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Research Article

Differential Effect of the PDE5 Inhibitors, Sildenafil and Zaprinast, in Aging- and Lipopolysaccharide-Induced Cognitive Dysfunction in Mice

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Strategy, Management and Health Policy				
Enabling Technology, Genomics, Proteomics	Preclinical Research	Preclinical Development Toxicology, Formulation Drug Delivery, Pharmacokinetics	Clinical Development Phases I-III Regulatory, Quality, Manufacturing	Postmarketing Phase IV

ABSTRACT In the present study, we have investigated the effects of exogenously administered cyclic GMP-specific PDE5 inhibitors, sildenafil and zaprinast, on cognitive performance in aged- and lipopolysaccharide (LPS)-treated mice using passive avoidance and plus-maze tasks. Aged and LPS-treated mice showed poor retention of memory in step-through passive avoidance and plus-maze task. Administration of PDE5 inhibitors reversed the age-induced retention deficits in both of the test paradigms. Also, PDE5 inhibitors on acute administration in young mice showed improvement in memory retention when tested in both of the paradigms. However, the effect of two PDE5 inhibitors was more pronounced in aged animals than that in young mice. PDE5 inhibitors enhanced the retention deficits in LPS-treated animals, which might be due to elevation of cGMP levels caused by sildenafil and zaprinast. In conclusion, these results indicated that differential effects of PDE5 inhibitors in aging and LPS-treated mice, but its prudential effect in aged mice may suggest new therapeutic use of these compounds. Drug Dev. Res. 63:66–75, 2004. © 2004 Wiley-Liss, Inc.

Key words: PDE 5 inhibitors; cognition; lipopolysaccharide; hippocampus

INTRODUCTION

The biological role of nitric oxide (NO) and cGMP as inter- and intracellular messengers in cardiovascular, endocrine, immune, and central nervous system (CNS) has been intensively investigated over the last decade [Chalimoniuk and Strosznajder, 1998]. Multiple signaling pathways, which increase cGMP formation in the brain, are implicated in various brain physiological functions [De Vente et al., 2001]. The levels of second messengers cAMP and cGMP are regulated by the specific phosphodiesterases (PDEs), which are the large group of structurally related enzymes that catalyze the hydrolysis of 3′, 5′-cyclic nucleotides to the corresponding inactive nucleoside 5′-monophosphate [Murthy, 2001].

The cGMP pathway is known to play a role in memory processes, besides its effect on vascular and visceral smooth muscle cells [Lincoln et al., 1990; Jim et al., 1993; Francis et al., 1998; Bernabeu et al., 1996, 1997; Murthy, 2001]. Recently, it has been reported that the PDE 5 inhibitor, sildenafil, improved the memory performance of mice in a passive avoidance task [Baratti and Boccia, 1999]. Zaprinast and sildenafil improved the object recognition task [Prickaerts et al., 1997, 2002b], and more recently intrahippocampal injection of the cGMP analog, 8-bromo-cGMP, improved memory performance in rats [Prickaerts et al., 2002a]. Incubation of hippocampal slices with zaprinast

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increased the nitric oxide-mediated cGMP response [Boulton et al., 1994; De Vente et al., 1996; Van Staveren et al., 2001].

The cognitive role of NO-cGMP is dependent on the amount formed. An excess of NO and cGMP may result in deleterious effects. NO produced from L-arginine by calcium-dependent cNO synthase (NOS) isoforms, including neuronal NOS (nNOS) and endothelial (eNOS), is necessary for the normal physiological effects (memory). A progressive decline of cGMP resulting from activation of cGMP specific-PDE in the brains of aged animals was linked to memory dysfunction [Chalimoniuk and Strosznajder, 1998].

Lipopolysaccharide (LPS), an endotoxin, is reported to induce various cytokines such as interleukin (IL)-1β, IL-6, interferon-α, tumor necrosis factor-α [Jo et al., 2001], inducible nitric oxide synthase (iNOS), and COX-2 [Liang et al., 1999]. These proinflammatory (IL-1β, TNF-α, and APP mRNA) mediators in turn activate astrocytes and microglia in the hippocampus, pituitary, and hypothalamus, resulting in degeneration of CA3 pyramidal neurons leading to impairments in spatial memory [Patil et al., 2003]. Proinflammatory cytokines [tumor necrosis factor (TNF)-α, IL-1β] produced by the microglial and astrocytes in the hippocampus can augment the immune/inflammatory reactions. These proinflammatory cytokines affect NO-dependent downstream cGMP signaling pathways. However, the effect of cGMP signaling on proinflammatory cytokine production is controversial, because N9 microglial studies showed inhibitory effects of cGMP-elevating agents on LPS-induced TNF-α product secretion [Liang et al., 1999; Paris et al., 2000], whereas Choi et al. [2002] showed stimulatory effects.

Based on the observations that cGMP-PDE activity is enhanced in aging and the differential effect of PDE5 inhibitors on LPS-induced cytokine production, the present study was designed to study the effect of sildenafil and zaprinast in aging- and lipopolysaccharide-induced memory impairment in mice using passive avoidance and plus-maze paradigms.

