Neuroscience 358 (2017) 227–237

NADPH OXIDASE CONTRIBUTES TO STREPTOZOTOCIN-INDUCED NEURODEGENERATION

KATHERINE GARCIA RAVELLI, [a] **BARBARA DOS ANJOS ROSÁRIO,** [a] **ANDREA RODRIGUES VASCONCELOS,** [b] **CRISTOFORO SCAVONE,** [b] **ROSANA CAMARINI,** [b] **MARINA S. HERNANDES** [c]* **AND LUIZ ROBERTO BRITTO** [a]

[a] *Department of Physiology and Biophysics, University of São Paulo, São Paulo, Brazil*

[b] *Department of Pharmacology, University of São Paulo, São Paulo, Brazil*

[c] *Division of Cardiology, Department of Medicine, Emory University, Atlanta, GA, United States*

Abstract—Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the progressive loss of memory. The neurodegeneration induced by AD has been linked to oxidative damage. However, little is known about the involvement of NADPH oxidase 2 (Nox2), a multisubunit enzyme that catalyzes the reduction of oxygen to produce reactive oxygen species, in the pathogenesis of AD. The main purpose of this study was to investigate the involvement of Nox2 in memory, in AD-related brain abnormalities, oxidative damage, inflammation and neuronal death in the hippocampus in the streptozotocin (STZ)-induced AD-like state by comparing the effects of that drug on mice lacking gp91$^{phox-/-}$ and wild-type (Wt) mice. Nox2 gene expression was found increased in Wt mice after STZ injection. In object recognition test, Wt mice injected with STZ presented impairment in short- and long-term memory, which was not observed following Nox2 deletion. STZ treatment induced increased phosphorylation of Tau and increased amyloid-β, apoptosis-inducing factor (AIF) and astrocyte and microglial markers expression in Wt mice but not in gp91$^{phox-/-}$. STZ treatment increased oxidative damage and pro-inflammatory cytokines' release in Wt mice, which was not observed in gp91$^{phox-/-}$ mice. Nox2 deletion had a positive effect on the IL-10 baseline production, suggesting that this cytokine might contribute to the neuroprotection mechanism against STZ-induced neurodegeneration. In summary, our data suggest that the Nox2-dependent reactive oxygen species (ROS) generation contributes to the STZ-induced AD-like state.

Keywords: Alzheimer's disease, streptozotocin, Nox2, cytokines.

INTRODUCTION

Alzheimer's disease (AD) is a degenerative disorder that usually begins with a subtle memory loss that slowly becomes more severe and, eventually, incapacitating. Other common clinical symptoms of AD include: confusion, poor judgment, language disturbance, agitation and hallucinations (Bird, 2008). In advanced stages of the disease, the deceleration of motor functions leads to a condition similar to parkinsonism (Selkoe, 2001). Several abnormalities were observed in the brain glucose metabolism of patients with sporadic AD. Both the impairment of neuronal insulin signaling and the impairment in the function of translation of insulin receptors have been associated with AD development (Moloney et al., 2010; Liu et al., 2011a,b; Bomfim et al., 2012; Talbot et al., 2012; Yarchoan et al., 2014).

The neuropathological changes of AD brain mainly affect neocortex, hippocampus, entorhinal cortex and some subcortical areas (Spires and Hyman, 2005) and include: the accumulation of amyloid plaques (composed primarily by β-amyloid peptide (Aβ)) (Hardy and Allsop, 1991) and the accumulation of neurofibrillary tangles, neuropil threads and dystrophic neurites containing hyperphosphorylated Tau protein. These changes are accompanied by astrocyte and microglial cell activation (Serrano-Pozo et al., 2011). When chronically activated, microglia generates reactive oxygen (ROS) and nitrogen (RNS) species (Wilkinson et al., 2012), in addition to cytokines, interleukins (IL) and other cytotoxic molecules (Heneka et al., 2015). The activation of microglial cells leads to NADPH oxidase (Nox)-dependent ROS production in the hippocampus resulting in significant neuronal death (Park et al., 2009; Bonda et al., 2010).

The intracerebroventricular (icv) injections of streptozotocin (STZ), a compound synthesized by *Streptomycetes achromogenes* (Szkudelski, 2001) have been used to induce AD-like state in rodents. STZ icv injections induce a progressive deterioration of cognitive function in parallel to changes in glucose and energy metabolism, oxidative stress (Grunblatt et al., 2004), accumulation of Aβ (Knezovic et al., 2015; Ravelli et al., 2017) and increased Tau phosphorylation (Chen et al., 2013; Ravelli et al., 2017). The most prominent brain abnormality in this model is neuroinflammation, reflected

*Corresponding author. Address: Department of Medicine, Division of Cardiology, Emory University, 101 Woodruff Circle, 3331 WMB, Atlanta, GA 30322, United States.
E-mail address: mshern2@emory.edu (M. S. Hernandes).

Abbreviations: 3-NT, 3-nitrotyrosine; 4-HNE, 4-hydroxy-2 nonenal; AD, Alzheimer's disease; Aβ, β-amyloid peptide; AIF, apoptosis-inducing factor; IL, interleukins; Nox, NADPH oxidase; RNS, reactive nitrogen species; ROS, reactive oxygen species; STZ, streptozotocin; Wt, wild type.

http://dx.doi.org/10.1016/j.neuroscience.2017.06.050
0306-4522/

by astrogliosis and microglial activation (Chen et al., 2013).

