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ORIGINAL PAPER

Preconditioning with a Novel Metallopharmaceutical NO Donor in Anesthetized Rats Subjected to Brain Ischemia/Reperfusion

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Abstract Rut-bpy is a novel nitrosyl–ruthenium complex releasing NO into the vascular system. We evaluated the effect of Rut-bpy (100 mg/kg) on a rat model of brain stroke. Forty rats were assigned to four groups (Saline solution [SS], Rut-bpy, SS+ischemia–reperfusion [SS+I/R] and Rut-bpy+ischemia–reperfusion [Rut-bpy+I/R]) with their mean arterial pressure (MAP) continuously monitored. The groups were submitted (SS+I/R and Rut-bpy+I/R) or not (SS and Rut-bpy) to incomplete global brain ischemia by occlusion of the common bilateral carotid arteries during 30 min followed by reperfusion for further 60 min. Thirty minutes before ischemia, rats were treated pairwise by intraperitoneal injection of saline solution or Rut-bpy. At the end of experiments, brain was removed for triphenyltetrazolium chloride staining in order to quantify the total ischemic area. In a subset of rats, hippocampus was obtained for histopathology scoring, nitrate and nitrite measurements, immunostaining and western blotting of the nuclear factor- κB (NF-κB). Rut-bpy pre-treatment decreased MAP variations during the transition from brain ischemia to reperfusion and decreased the fractional injury area. Rut-bpy pre-treatment reduced NF-κB hippocampal immunostaining and protein expression with improved histopathology scoring as compared to the untreated operated control. In conclusion, Rut-bpy improved the total brain infarction area and hippocampal neuronal viability in part by inhibiting NF-κB signaling and helped to stabilize the blood pressure during the transition from ischemia to reperfusion.

Keywords Brain · Ischemia–reperfusion · Nitric oxide · Nitrosyl–ruthenium complex · Nuclear factor-κB

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Introduction

Cerebral ischemia/reperfusion (I/R) is caused by a transient or permanent reduction in the brain blood flow and constitutes a major cause of human morbidity and mortality [1].

The pathophysiological process of brain I/R-induced neuronal damage is complex and involves nitric oxide (NO) pathways [2–5]. NO is a free radical associated with a multitude of physiological functions.

Nitric oxide (NO) produced by endothelial nitric oxidase (eNOS) reduces apoptosis and confers protection against stroke, whereas pathological concentrations of NO from inducible iNOS and neuronal nitric oxidase (nNOS) lead to apoptosis and are neurotoxic [6–8].

Nitric oxide (NO) donors are pharmacologically active substances that release NO into biological systems,

spontaneously or by induction, eliciting a response similar to that of endogenous NO or compensating for endogenous NO deficiency [9–11]. The administration of NO donors has broadened therapy strategies for neuronal protection, by improving vasodilation and blood perfusion to the brain tissue [12–14], especially when the blood flow and vascular endothelial system are compromised.

Over the past years much attention has been given to nitrosyl–ruthenium complexes and their potential pharmacological use, especially due to their rapid NO release [15] as well as low level of toxicity [16–18]. Several ruthenium compounds have been synthesized and purified, but so far none has been tested in studies of experimental brain ischemia and reperfusion.

The NO donor-Rut-bpy (cis-$[Ru(bpy)_2(SO_3)(NO)]PF_6$) can release nitric oxide through chemical, electro and photochemical reactions [11]. In biological medium, this complex can release NO activated by biological reducing agents [19].

Rut-bpy is a potent vasodilator capable of releasing intracellular NO and activating guanylate cyclase [20]. Besides producing higher maximum relaxation in aortic rings than sodium nitroprusside at similar molar basis, Rut-bpy is associated with higher levels of NO release without being photosensitive or releasing cyanide [20, 21].

In order to resemble a clinical condition prior to cerebrovascular surgery, when there is an increased risk of perioperative ischemia/reperfusion and stroke, we evaluate the preconditioning effect of a novel nitrosyl–ruthenium complex (Rut-bpy) on the total brain ischemic area and on variations of the mean arterial blood pressure following bilateral carotid occlusion and reperfusion in Wistar rats. In addition, we addressed whether this novel nitrosyl–ruthenium complex could protect hippocampal neurons with less NF-κB hippocampal expression.

Methods

All surgical procedures and animal handling were conducted in accordance with the Guide for the Care and Use of Laboratory Animals from the Brazilian College of Animal Experimentation, after approval by the local ethics committee (protocol #62). The study was designed to minimize the number of animals required for the experiments.

Male Wistar rats (*Rattus norvegius albinus*), weighing 280–300 g were under standard housing conditions with free access to water and chow, and were randomly assigned to four groups of fifteen animals each: saline solution (SS); Rut-bpy; SS followed by ischemia–reperfusion (SS+I/R); Rut-bpy followed by ischemia–reperfusion (Rut-bpy+I/R).

