Biomedicine & Pharmacotherapy 97 (2018) 458–472

Contents lists available at ScienceDirect

Biomedicine & Pharmacotherapy

journal homepage: www.elsevier.com/locate/biopha

Original article

Effect of the NADPH oxidase inhibitor apocynin on ischemia-reperfusion hippocampus injury in rat brain

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ARTICLE INFO

Keywords:
Reactive oxygen species
Cerebral ischemia
NADPH oxidase
Mitochondrial membrane potential
Apocynin
Inflammation
Apoptosis

ABSTRACT

Blockage along with sudden restoration of blood following ischemia, results in several cascading events, such as a massive ROS production which plays an important role in the pathophysiology of ischemia. NADPH oxidase complex in mitochondria complex is believed to be the major source for ROS production. The present study explores the therapeutic potential of apocynin, an NADPH oxidase inhibitor in attenuating the ROS production, and the resultant neuroinflammation and mitochondrial injury during cerebral ischemia in rats. Bilateral common carotid artery occlusion (BCCAO) model was chosen for the study where intracellular ROS and NO levels as well as the NADPH oxidase activity were found to be increased significantly post 7th day of ischemic injury. Enhanced glial activation was observed and an upregulated expression of GFAP and Iba-1 in hippocampus along with that of the transcription factor NFκB and inflammatory markers iNOS, IL-1α, IL-1β and TNF-α.The activity of mitochondrial electron transport chain (ETC) complexes I, II, IV and V were significantly decreased following ischemia. Consequently, there was a decrease in mitochondrial membrane potential (MMP) while an increased release of cytochrome c and upregulated apoptotic markers Bax, caspase-3 and 9 initiated the programmed neuronal death which was also reflected by the marked increase in TUNEL positive cells in the hippocampal region. The physiological functional alterations have been observed following ischemic injury i.e memory and motor deficits. The apocynin supplementation significantly reduced the NADPH oxidase activity and resulted in declined ROS production which in-turn prevented the glial activation and downregulated the inflammatory and pro-apoptotic markers. Apocynin also restored the MMP ($\Delta\psi_m$) and mitochondrial enzymes via inhibition of ROS vicious and relationship between NADPH oxidase and mitochondrial complexes. Apocynin treatment was also successfully reduced the behavioural deficits in ischemic animals. In conclusion, inhibiting the NADPH oxidase complex presumably attenuated the mitochondrial injury, neuroinflammation and apoptosis following ischemic injury in rat brain.

1. Introduction

Several studies have provided evidences for the involvement of reactive oxygen species (ROS) in the pathogenesis of ischemic lesions during transient focal/global ischemia in rodents. [1]. Under normal physiological conditions, ROS plays an important role in cell signalling, cell differentiation etc. [2], but under pathological conditions such as that of cerebral ischemia, there is an overproduction of ROS leading to oxidative stress. Several enzyme systems produce intracellular ROS, including xanthine oxidase [3], inducible nitric oxide synthase (iNOS) [4], Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [5], an enzyme system which is considered as one of the major producers of ROS within the cell and mitochondrial electron transport chain complexes [6]. These sources are interlinked and activation of one enzyme system may subsequently lead to the activation of others where mitochondria play the critical role [7]. It was reported that mitochondria are not only a target for superoxide anions produced by NADPH oxidase but also result in a significant increase in other ROS species [8], which in-turn under certain condition may stimulate NADPH oxidase [7].

Thus, mitochondria are not only considered to be the major source of intracellular ROS but are also prone to the oxidative stress itself [9]. The oxygen free radicals produced following reperfusion injury result in macromolecular damage of lipids, proteins and nucleic acids of both

Abbreviation: CAT, Catalase; ETC, Electron transport chain; IL, -Interleukin; iNOS, indicible nitric oxide synthase; MMP, Mitochondrial membrane potential; NADPH, Nicotinamide adenine dinucleotide phosphate; NFκB, -Nuclear factor kappa-light-chain-enhancer of activated B cells; NOS, Nitric oxide synthase; ROS/RNS, Reactive oxygen species/Reactive nitrogen species; SOD, Superoxide dismutase; TGCI, Transient global cerebral ischemia; TNF-α, -Tumor necrotic factor alpha

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http://dx.doi.org/10.1016/j.biopha.2017.10.123
Received 11 August 2017; Received in revised form 17 October 2017; Accepted 23 October 2017

cellular as well as sub-cellular organelles that can destabilise the cellular homeostasis [9]. Oxidative damage to mitochondria have also been shown to impair mitochondrial functions resulting in cell death via apoptosis which is seen in the cerebral ischemia, where the mitochondrial membrane potential (MMP/$\Delta\psi_m$) is dissipated which is otherwise very essential to maintain a proton gradient across the inner mitochondrial membrane and stimulating high energy by the adenosine triphosphate (ATP) synthase. Loss of this MMP ($\Delta\psi_m$) favours the initiation of the programmed cell death [8].

