

Research report

Effect of NXY-059 on secondary mitochondrial dysfunction after transient focal ischemia; comparison with cyclosporin A

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Abstract

The free radical trapping agents NXY-059 and α -phenyl-*N-tert.*-butylnitron (PBN) markedly reduce infarct volume, even when given 1 or 3 h after the start of recirculation, following 2 h of middle cerebral artery (MCA) occlusion in rats. Their anti-ischemic effects are shared by the two immunosuppressants cyclosporin A (CsA) and FK506. Interestingly, CsA causes an additional reduction in infarct volume when given after only 5 min of recirculation, possibly reflecting blockade of a mitochondrial permeability transition (MPT) pore. PBN, CsA and FK506 are known to ameliorate the secondary dysfunction of mitochondrial function, as assessed *in vitro*, which occurs during the first 4–6 h of recirculation. The present experiments were undertaken to assess whether NXY-059 reduces tissue damage by acting directly on mitochondrial membranes, and provided that this is the case, if blockade of an MPT is involved. The results were compared to those of CsA, which thus served as a reference compound. NXY-059 was given *i.v.* after 5 min and 1 h, and CsA after 5 min of recirculation. Both NXY-059 and CsA reduced infarct volumes to about 30% of control, prevented the secondary decline in mitochondrial respiratory function during recirculation, and reduced the mitochondrial release of cytochrome c after 6 and 24 h of recirculation. However, NXY-059 failed to block the effect of Ca^{2+} on mitochondrial swelling *in vitro*, as CsA did. Furthermore, NXY-059, given after 5 min of recirculation, did not reproduce the effects of CsA. The results thus suggest that NXY-059 exerts its effects on mitochondria by indirect mechanisms. © 2002 Elsevier Science B.V. All rights reserved.

Theme: Disorders of the nervous system

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1. Introduction

Reperfusion following transient focal ischemia is known to cause secondary, delayed tissue damage. After 2 h of middle cerebral artery (MCA) occlusion, this is evident by the fact that, in the first hour of reperfusion, the bioenergetic state of the tissue is extensively restored, but secondary deterioration is observed after 4 h of recovery [15]. In the MCA model used in these studies, this secondary bioenergetic compromise did not seem to be

caused by microcirculatory failure, as evidenced by data on local cerebral blood flow (CBF), or capillary patency [30,33,39]. Other results suggested a mitochondrial origin of the secondary damage. Thus, measurements of ADP-stimulated respiration and mitochondrial respiratory control ratios (RCRs) in tissue homogenates showed partial recovery after 1 h of recovery, but secondary deterioration thereafter [28,29,39].

In this model, *i.e.* 2 h of MCA occlusion with reperfusion, two classes of pharmacological agents have proven to be efficacious, even when given 1–3 h after the start of reperfusion. One comprises the free radical trapping agents α -phenyl-*N-tert.*-butylnitron (PBN) and its derivative NXY-059 [31,54], and the other group the immunosuppressants cyclosporin A (CsA) and FK506 [7,27,29,37,38]. The results, and the pharmacological

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window of opportunity observed, demonstrate that delayed reperfusion events play a major role in the final damage incurred. Two of the pharmacological agents tested, the immunosuppressants PBN and FK506, could be shown to ameliorate (or prevent) the secondary mitochondrial dysfunction [15,28,29,38]. In these experiments, mitochondrial dysfunction was assessed by measurements of the bioenergetic state or by recordings of ADP- and uncoupler-stimulated mitochondrial O_2 consumption in tissue homogenates.

In the present study, we explored whether NXY-059 exerted its anti-ischemic effects by preventing secondary mitochondrial failure, as this can be assessed by measurements of respiratory functions of mitochondria in homogenates from the focus and the penumbra, and from the release of cytochrome c from mitochondria to cytosol. The immunosuppressant CsA was used as a reference compound. When given 1 h after the start of reperfusion, following 2 h of MCA occlusion, NXY-059 and CsA have relatively similar effects on infarct size. However, when CsA was infused into the carotid artery 5 min after the start of reperfusion, its anti-ischemic effect was enhanced [50]. For that reason, we evaluated the effect of NXY-059 when given after either 5 or 60 min of reperfusion. Since CsA is known to block the mitochondrial permeability transition (MPT) pore, whose assembly may be a major determinant of the final tissue damage incurred after ischemia–reperfusion [5,12,20], we also compared the effects of CsA and NXY-059 in blocking the MPT pore which is assembled in isolated mitochondria in response to a calcium load. The results suggest that although NXY-059 and CsA appear to have effects on a common target they do so by different mechanisms.

2. Materials and methods

2.1. Animal preparation

Male Wistar rats, weighing 260–310 g, were purchased from Charles River Labs. (Wilmington, MA, USA). They were fasted overnight with free access to water before surgery. After induction of anesthesia by inhalation of 4% halothane in N_2O-O_2 (70:30, v/v), an operation was performed under artificial ventilation (Rodent ventilator, Ugo Basile, Italy) in 1.5% halothane in N_2O-O_2 . The tail artery was cannulated with a PE50 catheter to monitor arterial blood gases, pH, blood glucose, and blood pressure.

MCA occlusion was performed with a filament occlusion model [22,34] as described in a previous publication [50,51]. Briefly, the right common carotid artery (CCA), internal carotid artery (IC), external carotid artery (EC), and proximal branches of the EC were carefully dissected under a microscope. The EC at the most proximal portion and the proximal CCA were permanently ligated. Then, the

IC just distal to its bifurcation was temporarily clipped with a vascular clip. The filament, covered with silicon rubber with a round tip (0.3 mm in diameter), was inserted 19–21 mm distal from the bifurcation of the IC to occlude the origin of MCA, and fixed to prevent spontaneous withdrawing. After that, the animals were allowed to wake up and resume spontaneous breathing. Two hours after induction of ischemia, the filament was completely withdrawn through the same skin incision under reanesthesia.

