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Localization and distribution of superoxide dismutase-1 in the neural tube morphogenesis of chick embryo

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ABSTRACT

Superoxide dismutase 1 (SOD- 1) is an antioxidant enzyme that regulates the levels of Reactive oxygen species (ROS) by catalyzing the conversion of superoxide radical into hydrogen peroxide (H_2O_2) and oxygen. ROS are known to play a significant role in various cellular processes, via redox modification of a variety of molecules that participate in signaling pathways involved in this processes. As the levels of ROS in cells are controlled by the levels of antioxidant enzymes, thus SOD-1 may be indirectly involved in regulating different cellular processes by maintaining the required levels of H_2O_2. Therefore, in the present study we have investigated the possible involvement of SOD- 1 in the neurulation during the development of chick embryo. During gastrulation, SOD- 1 immunoreactivity was observed throughout the ectoderm and cauda mesoderm areas, however, its presence during neurulation was restricted to certain areas of neural tube particularly in the dorsal neural tube where neural tube closure takes place. Assaying enzyme activity revealed a significant increase in the SOD activity during neurulation. Further, inhibition of SOD- 1 by Diethyldithiocarbamate (DDC) induced abnormalities in the development of the neural tube. SOD- 1 inhibition specifically affected the closure of neural tube in the anterior region. Thus, here we report the presence of SOD- 1 mainly in the ectoderm and tissues of ectodermal origin during gastrulation to neurulation which suggests that it may be involved in the regulating the cellular processes during neural tube morphogenesis.

1. Introduction

Reactive oxygen species (ROS) are known to play a significant role in various cellular processes via redox modification of a variety of molecules that participate in signaling pathways (Covarrubias et al., 2008; Kamata and Hirata, 1999; Reth, 2002; Pi et al., 2010; Sauer and Wartenberg, 2005; Vieira et al., 2011). ROS like hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{-\bullet}$) and hydroxyl radical ($OH^{-\bullet}$) are free radicals generated during several metabolic processes (Sies, 1997). ROS, in excess, can affect biomolecules by their ability to cause lipid peroxidation, protein oxidation, DNA fragmentation and cell death (Mates, 2000; Qanungo and Mukherjea, 2000). However, to protect the cells from injury, these free radicals are neutralized by highly complex and integrated network of antioxidant defense system. Antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and γ-glutamylcysteine synthatase serve as a first line of defense against the ROS (Min-Jung et al., 2004). Superoxide dismutase functions by catalyzing the conversion of superoxide radical into hydrogen peroxide and oxygen (Abreu and Cabelli, 2010; McCord and Fridovich, 1969).

Levels of ROS can be indirectly analyzed by monitoring the expression of antioxidant enzymes. Relative increase in the activity of SOD, and other antioxidant enzymes, has been reported in the brain, lung and liver of the oviparous and metamorphosing vertebrates. This was mainly suggestive of its protective function against increasing ROS (Starrs et al., 2001).The presence of SOD and its oxidative stress-protective effect during embryogenesis of mammals, birds, reptiles, fishes and amphibianshas also been described (Frank and Grosecolose, 1984; Gaupale et al., 2012; Khan and Black, 2003; Montesano et al., 1989; Mourente et al., 1999; Qanungo and Mukherjea, 2000; Rizzo et al., 2007; Surai, 1999; Starrs et al., 2001; van Golde et al., 1998). These studies have mostly focused on the biochemical estimation of the enzyme activities during development. However, tissue specific distribution of the enzyme, and its fate from gastrulation to neurulation, has not been investigated. Moreover, being a hydrogen peroxide (H_2O_2) scavenger, SOD is known to indirectly modulate the H_2O_2 function in

Abbreviations: bec, body ectoderm; DDC, diethyldithiocarbamate; dnt, dorsal neural tube; ec, ectoderm; en, endoderm; fg, foregut; hec, head ectoderm; l, lateral neural tube; lpm, lateral plate mesoderm; n, notocord; nb, neuroblast; ncc, neural crest cells; nf, neural fold; ng, neural groove; np, neural plate; nt, neural tube; ov, optic vescicle; r, roof of neural tube; s, somite; sp, splanchnopleure; vnt, ventral neural tube.

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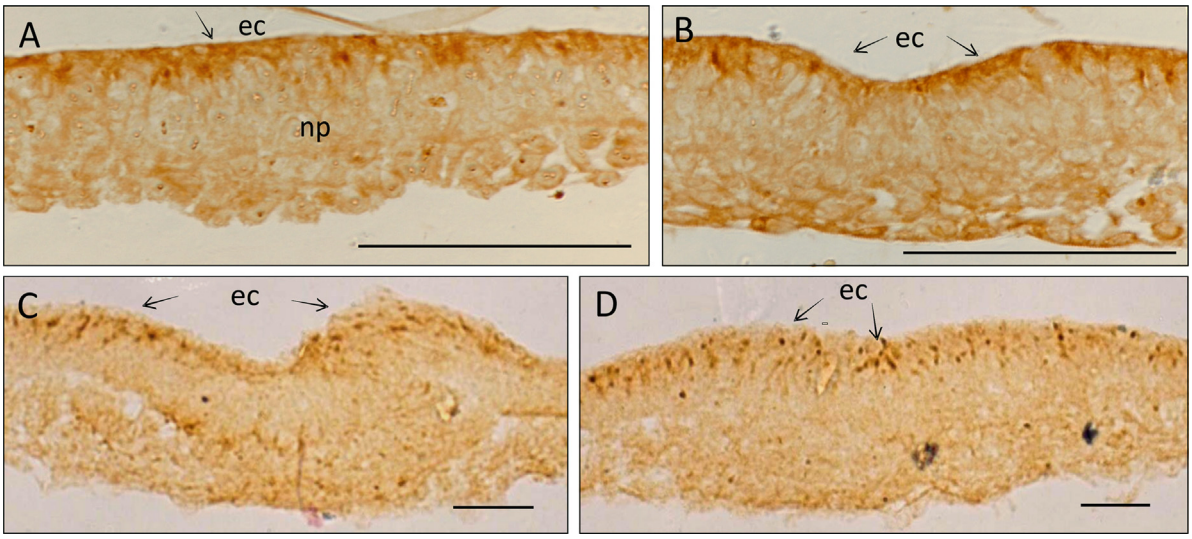