NADPH oxidases are a family of enzymes dedicated to the production of ROS. These enzymes are able to catalyze the production of superoxide anion by reducing an electron of molecular oxygen, using NADPH as an electron donor. The prototypical Nox, known as Nox2, is composed of three subunits ($p40^{PHOX}$, $p47^{PHOX}$ and $p67^{PHOX}$) present in the cytosol as a complex and two membrane subunits ($gp91^{phox}$ and $p22^{phox}$) composing the cytochrome b558. After a stimulus, the activation of a low-molecular weight G protein (Rac1 or Rac2) and phosphorylation of $p47^{phox}$ initiate migration of the cytosolic complex to the plasma membrane (Hernandes and Britto, 2012), where it associates with cytochrome b_{558} forming a functional Nox complex capable of reducing oxygen to superoxide (Hernandes et al., 2013).

Nox-derived ROS seem to mediate cerebrovascular dysfunctions induced by Aβ (Block, 2008). *In vitro* experiments demonstrated that Nox inhibition by apocynin, a nonspecific pharmacological Nox2 inhibitor, decreased the ability of the β-amyloid peptide to induce degeneration and to trigger morphological changes in neurons (Bruce-Keller et al., 2010). However, in a transgenic model of AD, apocynin treatment did not improve behavioral or neuropathological deficits despite causing a reduction in oxidative stress in the cerebral cortex (Dumont et al., 2011). Gp91ds-tat, a Nox2 peptide inhibitor, was able to decrease the generation of ROS induced by exogenous Aβ in the somatosensory cortex of mice. Moreover, exogenous Aβ was not able to trigger the production of ROS in mice lacking the catalytic subunit of Nox2 ($gp91^{phox}$). Furthermore, no evidence of oxidative stress was observed in transgenic mice overexpressing the amyloid precursor protein but lacking $gp91^{phox}$ (Park et al., 2005).

Nox2-derived ROS has emerged as an important mechanism in the pathogenesis of AD. However, there has been no study directly testing the involvement of Nox2 in cognitive impairment induced by STZ, nor has its role in Tau phosphorylation, oxidative damage, neuronal death and neuroinflammation induced by STZ been investigated. In light of those facts, the main purpose of this study was to investigate the involvement of Nox2 in short- and long-term memory, in AD-related brain abnormalities, inflammation and neuronal death in the hippocampus in the STZ-induced AD-like state by comparing the effects of that drug on mice lacking $gp91^{phox-/-}$ and wild-type mice (Wt) (C57BL/6).

EXPERIMENTAL PROCEDURES

Animals

Male $gp91^{Phox-/-}$ mice (25–30 g) were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and maintained on a C57BL/6 background. The animals were maintained on a 12: 12 h light–dark cycle and with free access to food and water. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Institute of Biomedical Sciences of the University of Sao Paulo, Brazil (protocol number: 098/2012).

Surgical procedures

Surgical procedures were performed as previously described (Ravelli et al., 2017). Mice were anesthetized initially with 5% isoflurane for induction and then maintained with 2–3% isoflurane throughout the duration of surgery. STZ (Sigma, St. Louis, MO) was dissolved in citrate buffer (0.05 mol/L, pH 4.5) immediately before injection. Mice were placed in a stereotaxic apparatus (Insight Ltda, SP, Brazil) and the STZ group was injected bilaterally in the lateral ventricles with STZ (3 mg/kg) in two divided doses (1.5 mg/kg each), on days 1 and 3. The concentration was adjusted so as to deliver 1.5 μl per injection. Control mice were injected with citrate buffer. The bregma coordinates used for injection were: AP: −0.5 mm; ML: ±1.1 mm; DV: −2.8 mm. The injections were performed using a Hamilton syringe (model 701) and conducted at a rate of 0.5 μL/min. The needle was left *in situ* for 3 min to prevent back flow. Clinical signs were monitored daily after the surgery, including general body condition and dehydration. The mice were euthanized for analysis 14 days after the surgery (Ravelli et al., 2017).

Real-time PCR

Tissue from the hippocampi from Wt mice ($n = 6$ for each group) were directly homogenized in 1 ml TRIzol (Invitrogen, Carlsbad, CA, USA) and total RNA was isolated following the manufacturer's suggested protocol. Following two chloroform extraction steps, RNA was precipitated with isopropanol and the pellet washed twice in 70% ethanol. Samples were resuspended in DEPC-treated water and RNA was quantified by measuring the optical density at 260 nm using a Nanodrop-1000 spectrophotometer. One microgram total RNA was reverse transcribed using the Promega Reverse Transcription System (Madison, WI, USA). Total RNA was incubated at 70 °C for 10 min. The solution was mixed with 4 μL of $MgCl_2$ (25 mM), 2 μL of 5 × RT buffer, 2 μL of dNTP mixture (10 mM), 0.5 μl of RNAsin inhibitor (40U/μl), 0.5 μl of AMV reverse transcriptase, and 1 μL of oligodT primer (0.5 μg). The reactions were incubated at 16 °C for 30 min, 42 °C for 30 min, 85 °C for 5 min and then kept at 4 °C. qPCR was carried out with SYBR Green Real-Time Selected Master Mix (Applied Biosystems, CA, USA) according to the user guide. The reaction volume was 20 μL with 2 μL diluted cDNA, 10 μL of SYBR Master Mix, and 500 nM of each primer. Amplification and PCR product detection were performed with the ABI prism 7500 real time-PCR System (Applied Biosystems, USA). The conditions for PCR were as follows: 50 °C for 2 min, 95 °C for 2 min, then 30 cycles of 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 15 s. The specificity of the SYBR® green assay was confirmed by melting-point analysis. Expression data were calculated from the cycle threshold (Ct) value using the ΔCt method for quantification (Dussault and Pouliot, 2006). Gene expression of GAPDH was used for normal-