Anesthesia and Blood Pressure Monitoring

Rats were anesthetized by an intramuscular injection of xylazine (10 mg/kg) and ketamine (90 mg/kg) solution. A polyethylene catheter filled with heparinized saline solution was introduced directly into the femoral artery and connected to a pressure transducer for continuous monitoring of mean arterial pressure (MAP) during the course of the experiment. The rectal temperature was also monitored with a digital thermometer and maintained at 35–37°C.

Drug

Rut-bpy (cis-$[Ru(bpy)_2(SO_3)(NO)]PF_6$) was synthesized and purified at the Department of Organic and Inorganic Chemistry of the Federal University of Ceará (Brazil), following procedures described elsewhere [14]. Thirty minutes before induction of ischemia, Rut-bpy (prepared from a working solution of 1.95 mM) was administered intraperitoneally at a dose of 0.15 mmol/kg (equivalent to 100 mg/kg). This dosage was based on previous experiments, which showed low toxicity to rats [17] and benefits in a model of septic shock [15].

Induction of Ischemia and Reperfusion

After anesthesia and femoral artery cannulation, experimental rats were submitted to anterior cervical incision followed by dissection of the common carotid arteries around the internal and external artery bifurcation (i.e., the clamping site), as described elsewhere [21–23]. The rats assigned to SS or Rut-bpy groups were spared of further interventions but subsequently euthanized. The rats assigned to ischemia–reperfusion were submitted to a similar bilateral occlusion of the carotid arteries for 30 min, followed by release of the vascular clamp and free cerebral blood flow for 60 min and then at the desired time after the onset of reperfusion, animals were sacrificed and sampled (brain) for 2% 2,3,5-triphenyl-tetrazolium chloride (TTC) stain, hippocampal histopathology, NO metabolite measurement, and NF-κB immunolabeling, as described below.

TTC Stain

Following decapitation, the brain was excised and 2 mm coronal sections were made in segments 0 to +6 mm of the Bregma, according to the stereotaxic coordinates [24]. Each section was incubated in saline solution containing 1% of TTC for 30 min at 37°C and repositioned after 15 min. Next, the slices were rinsed three times for 1 min with normal saline solution to remove excess TTC, as previously described [25]. Areas not stained red by TTC were considered as damaged tissue [26–29]. The slices

were scanned and digitized in high resolution (600 dpi) without color correction, image enhancement or linear gain [30]. The area of tissue damage of each section was calculated with the morphometry software UTHSCSA (The University of Texas Health Science Center at San Antonio). The damaged area/total area ratio was calculated for each section, and the sum of the ratios of the four sections from each brain (within the selected stereotactic coordinates) was taken as the total incidence of tissue damage of each animal.

Hippocampus Histopathology

The brain was removed from the skull and four coronal sections were made (2 mm each) and then immersion fixed in formalin for 24 h before being transferred to 70% ethanol solution, further processed, embedded in paraffin, and cut to generate 5-μm-thick coronal sections onto slides. The slides were stained using hematoxylin and eosin. The extent of the hippocampus damage was assessed by a neuropathologist, blindly, using a light microscope (Olympus, 400×), the number of pink acidophilic dead neurons (red neurons) were counted, as described by Kaku et al. [31]. Neuronal damage in the Ca1, Ca2/Ca3 areas of the hippocampus was expressed as the percentage of dead cells in the total 400× microscope-field cell population.

Immunohistochemistry

Immunohistochemistry for NF-κB p50 (NLS) was performed on brain tissue using the streptavidin–biotin-peroxidase method in formalin-fixed, paraffin-embedded tissue sections (4 μm thick), mounted on poly-L-lysine-coated microscope slides. The sections were deparaffinized and rehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (vv-1) hydrogen peroxide and washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4°C) with primary rabbit anti-NF-κB antibody diluted 1:200 in PBS plus bovine serum albumin (PBS–BSA). The slides were then incubated with biotinylated goat anti-rabbit; diluted 1:400 in PBS–BSA. After washing, the slides were incubated with avidin–biotin-horseradish peroxidase conjugate (Strep ABC complex by Vectastain® ABC Reagent and peroxidase substrate solution) for 30 min, according to the Vectastain protocol. NF-κB was visualized with the chromogen 3,3′diaminobenzidine (DAB). Negative control sections were processed simultaneously as described above but with the first antibody being replaced by PBS–BSA 5%. None of the negative controls showed NF-κB immunoreactivity. Slides were counterstained with Harry's hematoxylin, dehydrated in a graded alcohol series, cleared in xylene and cover slipped.