MATERIALS AND METHODS

Animals

Young (3 months) and aged (20–22 months old) male Swiss mice, weighing 20–25 and 35–40 g, respectively, were housed under standard laboratory conditions and kept under a 12:12-h natural light:dark cycle. The animals, procured from Central Animal House, Panacea Biotec Limited, Lalru, Punjab, were housed six per cage with free access to standard food and water. Mice were acclimatized to laboratory conditions before testing. Experiments were carried out between 9:00 a.m. and 6:00 p.m. All the experimental protocols were approved by the Institutional Animal Ethical Committee. Animals were divided into different groups for behavioral tests, i.e., passive avoidance test, plus-maze, and locomotion.

Drugs and Treatment Schedule

Sildenafil (Panacea Biotec Ltd., Lalru), zaprinast (Merck) and LPS from *Salmonella typhimurium* (Sigma, USA) were used in the study.

Sildenafil citrate was dissolved in distilled water, whereas zaprinast was dissolved in 0.05 M NaOH (in 0.9% NaCl) and the pH was adjusted to near neutral with 1 M HCl. Doses of 0.25, 0.5, and 1.0 mg/kg of sildenafil and 0.5, 1.0, and 2.0 mg/kg of zaprinast were used. The dose selection was based on the preliminary studies, which showed no effect on the blood pressure. Control animals received saline (0.9% NaCl, 10 mL/kg, i.p.) treatment. Animals were treated with test drug immediately after the learning trials (Day 0) in both the paradigms and transfer latency (TL) was observed on day 1.

In another study, LPS (50 μg/mouse, i.p.) was administered just after the learning trial (Day 0) in both the paradigms and TL was observed on day 1. Test drug was administered intraperitoneally, just after the LPS injection.

Passive Avoidance Performance Test (Step-Through Test)

The step through test was performed according to the method of Casamenti et al. [1993]. The apparatus (UGO BASILE, Italy) consisted of two compartments of grid floor 50×50 cm and 35-cm high walls, separated by a wall with a guillotine door 6×6 cm. One of the two chambers was illuminated with a 100-V bulb placed at 150-cm height and the other was dark. The test was conducted on 2 consecutive days at the same time of the day. On the day 0 (learning trial), each mouse was placed in the illuminated compartment of the apparatus. After 60 sec, the guillotine door was raised, allowing access to the dark compartment and when placed in the illuminated chamber, rapidly moved to the dark chamber. Once the mouse entered the dark compartment, it received an electric shock on the feet (2 mA, 2 sec) through the stainless steel grid floor. The time when mouse entered the dark chamber was recorded automatically and described as TL. On the second day (testing trial or transfer latency), the same test procedure was followed. The TL was recorded in testing trial.

Plus-Maze Test

The plus maze was used to evaluate spatial long-term memory, following the procedure as described [Reddy and Kulkarni, 1998]. Briefly, the apparatus consisted of two open arms (16 cm × 5 cm) and two closed arms (16 cm × 5 cm × 12 cm). The arms extended from a central platform (5 cm × 5 cm), and the maze was elevated to a height of 25 cm from the floor. On the first day, each mouse was placed at the end of an open arm. Transfer latency (TL), i.e., the time taken by the mouse to move into one of the enclosed arms, was recorded on the first day. If the animal did not enter any one of the enclosed arms within 90 sec, it was gently pushed into one enclosed arm and the TL was assigned as 90 sec. The mouse was allowed to explore the maze for 20 sec, and then returned to its home cage. Retention was examined 24 h after the first day trial. Each mouse was again placed at the end of one of the open arms of the maze and TL was recorded as described above. A long latency period to reach an enclosed arm indicated poor retention as compared to significantly shorter latencies.

Locomotor Activity Test

The locomotor activity was counted using an activity meter (IMCORP, India). Before subjecting the animals to cognitive tasks, they were individually placed in a Plexiglas cage (40 × 15 × 15 cm) and the total activity count registered for 5 minutes was recorded. The locomotor activity was expressed in terms of the total beams count/5 min per animal [Reddy and Kulkarni, 1998].

Noninvasive BP recorder in mice

Blood pressure was recorded in mice using a noninvasive tail-cuff method (Ugo Basile, Italy). The blood pressure was recorded at 30 min, 1, 2, and 4 h after the treatment of drug at the highest dose.

Statistical Analysis

The results are expressed as mean ± S.E.M. The data for step-through latency (STL), TL, and locomotor activity were subjected to one-way analysis of variance followed by Dunnett's test. In all the tests, the criterion for statistical significance was $P < 0.05$.

