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Pharmacological Preconditioning by Milrinone: Memory Preserving and Neuroprotective Effect in Ischemia-Reperfusion Injury in Mice

Reetu Saklani, Amteshwar Jaggi, and Nirmal Singh

Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala-147002, India

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We tested the neuroprotective effect of milrinone, a phosphodiesterase III inhibitor, in pharmacological preconditioning. Bilateral carotid artery occlusion for 12 min followed by reperfusion for 24 h produced ischemia-reperfusion (I/R) cerebral injury in male Swiss albino mice. Cerebral infarct size was measured using triphenyltetrazolium chloride staining. Memory was assessed using the Morris water maze test, and motor coordination was evaluated using the inclined beam walking test, rota-rod test, and lateral push test. Milrinone (50 μg/kg & 100 μg/kg *i.v.*) was administered 24 h before surgery in a separate group of animals to induce pharmacological preconditioning. I/R increased cerebral infarct size and impaired memory and motor coordination. Milrinone treatment significantly decreased cerebral infarct size and reversed I/R-induced impairments in memory and motor coordination. This neuroprotective effect was blocked by ruthenium red (3 mg/kg, *s.c.*), an intracellular ryanodine receptor blocker. These findings indicate that milrinone preconditioning exerts a marked neuroprotective effect on the ischemic brain, putatively due to increased intracellular calcium levels activating calcium-sensitive signal transduction cascades.

Key words: Milrinone, Phosphodiesterase, Pharmacological preconditioning, Cerebral ischemia, Ruthenium red, Memory, Morris water maze

INTRODUCTION

Brain ischemia causes significant morbidity and mortality in multiple disease states, including cardiac arrest, stroke, and traumatic brain injury. Little progress has been made in developing novel neuroprotective strategies. Ischemic preconditioning (IPC) is a phenomenon by which brief episodes of ischemia increases the ability of an organ to tolerate subsequent prolonged periods of ischemic injury. IPC was first discovered by Murray et al. (1986) for the ischemic myocardium and later applied by Kitagawa et al. to ischemic neuronal injury (Kitagawa et al., 1990). Neuronal ischemic preconditioning is an endogenous neuroprotective strategy that provides sustained and robust ischemic tolerance (Davis and Patel, 2003). IPC occurs in several species, including human, rat, mouse, rabbit, and pig, and in several organs, including brain, spinal cord, heart, kidney, and intestine (Edwards et al., 2000). Other forms of preconditioning have been investigated, such as pharmacological, thermal, and gas inhalation. Thermal preconditioning involves episodes of hyperthermia (McCormick et al., 2003) as well as hypothermia (Gluckman et al., 2005) before ischemic insult that subsequently provide protection against prolonged ischemic injury. Anesthetic preconditioning protects tissues against sustained ischemic injury after the exposure of gaseous or liquid anesthetics (Warner et al., 1993; Zheng and Zuo, 2004; Rehni and Singh, 2007; Rehni et al., 2007, 2008a; Pateliya et al., 2008; Kaur et al., 2009a). Remote ischemic preconditioning is a novel method where ischemia followed by reperfusion of one organ protects remote organs, either due to release of biochemical messengers in the circulation or activation of nerve pathways (Peralta et al., 2003; Loukogeorgakis et al., 2005; Lang et al., 2006). Ischemic post-conditioning explains how brief repetitive cycles of ischemia with intermittent reperfusion following prolonged ischemia elicit tissue pro-

Correspondence to: Nirmal Singh, Department of Pharmaceutical Sciences and Drug Research, Punjabi University, Patiala-147002, Punjab, India
Tel: 91-0175-3046255, Mobile No: 919815129884
E-mail: nirmal_puru@rediffmail.com

tection (Yang et al., 2004; Somers et al., 2007).