Western Blotting

Immediately after reperfusion time, total hippocampus was carefully removed and rapidly frozen in liquid nitrogen and stored at −80°C. Thawed specimens were pulverized with an electric homogenizer (ultra-Turrax homogenizer, Sigma, St. Louis, MO, USA), containing lysis buffer and then transferred to test tubes with a protease inhibitor cocktail and centrifuged at 14,000 rpm. Supernatants were assayed using the bicinchoninic acid method, BCA Protein Assay Kit (Pierce, Rockford, IL, USA) to standardize 50 μg of protein product. Samples were loaded into 15% denaturating polyacrylamide mini gels (Bio-Rad, Hercules, CA, USA), and gels were transferred overnight and then blotted onto nitrocellulose membranes. Membranes were incubated with rabbit anti-NF-κB antibody at dilution of 1:300, (Santa Cruz Biotechnology, CA, USA) for 2 h and then rinsed three times in rinsing buffer, then incubated in a secondary antibody and rinsed as described above. Each membrane was washed and exposed to Kodak X-Omat AR film (Kodak, Rochester, NY, USA). Densitometry results were normalized with β-actin, as an internal control.

Measurement of NO Metabolites in Hippocampus

Immediately after reperfusion time, total hippocampus was carefully removed and rapidly frozen in liquid nitrogen and stored at −80°C until NO metabolite analyses. Briefly, hippocampus tissue was minced and homogenized in sucrose buffer. Homogenates were centrifuged at 1,000× *g* for 10 min. Supernatants were incubated in a microplate with nitrate reductase (0.016 U*well^{-1}) for 12 h to convert NO_3–NO_2. The accumulation of nitrite (NO_2) in the supernatant, an indicator of the production of NO, was determined with a colorimetric assay with Greiss reagent (0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) as described by Green et al. [32]. Equal volumes of supernatant and Greiss reagent were mixed; the mixture was incubated for 10 min at room temperature in the dark. Calibration curves were made with sodium nitrite (5–50 M) in distilled water. Absorbance was measured in an ELISA plate reader at 540 nm by a spectrophotometer. Results were expressed as micromoles of nitrite using the internal standard curve.

Statistical Analysis

Data (TTC and western blotting) were expressed as mean ± SD. After a Kolmogorov–Smirnov test of normality, data were submitted to one-way analysis of variance followed by Tukey's multiple comparison test. MAP was expressed as mean + SD. MAP data were submitted to two-way analysis of variance followed by Bonferroni post-tests. Nonparametric data (histopathology) were expressed as medians and

percentiles (25–75), and analyzed by Mann–Whitney test. The level of statistical significance was set at 5%.

Results

Ischemia–Reperfusion Effect on Cerebral Tissue

The amount of TTC unstained cerebral tissue was greater in the SS+I/R group (administration of SS prior to ischemia–reperfusion) than in the group receiving SS alone (damaged/total area ratio, $P < 0.01$) (Fig. 1). The histopathological examination (H&E) of target hippocampus regions also revealed a greater number of damaged nerve cells (red nerve cells) in the SS+I/R group than in the group receiving SS alone ($P < 0.02$) (Figs. 2, 3).

Neuroprotective Effect of Rut-bpy

The total TTC unstained area was significantly smaller in the Rut-bpy+I/R group as opposed to the SS+I/R group

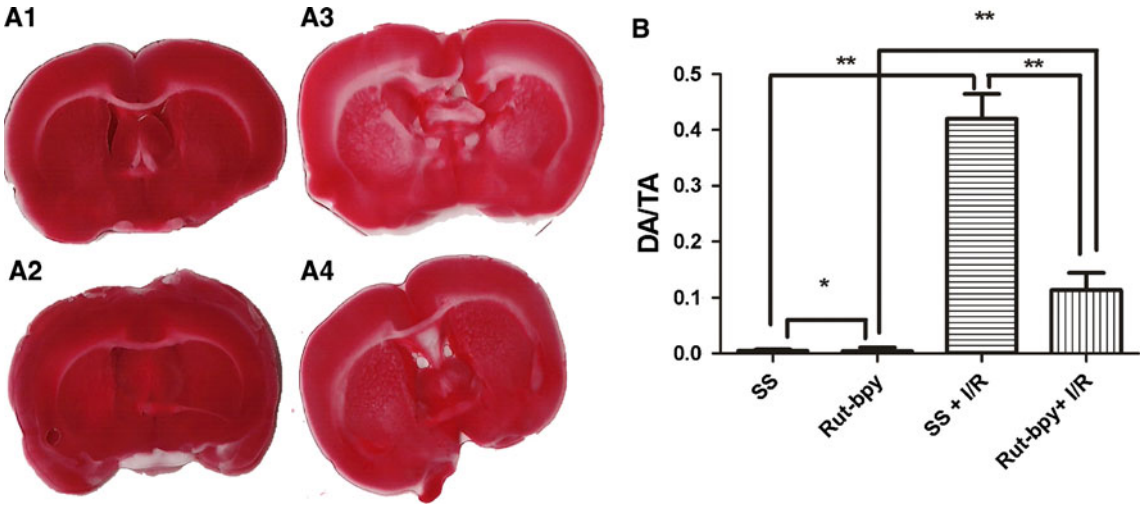

Fig. 1 **A** Representative TTC-stained-coronal brain sections from SS (*A1*), Rut-bpy (*A2*), SS+I/R (*A3*), and Rut-bpy+I/R (*A4*). **B** Damaged area/total area ratio (DA/TA) of rat brain sections (sum of the section ratios)—TTC stain. $^{**}P < 0.01$ for SS versus SS+I/R, SS+I/R versus Rut-bpy+I/R; $^{*}P > 0.05$. SS = saline solution; Rut-bpy = cis-$[Ru(bpy)_2(SO_3)(NO)]PF_6$; I/R = ischemia/reperfusion