While mitochondria have a role in energy production and maintenance of brain functions, the glial cells which are central to the brain homeostasis, are also involved in progression of various insults to the nervous systems [10]. The glial cells are central in providing brain homeostasis. Their activation is however protective to the host, as they enable the cell in the removal of debris and also the killings of pathogens [10,11]. However, their excessive or chronic activation can lead to phagocytic activation and kill the neighbouring neurons. Among the glial cells, the astrocytes and microglial cells are mostly involved in such ischemic insults. The glial activation also leads to the activation of NADPH oxidase [12]. Among various homologs (NOX1-NOX5) of the catalytic subunit of the NADPH oxidase enzyme, NOX2 isoform is a membrane-bound complex consisting of two membrane bound (gp91phox and p22phox) and three cytosolic subunits (p40phox, p47phox, p67phox) [13,14]. Initially being discovered as the enzyme responsible for the oxidative burst by which leukocytes kill bacteria [15], this enzyme complex when activated, rapidly produces high levels of superoxide extracellulary also, which may either dismutase to produce hydrogen peroxide or react with NO to produce peroxynitrite [11] for cellular pathogens.

Studies have shown that treatment with apocynin, an inhibitor of NOX2 significantly attenuate the ischemic injury [16]. Apocynin has been reported to protect against oxidative stress by inhibiting the extracellular signal-regulated kinase (ERK)-dependent phosphorylation and membrane translocation of p47phox [17].However, the role of apocynin in inhibiting the mitochondrial injury is not established. Therefore, the present study is designed to look into the role of apocynin, to attenuate the mitochondria injury, thus interlinking the two different ROS sources (NADPH oxidase and mitochondrial electron transport chain) during global cerebral ischemia. Recent investigations have shown that hippocampus is more vulnerable in brain injury during ischemic assault. The hippocampus is the centre of cognitive activity and the increase of dementia prevalence in stroke survival is approximately reported to be 30%, which increases exponentially with age [18]. Further, in hippocampus, CA1 region is most vulnerable to hypoxia [19] and thus the present study in hippocampus following cerebral ischemia, and attenuation by NADPH oxidase inhibitor, apocynin assumes a major attempt in understanding the molecular pathology of ischemia and possible therapeutic intervention.

2. Material and methods

2.1. Experimental animals

Male wistar rats (250–300 g) were procured from the Central Animal House Panjab University, Chandigarh, India. The animals were housed in polypropylene cages under ambient conditions of humidity and temperature and got acclimatized for 1 week. They were provided with food and water ad libtium throughout the experimental period. All the protocols were done in accordance with ethical guidelines as provided by Institutional Animal Ethics Committee (IAEC) of Panjab University.

2.2. Chemicals

All the chemicals used in the study were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA), Genei (Banglaore, India), Santa cruz biotechnology (USA), Merck (Mumbai, India), and Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Apocynin purchased from Sigma chemicals, is a natural organic compound which is also known as acetovanillone(with IUPAC name 1-(4-Hydroxy-3-methoxyphenyl)ethan-1-one). It was isolated from Picrorhizakurroa, a small plant that grows at high altitudes in the Western Himalayas.

2.3. Induction of transient global cerebral ischemia

Transient global cerebral ischemia (TGCI) was induced by the modified method of Speetzen [20]. For the surgical procedure, rats were anesthetized by 10% chloral hydrate (300 mg/kg body weight, i.p.) and were fixed in supine position while a middle incision was made in neck. Both common carotid arteries were exposed and separated carefully from the vagus nerve. Ischemic insult was introduced by occlusion of both common carotid arteries with an occlusion clamp for a period of 20 min followed by the recirculation by removing the clamps. The animals which were subjected to the same surgery without occlusion of common carotid arteries served as sham animals. They were allowed to recover from anaesthesia by placing at a temp. near37 °C (by using heating pad) because anaesthesia lowered the body temperature which may lead to hypothermia.

2.4. Experimental design

The rats were randomly divided into 4 groups with 10-12 animals per group. **Sham:** Animals were sham operated and received normal saline (0.9%) daily for 7 days; **TGCI:** Animals undergo transient global cerebral ischemia (TGCI); **TGCI + Apo:** Animals undergo TGCI and administered with apocynin (5 mg/kg b.wt; i.p) 30 min before surgery and then daily up to 7 days; **Apo:** Animals were administered with Apocynin (5 mg/kg b.wt; i.p)daily for 7 days. The apocynin dose was prepared in normal saline (0.9%).

2.5. Biochemical analysis for oxidative stress markers and antioxidant enzymes

2.5.1. Preparation of sample

Animals were sacrificed and brains dissected out. The hippocampus was isolated anda 10% (W/V) homogenate was prepared in PBS (phosphate buffer saline; pH 7.4). The homogenate was centrifuged at 10,000 × *g* for 30 min and the supernatant was collected called the post mitochondrial fraction (PMF).

2.5.2. Protein estimation

The protein contents in various sections of the brain samples were estimated by the method of Lowry [21]. This method is based on the formulation of the intense blue coloured cupric protein complex upon the treatment of the protein sample with alkaline copper tartarate, resulted from the reduction of phosphomolybdic acid and phosphotungstic acid by the aromatic amino acids and by cupric amino acids complex. Briefly, the protein sample was mixed with Lowry reagent. Following incubation of 10 min, 0.3 ml of Follin's Reagent was added to the tubes, the reaction mixture was then incubated at 37 °C for 30 min and the absorbance was measured at 620 nm. BSA was used as the standard to estimate the protein content.

2.5.3. Reactive oxygen species (ROS)

ROS levels were estimated by method of Best [22], which is based on the deacetylation of 2′7′-dichlorofluorescceine diacetate (DCFH-DA) following ROS mediated oxidation leading to a fluorescent product i.e DCF. The fluorescence was measured with a Perkin Elmer fluorescence spectrometer at an excitation/emission wavelength of 488/525 nm. The units were expressed as AFU/mg of protein where AFU: Arbitrary fluorescence units.