Pharmacological activation can lead to a preconditioning-like protective effect that lasts beyond agent elimination, or pharmacological preconditioning (PP) (Rehni et al., 2008b; Kaur et al., 2009b), a clinically feasible paradigm (Yellon and Dana, 2000). PP can be elicited by receptor agonists for adenosine, *aaa*-adrenergic, bradykinin, calcitonin gene related peptide (CGRP), opioid receptors (Schulz et al., 1998, 2001; Rehni et al., 2008b) and ryanodine receptors (Kaur et al., 2009b). We also showed (Rehni et al., 2008b; Kaur et al., 2009b), as did others (Zhao et al., 2007), that pharmacological preconditioning exerts beneficial effects on memory dysfunction associated with cerebral ischemia/reperfusion injury.

Calcium ions modulate protective phenomenon like IPC and PP (Miyawaki and Ashraf, 1997). Phosphodiesterase mediates metabolism of the second messenger, cyclic-adenosine-monophosphate (cAMP), and several isoforms exist (Conti, 2000; Lugnier, 2006). Phosphodiesterase type III inhibitors like milrinone and amrinone are used in heart failure as inodialators, and have preconditioning-like effects in the heart (Saltman et al., 2000) and liver (Kume et al., 2006). Milrinone also has beneficial effects in the treatment of cerebral vasospasm (Fraticelli et al., 2008), but its PP activity is unclear.

Therefore, we tested the neuroprotective effects of milrinone by PP. Ruthenium red is a ryanodine receptor blocker (Lukyanenko et al., 2000) that inhibits calcium induced calcium release (CICR) from ryanodine-sensitive intracellular calcium stores in the sarcoplasmic reticulum.

MATERIALS AND METHODS

Animals

Male albino Swiss mice weighing 25 ± 2 g, maintained on standard laboratory diet (Kisan Feeds Ltd.) and having free access to tap water were housed in the departmental animal house under a 12-h light/dark cycle. The experiments were conducted in a semi-sound proof laboratory. The experimental protocol was approved by the Institutional Animal Ethics Committee (IAEC) and care of the animals was performed per the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India (Reg. No.-107/ 1999/ CPCSEA).

Drugs and chemicals

Milrinone (Sanofi-Synthelabo), ruthenium red (Loba Chemie) and chloral hydrate (Riedel-deHaen) were dissolved in normal saline. All other chemicals were of analytical quality. All drug solutions were freshly prepared before use.

Ischemia-reperfusion induced cerebral injury

Mice were anaesthetized using chloral hydrate (350 mg/kg, *i.p.*). A midline ventral incision was made in the neck to expose the right and left common carotid arteries isolated from the surrounding tissue and vagus nerve. A cotton thread was passed below the carotid artery. Global cerebral ischemia was induced by occluding the carotid arteries. After 12 min of global cerebral ischemia, the incision was sutured back in layers and reperfusion was allowed for 24 h (Rehni and Singh, 2007). The sutured area was cleaned with 70% ethanol and was sprayed with antiseptic dusting powder. The animals were housed individually during recovery. Milrinone (50 or 100 μg/kg, *i.v.*) was given 24 h prior to the global cerebral ischemia for PP.

Assessment of cerebral infarct size

After 24 h of reperfusion, animals were sacrificed by spinal dislocation and the brain was removed and placed immediately in ice cold saline for 10 min before preparation of uniform coronal sections of about 1 mm thickness. The slices were incubated in 1% triphenyltetrazolium chloride (TTC) at 37°C in 0.2 M tris buffer (pH 7.4) for 20 min (Bochelen et al., 1999). TTC is converted to a red formazan pigment by nicotinamide adenine dinucleotide (NAD) and lactate dehydrogenase (LDH) to stain viable cells deep red. Infarcted cells have lost the enzyme and cofactor and thus remained an unstained dull yellow. Brain slices were placed over a glass plate and covered with a transparent plastic grid with 100 squares in 1 cm^2. The average area of each brain slice was calculated by counting the number of squares on either side, and the infarct area was counted as squares falling over non-stained dull yellow areas. The infarcted area is expressed as a percentage of total brain volume. Whole brain slices were weighed, then weighed again after the infarcted area was removed. Infarct size is expressed as a percentage of total brain wet weight.