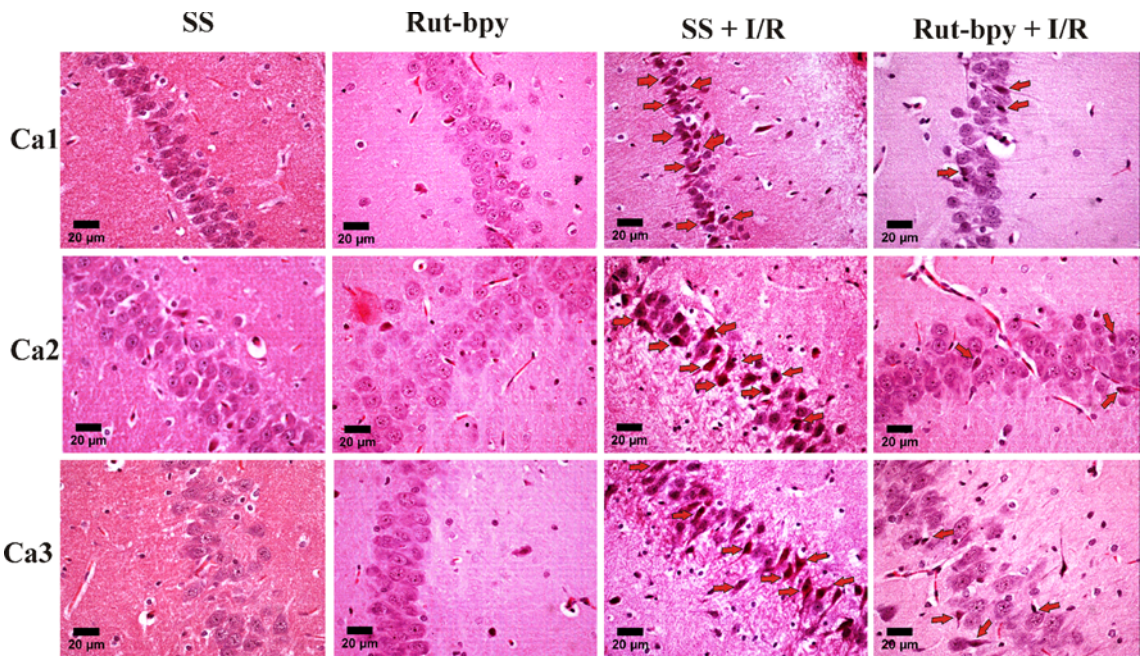

Fig. 2 Histological changes in the **CA1**, **CA2**, and **CA3** hippocampal regions. Representative hematoxylin and eosin-stained pyramidal neurons from SS, Rut-bpy, SS+I/R, and Rut-bpy+I/R groups. Magnification ×400. SS = saline solution; Rut-bpy = cis-$[Ru(bpy)_2(SO_3)(NO)]PF_6$; I/R = ischemia/reperfusion; *Arrows* indicate red neurons (acidophilic dead neurons)