Fig. 1. Transverse section of chick embryo from stage 4 and stage 6 (A–D). (A and B): Transverse sections through Primitive streak stage 4. Strong SOD- 1 positive reaction was seen in the neural plate (np), embryonic ectoderm (ec) (C and D): Transverse section of stage 6. SOD- 1 positive cells in the embryonic ectoderm (ec) cells. Scale bar: A–D, 100 μM.

signal transduction during cell proliferation and cell death (Hanada et al., 1997; Kashiwagi et al., 1999; Rizzo et al., 2007; Ruiz-Gines et al., 2000). Inspite of its wider distribution and presence across the vertebrate phyla, tissue specific distribution, localization and direct involvement of SOD in early embryonic development have not been reported in any of the vertebrate species. Herein, we test the hypothesis that SOD-1 is one of the crucial enzymes regulating the morphogenesis of neural tube during early development. The study is likely to provide new insights into the early development of vertebrates. In the present study, using chick as a model organism, we investigate the spatio-temporal distribution of SOD and it's relevance during gastrulation to neurulation. Biochemical analysis of SOD was also performed to reveal its activity profile during this phase. Further, the effect of SOD −1 inhibitor on neural tube development was investigated to test the possible involvement of SOD in the neural tube morphogenesis. Chick embryo served as an excellent model, in view of its easy availability and facility, in experimental manipulation.

2. Materials and methods

Fresh fertilized eggs were procured from Vyankateshwara hatcheries, Urali kanchan, Pune (India). All the experiments were performed following ethical guidelines established for animal usage by Institutional Animal Ethics Committee (IAEC) Savitribai Phule Pune University and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India (Registration no. 538/02/c/CPCSEA). Fresh fertilized White Leghorn chicken eggs were incubated at 37.5 °C and staging were carried out according to Hamburger and Hamilton (1951). Embryos at the required developmental stages from gastrulation till neurulation (stage 4–11) were harvested by the filter paper ring technique (Olzanska and Lassota, 1980). For immunohistochemical study, chick embryos (stage 4–11) were washed in saline and fixed in Bouin's fixative for 24 h. Embryos were washed to remove the fixative and then dehydrated in alcohol, embedded in Paraffin and serially sectioned (6 μm) in the transverse plane. Sections were mounted on poly-L-Lysine coated slides and were stored for further use. For Immunoblot analysis and enzyme assays, chicken embryos (stage 4–11) were harvested and frozen in liquid nitrogen and immediately stored at −80 °C until further experimentation.

2.1. Immunohistochemistry

Sections were used for the immunohistochemical localization of superoxide dismutase following the standard protocol (Gaupale et al., 2012). Briefly, the sections were deparaffinized, hydrated and then washed with phosphate buffered saline (PBS) thrice and treated with the 0.3% H_2O_2 in methanol for 45 min. Sections were then washed in PBS and incubated with blocking agent containing 0.5% BSA (Bovine serum albumin) and 0.5% gelatin in PBS. After washing, sections were incubated for 60 min in normal goat serum. After incubation, excess goat serum was blotted out and then sections were incubated with rabbit polyclonal antibody against full length human SOD-1 protein (1:50, ab13498, Abcam, United Kingdom) for overnight at 4 °C in a humid chamber. Sections were then washed in PBS and incubated with biotinylated goat anti-rabbit IgG antibody (1:200, Vectastain, Burlingame, CA, USA). Sections were then washed with PBS thrice and incubated with ABC reagent for 60 min (Vectastain, ABC Kit, Burlingame, CA, USA 1:100). After washing thrice with PBS, sections were reacted in dark for 5–6 min with 3,3-diaminobenzidine tetra hydrochloride (DAB) in tris buffer (0.05 M, pH 7.2) containing 0.02% H_2O_2. Slides were washed in distilled water, dehydrated, cleared in xylene, mounted in D.P.X. Sections were observed under microscope (Carl Zeiss, Axioskop 40) and images were captured using Nikon camera (Coolpix 4500). Control sections were incubated with PBS without primary antibody.

2.2. Immunoblot analysis

Immunoblot analysis was carried out from crude extracts of 40 chicken embryos of stage 10 following standard protocol. Briefly, the embryos were harvested using filter paper ring method and were homogenized in homogenizing buffer containing 50 mM Tris–HCl (pH 7.5), 50 mM $MgCl_2$, 1 mM EDTA, 0.1% Triton X-100 and 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor (PI) cocktail. The homogenate was centrifuged at 10,000 rpm for 20 min to eliminate cell debris and supernatant was used. Protein estimation was carried out by Bradford's method (1976). 50 μg protein was loaded per well. Proteins were resolved on 12% SDS–polyacrylamide gel electrophoresis. Protein bands were transferred to PVDF membrane by electro-blotting unit in transfer buffer (192 mM glycine, 25 mM Tris, 0.1% SDS, 20% methanol) overnight at 75 mA. Then the membrane was incubated with blocking solution