Memory assessment using the morris water maze test

The Morris water maze (MWM) test was used to assess learning and memory (Morris, 1984; Parle and Singh, 2004). MWM consisted of a large circular pool (150 cm in diameter, 45 cm in height, filled to a depth of 30 cm with water at 28 ± 1°C). The water was made opaque with a nontoxic white dye. The pool was divided into four equal quadrants using two threads, fixed

at a right angle to each other on the rim of the pool. A submerged platform (10 cm^2), painted white was placed in the target quadrant 1 cm below the surface to provide an escape area. The position of platform was not altered throughout the training session.

Acquisition trial

Each mouse was subjected to four trials on each day. A rest period of 5 min was allowed between each trial. Four trials per day were repeated for four consecutive days. The starting position on each day was rotated (see below), but quadrant Q4 was the target quadrant in all acquisition trials.

Day 1	Q1	Q2	Q3	Q4
Day 2	Q2	Q3	Q4	Q1
Day 3	Q3	Q4	Q1	Q2
Day 4	Q4	Q1	Q2	Q3

Escape latency time (ELT) to locate the hidden platform in water maze was noted and day 4 ELT served as an index of acquisition or learning.

Retrieval trial

On fifth day the platform was removed. Each mouse was placed in the maze and allowed to explore the maze for 120 sec. Each animal was subjected to four trials, each starting from a different quadrant. The mean time spent in all quadrants was recorded. Time in Q4 served as an index of retrieval. The experimenter always stood at the same position, and other visual cues in the room were not disturbed. All trials were completed between 10.00 to 16.00 h. After recording day 4 ELT, the animal was subjected to the surgical procedure and then tested on day 5 for the retrieval test.

Assessment of motor coordination

Rota-rod test

The rota-rod evaluates motor coordination by testing the ability of mice to remain on a revolving rod (Dunham and Miya, 1957). The apparatus consisted of horizontal rough metal rod, 3 cm diameter, attached to a motor with variable speed. This 70 cm long rod was divided into four sections by wooden partitions. The rod was placed at a height of 50 cm to discourage jumping. The rate of rotation was adjusted to allow normal mice to stay on it for five minutes. Each mouse was given five trials, and animals that remained on the rod for 5 min were selected for surgery. The final test was 24 h after surgery.

Inclined beam-walking test

The inclined beam walking test was used to evaluate fore and hind limb motor coordination (Feeney et al., 1981). Each animal was individually placed on a metallic bar, 55 cm long and 1.5 cm wide, inclined 60° from the ground. Mouse motor performance was graded on a scale ranging from 0 to 4. A grade of 0 indicated that the animal could readily traverse the beam, grade 1 indicated mild impairment, grade 2 indicated moderate impairment, grade 3 indicated severe impairment, and grade 4 indicated complete inability to walk on the beam. The inclined beam-walking test was performed before and 24 h after global cerebral ischemia and reperfusion.

Lateral push test

Motor coordination was also evaluated by percentage of mice showing resistance to lateral push (Bederson et al., 1986). A mouse was placed on a rough surface for firm grip and evaluated for resistance to lateral push from either shoulder. The test was performed before global cerebral ischemia and 24 h after global cerebral ischemia and reperfusion.

Experimental protocol

The seven groups each consisted of 7 animals.

Group I (Sham group): Surgical procedure with a thread passed below the carotid arteries, but they were not occluded. After 12 min, threads were removed and the animal was sutured and allowed to recover for 24 h.

Group II (Control group): 12 min global cerebral ischemia followed by reperfusion for 24 h.

Group III (50 μg/kg Milrinone preconditioning group): Milrinone (50 μg/kg, *i.v.*) 24 h prior to 12 min of global cerebral ischemia followed by reperfusion for 24 h.

Group IV (100 μg/kg Milrinone preconditioning group): Milrinone (100 μg/kg, *i.v.*) 24 h prior to 12 min of global cerebral ischemia followed by reperfusion for 24 h.