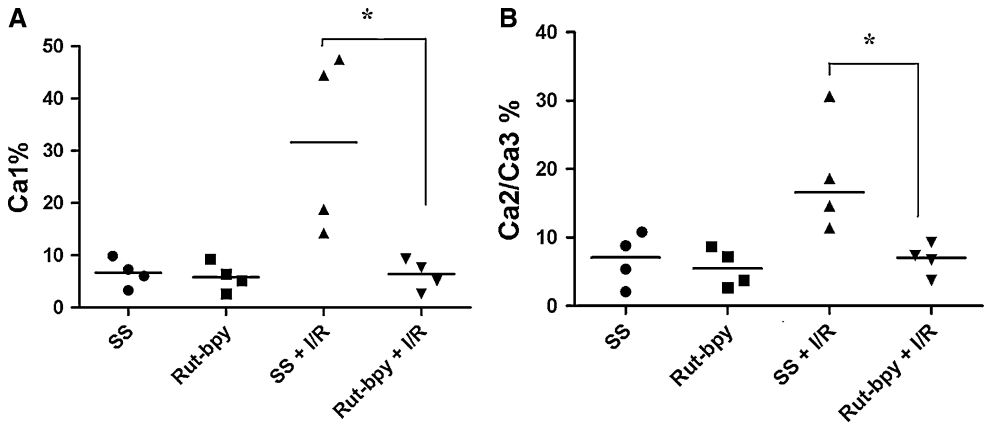

Fig. 3 Percentage of dead cells in the total pyramidal cell population counted from high-magnified fields of hippocampal sections. **a** Percentage of dead cells in the CA1 total pyramidal cell population counted from high-magnified fields. $*P < 0.05$ **b** Percentage of dead cells in the CA2 plus CA3 total pyramid cell population counted from high-magnified fields, $*P < 0.05$ (group SS+I/R vs. Rut-bpy+I/R). SS = saline solution; Rut-bpy = cis-$[Ru(bpy)_2(SO_3)(NO)]PF_6$; I/R = ischemia/reperfusion

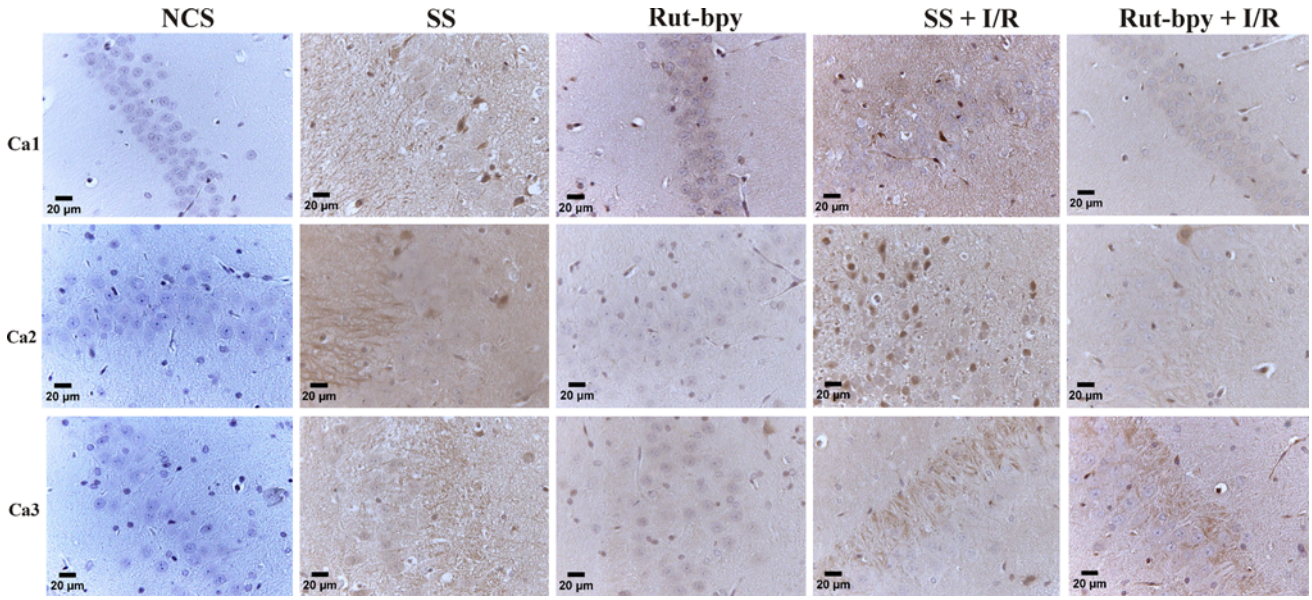

Fig. 4 Representative hippocampal NF-κB p50 (NLS) immunohistochemistry in **CA1**, **CA2** and **CA3** regions obtained from Rut-bpy, SS+I/R, and Rut-bpy+I/R groups. Cells with *brown/dark* staining are NF-κB-positive. None of the negative controls sections showed NF-κB immunoreactivity. Magnification ×400. SS = saline solution; Rut-bpy = cis-$[Ru(bpy)_2(SO_3)(NO)]PF_6$; I/R = ischemia/reperfusion; NCS = negative control sections (Color figure online)

(0.11 ± 0.03 vs. 0.42 ± 0.04; $P < 0.01$) (Fig. 1). The SS and Rut-bpy groups did not differ significantly ($P > 0.05$). However, we found differences between SS versus Rut-bpy+I/R and Rut-bpy versus Rut-bpy+I/R groups, $P < 0.01$.

The histopathological examination (H&E) of the hippocampal regions revealed a significantly lower number of damaged neuronal cells (eosinophilic neuron) in the Rut-bpy+I/R group (Ca1: 6.42, range 3.26–8.92; Ca2/Ca3: 7.05, range 4.50–8.81) as compared with the SS+I/R group (Ca1: 31.60, range 15.40–46.68; Ca2/Ca3: 16.60, range 12.22–27.64) ($P < 0.02$) (Figs. 2, 3). SS and Group Rut-bpy groups did not differ significantly ($P > 0.05$) (Figs. 2, 3).