NXY-059, kindly donated to us by Centaur Pharmaceuticals (CA, USA), was continuously infused as described in a previous communication [31]. A PE50 tube was inserted 3 cm into the right jugular vein, placed in the subcutaneous layer around the neck and externalized through the skin of the head, with fixation to the skin. NXY-059 was administered as a 30 mg kg^{-1} i.v. bolus injection 1 h after ischemia followed by a 30 mg $kg^{-1} h^{-1}$ continuous i.v. infusion through the jugular vein into the superior vena cava for a maximal period of 24 h of reperfusion using a CMA/100 microinjection pump (Carnegie Medicine). The vehicle (saline) was administered by the same route, i.e. it was given as a bolus injection followed by continuous infusion of the same volume of fluid as in the NXY-059 experiments. CsA (Sandimmune Novartis), diluted six times with saline, was infused directly into the carotid artery [50,51]. At 5 min after withdrawing the filament, it was secured so that there was back flow from the distal end of the IC, and a 0.28 mm polythene tubing (Portex) was inserted into the IC. Recognition of back flow in the tube allowed administration of CsA in a dose of 10 mg kg^{-1} over 5 s during temporary occlusion of the ipsilateral pterygopalatine artery. This infusion rate was well below the threshold that could result in hypertensive opening of the BBB [43]. We gave the control animals the same amount of saline vehicle, using a similar protocol. Furthermore, since CsA in the Sandimmune preparation is dissolved in Cremaphore (polyoxyethylated castor oil) and alcohol, these animals were given that vehicle i.c., after dilution with saline. The results were identical to those obtained with saline alone.

Sham rats underwent the entire initial surgical procedure with the insertion of the filament into the internal carotid artery, but the filament was not advanced to occlude the MCA. It was completely withdrawn 2 h after insertion, i.e. the procedures were similar to those followed in animals subjected to MCA occlusion.

During operation, the core temperature was kept at about 37.0 °C by a heating mat (temperature control unit HB101/2, LSI), monitored with an electrical temperature probe. The rats with high temperature after operation were, if necessary, controlled in a cold box with an air-cooling system, from the induction of ischemia up to 4 h of recirculation [53]. Heparin (0.2 ml, 250 units ml^{-1}) was administered just before induction of ischemia.

The neurological status of each rat after MCA occlusion was evaluated from awaking to 1 h after ischemia accord-

ing to the neurologic examination grading system described by Bederson et al. [2]. All animals showing grade 0, 1 or 2 were excluded from this study. The grade 3 animals, i.e. those showing consistent circling counter-clockwise were the majority, constituting 91% of animals originally subjected to MCA occlusion.

2.2. Evaluation of infarct volume by TTC staining

To evaluate the effect of NXY-059 on cerebral ischemia, we compared the rats treated with NXY-059 to controls given vehicle by the same route. Rats were decapitated under deep anesthesia 48 h after induction of ischemia, and the brains were carefully removed. The whole brains were cooled on ice for a few min and then coronally sectioned in 2-mm thick sections from 2 mm caudal to the frontal tip with a tissue slicer. The slices were immediately stained with 1% 2,3,5-triphenyltetrazolium chloride (TTC) (Sigma) at room temperature for 30 min, and fixed by immersion in 3.7% phosphate-buffered formalin solution [3]. The six sections obtained were scanned with a CCD camera (Leaf Lumina) and quantified with NIH IMAGE (version 1.6). The total infarct volume was determined by summing up the infarct areas of the six sections and recalculated to yield the percentage volume of the contralateral hemisphere.

2.3. Measurements of mitochondrial respiratory activities

We measured ADP-stimulated O_2 consumption using the method of Sims and Blass [45] as described in previous publications from the laboratory [28,38]. The procedures were as follows. Brains from animals subjected to 2 h of ischemia or to 1 or 4 h of recirculation, as well as those obtained from animals given NXY-059 or CsA, allowed 4 h of recirculation, ($n=6$ in each group) were rapidly removed and transferred to a cold isolation buffer, containing 0.32 M sucrose, 1 mM K-EDTA, and 10 mM Tris base. The forebrain was cut with a tissue slicer in the coronal plain, yielding a 4-mm thick slice 5 mm caudal to the frontal tip. The tissue samples were divided into three portions, representing the focus and the penumbra, as defined by Folbergrová et al. [15], as well as neocortex from the contralateral side. The samples were homogenized in the isolation buffer (on ice) and homogenized using ten strokes in 500 μ l of the buffer.

ADP-stimulated oxygen consumption in the homogenates was measured polarographically with an oxygen microelectrode (YSI model 5300 oxygen monitor) in a closed and magnetically stirred chamber, maintained at 28 °C. The tissue sample (25 μ l) was added to the reaction buffer (350 μ l) which contained 100 mM KCl, 75 mM mannitol, 25 mM sucrose, 5 mM Tris-phosphate, 0.05 mM K-EDTA, and 10 mM Tris-base. Substrates that consisted of 4 μ l of 0.5 M glutamate and 0.5 M malate

(neutralized with KOH) were added to this solution. ADP-stimulated respiration was initiated by the addition of 0.1 M ADP (0.3 or 0.5 μ l). Non-stimulated (–ADP) respiration was measured from tracings obtained after this ADP was depleted and the rate declined to a constant value. The respiratory control ratio (RCR) was calculated as the ratio of stimulated to non-stimulated respiration. An aliquot of samples which were stored frozen were used to measure the protein content with the Dc Protein Assay Kit (Bio-Rad) using a spectrophotometer (DU650, Beckman).

2.4. Measurement of the Ca^{2+} -induced MPT *in vitro*

The homogenate of a forebrain with isolation buffer was centrifuged at 1330 g for 3 min twice, and 21 200 g once for 10 min. The pellet resuspended in 15% Percoll was layered onto 23% and 40% Percoll. The gradients were centrifuged at 31 700 g for 10 min. The mitochondrial fraction located between the bottom two layers was carefully removed, and centrifuged with isolation buffer at 167 00 g for 10 min. This pellet was resuspended in buffer containing 320 mM sucrose, 0.1 mM K_2 EDTA, and 10 mM Tris-HCl (pH 7.4), and the suspension with bovine serum albumin and was finally centrifuged at 6900 g. The protein concentrations of the mitochondrial fraction were measured using the assay kit.