Group V (Ruthenium red control group): Ruthenium red (3 mg/kg, *s.c.*), a ryanodine receptor blocker, was administered 1 h, 6 h, 12 h, 18 h, and 24 h prior to 12 min global cerebral ischemia followed by reperfusion for 24 h.

Group VI (Ruthenium red 50 μg/kg milrinone preconditioning group): Ruthenium red (3 mg/kg, *s.c.)* was administered 1 h before and 6 h, 12 h, 18 h, and 24 h following milrinone (50 μg/kg, *i.v.*), then treated as for group III.

Group VII (Ruthenium red 100 μg/kg milrinone preconditioning group): Ruthenium red (3 mg/kg,

s.c.) was administered 1 h before and 6 h, 12 h, 18 h, and 24 h following milrinone (100 μg/kg, *i.v.*), then treated as for group III.

Statistical analysis

Results are expressed as mean ± S.E.M. of means, Statistical analysis was done using one-way ANOVA followed by Tukey's multiple range tests. The results of the inclined beam walking test and lateral push test were analyzed using the Wilcoxon rank sum test and Chi square test, respectively. $P < 0.05$ was considered statistically significant.

RESULTS

Effect on cerebral infarct size

Global cerebral ischemia for 12 min followed by reperfusion for 24 h (I/R) increased ($p < 0.05$) cerebral infarct volume compared to sham group. PP with milrinone 24 h prior to I/R significantly blocked ($p < 0.05$) increases in cerebral infarct size. Pretreatment of ruthenium red alone did not affect I/R-induced infarction, but significantly attenuated ($p < 0.05$) the effects of milrinone (Fig. 1A and B).

Effect on memory

Escape latency time (ELT) decreased during MWM training, indicating normal learning abilities (Table I). Sham control mice spent more time in the target quadrant (Q4) searching for the platform during the retrieval test on day 5 than other quadrants ($p < 0.05$), reflecting normal memory capacity (Fig. 2). Global cerebral I/R significantly reduced day 5 time spent in the target quadrant (TSTQ) compared to the sham control animals, reflecting memory impairment. Milrinone significantly attenuated ($p < 0.05$) the I/R-induced decrease in day 5 TSTQ of controls, indicating reduced memory impairment. Pretreatment with ruthenium red in control animals did not affect I/R-induced memory impairment, but did abolish milrinone effects on memory (Fig. 2).

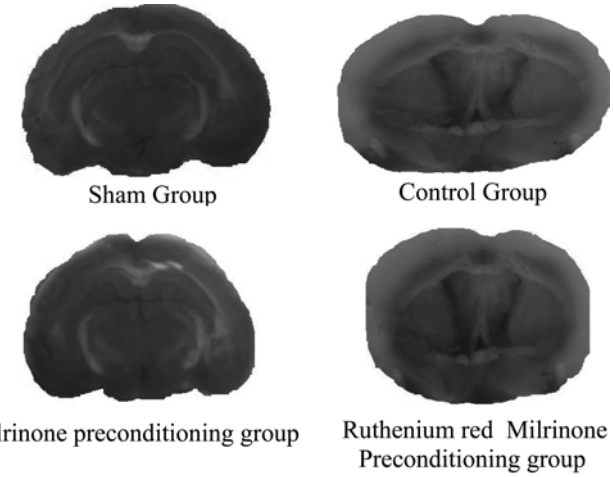

Fig. 1B. Representative TTC-stained images of brain showing infarction. Area stained in red is viable tissue, and non-stained areas indicate infarcted tissue.