Furthermore, the NF-κB p50 (NLS) immunohistochemical staining was less striking in Rut-bpy+I/R than in the SS+I/R group (Fig. 4).

Mean Blood Pressure Monitoring

During the first 15 min of reperfusion, blood pressure variation was significantly smaller in the Rut-bpy+I/R group as compared with the SS+I/R group (6.49 ±

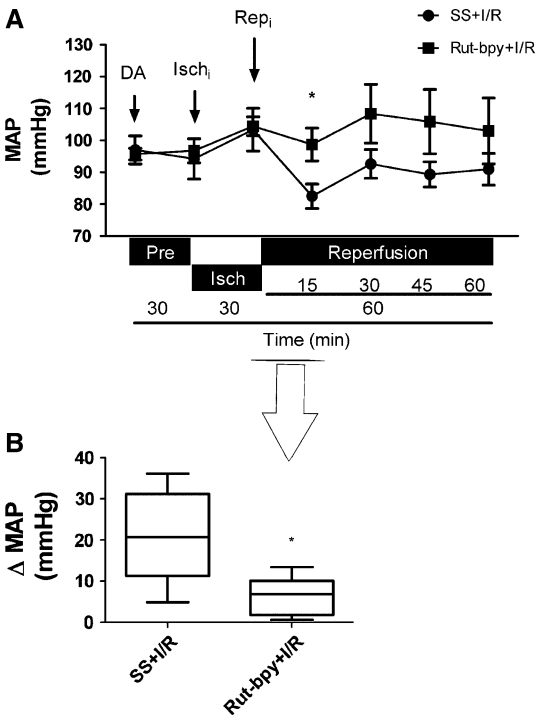

Fig. 5 **a** Mean arterial pressure (MAP) from experimental rats during induced-ischemia (30 min) ($P > 0.05$, SS+I/R vs. Rut-bpy+I/R) and during reperfusion (*$P < 0.05$, SS+I/R vs. Rut-bpy+I/R). **b** Differences in mean arterial pressure (ΔMAP) between ischemia (30 min) and the first 15 min of reperfusion. *$P < 0.05$ for SS+I/R versus Rut-bpy +I/R. SS = saline solution; Rut-bpy = cis-[Ru(bpy)$_2$(SO$_3$)(NO)]PF$_6$; Isch$_i$ = beginning of the isquemia; Rep$_i$ = beginning of the reperfusion; DA = drug administration; Pre = preconditioning with Rut-bpy (Rut-bpy+I/R group) or SS (SS+I/R group); Rep = reperfusion phase

4.6 mmHg vs. 20.89 ± 11.77 mmHg; $P < 0.04$) (Fig. 5). The groups did not differ significantly during the ischemia phase ($P > 0.05$).

NO Metabolite Concentration

We found reductions in the nitrate concentration in the SS+I/R hippocampus when compared with SS (these result was in agreement with the reduced level of NO reported earlier in cerebral tissues after I/R injury [12, 33]. The Rut-bpy+I/R group showed greater hippocampal nitrate concentration than the SS+I/R group. However, SS versus Rut-bpy+I/R did not differ significantly ($P > 0.05$). The group Rut-bpy showed the highest level of NO metabolites than all the others groups ($P < 0.01$; Rut-bpy vs. SS, SS+I/R, Rut-bpy+I/R) (Fig. 6).

Hippocampal Expression of NF-κB

As shown in Fig. 7a, b, NF-κB densitometry in SS+I/R group was markedly increased. Preconditioning of Rut-bpy reduced NF-κB protein levels in the hippocampus.

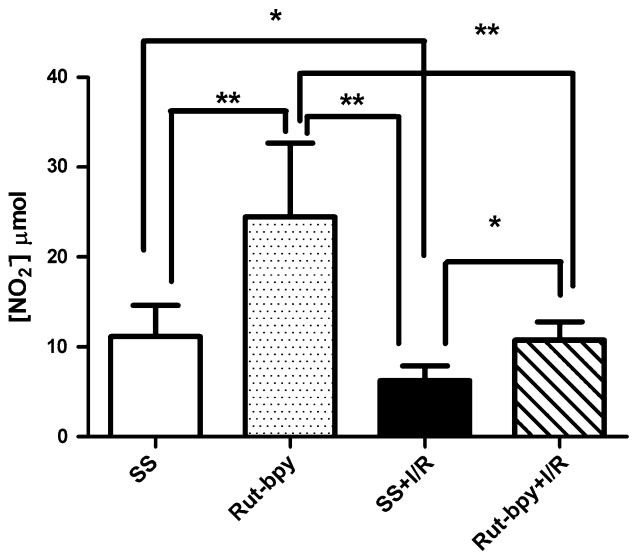

Fig. 6 Effect of Rut-bpy preconditioning on NO_2^- levels in the hippocampus after 30 min of ischemia and 60 min of reperfusion. Results are expressed as NO_2^- tissue level (µg/ml) and data are presented as mean ± SD from duplicate measurements in two different sets. *$P < 0.05$, **$P < 0.01$