RESULTS

Effect of PDE 5 Inhibitors on Aging-Induced Impairment of Passive Avoidance Performance (Step-Through)

Adult mice (control) on day 1 showed a significant increased in STL as compared to respective control (day 0). However, aged mice (control) failed to recall the learned task after 24 h, indicating an age-related memory impairment. Sildenafil (0.5 and 1.0 mg/kg, i.p.), or zaprinast (1.0 and 2.0 mg/kg, i.p.) on day 1 showed an increase in STL in young adult mice in comparison to control mice (day 0) (Fig. 1a,b). Aged mice on acute administration of sildenafil (0.25, 0.5, and 1.0 mg/kg, i.p.), or zaprinast (0.5, 1.0, and 2.0 mg/kg, i.p.) on day 1 showed an increase in the STL in comparison to control mice (day 0) (Fig. 2a,b). However, sildenafil and zaprinast showed greater increases in STL in aged mice as compared to young mice at all doses (data not shown).

Effect of PDE5 Inhibitors on the Aging-Induced Impairment of Elevated Plus-Maze Performance

Adult mice (control) on day 1 showed a significant increase in the STL as compared to respective control (day 0). However, aged mice (control) failed to recall

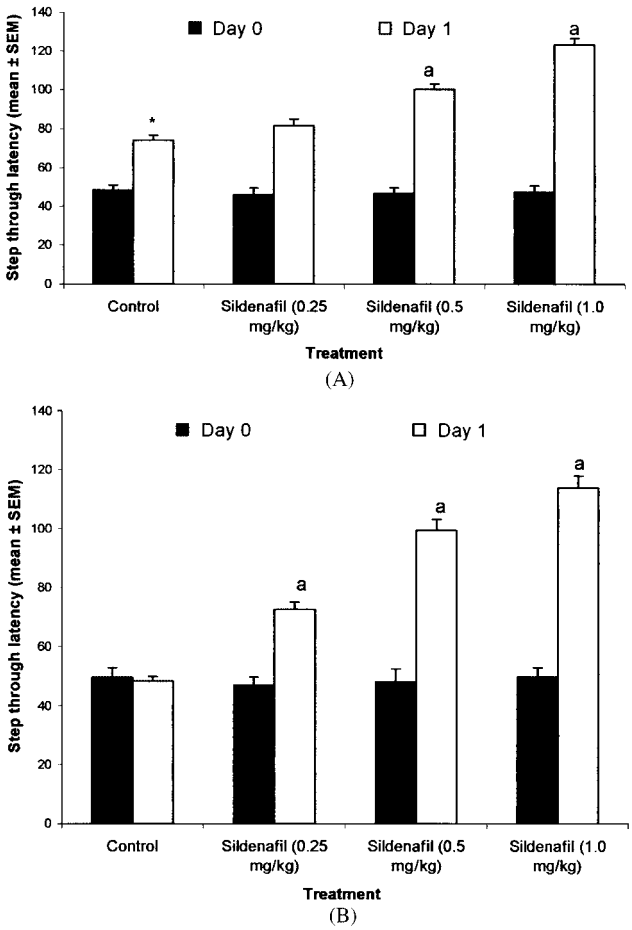

Fig. 1. Effect of sildenafil (0.25, 0.5, and 1.0 mg/kg, i.p.), on transfer latency in **(a)** young and **(b)** aged mice during retention test on passive avoidance (step-through) task. The transfer latency of each group of mice is expressed as mean ± S.E.M. (n=6–8). *$P < 0.05$ compared with step-through latency of control (saline) on day 0; [a]$P < 0.05$ compared with step-through latency of control (saline) on day 1.

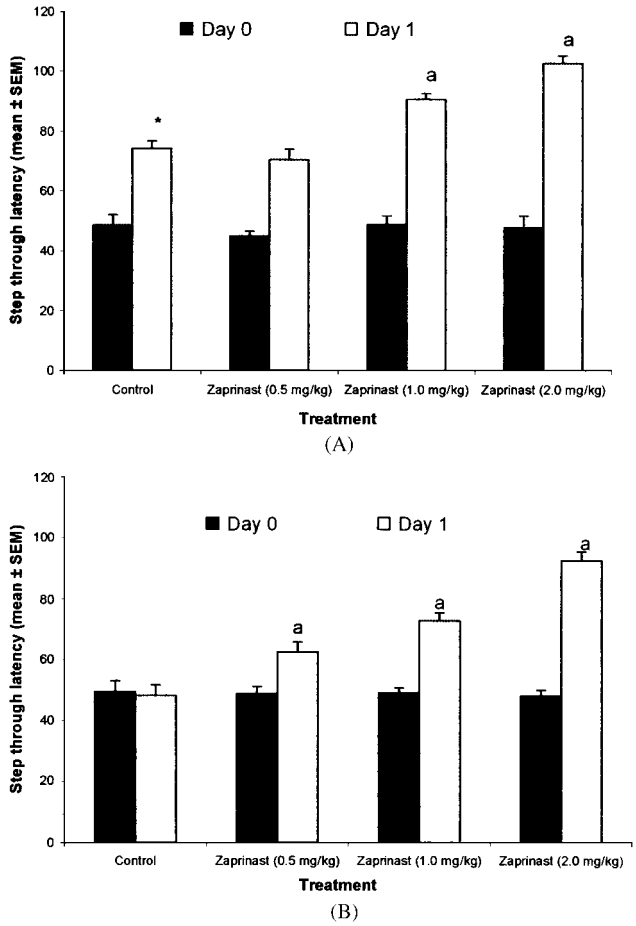

Fig. 2. Effect of zaprinast (0.5, 1.0, and 2.0 mg/kg, i.p.), on transfer latency in **(a)** young and **(b)** aged mice during retention test on passive avoidance (step-through) task. The transfer latency of each group of mice is expressed as mean ± S.E.M. (n=6–8). *$P<0.05$ compared with step-through latency of control (saline) on day 0; [a]$P<0.05$ compared with step-through latency of control (saline) on day 1.

