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Original article

Peroxynitrite enhances self-renewal, proliferation and neuronal differentiation of neural stem/progenitor cells through activating HIF-1α and Wnt/β-catenin signaling pathway

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ABSTRACT

Hypoxic/ischemic stimulation could mediate growth and differentiation of neural stem/progenitor cells (NSCs) into mature neurons but its underlying mechanisms are largely unclear. Peroxynitrite formation is considered as a crucial pathological process contributing to cerebral ischemia-reperfusion injury. In the present study, we tested the hypothesis that peroxynitrite at low concentration could function as redox signaling to promote the growth of NSCs under hypoxic/ischemic conditions. Increased NSCs proliferation was accompanied by peroxynitrite production in the rat brains with 1 h of ischemia plus 7 days of reperfusion *in vivo*. Cell sorting experiments revealed that endogenous peroxynitrite level affected the capacity of proliferation and self-renewal in NSCs *in vitro*. Hypoxia stimulated peroxynitrite production and promoted NSCs self-renewal, proliferation and neuronal differentiation whereas treatments of peroxynitrite decomposition catalysts (PDCs, FeTMPyP and FeTPPS) blocked the changes in NSCs self-renewal, proliferation and neuronal differentiation. Exogenous peroxynitrite treatment revealed similar effects to promote NSCs proliferation, self-renewal and neuronal differentiation. Furthermore, the neurogenesis-promoting effects of peroxynitrite were partly through activating HIF-1α correlated with enhanced Wnt/β-catenin signaling pathway. In conclusion, peroxynitrite could be a cellular redox signaling for promoting NSCs proliferation, self-renewal and neuronal differentiation and peroxynitrite production could contribute to neurogenesis in ischemic/hypoxic NSCs.

1. Introduction

Targeting neural stem/progenitor cells (NSCs) for adult neurogenesis becomes a new therapeutic strategy for brain repair and recovery of neurological functions in treatments of stroke and neurodegenerative diseases [1,2]. Accumulated evidences indicate that ischemia and/or hypoxia could stimulate the proliferation and differentiation of NSCs. Hypoxia/ischemia-mediated neurogenesis has been reported in different experimental systems including the *in vitro* hypoxic neural stem/progenitor cells (NSCs) [3,4], the experimental stroke animal models of neonatal mice [5] and adult rats [6] as well as stroke patients [7]. For examples, exposure to 1–5% O_2 improved NSCs proliferation, survival and stimulated the development of dopaminergic neurons [8,9]. Enhanced NSCs proliferation and newly generated neurons were found in the rat hippocampal dentate gyrus after transient brain ischemia [10,11]. However, the underlying mechanisms of hypoxia/ischemia-induced neurogenesis remain obscure.

Free radicals, including reactive oxygen species (ROS) and reactive nitrogen species (RNS), appear to have dual roles in cerebral ischemia-reperfusion injury. Ischemia/hypoxia insults mediate free radical generation, triggering numerous molecular cascades and leads to blood brain barrier hyper-permeability, brain edema, hemorrhage, inflammation and neuronal cell death [12–16]. On the other hand, free radicals could be redox signaling for triggering neurogenesis and brain repair. The neurogenesis-promoting or cytotoxic effects of free radicals appear to be dependent on the concentration, sources and their microenvironments. For example, nitric oxide (NO), a representative RNS, could promote NSCs proliferation and neuronal differentiation [17–19]. Low dose of NO donor NOC-18 (10 μM) increased NSCs proliferation but high concentrations (100 μM) inhibited NSCs proliferation [20]. Inflammatory NO production inhibited NSCs proliferation and induced astrocyte differentiation [21,22]. Similarly, superoxide could also

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mediate NSCs proliferation and neuronal differentiation [23,24]. Notably, NO and superoxide are simultaneously produced in hypoxic/ischemic brains and they react extremely rapidly at closely diffusion limit to form peroxynitrite ($ONOO^-$). The reaction is much fast than the reactions of NO and superoxide with other biomolecules [16]. Since the roles of NO and superoxide in mediating neurogenesis are well addressed and $ONOO^-$ has far more reactive than its precursors, we logically explore the roles of $ONOO^-$ at low concentration in regulation of neurogenesis in hypoxic/ischemic brains.

Peroxynitrite formation is widely accepted as an important neurotoxic pathway in ischemic brain injury [15,25]. Increased 3-nitrotyrosine (3-NT, a footprint of the reaction of $ONOO^-$ with protein tyrosine residue) was found in the brain tissues of experimental stroke animal models as well as the blood and brain samples of stroke patients [26–29]. Peroxynitrite decomposition catalyst (PDCs, FeTMPyP and FeTPPS) has showed robust neuroprotective effects against ischemic brain injury in different experimental stroke animal models [30–33]. Peroxynitrite could be a redox signaling to stimulate endothelial cell proliferation, tube formation and blood vessel growth [34] and increase hypoxia-induced proliferation of pulmonary artery smooth muscle cells [35]. Interestingly, 3-morpholinylsydnoneimine chloride (SIN-1, an $ONOO^-$ donor), promoted embryonic NSCs proliferation [36]. Those results provide a clue that $ONOO^-$ might also have adult neurogenesis-promoting effects.

In the present study, we firstly detected the correlation of $ONOO^-$ production and neurogenesis in post-ischemic rat brains *in vivo*. By using our newly developed $ONOO^-$ specific fluorescent probe HKYellow-AM [42] and the *in vitro* cultured NSCs, we investigated the cause-consequence relationship between $ONOO^-$ formation and the self-renewal and proliferation of NSCs under hypoxic condition. Furthermore, we investigated the underlying mechanisms of $ONOO^-$-mediated neurogenesis and found to be related to activate HIF-1α correlated with enhanced Wnt/β-catenin signaling pathway.