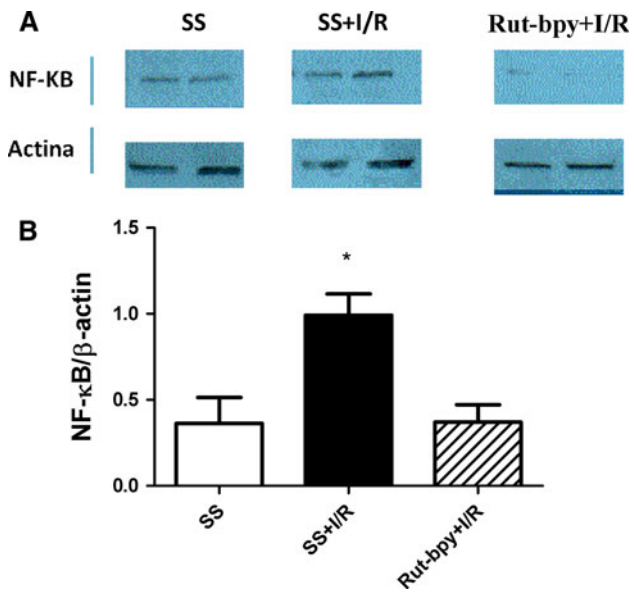

Fig. 7 Rut-bpy preconditioning decreased NF-κB expression in hippocampus. **a** Representative Western blot analysis. **b** Quantitative analysis of the protein levels of NF-κB from four rats in each group. The data are normalized to the loading control β-actin. Values are expressed as mean ± SD ($n = 4$). *$P < 0.05$, as compared SS+I/R with SS or Rut-bpy+I/R

Discussion and Conclusions

Over the past decade much has been discovered concerning the pathophysiology of cerebral ischemia/reperfusion [34–36] and many new forms of treatment have been

proposed based on the pharmaceutical effects of nitric oxide made available by NO donors [11–13, 37, 38] or NO synthase (NOS) inhibitors [4, 11, 39, 40].

The only metallopharmaceutical NO donor used clinically today is sodium nitroprusside, with a disadvantage to be photosensitive and to release cyanide. In contrast, the newly discovered nitrosyl–ruthenium complex Rut–bpy (cis-$[Ru(bpy)_2(SO_3)(NO)]PF_6$) has neither of these disadvantages besides being soluble in water [14].

To the best of our knowledge, the present study is the first to demonstrate that Rut-bpy, with a dose smaller than the lethal dose (LD_{50}) used elsewhere [17], can markedly reduce damage to hippocampal neurons caused by ischemia/reperfusion, with relatively small changes in blood pressure during transition from ischemia to reperfusion.

To evaluate the effect of pharmacological preconditioning on cerebral ischemia/reperfusion in anesthetized rats, the animals were treated with a combination of xylazine and ketamine at a dose capable of reducing cerebral blood flow and the oxygen partial pressure in the cerebral tissue [41] and in regard to the body temperature, Stein [42] did no found differences in body temperature values when compared with the control group in their studies in rats under xylazine and ketamine anesthesia. In this model, previously used by a number of researchers [21, 22, 24, 36, 43, 44], damage to the cerebral tissue is caused by the occlusion of the common carotid artery. In the current study, however, the two carotid arteries were occluded simultaneously to induce ischemia and unblocked to restore blood flow. The model is easy to use and effectively impairs blood supply to the brain [44, 45] and reduces ATP-forming oxidative phosphorylation [21]. In addition, it simulates the consequences of perioperative ischemia/reperfusion when the blood flow in the carotid artery needs to be interrupted, as during surgery for tumors, aneurisma and post-trauma correction of the carotids.

Rut-bpy has been shown to induce relaxation in vitro in aortic rings (without endothelium) by the direct release of NO into the smooth musculature [18]. In the present study, using an incomplete global ischemia model in vivo, Rut-bpy reduced neuronal damage caused by ischemia/reperfusion. The evaluation took into account the intensity of staining with TTC—a proton acceptor for many pyridine nucleotide-linked dehydrogenases which, along with the cytochromes, form an integral part of the inner mitochondrial membrane and make up the electron transport chain [46], thereby reflecting mitochondrial functional activity [29]. However, the finding of the transient reduction of dehydrogenase activity is uncertain after a short period of reperfusion [47, 48]. Therefore, in addition to TTC, we analyze the hippocampus tissue, a well-known vulnerable brain region to the ischemic–reperfusion insult [23].

In our studies, histopathological examination of the hippocampus provided clear evidence of the protective effect of Rut-bpy when administered in vivo prior to ischemia/reperfusion (Figs. 2, 3). It also suggests that NF-κB downregulation played a role in rut-bpy-related hippocampal neuroprotection (Figs. 4, 7). Similar findings have been reported with NO donors in focal ischemia models [11].