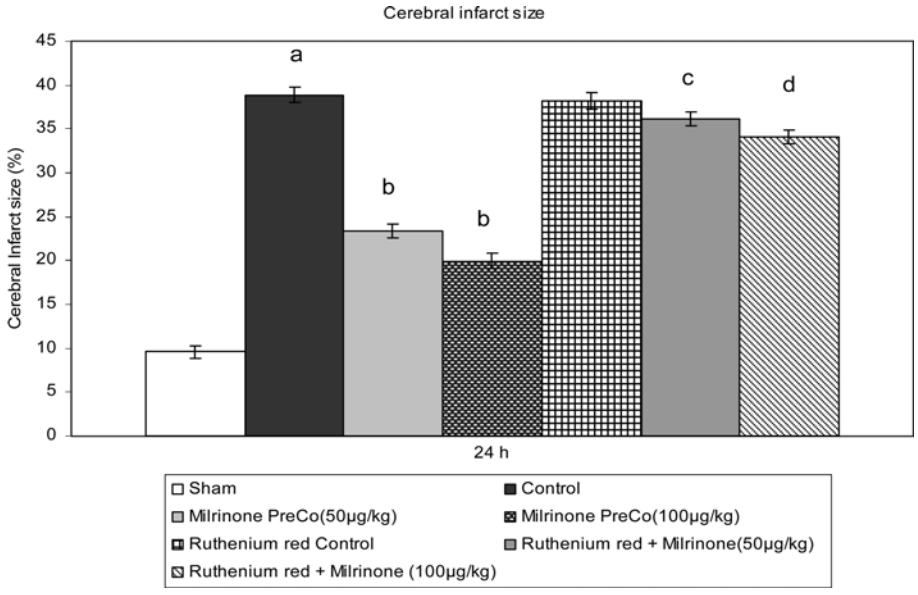

Fig. 1A. Effect of pharmacological preconditioning and interventions on ischemia/reperfusion-induced cerebral infarct size. Values are mean ± S.E.M. One way ANOVA followed by Tukey's test. DF (6, 28), F value = 262.492. a = $p < 0.05$ *vs* Sham group. b = $p < 0.05$ *vs* Control group. c = $p < 0.05$ *vs* Milrinone (50 μg/kg) preconditioning group. d = $p < 0.05$ *vs* Milrinone (100 μg/kg) preconditioning group

Table I. Escape latency time (ELT) of animals in the Morris water maze

Group	Day 1 ELT (s)	Day 4 ELT (s)
Sham	82.2 ± 3.8	16.5 ± 2.4[a]
Control	90.0 ± 3.1	18.1 ± 2.0[a]
Milrinone preconditioning (50 μg/kg)	87.5 ± 3.6	17.6 ± 2.1[a]
Milrinone preconditioning (100 μg/kg)	86.5 ± 3.0	21.5 ± 2.5[a]
Ruthenium red control	88.5 ± 3.5	19.3 ± 2.8[a]
Ruthenium red + Milrinone (50 μg/kg)	79.4 ± 3.3	23.4 ± 2.2[a]
Ruthenium red Milrinone (100 μg/kg)	83.0 ± 3.7	20.5 ± 2.6[a]

Values are mean ± S.E.M.
[a]$p < 0.05$ *vs* day 1 ELT
One way ANOVA followed by Tukey's test
1st day ELT, DF (6, 28), F value = 11.759; day 4 ELT, DF (6, 28), F value = 5.219

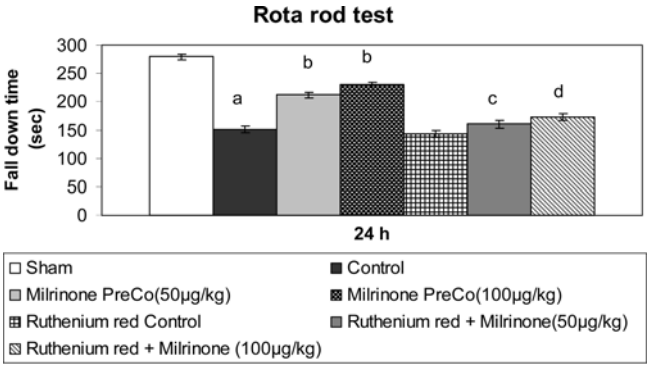

Fig. 3. Effect of pharmacological preconditioning and interventions on ischemia reperfusion-induced changes in motor performance (falling time) in mice with the rota-rod test. Values are mean ± S.E.M. One way ANOVA followed by Tukey's test. DF (6, 28), F value = 725.478. a = $p < 0.05$ *vs* Sham group. b = $p < 0.05$ *vs* Control group. c = $p < 0.05$ *vs* Milrinone (50 μg/kg) preconditioning group. d = $p < 0.05$ *vs* Milrinone (100 μg/kg) preconditioning group