In order to study the effect of NXY-059 and CsA on calcium-induced mitochondrial swelling brain mitochondria (0.5 mg) were resuspended in buffer containing 125 mM KCl, 1 mM $MgCl_2$, 2 mM K_2HPO_4 , 5 mM malate, 5 mM glutamate, 20 mM Tris-HCl (pH 7.2) in the presence of NXY-059 (1, 10 and 100 μ M) or CsA (2.5 μ M). Swelling was induced by addition of 40 nmol Ca^{2+} per mg protein. As described in a previous publication [23], light intensity before calcium addition is marked as 100 on the ordinate in the figure shown below, while the zero point (maximal swelling) was obtained by addition of alamethicin (40 μ g per mg of mitochondrial protein).

2.5. Subcellular fractionation and Western blotting

Subcellular fractions were prepared from sham-operated animals and from those in the experimental groups. Brain tissues were homogenized by hand (ten strokes) in 10 volumes of ice-cold isolation buffer (as mentioned above) with the proteinase inhibitor cocktail (Sigma). The homogenates were then centrifuged at 800 g at 4 °C for 10 min. The pellets were discarded and the supernatants were centrifuged at 8000 g at 4 °C for 20 min to get the mitochondrial pellets. The supernatants were centrifuged at 32 000 g to obtain the cytosolic fraction. The protein concentration was determined by using the assay kit.

Western blot analysis of cytochrome c release was carried out using 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli [32]. The mitochondrial samples (10 μ g of protein) and cytosolic

fractions (50 μg) were applied to each lane in a slab gel of SDS–PAGE. Following electrophoresis, proteins were transferred to an immobilon-P membrane (Millipore). The membrane was incubated overnight at 4 °C with primary antibodies against cytochrome c at a dilution of 1:2000. Monoclonal cytochrome c antibody was obtained from PharMingen (San Diego). The membranes were then incubated for 45 min at room temperature with horseradish peroxidase conjugated secondary antibodies. The blots were developed using the ECL detection method (Amersham).

2.6. Statistical analysis

We used the percentage of the infarct volume, related to the contralateral hemisphere, for statistical analysis. Analysis of variance (ANOVA) followed by Fischer's protected least significant difference (PLSD) was used for comparison of RCR, and physiological variables, and that followed by Scheffe's *F*-test was used for comparison of the infarction volumes. Paired *t*-test (95% confidence interval) was used to evaluate the efficacy of NXY-059 and CsA. Differences were considered significant at $P < 0.05$ or 0.01.

3. Results

3.1. Physiological variables

We measured core temperature as well as arterial pH, P_{O_2} , P_{CO_2} , glucose concentration, and blood pressure at the induction of ischemia, after 0.5, 1, 1.5 and 2 h of ischemia, and after 1, 2, 3 and 4 h of recirculation. The values were similar in the experimental groups, and only one significant difference in the temperature was detectable between NXY and control at 4 h after ischemia. However, this cannot conceivably have influenced the result. The data have not been presented in table form but are available on request.

3.2. Effect of NXY-059 on infarct volume

As stated above, CsA, when infused into the carotid artery after 1 h of recirculation, reduces infarction volume to about 40% of control, but when similarly infused after only 5 min of recirculation, the drug reduces infarct volume to less than 10% of control [50]. This suggests that CsA, when given 1 (or 3) h after the start of reperfusion, suppresses postischemic reactions which lead to an infarct after the 2-h transient ischemia, but that the drug, when given in the immediate postischemic recovery period, blocks additional reactions of a potentially detrimental effect. As a speculative working hypothesis, such reactions

could encompass the assembly of an MPT pore (see Discussion).

The question arose as to whether NXY-059 acts similarly. Fig 1 shows a reduction of infarct volume observed when 30 mg kg^{-1} NXY-059 was given i.v. 5 or 60 min after the start of reperfusion, following 2 h of MCA occlusion, followed by continuous infusion of NXY-059 in a dose of 30 $\text{mg kg}^{-1} \text{h}^{-1}$. Infusion of NXY-059 after these postischemic periods similarly reduced infarct volume ($11.9 \pm 9.8\%$ given after 5 min, and $10.3 \pm 4.4\%$ given after 60 min) to about 30% of control (32.8% of hemispheric volume, $P < 0.01$). The results corroborate those of Kuroda et al. [31], obtained by infusion of NXY-059 60 or 180 min after the start of recirculation, but they failed to show a more robust effect following infusion after only 5 min of reperfusion.

3.3. Mitochondrial respiration in vitro

Table 1 shows ADP-stimulated (state 3) and non-stimulated (state 4) respiratory rates. In general, ischemia was associated with a reduction in state 3 respiration, recirculation for 1 h virtually restored normal respiratory rates, but continued recirculation (4 h) gave rise to a secondary decline in state 3 respiration. Both NXY-059 and CsA prevented this secondary decline. The changes in mitochondrial respiration are even better exemplified by graphs showing the changes in RCRs, calculated as state 3 divided by state 4 respiratory rates (Fig. 2). On the contralateral