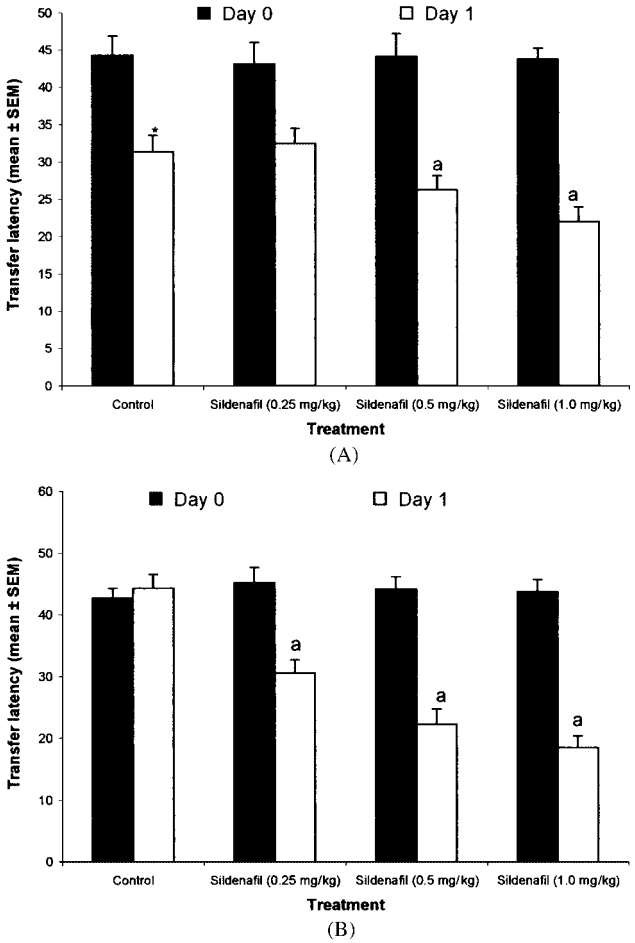

Fig. 3. Effect of sildenafil (0.25, 0.5, and 1.0 mg/kg, i.p.), on transfer latency in **(a)** young and **(b)** aged mice during retention test on elevated plus-maze task. The transfer latency of each group of mice is expressed as mean ± S.E.M. (n=6–8). *$P<0.05$ compared with step through latency of control (saline) on day 0; [a]$P<0.05$ compared with step-through latency of control (saline) on day 1.

the learned task after 24 h, indicating age-related memory impairment. Sildenafil (0.5 and 1.0 mg/kg, i.p.), or zaprinast (1.0 and 2.0 mg/kg, i.p.) on day 1 showed an increase in STL in young adult mice in comparison to control mice (day 0) (Fig. 3a,b). Aged mice on acute administration of sildenafil (0.25, 0.5, and 1.0 mg/kg, i.p.), or zaprinast (0.5, 1.0, and 2.0 mg/kg, i.p.) on day 1 significantly increased the STL in comparison to control mice (day 0) (Fig. 3a,b). However, sildenafil and zaprinast showed greater increases in STL in aged mice as compared to young mice at all the tested doses (data not shown).

Effect of PDE5 Inhibitors on LPS-Mediated Cognitive Impairment in Mice

Acute administration of LPS (50 μg/mouse, i.p.) produced significant decrease in latency (TL) in comparison to control animals, indicating LPS-induced dementia in step-through and plus-maze test paradigms. Acute administration of sildenafil (0.5 and 1.0 mg/kg, i.p.), or zaprinast (1.0 and 2.0 mg/kg, i.p.) potentiated the retention deficits in both of the tests (Fig. 5a,b and Fig. 6a,b).

Effect of PDE5 Inhibitors on the Locomotor Activity

To assess locomotor performance of aged and adult mice, animals were subjected to a 5-min activity test. Sildenafil (0.5 and 1.0 mg/kg, p.o.) and zaprinast (1.0 and 2.0 mg/kg, p.o.) did not produce any significant alteration in the locomotor activity of both aged and young adult mice (Table 1a,b).