Effect on Motor Performance

Effect on falling time in the rota-rod test

Global cerebral ischemia for 12 min followed by reperfusion for 24 h significantly ($p < 0.05$) reduced falling time in the rota rod test compared to the sham group. Milrinone significantly ($p < 0.05$) reversed this effect on falling time. Ruthenium red in control animals did not affect I/R-induced reductions in falling time, but significantly ($p < 0.05$) blocked the effects of milrinone on falling (Fig. 3).

Effect on motor coordination score using the inclined beam walking test

Global cerebral ischemia for 12 min followed by reperfusion for 24 h decreased ($p < 0.05$) motor coordination score on the beam walking test compared to the sham group. Milrinone administered 24 h prior to ischemic insult significantly ($p < 0.05$) attenuated the I/R induced changes in motor coordination. Ruthenium red alone did not affect I/R-induced changes in motor coordination, but significantly ($p < 0.05$) abolished milrinone activity (Fig. 4).

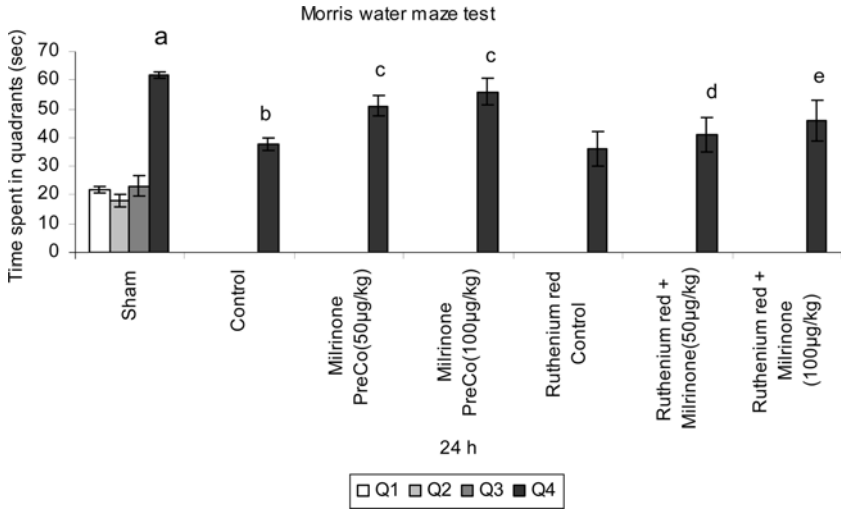

Fig. 2. Effect of Pharmacological preconditioning and interventions on ischemia reperfusion-induced decreases in time spent in target quadrant (TSTQ) using Morris water maze test. Values are mean ± S.E.M. One way ANOVA followed by Tukey's test. DF (6, 28), F value = 151.882. a = $p < 0.05$ *vs* time spent in other quadrants i.e. Q1, Q2, Q3 in Sham group. b = $p < 0.05$ *vs* time spent in target quadrant i.e. Q4 in Sham group. c = $p < 0.05$ *vs* time spent in target quadrant in Control group. d = $p < 0.05$ *vs* time spent in target quadrant in Milrinone (50 μg/kg) preconditioning group. e = $p < 0.05$ *vs* time spent in target quadrant in Milrinone (100 μg/kg) preconditioning group