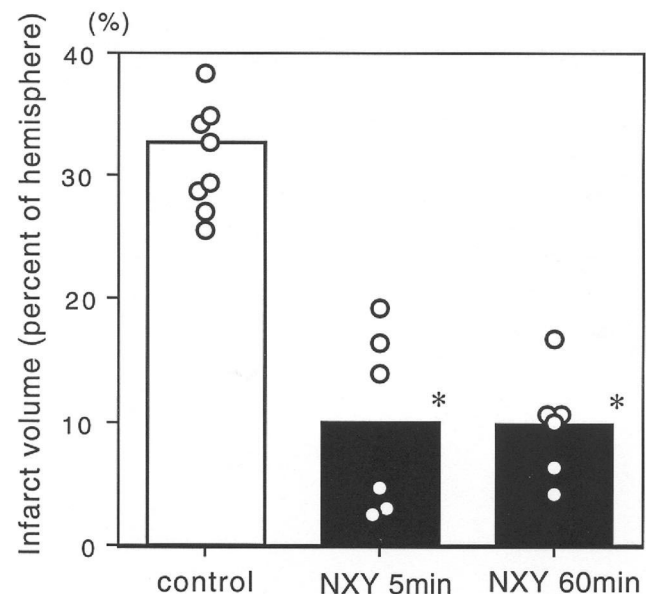


Fig. 1. Effect of NXY-059 on infarct volumes in rats subjected to 2 h of MCA occlusion, and allowed 48 h of reperfusion before TTC staining was performed. NXY-059 was given as a bolus injection of 30 mg kg^{-1} into the internal jugular vein, either 5 min or 60 min following the start of reperfusion, followed by a continuous infusion of 30 $\text{mg kg}^{-1} \text{h}^{-1}$ for 24 h. NXY-059 reduced brain damage significantly against control; *, $P < 0.01$.

Table 1
ADP-stimulated and non-stimulated respiration in mitochondria

	Control	Ischemia	1 h R	4 h R+S (i.c.)	4 h R+CsA(i.c.)	4 h+S (i.v.)	4 h R+NXY (i.v.)
<i>Focus</i>							
–ADP	3.97±1.12	5.38±1.58	4.02±1.29	4.98±1.36	4.60±1.51	3.03±0.43	3.29±0.46
+ADP	13.78±2.65* ¹	8.34±3.46* ^{1,2}	12.54±1.9* ²	10.10±3.02† ³	13.77±2.47† ³	6.88±1.13† ⁴	10.86±1.96† ⁴
<i>Penumbra</i>							
–ADP	3.68±0.62	5.69±2.56	4.66±1.44	4.35±2.48	5.24±2.10	2.92±0.64	3.30±0.81
+ADP	13.26±1.47	10.59±3.75* ⁵	15.29±1.90* ⁵	12.16±3.73	16.36±4.84	7.94±2.23† ⁶	11.66±2.69† ⁶
<i>Contralateral cortex</i>							
–ADP	4.01±1.93	3.52±1.26	3.75±1.07	3.82±0.94	3.78±0.96	2.78±0.51	2.49±0.43
+ADP	13.88±4.12	12.62±3.82	14.36±2.93	15.30±4.30	13.58±3.24	11.22±1.33	9.91±1.12

Values are mean±SD (nmol O₂/min/mg protein). *, $P < 0.05$ vs. control and 1 h R groups (*, ANOVA followed by Fisher's PLSD); †, $P < 0.05$ (unpaired *t*-test). Statistically significant differences were performed between the same numbers.

side, the RCRs were similar in all groups (data not shown). In both focal and penumbral tissues of the ipsilateral side, though, the results showed an initial recovery, a delayed deterioration in vehicle-induced animals, and the improvement of RCRs in drug-infused animals. The results thus demonstrate that mitochondrial function, as assessed in tissue homogenates *in vitro*, shows a secondary, post-ischemic deterioration, which is ameliorated by both NXY-059 and CsA.

3.4. Effects of CsA and NXY-059 on the Ca²⁺-induced MPT *in vitro*

CsA is a known blocker of the MPT pore *in vitro*, as this can be assessed in mitochondria exposed to a calcium load, or other adverse events [6,20,40]. We attempted to reproduce this effect by studying the effect of CsA on the swelling of non-synaptic mitochondria in response to a Ca²⁺ load, and compared the effect to that of NXY-059. Fig. 3 shows that, depending on the dose, CsA either reduced or completely inhibited the Ca²⁺-induced mitochondrial swelling. This confirms results previously published from the laboratory [23]. However, NXY-059 in doses of 1, 10, or 100 μM had no effect. Similar results were obtained with other nitrones, including PBN [Kristián et al., unpublished data]. It seems likely, therefore, that NXY-059 (and PBN) ameliorates focal ischemic damage by other mechanisms than that associated with the blocking of an MPT pore.

3.5. Cytochrome *c* release

Fig. 4 shows Western blots of cytochrome *c* in mitochondrial and cytosolic fractions. Cytosolic cytochrome *c* was not detected in sham animals, or in animals subjected to ischemia and 1 h of reperfusion (data not shown). However, it increased markedly at 6 h and further increased at 24 h of reperfusion in the ischemic focus. An increase of cytochrome *c* was also found in the penumbra area, but the changes were less pronounced compared to the focus (Fig. 4). There were no significant changes of

cytosolic cytochrome *c* in the brain tissue from contralateral cortex. NXY-059 attenuated the increase in cytosolic cytochrome *c* at 6 h and 24 h of reperfusion after ischemia. Also CsA, given in a dose of 10 mg kg⁻¹ by intracarotid injection, significantly decreased the cytochrome *c* release in both focus and penumbra (Fig. 5).

