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Research report

Sildenafil provides sustained neuroprotection in the absence of learning recovery following the 4-vessel occlusion/internal carotid artery model of chronic cerebral hypoperfusion in middle-aged rats

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ABSTRACT

In this study, we tested whether the phosphodiesterase-5 inhibitor sildenafil protects against neurodegeneration and facilitates recovery from learning deficits examined long after chronic cerebral hypoperfusion (CCH) induced by the 4-vessel occlusion/internal carotid artery (4-VO/ICA) model in middle-aged rats. Male Wistar rats (12–15 months of age) were subjected to permanent 3-stage 4-VO/ICA with an interstage interval of 4 days. Sildenafil (3 mg/kg, p.o.) was administered at one dose per day for 10 days, beginning soon after the first occlusion stage. Three months later, learning in a non-food-rewarded, eight-arm radial maze task was tested. Learning performance is expressed as the latency to find a goal box and the number of reference or working memory errors. Histological examination was performed 1–3 days after behavioral testing. In the vehicle-treated group, permanent 4-VO/ICA markedly disrupted learning performance and caused moderate-to-severe neurodegeneration in the CA1-CA4 subfields of the hippocampus (56.2%), dentate gyrus (DG; 19.2%), retrosplenial cortex (RS cortex; 47.4%), and parietal association cortex (PtA cortex; 38.2%). Sildenafil treatment did not prevent 4-VO/ICA-induced learning deficits, whereas neurodegeneration was significantly reduced in the CA1-CA4 subfields (30.5%), DG (7.2%), RS cortex (11.8%), and PtA cortex (6.5%). Advancing previous findings from our laboratory, this study suggests that while sildenafil can provide important neuroprotection in different brain regions of middle-aged rats subjected to CCH, such histological effect does not translate into cognitive recovery.

1. Introduction

Ample experimental and clinical observations support the hypothesis that a state of chronic cerebral hypoperfusion (CCH) represents an etiologic factor of age-related neurodegenerative diseases and dementia (de la Torre et al., 2002). Prevention against CCH-related risk factors, mainly hypertension and chronic heart disease, constitutes the primary recommendation to reduce the prevalence of neurodegenerative disease associated with CCH (de la Torre, 2009). However, once CCH occurs and evolves, the question arises whether the progression of brain damage and cognitive impairment can be mitigated pharmacologically. Drugs such as sildenafil (Viagra) possess pharmacological properties by which their therapeutic indication transcends the area of erectile dysfunction and pulmonary hypertension to include the treatment of vascular-related CNS disorders among other conditions (Vlachopoulos et al., 2009). Sildenafil acts primarily by selectively inhibiting phosphodiesterase type-5 (PDE5) and increasing intracellular cyclic guanosine monophosphate (cGMP) that in turn leads to vascular smooth muscle relaxation, vasodilation, and perhaps overall cerebral circulation improvement (Ghofrani et al., 2006). Beyond its direct action on vascular smooth muscles, sildenafil exerts important preconditioning and antiapoptotic actions in the myocardium, followed by reduced heart infarct size and improved myocardial function after experimental ischemia (Das et al., 2005; Kukreja et al., 2005). As defined elsewhere, "pharmacological preconditioning refers to the action of compounds that trigger the preconditioning signaling cascades without a physical stimulus" (Dimagl et al., 2009). Whether sildenafil can induce preconditioning in the brain is unknown. However, sildenafil modulates the nitric oxide (NO)/cGMP pathway, the signaling of which promotes angiogenesis, neurogenesis, axonal outgrowth, and synaptic plasticity during development and in the adult animal (Prickaerts et al., 2002; Zhang et al., 2005). In a rat model of stroke, sildenafil induced

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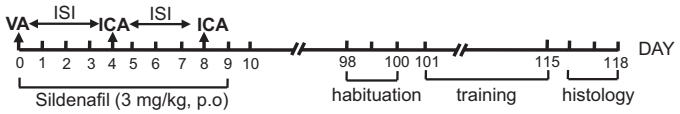

Fig. 1. Schematic representation of the experimental protocol that delineates the phases of the 3-stage 4-VO/ICA model with an ISI of 4 days, periods of sildenafil administration, habituation to the test environment, acquisition training, and histology. VA, vertebral artery; ICA, internal carotid artery; ISI, interstage interval.

angiogenesis, neurogenesis, and axonal remodeling, improved cerebral blood flow in the periinfarct area, and facilitated functional recovery (Zhang et al., 2006; Li et al., 2007; Ding et al., 2011). Acutely, sildenafil improves learning and memory function in several rodent species tested in different behavioral tasks (Reneerkens et al., 2009).