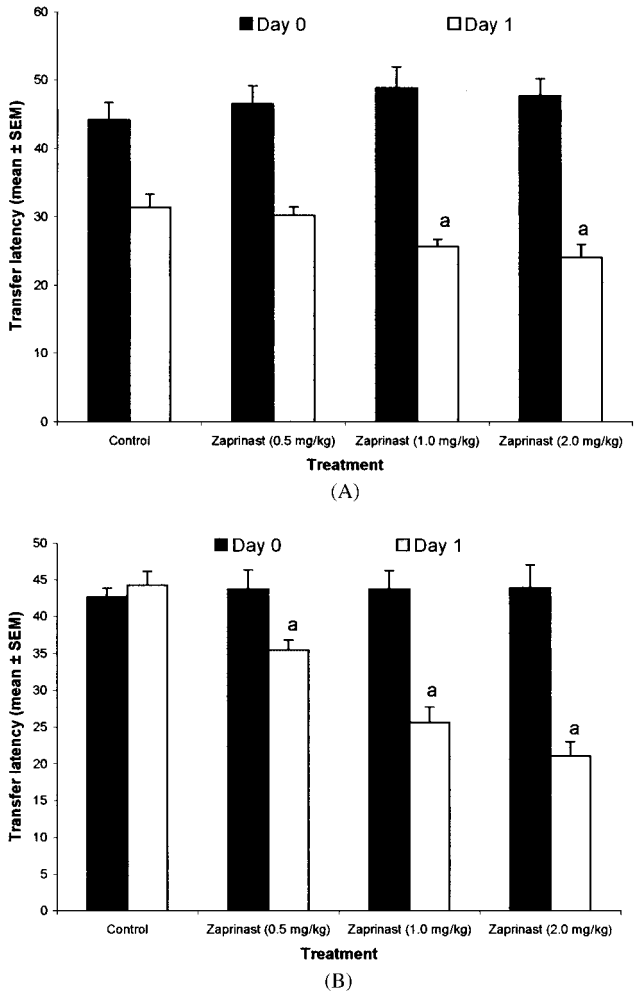

Fig. 4. Effect of zaprinast (0.5, 1.0, and 2.0 mg/kg, i.p.), on transfer latency in **(a)** young and **(b)** aged mice during retention test on elevated plus-maze task. The transfer latency of each group of mice is expressed as mean ± S.E.M. (n=6–8). *$P<0.05$ compared with step through latency of control (saline) on day 0; $^{a}P<0.05$ compared with step through latency of control (saline) on day 1.

Effect of PDE5 Inhibitors on the Peripheral Blood Pressure

The highest test dose of sildenafil (1 mg/kg, i.p.) and zaprinast (2 mg/kg, i.p.) on acute administration did not produce any alteration in the mean arterial blood pressure (Table 2a,b).

DISCUSSION

Biochemical and immunological studies have provided evidence that activation of soluble guanylyl cyclase and subsequently cGMP formation in the rat brain plays an important role in animal models of memory studies [De Vente et al., 1990; Garthwaite, 1991; Southain and Garthwaite, 1993]. Several PDE types have been localized in the rat hippocampus as shown by in situ hybridization or immunocytochemistry, i.e., that PDE1, PDE2, PDE3, and PDE4 are present in this brain area of the rat. Recently, the presence of PDE5 mRNA in hippocampus, cerebellum, medulla, spinal cord, substantia nigra, and subthalamic nucleus was shown by Northern blot analysis in human brain. However, the expression of PDE5 in hippocampus was low, especially when compared to cerebellum [Loughney et al., 1998]. Also, lower levels of PDE5 signals were found in the hippocampus of rat brain analyzed using Northern blots or immunocytochemistry [Kotera et al., 1997, 2000]. These findings suggest that hippocampal PDE5 enzymes are responsible for the degradation of cGMP in the brain and for the impairment of memory. Recent studies showed that sildenafil, vardenafil, or zaprinast improved the memory performance of rodents in a one-trail learning task such as the object recognition task [Prickaerts et al., 1997, 2000b] and passive avoidance-learning task [Baratti and Boccia, 1999].

The lipophilic nature of the PDE5 inhibitors enables them to penetrate tissues very easily, including the brain. [^{14}C]-Sildenafil (4 mg/kg, i.v.) showed distribution to all tissues 6 min after dosing when studied by autoradiographically supporting the central effect of PDE5 inhibitors, which, in increasing hippocampal cGMP level, may be responsible for improved memory performance in one trail-learning tasks. Other reports show physiological disturbances (aggressive behaviors) after sildenafil administration [Schultheiss et al., 2001; Milman and Arnold, 2002].

Effect of PDE 5 Inhibitors on Aging-Induced Dementia in Mice

Age-related changes in the cGMP levels have been demonstrated in the hippocampus and cerebellum of aged and adult rats. In the present study, PDE5 inhibitors, sildenafil and zaprinast, were found to improve the memory performance in young and aged mice. Sildenafil and zaprinast bind to the noncatalytic, allosteric site of type 5 PDEs, leading to increases in cGMP. The effect of both PDE5 inhibitors was more pronounced in aged as compared to young mice, which may be due to increased activity of PDE5 enzymes during aging. As noted, aging coincides with decreased basal levels of cGMP as a consequence of a more active degradation of cGMP by PDE5 in hippocampus and cerebellum [Chalimoniuk and Strosznajder, 1998].