4. Discussion

As stated in the introduction, the free radical trapping compounds NXY-059 and PBN and the immunosuppressants CsA and FK506 exert their anti-ischemic effects within a wide window of therapeutic opportunity, which seems to be at least 3 h after the start of reperfusion, following 2 h of MCA occlusion. This remarkable amelioration of the tissue damage incurred after a long period of focal ischemia does not simply represent a delay in the maturation of the damage, as observed in tissues stained with TTC after 48 h of recovery. Thus, the effect of NXY-059 was similar when selective neuronal necrosis (SNN) and infarction were evaluated with histopathological techniques after 7 days of recovery [31]. The results raise the question whether the two disparate classes of drugs share common mechanisms of postischemic damage protection. An anti-ischemic effect of CsA was suggested by the studies of Shiga et al. [44] who pretreated rats with the immunosuppressant for 5 days before subjecting the animals to 90 min of MCA occlusion. Although CsA pretreatment seemed to reduce ischemic edema and to ameliorate the ischemic damage, recovery was limited to 24 h. Further studies with CsA in ischemic conditions were hampered by the documented low permeability of the BBB to CsA, which was assumed to reflect counter-transport of the lipophilic CsA from brain to blood [4,9]. In 1995, it was unexpectedly found that CsA, when given to animals in which the BBB permeability was enhanced by the insertion of a needle into the hippocampus on one side, was dramatically protective against SNN in the hippocampal CA1 region in animals subjected to 7 or 10 min of forebrain ischemia [48,49]. Similar effects were seen in

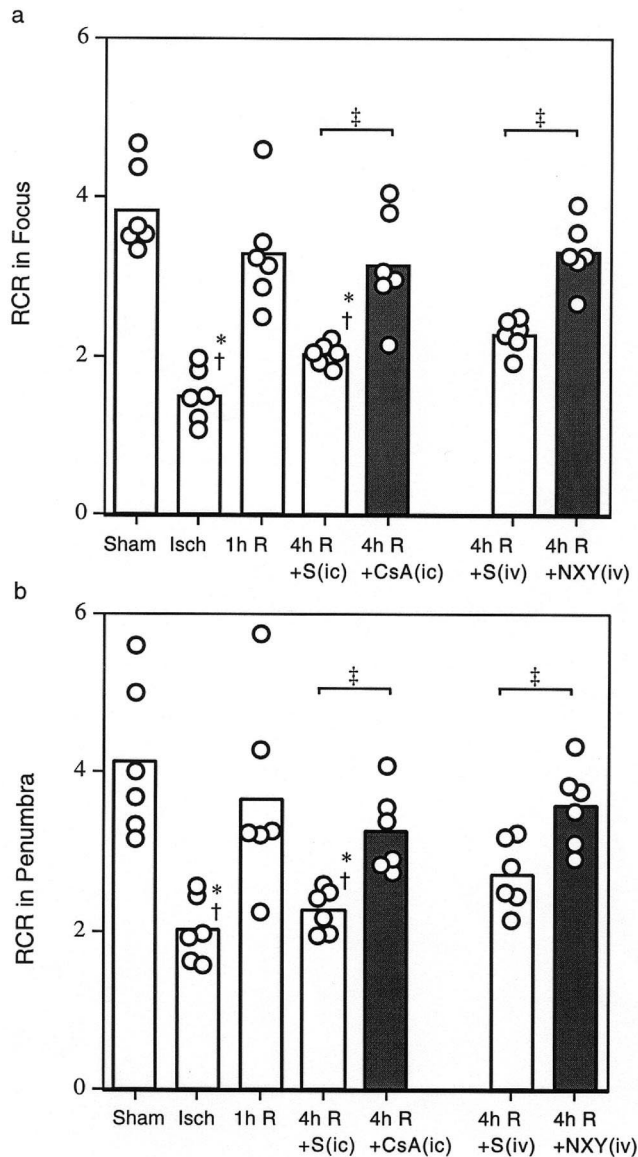


Fig. 2. Effect of 2 h of ischemia (Isch), as well as of 1 or 4 h of recirculation (R) on the mitochondrial respiratory control ratio (RCR) in focus (A) and penumbra (B); *, $P < 0.01$ against sham control; †, $P < 0.01$ against 1 h R. Open bars illustrate changes occurring in vehicle-injected animals, filled bars those observed in animals given CsA or NXY-059. CsA was infused i.c. 5 min of reperfusion. The animals were compared with those given saline (S) according to the same protocol. NXY-059 or saline was given i.v. (see Materials and methods). RCR values in animals given CsA or NXY-059 were significantly improved compared to those obtained in vehicle-injected animals at 4 h R (‡, $P < 0.01$).

hyperglycemic animals, in which damage occurs also in other regions than the CA1 sector, and CsA also proved to abort the postischemic seizures which are observed in these animals [33].

At the time when these results emerged it was known that under adverse conditions such as oxidative stress and exposure to Ca^{2+} , the inner membranes of mitochondria derived from heart muscle and other extracranial tissues could assemble a transition pore which was indiscriminate-

ly permeable to H^+ , Ca^{2+} and other ions [19]. This pore, which caused the electrochemical potential for H^+ to collapse, gave rise to mitochondrial failure of a type which was considered to jeopardize the survival of the cell in postischemic state. The pore opening, i.e. the MPT was more or less selectively blocked by CsA and some of its derivatives, e.g. MeVal CsA. However, since the immunosuppressant FK506, also known to be an antiischemic agent in focal ischemia [7], did not block the MPT in vitro it seemed likely that the two immunosuppressants (CsA and FK506) exerted their antiischemic effects by two mechanisms: one which was related to blocking of an MPT, and one which acted on another mechanism, probably related to the activation of calcineurin.

Results obtained in focal ischemia are less clear-cut than those obtained with forebrain ischemia possibly because recirculation after long periods of MCA occlusion may enhance, in an unpredictable way, blood-to-tissue transfer of CsA. Butcher et al. [7] observed that CsA, given i.v. in a dose of 20 mg kg^{-1} , reduced infarct size in animals in which MCA occlusion was induced by the intracerebral injection of endothelin. Since this injection could have affected the permeability of the BBB to CsA, it is difficult to deduce why CsA was efficacious. However, in a recent study Matsumoto et al. [37] reported that, when 20 mg kg^{-1} of CsA was given i.p. at the time of reperfusion, CsA reduced infarct volume to about 30% of control, following 2 h of MCA occlusion. Interestingly, they also found that MeValCsA, a nonimmunosuppressive analogue of CsA, had similar effects. Since MeValCsA blocks the MPT channel the results suggested that CsA exerts its antiischemic effects by blocking a postischemic MPT.