2. Material and methods

2.1. Cerebral ischemia-reperfusion model and BrdU incorporation in vivo

Male adult Sprague-Dawley rats (280–300 g) were obtained from Laboratorial Animal Unit, the University of Hong Kong. All the experimental protocols have been implemented in compliance with the regulation of Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong. Middle cerebral artery occlusion (MCAO) was used to induce cerebral ischemia-reperfusion injury *in vivo* as our previously described [37]. Briefly, adult SD rats were anaesthetized by inhalation of isoflurane (5% for induction, 2% for maintenance) in a mixture of 70% N_2 and 30% O_2. The left common carotid artery was isolated from the surrounding nerves and clipped transiently using a microvascular clip. A piece of 3/0 monofilament nylon suture (Ethicon Johnson-Johnson, Brussels, Belgium) coated with silicon was inserted *via* lumen of left external carotid artery stump into the left internal carotid artery until its tip occluded the origin of the left middle cerebral artery. After MCAO ischemia for 0.5 or 1 h, reperfusion was induced by carefully withdrawal of the suture from the left anterior cerebral artery. BrdU (50 mg/kg) was intraperitoneally injected into rats for 7 days to label actively proliferation cells. After 7 day reperfusion, rats were anesthetized with Xylazine (5 mg/kg) and Ketamine (100 mg/kg), and then transcardially perfused with cold PBS for harvesting brain tissues for western blot analysis. For immunostaining studies, rats were then perfused with 4% paraformaldehyde. The brains were then penetrated with 30% sucrose solution, followed by embedding in OCT and cyto-sectioned into 20 μm thicknesses sections. The brain sections were prepared in “1 in 6”. In detail, one section was picked up from every 6 consecutive sections by 100 μm intervals to avoid repeated counting of cells.

2.2. Isolation of rat NSCs

Primary NSCs were prepared from fetal Sprague–Dawley rats (embryonic days E14-15) as previously described [38]. Briefly, cells were dissociated from the cerebral cortex of embryonic rats. The dissociated cells (1×10^5 cells/ml) were suspended in DMEM/F12 medium replenished with 2% B27 supplement, recombinant human bFGF (20 ng/ml), and EGF (20 ng/ml). The B27 minus antioxidant (AO) was applied in all the experiments. The formed neurospheres were dissociated into single cell and suspended for sub-culture. NSCs during 2–5 passages were used for experiments. For NSCs differentiation study, 2–5 passages of NSCs were mechanically dissociated as single cells and directly plated onto poly-L-lysine coated coverslips in the culture medium withdraw growth factors for several days.

2.3. Peroxynitrite synthesis

Peroxynitrite was synthesized according to previous description [39]. Briefly, a mixture of sodium nitrite (0.6 M) and H_2O_2 (0.7 M) was acidified with hydrochloric acid (0.6 M), and then sodium hydroxide (1.5 M) was added within 1–2 s to make the solution alkaline. Excess H_2O_2 was removed by passing the solution through a short column of manganese dioxide. The solution was split into small aliquots and stored at − 80 °C. The aliquots were thawed on ice immediately before use, and the concentration of $ONOO^-$ was determined by measuring the absorption of the solution at 302 nm [C_{ONOO^-} = Abs302 nm/1.67 (mM)]. The control wells were exposed to equal volumes of 0.1 M NaOH solution.

2.4. Hypoxia-mediated endogenous $ONOO^-$ induction and extraneous $ONOO^-$ treatment in cultured NSCs

Two to five passages of NSCs were mechanically dissociated as single cells and directly plated onto poly-L-lysine coated coverslips in the culture medium with or without growth factors. FeTMPyP or FeTPPS (10 μM), the representative PDCs, were added to the medium at 10 min before hypoxia. Cells were then placed into a chamber flushed with 2.5% O_2 plus 5% CO_2 balanced 92.5% N_2 for hypoxia exposure. Oxygen concentration was monitored with PA-10a oxygen analyzer. NSCs were maintained in the hypoxia chamber at 37 °C for 4 days. For extraneous $ONOO^-$ treatment, 2–5 passages of NSCs were mechanically dissociated and plated as mentioned above, fresh synthesized $ONOO^-$ (PN 20 nM, 200 nM) or $ONOO^-$ donor 3-morpholinosydnonimine (SIN-1, 10 μM) were added to medium once a day for 4 days. Cells were then subjected to different measurement. Cells were harvested 4 h after last adding ONOO-.

2.5. Peroxynitrite detection by HKYellow-AM

HKYellow-AM (10 μM), a high selective $ONOO^-$ probe, was added to medium immediately after taking plates out of the hypoxia chamber for incubation 30 min and images were acquired by a fluorescent microscope (Carl Zeiss, Wavelength 568) with Axio Vision digital imaging system. Quantification of fluorescent intensity was conducted by Image J software.

2.6. NSCs sorting by HKYellow-AM

NSCs were dissociated by StemPro® Accutase® (ThermoFisher) into single cell suspension in PBS. HKYellow-AM (10 μM) was added for staining $ONOO^-$ and incubated with the cells for 30 min. Live cell sorting was performed with FACSria SORP (BD Biosciences). Sort gates were first set by side and forward scatter to eliminate dead and aggregated cells and then by HKYellow-AM fluorophores to separate cells into PN^{Hi} (cells with the top 10% HKYellow-AM intensity) and PN^{Low} (cells with the bottom 10% HKYellow-AM intensity) NSCs. After

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