Whether cGMP-induced improvement in cognition is a pre- or postsynaptic event or early or late phase signal transduction is unknown. Zhuo et al. [1994] suggested a presynaptic role of the cGMP/PKG path-

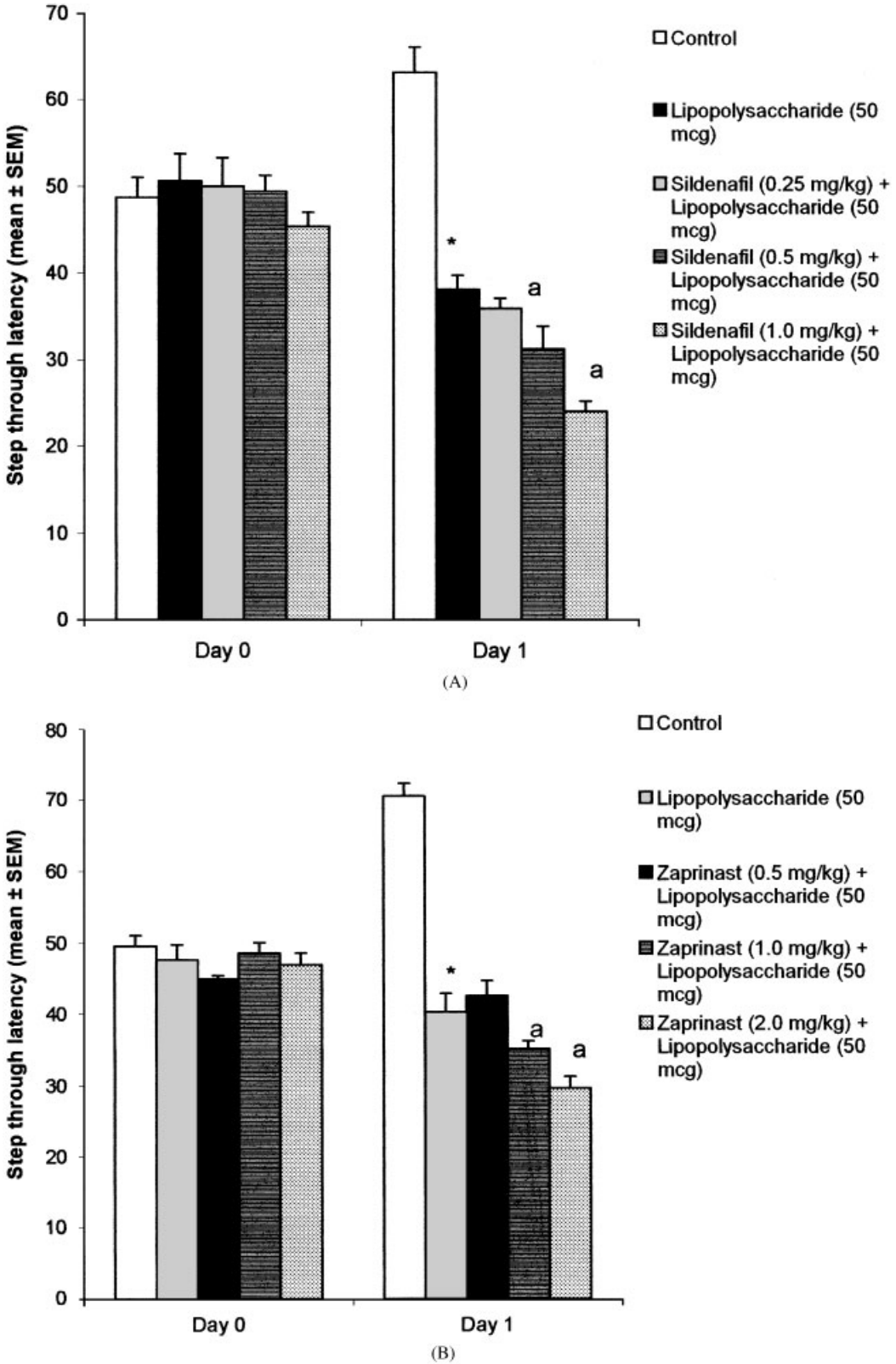

Fig. 5. Effect of PDE5 inhibitors **(a)** sildenafil (0.25, 0.5, and 1.0 mg/kg, i.p.), and **(b)** zaprinast (0.5, 1.0, and 2.0 mg/kg, i.p.) on lipopolysaccharide (LPS) (50 μg/mouse)-induced retention deficits (transfer latency) in mice on passive avoidance (step-through) task. The transfer latency of each group of mice is expressed as mean ± S.E.M. (n=6–8). $^{*}P<0.05$ compared with step-through latency of control (saline); $^{a}P<0.05$ compared with transfer latency of LPS-treated animals on day 1.