Before the results of Matsumoto et al. [37] were known to us, we attempted to circumvent the low BBB permeability to CsA by injecting the immunosuppressant directly into the carotid artery [50]. When injected i.c., in a dose of 10 mg kg^{-1} after 60 min of reperfusion, CsA was found to reduce infarct size to about 40% of control. This effect seemed similar to that previously reported for PBN, NXY-059, and FK506. However, when CsA was infused by the i.c. route after only 5 min of reperfusion the anti-ischemic effect was very pronounced since infarct size was reduced to $< 10\%$ of control. We tentatively concluded that CsA is more efficacious when injected after 5 min of reperfusion, as compared to 60 min because it blocks the assembly of an MPT.

The free radical trapping agent NXY-059 reduced mean infarct size to $< 40\%$ of control, when infused after 1 h of recirculation, and efficacy was preserved when the drug was infused after 3 h, i.e. 5 h after the start of ischemia [31]. When the effect was retested in a Wistar strain of rats, available to us in Honolulu, the results were similar, although we did not find 'zero animals', i.e. those showing no detectable damage. However, in contrast to CsA, NXY-059 did not further reduce the ischemic damage when infused after 5 min of reperfusion. It seems possible,

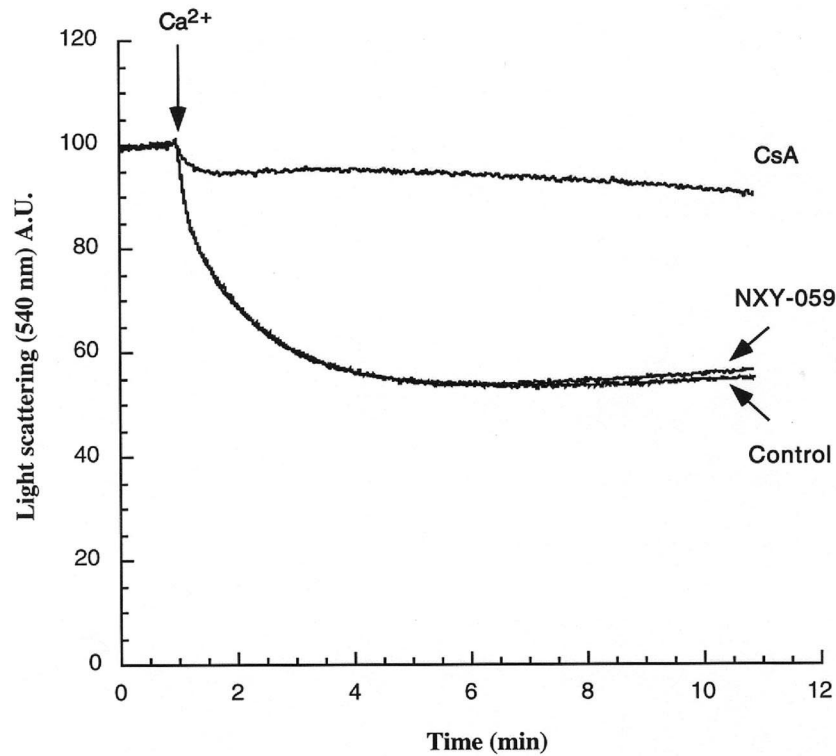


Fig. 3. Effect of NXY-059 and CsA on calcium-induced swelling of non-synaptic brain mitochondria. Mitochondria (0.5 mg) were incubated in a buffer containing 125 mM KCl, 1 mM MgCl₂, 2 mM K₂HPO₄, 1 μM EGTA, 20 mM Tris, pH 7.2, 5 mM malate and 5 mM glutamate for 4 min before calcium addition (40 nM Ca/mg protein). Calcium induced a rapid swelling of the mitochondria as assessed by recording of the changes in light scattering at 540 nm (Control). This swelling was prevented by 2.5 μM of CsA, but presence of 100 μM NXY-059 did not have any effect on the calcium-induced mitochondrial swelling. Light intensity after alamethicin treatment represents 0, and the light intensity before calcium addition is marked as 100 on the ordinate.

therefore, that infusion of CsA after only 5 min of reperfusion discloses mechanisms of protection, which are not shared by NXY-059 (or by PBN and FK506). Clearly, those mechanisms may encompass a blockage of the MPT

pore, assumed to be assembled during reperfusion in other tissues than the brain. The results of Matsumoto et al. [37] support this interpretation.