However, whether sildenafil is effective against the neurohistological and behavioral outcomes of CCH has not yet been systematically investigated (Romanini et al., 2010). In that study, young rats were subjected to a 4-vessel occlusion/internal carotid artery (4-VO/ICA) model of CCH, which is based on the permanent, stepwise occlusion of the vertebral arteries (VAs) and internal carotid arteries (ICAs; Neto et al., 2005; Barros et al., 2009). Sildenafil was found to reduce both the rate of mortality and hippocampal neurodegeneration, but its effect on behavior was not evaluated. Unexpectedly, permanent 4-VO/ICA did not impact the ability of young rats to perform the radial maze task. When imposed to middle-aged rats, however, permanent 4-VO/ICA caused not only hippocampal and cortical neurodegeneration, but also learning and memory deficits (Ferreira et al., 2011). Therefore, and given continuity to our previous investigation, in the present study we sought to examine whether the treatment with sildenafil could provide both neurohistological protection and behavioral recovery after permanent 4-VO/ICA in middle-aged rats.

2. Materials and methods

2.1. Subjects

Seventy-one middle-aged (12–15-month-old, 490–600 g body weight) male Wistar rats (inbred strain) were assigned to the following three groups: sham operation ($n = 13$), 4-VO/ICA + vehicle ($n = 34$), and 4-VO/ICA + sildenafil ($n = 24$). The rats were housed at a controlled temperature ($22 \pm 1\,^{\circ}C$) on a 12 h/12 h light/dark cycle (lights on at 7:00 AM). Food and water were provided *ad libitum* throughout the experiment. The materials and methods were approved by our Ethics Committee on Animal Experimentation (protocol no. 044/2008).

2.2. Surgery

Fig. 1 provides a schematic of the entire experimental protocol. The animals were anesthetized with ketamine (15 mg/kg) plus xylazine (1.0 mg/kg) administered intramuscularly. Permanent 4-VO/ICA or sham surgery was performed gradually in three stages according to the sequence VA → ICA → ICA, with an interstage interval (ISI, →) of 4 days. For bilateral occlusion of the VAs, the tip of a unipolar electrode was inserted into the alar foramen of the first cervical vertebra and gently rotated until the presence of hemorrhage ensured vessel rupture. The hemorrhage was then immediately staunched by a 3–4 mA electrical current. This procedure ensures complete and irreversible VA occlusion. The ICAs were carefully dissected from adjacent tissues and permanently ligated using cotton thread. After each occlusion stage, the incision was sutured, and the animal was returned to its home cage until the next surgery. Rectal temperature was monitored with a digital thermometer (Minipa, APPA MT-520, São Paulo, Brazil) using a rectal probe inserted to a depth of approximately 6 cm. Core temperature was controlled only during surgery and maintained at approximately 37.5 °C by a heating blanket. The animals assigned to the sham surgery group were subjected to the same surgical procedures as their counterparts but did not receive vessel occlusions.

2.3. Drug preparation and treatment

Sildenafil citrate (1-[4-etoxido-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1*H*-pirazolo[4,3-*d*]pyrimidine-5-yl)phenylsulfonyl]-4-methylpiperazine citrate; Gamma Pharmaceuticals, Beijing, China) was dissolved in sterile 0.9% saline (vehicle) and administered by gavage at 3.0 mg/kg in a volume of 1.0 ml/kg. We choose this dose because it effectively affords neuroprotection in young rats (Romanini et al., 2010). Moreover, 3 mg/kg was found to be at the peak of the dose-response curve for the effects of sildenafil on performance in rats tested in the object recognition task (Prickaerts et al., 2005). Drug administration began soon after the first occlusion stage (VA) and continued for 9 days, with one dose per day. Sildenafil solution was freshly prepared before administration.