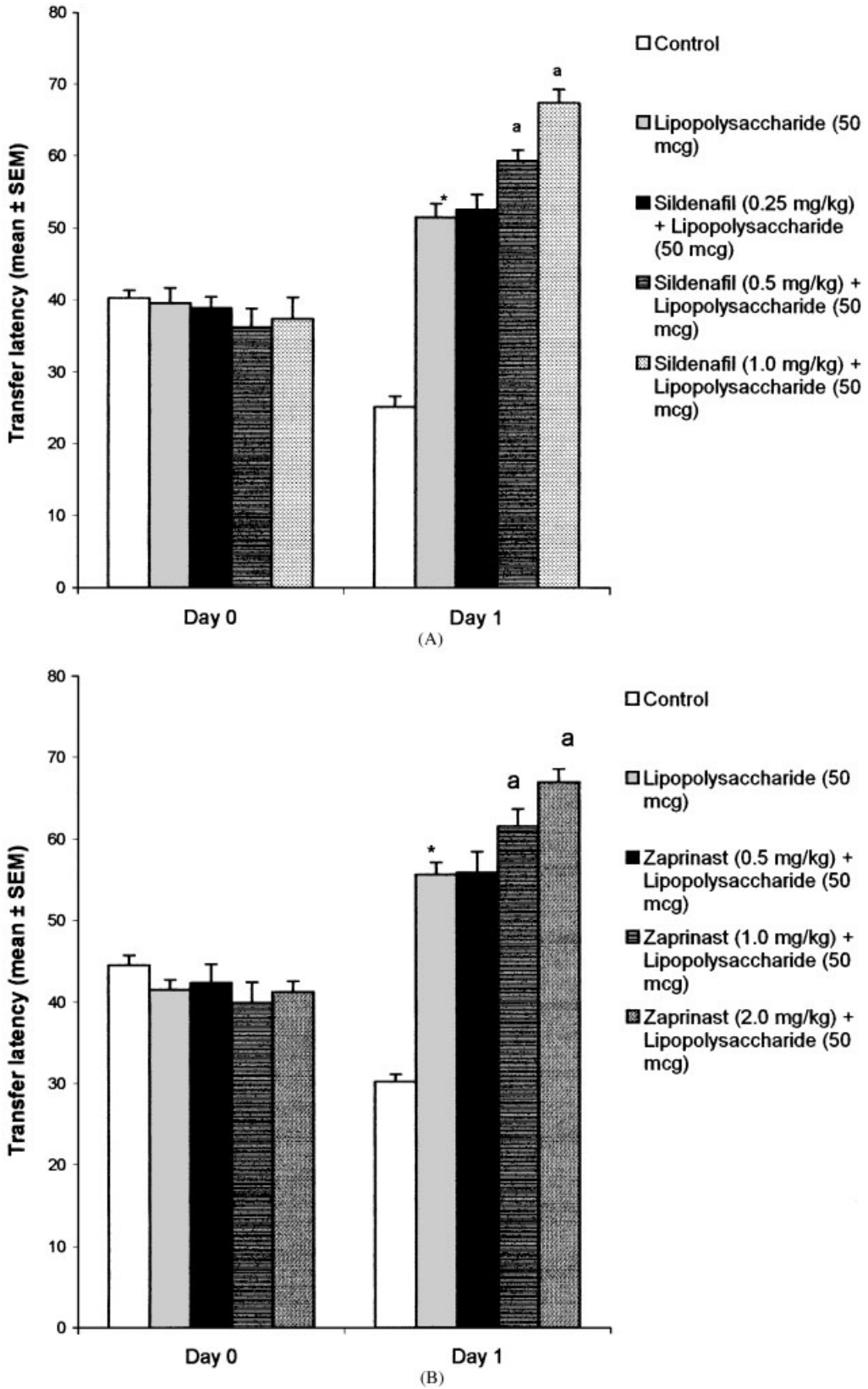

Fig. 6. Effect of PDE5 inhibitors **(a)** sildenafil (0.25, 0.5, and 1.0 mg/kg, i.p.), and **(b)** zaprinast (0.5, 1.0, and 2.0 mg/kg, i.p.) on lipopolysaccharide (50 μg/mouse)-induced retention deficits (transfer latency) in mice on elevated plus-maze task. The transfer latency of each group of mice is expressed as mean ± S.E.M. (n=6–8). $^{*}P<0.05$ compared with transfer latency of control (saline); $^{a}P<0.05$ compared with transfer latency of LPS-treated animals on day 1.

TABLE 1a. Effect of Sildenafil and Zaprinast on Locomotor Activity in Young Mice

Treatment (mg/kg, i.p.)	Day 0	Day 1
Control (saline)	149.8±6.9	144.4±4.1
Sildenafil (0.25)	152.6±3.9	159.1±6.6
Sildenafil (0.5)	150.2±5.3	155.8±4.9
Sildenafil (1.0)	142.8±1.9	147.8±6.2
Zaprinast (0.5)	139.9±2.3	149.8±9.6
Zaprinast (1.0)	147.8±7.2	43.9±3.5
Zaprinast (2.0)	148.9±6.1	158.7±7.7