The observed neurovascular protective mechanism of NO indicates that NO donors are most effective when an appropriate drug amount is delivered by a suitable source at the right time window and in suitable environment [12, 49].

There is much evidence to support that brain ischemia and reperfusion can lead to dysfunction in the cerebral vascular endothelium, possibly due to an increased production of vasoconstrictors and/or reduced availability of vasodilators, like NO [50]. In fact, NO donors can reduce tissue damage following brain ischemia and reperfusion [3, 11, 13, 37] through vasodilation and increased cerebral blood flow [11]. The NO donor Rut-bpy has been shown to induce vasodilation through cGMP [18] and show benefits in amplifying hippocampal evoked potentials in vitro, directly correlated with NO release [51]. In the present work, as expected, we found increased NO metabolites in hippocampus, confirming the NO release and metabolization.

Due to their well-developed circle of Willis, rats have their cerebral blood supply reduced by 50% only when submitted to ischemia through the clamping of both carotids [44]. Thus, the presence of a vasodilator, such as NO, favors blood flow in any unoccluded vessels of the circle of Willis (vertebral arteries). In addition, studies on cerebral angiography have shown that even with a thoroughly cauterization of the vertebral arteries, a number of unoccluded vessels may be left and the brain tissue may receive blood from the collateral circulation even when all extracranial cerebral vessels have been occluded [52], thus justifying the use of NO to increase blood flow.

De La Torre and Aliev [53] reported that animals treated with selective endothelial NO synthase (eNOS) inhibitors experienced increased cerebral edema during cerebral hypoperfusion, suggesting that eNOS-derived NO plays a critical role in the maintenance of cerebral blood flow and justifying the administration of NO donors.

Interestingly, in a study by Greco [54], pretreatment with the selective eNOS inhibitor, L-N-(1-iminoethyl)-ornithine, at doses high enough to induce ischemic brain damage caused a significant reduction of the IκB-alpha expression in the ischemic cortex, suggesting that eNOS-derived NO can prevent ischemic damage by inhibiting NF-κB [47].

The activation of NF-κB plays dual roles in cells death and survival, depending on the cell type, developmental stage, and apoptotic stimuli [55]. In normals cells, NF-κB

is associated with inhibitory IκB proteins that inhibit nuclear localization and DNA binding of NF-κB. In response to stimuli, including free radicals, which have been implicated in the pathogenesis of cerebral ischemia, IκBs are phosphorylated by the IκB kinase, and subsequently degraded, releasing NF-κB to translocate into the nucleus where it binds to a κB-specific DNA motif and regulates transcription of target genes, including immunological NOS (iNOS), which are known to be induced in cerebral ischemia and capable of mediating the deleterious effect [56, 57]. Moreover, NF-κB is activated at earlier reperfusion time points and that early NF-κB inhibition in transient focal ischemia has a beneficial effect [58].

In this study, when a NO donor (Rut-bpy) was administered prior to ischemia/reperfusion the expression of activated NF-κB was reduced in the hippocampal regions Ca1, Ca2 and Ca3, suggesting that Rut-bpy inhibited NF-κB, probably through NO release from the ruthenium–nitrosyl complex. Further studies are required to evaluate the upstream cytokine network involved in this protective mechanism.

Paradoxically, but not uncommonly, when the blood flow is restored from ischemia, and viable brain cells and tissues are reoxygenated, free radicals tend to rise in neuronal, endothelial and glial cell environment [59, 60] leading to faster and farther cell damage, producing the so-called ischemia/reperfusion lesion.

Some free radicals are derived from NO produced by neuronal or immunological NOS (nNOS and iNOS, respectively), which form highly reactive peroxynitrite, responsible for cell membrane lipid peroxidation [61], including mitochondrial membranes. Noteworthy is that, the administration of the NO donor Rut-bpy may not cause significant lipid peroxidation, since pharmacologically increases in NO levels reported in several studies fail to exacerbate free radical production [11, 37] and because the mechanisms of neuronal protection during events of ischemia and reperfusion necessarily involve both NO production and NOS inhibition.

In addition, in animals receiving Rut-bpy, blood pressure (BP) was more stable during reperfusion and during the transition from ischemia to reperfusion. Increased BP stability may have enhanced the protective effect of Rut-bpy administration, but more studies are needed to test the possible association between BP stability and cerebral damage in Rut-bpy pretreatment models.

In conclusion, when used in vivo, preconditioning with NO donor Rut-bpy was found to improved the total brain infarction area and hippocampal neuronal viability, in an early phase of ischemia/reperfusion, in part by inhibiting NF-κB signaling and helped to stabilize the blood pressure during the transition from ischemia to reperfusion. It may therefore be considered an attractive candidate for further investigation using experimental stroke models.

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Conflict of interest There are no actual or potential conflicts of interests.

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