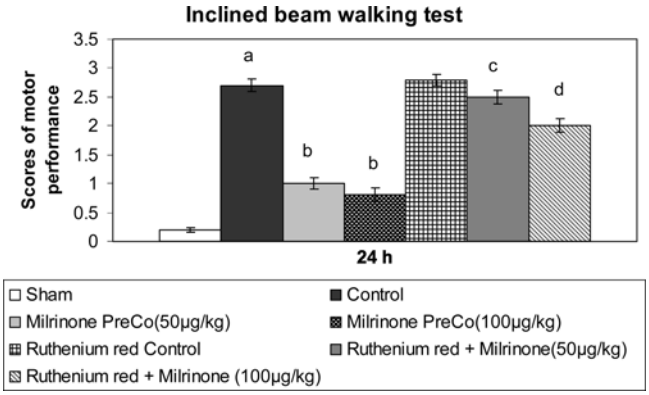

Fig. 4. Effect of pharmacological preconditioning and interventions on ischemia reperfusion-induced changes in motor performance (score) in mice using the inclined beam walk test. Values are mean ± S.E.M. The Wilcoxon rank sum test was used to analyze statistical differences. a = $p < 0.05$ *vs* Sham group. b = $p < 0.05$ *vs* Control group. c = $p < 0.05$ *vs* Milrinone (50 µg/kg) preconditioning group. d = $p < 0.05$ *vs* Milrinone (100 µg/kg) preconditioning group

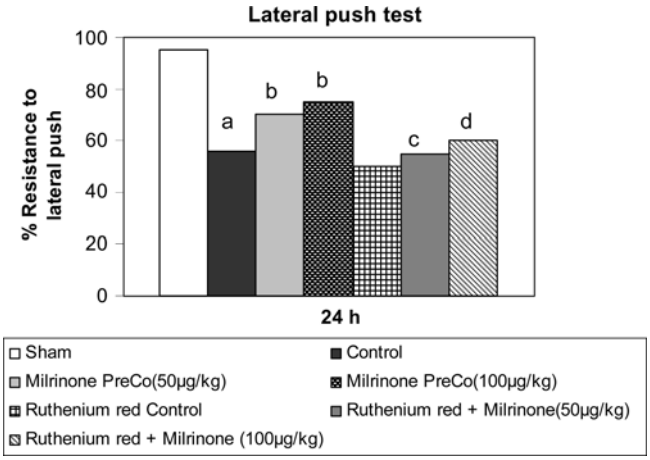

Fig. 5. Effect of pharmacological preconditioning and interventions on ischemia reperfusion-induced changes in motor performance in mice with the lateral push test. Values are percentage of mice showing resistance to lateral push. Chi-square test was used to test statistical significance. a = $p < 0.05$ *vs* Sham group. b = $p < 0.05$ *vs* Control group. c = $p < 0.05$ *vs* Milrinone (50 µg/kg) preconditioning group. d = $p < 0.05$ *vs* Milrinone (100 µg/kg) preconditioning group

Effect on resistance to lateral push

Global cerebral ischemia for 12 min followed by reperfusion for 24 h significantly ($p < 0.05$) decreases the percentage of mice with resistance to lateral push compared to the sham group. Milrinone significantly ($p < 0.05$) attenuated this I/R-induced decrease in lateral push resistance. Ruthenium red alone had no effect, but significantly blocked ($p < 0.05$) milrinone activity (Fig. 5).

DISCUSSION

We used male mice because high estrogen levels can protect against ischemia reperfusion injury (Zhai et al., 2000). Our global ischemia/reperfusion model simulates clinical findings in cerebral ischemia (Neumar, 2000). Cerebral ischemia impairs memory because hippocampal neurons are injured by ischemia/reperfusion and the hippocampus is involved in memory regulation (Jenkins et al., 1981). Cerebral ischemia also impairs motor ability (Dobkin, 1991). We therefore used the MWM test to assess memory, and assessed motor coordination via the rota-rod test, inclined beam walk test, and lateral push test. Our model of global cerebral ischemia and reperfusion increased infarct size and impaired memory as well as motor coordination, consistent with other reports (Rehni and Singh, 2007; Rehni et al., 2007, 2008a, 2008b; Pateliya et al., 2008; Kaur et al., 2009a, 2009b). However, milrinone, a phosphodiesterase III inhibitor (Baim et al., 1983), given 24 h prior to global cerebral ischemia, dose dependently reduced infarct size and improved memory and motor coordination. Pretreatment with ruthenium red, a ryanodine receptor blocker, blocked milrinone activity.