TABLE 1b. Effect of Sildenafil and Zaprinast on Locomotor Activity in Aged Mice

Treatment (mg/kg, i.p.)	Day 0	Day 1
Control (saline)	29.6±5.5	135.6±3.9
Sildenafil (0.25)	129.6±5.5	133.9±6.9
Sildenafil (0.5)	135.8±3.9	138.9±6.5
Sildenafil (1.0)	144.8±7.8	140.9±5.9
Zaprinast (0.5)	149.9±2.6	155.6±9.6
Zaprinast (1.0)	139.8±6.1	129.5±4.5
Zaprinast (2.0)	125.9±8.9	137.5±2.9

TABLE 2a. Effect of Sildenafil and Zaprinast on Mean Arterial Blood Pressure in Young Mice

Treatment (mg/kg, i.p.)	Mean arterial BP of young mice (mean±SEM) expressed as mm of Hg				
	0	30	60	120	240
Sildenafil (1 mg/kg)	86.0±2.91	88.2±3.30	89.0±3.67	94.0±4.57	83.0±2.0
Zaprinast (2 mg/kg))	83.4±2.37	83.0±3.64	88.0±2.79	90.0±1.51	82.6±3.17

TABLE 2b. Effect of Sildenafil and Zaprinast on Mean Arterial Blood Pressure in Aged Mice

Treatment (mg/kg, i.p.)	Mean arterial BP of aged mice (mean±SEM) expressed as mm of Hg				
	0	30	60	120	240
Sildenafil (1 mg/kg)	90.5±4.99	93.6±2.91	99.5±4.1	94.0±4.57	89.6±2.6
Zaprinast (2 mg/kg))	95.6±3.6	92.6±3.91	102.5±4.99	97.5±3.69	92.5±2.77

way in the early phases of hippocampal signal transduction [Schultheiss et al., 2001]. However, other findings suggest a postsynaptic role of the cGMP/PKG/CREB pathway in late phases of signal transduction via phosphorylation of CREB (cyclic AMP responsive element binding protein) [Lu et al., 1999]. Also, the postsynaptic cGMP pathway involved in late phase signal transduction is thought to occur at the level of fibers (axons and dendrites) as NO-mediated cGMP response to PDE5 inhibitors are also found in neuronal

fibers of the hippocampal CA2/CA3 region [De Vente et al., 1996; Prickaerts et al., 2002b; Van Staveren et al., 2001].

The PDE5 inhibitors used in the present study can cause vasodilation, resulting in cGMP-dependent hypotension [Dundore et al., 1993; Rees et al., 1989], which could be responsible for the memory-improving effect by increasing blood flow with a consequent increase in glucose metabolism [Kopf and Baratti, 1996]. However, this is unlikely because sildenafil and zaprinast did not produce any change in blood pressure nor alter the locomotion (data not shown) as observed in our study.

Effect of PDE5 Inhibitors on LPS-Induced Dementia in Mice

LPS, a potent activator of microglia, plays a significant role in neuroinflammation and induces phosphorylation of proteins. Furthermore, LPS injected into rat brain increases activated astrocytes and microglia, induces IL-1β, TNF-α, and APP mRNA levels in the basal forebrain, and significantly impairs spatial memory by degeneration of CA3 pyramidal neurons. Glial cells in the CNS are abundant source of cytokines that lead to the production of proinflammatory cytokines, TNF-α, and interleukin-1β (IL-1β) when stimulated by pathological signals, such as LPS [Benveniste, 1997, 1998]. These cytokines in turn augment the pathologic activation of glial cells to produce nitric oxide (NO) [Murad et al., 1978; Murphy et al., 1993; Murphy, 2000], which activates soluble guanylyl cyclase [Bernabeu et al., 1996; Loughney et al., 1998] to produce cGMP. Therefore, increased cellular cGMP concentration is involved the repertoire of glial reactions.

Our results indicate that both PDE5 inhibitors accentuated LPS-induced dementia in both the models of cognition. The effect may be due to the positive effect of sildenafil and zaprinast on TNF-α and IL-1β production in hippocampal astrocytes and microglia. Zaprinast enhances the LPS-induced secretion of TNF-α and IL-1β and the expression of iNOS and MHC class II molecules in rat microglial cells [Choi et al., 1999], an effect associated with cGMP/PKG signaling, which can augment central immune/inflammatory reactions, possibly via the increased production of TNF-α and IL-1β by microglia and astrocytes.

Hence, the results from the present study indicate the beneficial as well as deleterious effect of PDE5 inhibitors in aging and LPS-induced neurodegeneration, respectively. As in aging, the downregulation of the NO-cGMP pathway may be treated with PDE5 inhibitors; in contrast, it aggravates the effect of LPS by increasing iNOS activity, which subsequently increases NO, and cGMP. The amnesic effect of PDE5 inhibitors in LPS-treated animals might be of importance in the development of new proamnesic drugs.

In conclusion, these results indicate the differential effects of PDE 5 inhibitors in aging- and LPS-treated mice. In young and aged mice, sildenafil and zaprinast improved the memory performance, but the effect of these drugs appeared to be more pronounced in aged animals as compared to young adult mice, indicating that these compounds may offer new therapeutic strategies for the treatment of learning and memory deficits. However, there may be some limitations, such as hypotension, which may interfere under certain pathological conditions.

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