2.4. Behavioral testing

Learning and memory performance was measured in the aversive, eight-arm radial maze (AvRM) model, which works on the basis of the rat's natural behavior of avoiding open and illuminated areas and its preference for darkened and enclosed places (in this case, a shelter or goal box). A detailed description of the AvRM apparatus has been provided elsewhere (Romanini et al., 2010; Ferreira et al., 2011). After habituation, acquisition training began according to a schedule of three trials per session and one session per day for 15 days (5 days per week). In each trial, the rat was placed into the center of the arena, with all arms closed and the video camera turned on. Thirty seconds later, the arms were opened simultaneously, and the animal was allowed to explore the entire maze. When the rat entered halfway down a non-rewarded arm (*i.e.*, an arm that contained a false goal box), the guillotine doors of the remaining arms were lowered simultaneously. Upon the rat's return to the central area, the newly visited arm was closed immediately, and the animal was again confined in the arena for a further 10 s (delay). When the rat found and entered halfway down the rewarded arm (*i.e.*, the arm that contained the true goal box), the guillotine door of that arm was lowered, forcing the animal to enter the correct goal box where it was left for 1 min. If the rat did not find the correct arm within 4 min, then it was placed into it and gently introduced into the shelter. If the rat inserted only its head into an incorrect opening and remained there for more than 1 min, then it was repositioned to the center of the maze, and the trial was restarted. If such atypical behavior persisted for more than four consecutive sessions (days), then the animal was excluded. Between trials, the rat was moved from the maze (or goal box) to its individual home cage where it was left in a separate room until the maze was cleaned of excrement and randomly rotated on its central axis. The goal box was randomly moved to any of the other seven arms, although its spatial position in relation to the extra-maze cues remained unchanged across trials and sessions and was the same for all of the rats. This procedure took approximately 90 s, after which the subsequent trial began. Learning and memory performance was measured by three parameters: (i) the latency to find the goal box, (ii) the number of reference memory errors, and (iii) the number of working memory errors. Within a given trial, a reference error was counted when the rat visited an arm that contained a false goal box for the first time. However, if the rat returned to an arm that had been visited previously during that trial, then a working memory error was recorded. The animal was considered to have left an arm when it placed all four paws on the central platform.

2.5. Histology

Under anesthesia (Thiopental, 50 mg/kg, i.v.), the animals were transcardially perfused with 0.9% saline followed by Bouin's fixative (20 ml/min for 5 min). Following decapitation, the head was immersed in crushed ice (1–2 °C) for at least 2 h to avoid the appearance of so-called "dark" neurons. The brain was then removed and immersed in the same fixative for 2–3 h. The forebrain was sectioned into two parts and conserved in the same fixative for an additional 3–5 days, after which the parts were embedded in paraffin. For each brain, 12 coronal sections (7 μm thick, 70 μm apart) were cut at the medial level of the hippocampus (−3.60 to −4.30 mm) and processed for Nissl staining. Pyramidal cell loss was quantified bilaterally across the CA1–CA4 subfields of the hippocampus, in the retrosplenial (RS) cortex, and in the parietal association (PtA) cortex. Neurodegeneration (or atrophy) was also examined in the hippocampal dentate gyrus (DG). Because the high density of granular cells in this region precludes the reliable determination of cell number, the thickness of the granular cell layer was measured in the suprapyramidal blade, infrapyramidal blade, and crest of the DG. In both the hippocampus and cerebral cortex, the number of pyramidal cells and thickness of the DG granular cell layer averaged from the various measurements in each individual were transformed into a percentage. The mean of the sham-operated group was considered 100%. Each individual value was then normalized with respect to the mean of the sham. The identity of the groups was not revealed during histological assessment.

2.6. Statistical analysis

When learning and memory performance was analyzed across the five session blocks, a normal distribution (D'Agostino and Pearson omnibus test), sphericity (Mauchly's test), and homocedasticity (Lavene's test) were not consistently achieved among the various groups, within the same group, or among the different parameters (*i.e.*, latency, reference memory errors, and working memory errors). In this case, nonparametric Friedman's analysis of variance (ANOVA) was used to quantify within-group differences, which tested the slope of the learning curve for each individual group. If a global significant value was found, then Dunn's *post hoc* test was applied to localize the session block when the reduction of the latency or number of errors (*i.e.*, learning) was significant. Subsequently, the Kruskal–Wallis ANOVA was used for between-group comparisons in each session block. However, when