic chick following complete retina removal at embryonic day (E) 4–4.5 using ectopic factors such as fibroblast growth factor 2 (FGF2) which activates retinal stem/progenitor cells present in the CM of the eye or induces transdifferentiation of the retinal pigmented epithelium (RPE) (Spence et al., 2004). Here, we report that NAC is able to induce retina regeneration in the absence of any exogenous factor. Even though NAC decreases the level of ROS induced by injury, its regenerative potential is not dependent on its free radical scavenging ability. Our results support a model in which NAC activates the MAPK pathway independently of FGF receptor signaling, through its thiol-disulfide exchange activity.

2. Results

2.1. Redox status changes in the CM in response to injury

Since low levels of ROS have been shown to create/maintain an optimal redox status conducive for stem cell self-renewal (Urao and Ushio-Fukai, 2013), we first investigated the redox status in the CM (the retinal stem/progenitor cell niche of the embryonic chick) following retinectomy. Changes in redox status were documented by measuring levels of immunofluorescence when using an antibody against 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) which covalently binds to oxidized adducts in proteins. Previous work in our laboratory determined that activation of transcription factors necessary for induction of regeneration occurs in response to injury by 6 hours (h) post-retinectomy (PR) (Luz-Madrigal et al., 2014). Therefore, we investigated changes in the redox status within the CM at both 6h PR and 24h PR to be certain measurements were within the window of induction. DMPO immunofluorescence shows the level of oxidized proteins is increased significantly in the CM of retinectomized eyes at both 6h and 24h PR compared to uninjured developing eyes at E4 and E5 respectively (Fig. 1 B-E, N). We, then, added various antioxidants to determine their effect on the increased ROS that occurs in response to injury. The addition of either NAC, XJB 5–131, or Vitamin C at the time of retinectomy leads to a reduction in the level of ROS compared to retinectomy only, with the reduction by NAC and XJB 5–131 being significant after quantification of the DMPO immunofluorescence (Fig. 1 F-K, O). Interestingly, FGF2 also decreased (marginally significant) the level of oxidized proteins at 6 and 24h PR compared to eyes receiving retinectomy only (Fig. 1L, M, O). This suggests that changes in the redox status are necessary for induction of regeneration since regeneration will not occur in retinectomized eyes without the addition of exogenous factors such as FGF2. The redox status results for NAC were corroborated with the fluorescent probe, (6)-carboxy-2′,7′dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA), which oxidizes in the presence of ROS (Fig. S1).

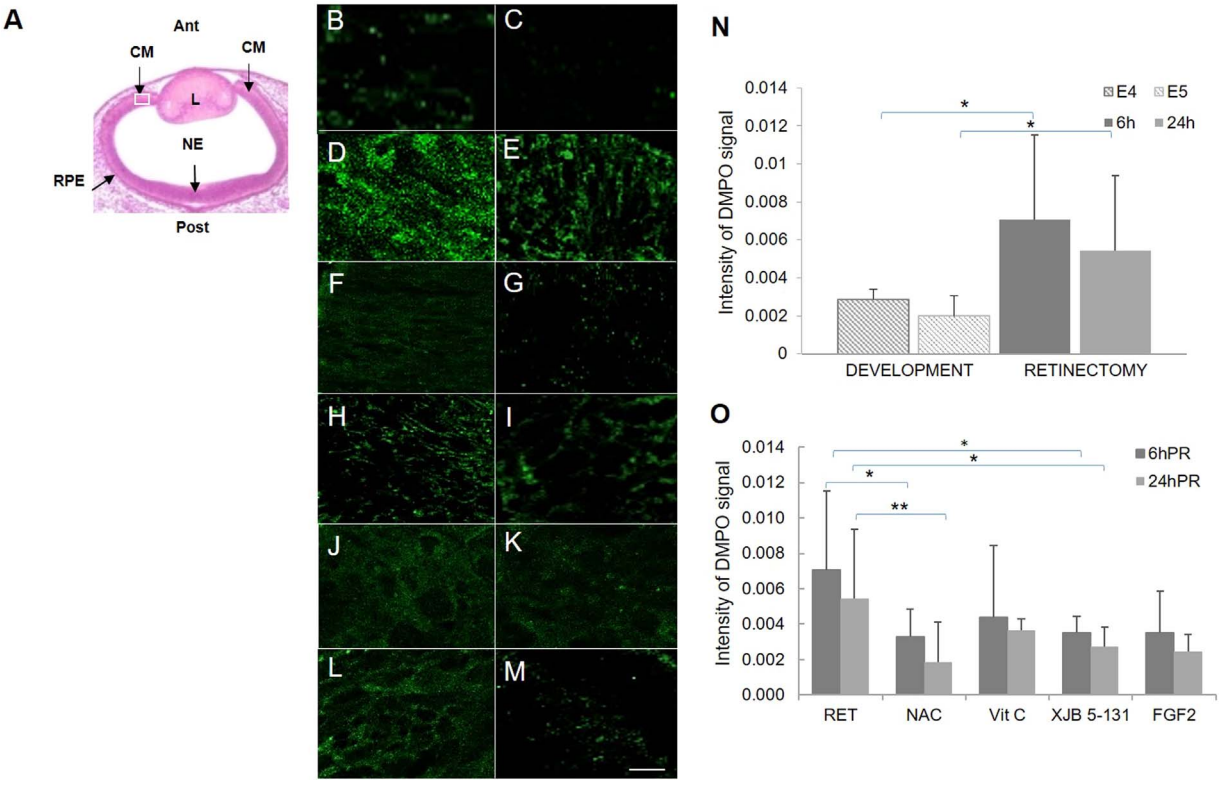

Fig. 1. Redox status in the chick ciliary margin post- retinectomy (PR). (A) Histological section of an embryonic day 4 (E4) chick eye showing the structures of the eye. Anterior (Ant) and posterior (Post) regions. L: Lens; CM: Ciliary margin; NE: neuro epithelium; RPE: Retinal pigmented epithelium. (B-M) Immunohistochemistry using the immunospin trap 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) antibody on sections of the CM of chick eyes at (B) E4, (C) E5, (D) 6h and (E) 24h post-retinectomy (PR) only, as well as with the following treatments: (F, G) NAC at 6h and 24h PR respectively, (H, I) Vitamin C at 6h and 24h PR respectively (J, K) XJB 5–131 at 6h and 24h PR respectively, and (L, M) FGF2 at 6h and 24h PR respectively. Scale bar in M is 125 μm and applies to all. (N, O) Graphical representations of the ratio (intensity/area) of the signals detected in B-E and F-M respectively. Statistical analysis was performed using Dunnett multiple comparisons. The first analysis compared developmental samples and retinectomy samples and are shown in (N). Retinectomy significantly increased the level of oxidized proteins at both 6h and 24h PR (Dunnett 6h * = 0.05 and 24h * = 0.04). The second analysis compared NAC, Vitamin C, XJB 5–131, and FGF2 to Ret and results are shown in (O). NAC significantly reduced the level of oxidized proteins at both 6h and 24h PR (For NAC compared to Ret, Dunnett 6h * = 0.04 and 24h ** = 0.005; for XJB 5–131 compared to Ret, Dunnet 6h *= 0.05 and 24h *= 0.05). As an aside test, FGF2 was compared to Ret and showed marginal significant differences (Dunnett 6h = 0.07 and 24h = 0.06).