Our results demonstrate that, like PBN and FK506, the

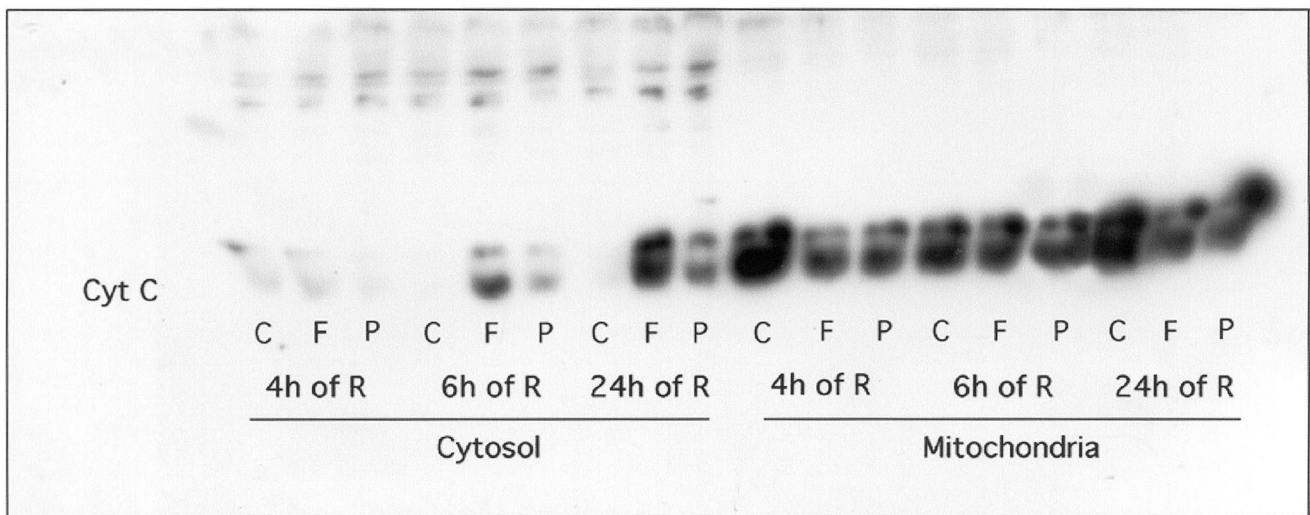


Fig. 4. Western blot analysis of cytochrome (cyt) c in cytosolic (50 μg) and mitochondrial (10 μg) fractions after 4, 6 and 24 h of recirculation (R). The expression of cyt c was markedly increased at 6 h and further increased at 24 h of recirculation in the ischemic focus (F), the changes being somewhat less pronounced in the penumbra (P). Cyt c in the contralateral cortex (C) could hardly be detected, even after 24 h of recirculation.

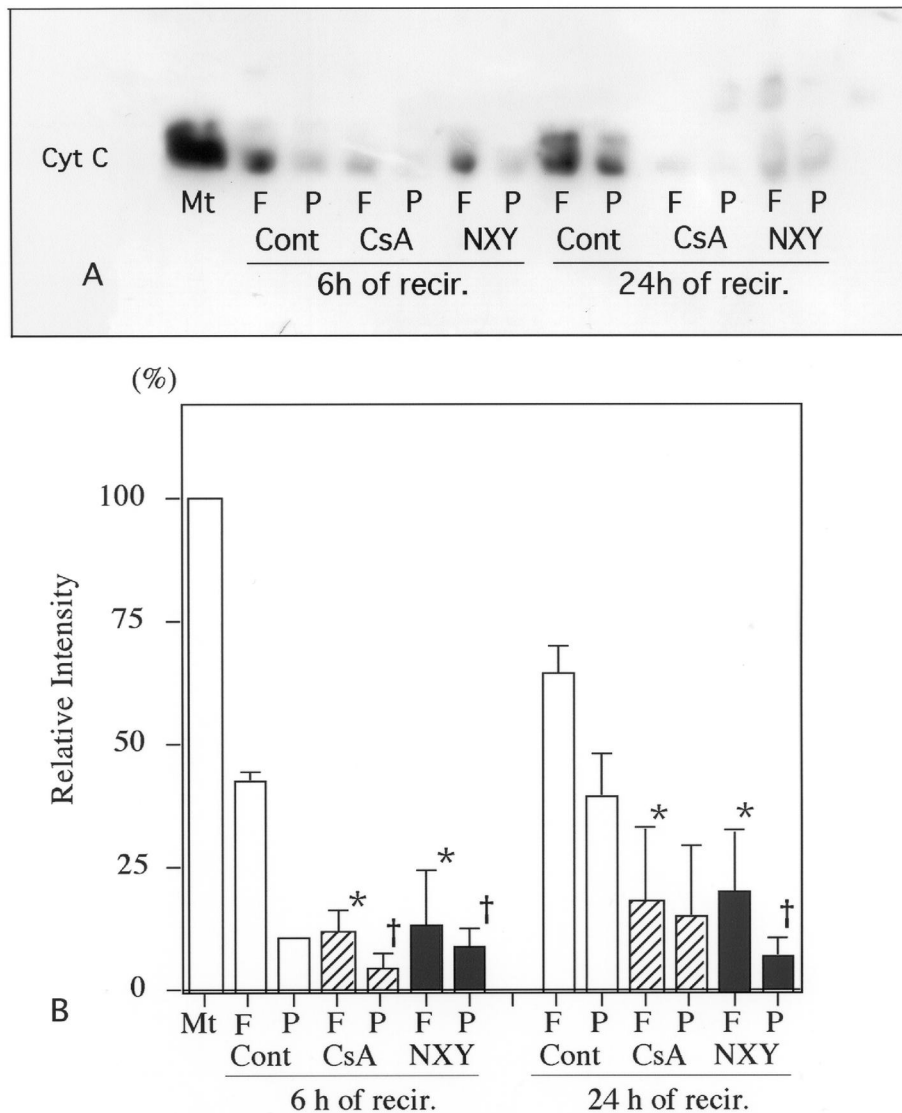


Fig. 5. Comparison the expression of cytosolic cytochrome (cyt) c in control (Cont) to those of animals given CsA or NXY-059 on western blot analysis (A). Both CsA and NXY-059 markedly decreased the cyt c release in both focus (F) and penumbra (P) at 6 and 24 h of recirculation. The relative intensity of Western blot analysis was calculated on the basis of the intensity of mitochondrial fraction (Mt) (B). The relative intensities of cyt c in the animals given CsA or NXY-059 were compared to those of controls in the same recirculation period. *, P was calculated against the intensity of focus and †, was against that of penumbra ($P < 0.01$).

drugs studied in the present experiments, i.e. NXY-059 and CsA, ameliorate the secondary deterioration of mitochondrial respiratory functions which is observed in vehicle-injected animals. This suggests that all four drugs act, directly or indirectly, on a final common pathway which obviously involves mitochondrial dysfunction, as evidenced by a decrease in ADP-stimulated mitochondrial respiration. It is tempting to assume that the decline in mitochondrial respiratory functions is due to inactivation of one or several of the respiratory complexes. However, in a recent series of experiments, we found that the activities of complexes I–V did not decrease during the first 4 h of reperfusion following 2 h of MCA occlusion [8].