Pre-ischemic administration of phosphodiesterase III inhibitors in different species (dogs, rabbits, rats) and organs (heart, liver) protects against ischemia-reperfusion injury (Kobayashi et al., 2002; Kucuk et al., 2009). Milrinone or olprinone, when administered before ischemia-reperfusion, was cardioprotective and reduced infarct size in dogs (Setoyama et al., 2006). Amrinone and milrinone decrease infarct size in rabbit hearts with coronary artery branch occlusion via vasodilation to increase myocardial perfusion in the tissue surrounding the ischemic zone (Rump et al., 1994). Olprinone and cilostazol, both selective phosphodiesterase III inhibitors, are neuroprotective (Ueda et al., 2003; Ye et al., 2007), indicating that phosphodiesterase III may be broadly neuroprotective.

We report, for the first time, that milrinone is neuroprotective in a pharmacological preconditioning paradigm. Milrinone competitively inhibits phosphodiesterase III and elevates intracellular cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase (PKA) to increase calcium levels (Evans, 1989). Calcium-induced calcium release (CICR) is allows cells to amplify Ca^{2+} signals by increasing cAMP levels and PKA activity to open L-type calcium channels, resulting in calcium entry into the cell. Calcium entry provides the stimulus for calcium release via the ryanodine receptor in the sarcoplasmic reticulum

(Wang et al., 2001). Both L-type calcium channels and intracellular ryanodine receptors control intracellular calcium kinetics. In cardiac cells, this mechanism is operated between voltage gated L-type calcium channels in the plasma membrane and calcium release channels (ryanodine receptors) in the sarcoplasmic reticulum (Fabiato, 1985; Mcgarry and Williams, 1993). Neurons allow calcium entry via voltage-gated calcium channels on the membrane (Budde et al., 1998) and ryanodine receptors present in sarcoplasmic reticulum. However, PKA may also affect sarcoplasmic reticulum calcium release independent of CICR by a voltage-sensitive release mechanism that is independent of L-type calcium channels.

Calcium can be neuroprotective following ischemia depending on the amount and route of calcium elevation (Friedman, 2006). Calcium also regulates synaptic transmission and intracellular functions such as neurotransmitter release, enzyme activity, membrane excitability, and gene expression (Finkbeiner and Greenberg, 1998; Carafoli et al., 2001), as well as ischemic preconditioning and pharmacological preconditioning (Miyawaki and Ashraf, 1997; Schulz et al., 2001). We found that L-type calcium channels and ryanodine regulate the neuroprotective effect of digoxin-induced pharmacological preconditioning (Rehni et al., 2008b). A similar calcium-dependent mechanism may mediate milrinone activity, as ruthenium red, a ryanodine receptor blocker, abolished the neuroprotective effects of milrinone in pharmacological preconditioning.

Calcium channel antagonists improve memory and are neuroprotective in ischemia-reperfusion injury (Yanpallewar et al., 2004; Iwasaki et al., 2007). In contrast, preconditioning-induced protective effects depend on increases in intracellular calcium (Miyawaki et al., 1996; Miyawaki and Ashraf, 1997; Kaur et al., 2009b). These different roles for calcium may result from differences in calcium levels during sustained ischemic insults *vs* ischemic preconditioning. During sustained ischemic insult, a massive intracellular accumulation of calcium ions triggers deleterious biochemical events, subsequently leading to cell death and memory loss. Calcium channel blockers can prevent this massive rise in intracellular calcium. In contrast, ischemic preconditioning causes an optimal increase in calcium to mediate the protective effects of preconditioning. Milrinone-induced pharmacological preconditioning may also increase intracellular calcium levels via phosphodiesterase inhibition to protect against ischemia/reperfusion injury.

Milrinone shows pharmacological preconditioning of the brain in mice by increasing intracellular calcium levels via ryanodine receptors.

CONCLUSION

Milrinone shows pharmacological preconditioning of the brain in mice to protect against memory loss and ischemic injury by increasing intracellular calcium levels via ryanodine receptors.

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