An alternative mechanism of postischemic mitochon-

drial damage is one which affects the permeability of the inner (or outer) mitochondrial membranes. This is potentially detrimental since an increased permeability may allow mitochondrial constituents to be released, with potentially adverse effects. Thus, it has been suggested that, in a variety of cells and tissues, mitochondrial membrane dysfunction is what initiates the sequence of events which leads to apoptotic cell death [19,26,52]. The cascade is believed to be elicited by initiation factors which, directly or indirectly, affect the permeability of mitochondrial membranes so that proapoptotic factors are released. These include the apoptosis-initiating factor (AIF) [25] and cytochrome c [19]. The former, suggested to be a protease, could act by degrading cytoskeletal and other proteins. Cytochrome c, on the other hand, acts as a

trigger of a sequence of reactions, which constitutes a death pathway. Thus, when cytochrome *c* combines with the apoptosis activating factor 1 and dADP the result is activation of procaspase 9, which secondarily causes activation of caspase-3, an enzyme which has been named the executioner of apoptotic cell death. This is because caspase-3 activates several enzymes causing degradation of cytoskeletal proteins, and of DNA [39,46].

Studies using the present model, involving 2 h of MCA occlusion with reperfusion, demonstrated that recirculation for 1 h is associated with a release of the mitochondrial isoform of aspartate amino transferase to the cytosol, suggesting mitochondrial membrane damage [41]. It was also shown by Fujimura et al. [16] that cyt *c* release can be detected already 4 h after focal ischemia of 20 min duration. For reasons which are not known, but which may be related to differences in the density of ischemia, we found no release after 4 h, and a very moderate release after 6 h. We thus have evidence to prove that mitochondrial membrane permeability is altered in the recirculation period, but no solid data to show that this is a crucial event during the first 4–6 h of recirculation.

It was shown by Fujimura et al. [17] that mitochondrial MnSOD-deficient mice had a significant release of cytochrome *c* after permanent MCA occlusion and that over-expression of CuZnSOD can reduce cytochrome *c* release in mice after transient focal ischemia [18]. These results raise the question as to whether NXY-059 and CsA act by scavenging free radicals. This does not seem likely since nitrones (NXY-059 and PBN) are relatively weak free radical scavengers. Furthermore, neither CsA nor FK506, which also prevent secondary mitochondrial damage, are known to act as free radical scavengers. As will be discussed below, another possibility is that the pharmacological agents discussed act on stops on the signal transduction pathway which secondarily influence mitochondrial function and structural integrity [51].

This general hypothesis of apoptotic cell death has withstood rigorous testing during the last 2–3 years of research. However, it should be accepted with the following caveats. First, the initiators of cell death, which may arise from the interaction of agonists and plasma membrane receptors or be products of intermediary metabolism, do not necessarily act directly on mitochondrial membranes, but may instead modulate the expression of genes of the Bcl-2 family of proteins which have been found to modulate mitochondrial membrane permeability [10,21,36,42] and, more specifically, to regulate cytochrome *c* release [14]. Second, it is no longer obvious that the death pathway initiated at the mitochondrial level by external stimuli, or internal signals, leads to apoptosis, as defined by classical criteria [35]. Thus, release of cytochrome *c* may compromise mitochondrial ATP production and, thereby lead to bioenergetic failure, and necrosis. This is to say that when the death pathway is initiated, the result may be either apoptosis or necrosis [24,46]. Clearly,

release of cytochrome *c* from energetically compromised mitochondria is a sign of mitochondrial distress, but it may be one which either leads to apoptosis or necrosis. Third, although the release of cytochrome *c* and the initiation of an apoptotic cascade were initially believed to be triggered by an MPT, it has now been established that release of cytochrome *c* can occur in the absence of mitochondrial membrane depolarization, and of an MPT [24–26,46,55]. Since cytochrome *c* is located on the outer side of the inner mitochondrial membrane, its release into the cytosol only requires that it is detached from the binding sites, and that a channel is formed in the outer mitochondrial membrane. It is, therefore, of interest that phosphorylation of Bad, a proapoptotic member of the Bcl-2 family of proteins, has been reported to regulate the mitochondrial release of cytochrome *c* by an effect on VDAC, the voltage-dependent anion channel in the outer mitochondrial membrane [13,49].

It is clear from these arguments that pharmacological agents which improve mitochondrial function in the recirculation periods do not necessarily act directly on mitochondrial membranes but may do so by indirect effects, e.g. by influencing a variety of mediators, such as the Bcl-2 class of proteins, which then act by regulating mitochondrial membrane permeability. Since any such effect seems to be exerted during the first 4–6 h of recirculation it seems less likely that changes in gene expression are involved. A more likely cause is a change in phosphorylation of proteins.

At least tentatively, therefore, the present results can be interpreted in light of these observations and considerations. Thus, any of the two drugs tested in the present experiments, or those studied previously (PBN and FK506) could act by influencing mitochondrial membrane permeability, either directly (like CsA, which blocks the MPT pore) or indirectly, by influencing the expression of gene products which modulate mitochondrial membrane permeability, such as Bad (see above). Furthermore, since cytochrome *c* can be released via a pore in the outer mitochondrial membrane it is not necessary to invoke the assembly of an MPT pore [1]. What speaks in favor of this assumption is that NXY-059, which does not block the pore *in vitro* (see Results), has been shown to upregulate the expression of Akt, a kinase which influences the phosphorylation of Bad [Yoshimoto et al., in preparation].

In summary, the present results have shown that the antiischemic spin trapping agent NXY-059, when given after 1 h of recirculation following 2 h of focal ischemia, ameliorates the mitochondrial dysfunction which is observed *in vitro* during the first 6 h of recirculation, and reduces the release of cytochrome *c* from mitochondria to cytosol after 6 and 24 h of recirculation. Since CsA had similar effects, when injected into the carotid artery, the results suggest that both drugs act, directly or indirectly, to preserve postischemic mitochondrial function. However, in contrast to CsA, NXY-059 does not accentuate the antiis-

chemic effect when given after 5 min of recirculation and it does not block a Ca^{2+} induced MPT in vitro. Clearly, it seems justified to further explore by what mechanisms anti-ischemic drugs preserve mitochondrial function and structural integrity.

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