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Early-onset tolerance in rat global cerebral ischemia induced by a mitochondrial inhibitor

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Abstract

It was studied whether a subtoxic dose of the mitochondrial neurotoxin, 3-nitropropionic acid (3-NPA), can initiate early-onset tolerance induction for subsequent ischemic injury. Wistar rats were pretreated for 3 h by intraperitoneal 3-NPA (20 mg/kg body weight; $n = 13$) or solvent ($n = 12$). Fifteen minutes global cerebral ischemia was induced by bilateral carotid artery occlusion and hypobaric hypotension. rCBF and tissue hemoglobin oxygen saturation were measured by laser Doppler scanning and a microspectrophotometric method. Ischemic insult and brain temperature were identical in both groups. Body weight and neurological scores recovered in the pretreated group but further deteriorated in the non-treated group ($P < 0.05$). Quantitative histology demonstrated a better neuronal density in neocortex and hippocampal CA2, CA3, and CA4 of pretreated animals ($P < 0.05$).

Keywords: 3-Nitropropionic acid; Preconditioning; Tolerance induction; Global cerebral ischemia; Rat

A brief ischemic episode can induce neuroprotection against subsequent severe ischemia. Pretreatment with 2-min of forebrain ischemia 3–4 days prior to 5-min bilateral carotid artery occlusion has been reported to protect CA1 neurons from delayed death in gerbil brain [8,9]. Here, pretreatment with a mitochondrial inhibitor, 3-nitropropionic acid (3-NPA), is used to induce tolerance to global cerebral ischemia in rats. 3-NPA is a fungal toxin which causes irreversible inhibition of succinate dehydrogenase, a part of both the Krebs cycle and complex II of the mitochondrial electron transport chain [2]. Hamilton and Gould [4] studied the toxicity of repeated high doses of 3-NPA in rats and found lesions in caudate-putamen, hippocampus and thalamus, but not in the cerebral cortex. Recently, Riepe et al. [12] reported that a low dosage of 3-NPA (20 mg/kg) can protect mitochondrial oxidation in rat hippocampal slices by a mechanism similar to preconditioning. Studies of the time course of 3-NPA induced tolerance to hypoxia showed that functional recovery of the population spike amplitude in hippocampal slices were better after hypoxia if a single i.p. injection of 3-NPA had been applied up to 4 days prior to hypoxia [13]. Recently the preconditioning effect of 3-NPA has been confirmed in mice, where 20 mg/kg 3-NPA were more efficient than 5, 10 or 40 mg/kg [5]. In the gerbil only a lower dose (3 but not 10 mg/kg) appears effective to induce tolerance [17]. Moreover, in that report using the gerbil 5 min global ischemia model hippocampal protection was only seen 2–3 days after 3-NPA but not after 1 day [17]. In focal cerebral ischemia 3-NPA only induced tolerance if applied 3 days before ischemia but not after preconditioning intervals of 15 min, 12 h, 24 h, 2 days or 5 days [18]. The published data are hence inconclusive as to the onset of protection afforded by 3-NPA pretreatment. Aim of the present study, therefore, was to investigate whether 3-NPA in vivo can induce early-onset tolerance - i.e. after 3 h - against global cerebral ischemia of longer duration as suggested by the initial ex vivo study [13].

To do so, male Wistar rats (270–370 g) were pretreated with 3-NPA (20 mg/kg b.w. intraperitoneally (i.p.), dissolved in 1.8 ml pure H_2O with 200 μl 1 M NaOH; $n = 13$) or only physiological saline (adjusted to the same

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pH as used for treated animals; $n = 12$) in a blinded protocol. Seven additional rats served as sham-operated controls. Clinical observation showed that all rats given the drug or solvent i.p. reacted normally including gait and spontaneous movement, and clinical signs of acute intoxication such as uncoordinated gait or padding movement were not apparent, although twelve rats (three in the non-treated group and nine rats in the treated group) were moderately inactive. One hour after drug application, the animals were premedicated with 0.5 mg atropine and anesthetized by i.p. injection of chloral hydrate (36 mg/100 g b.w.). During the experiment, spontaneous ventilation was maintained, and rectal temperature was controlled at 37°C. Carotid arteries were exposed bilaterally and a 6–0 monofilament thread was looped around the right carotid artery for later occlusion. A polyethylene catheter was inserted into the left carotid artery for continuous registration of arterial blood pressure and blood gases. PaO_2, $PaCO_2$, and arterial pH were measured using an ABL3 blood gas analyzer (Radiometer). Rats were mounted on a stereotactic frame. A cranial window (3 × 4 mm) was drilled over the right fronto-parietal region. During the craniotomy, the drill tip was cooled continuously with physiologic saline. The dura was left intact. Thereafter, saline with fixed temperature (37°C) was superfused continuously over the dura. Temperatures of the perfused saline and the temporal muscle were monitored.

Just 3 h after i.p. injection of 3-NPA or solvent, carotid arteries were occluded by attaching a 15 g weight to the loose carotid artery ligature, and mean arterial blood pressure (MABP) was reduced by hypobaric hypotension to 35 mmHg [14]. MABP was brought to the intended level immediately and was then maintained constant for 15 min during this plateau phase. MABP during induced hypotension dropped to 36.4 ± 2.5 mmHg from the baseline 82.5 ± 10.8 mmHg in the non-treated group, and to 36.6 ± 1.5 mmHg from the baseline 80.8 ± 12.5 mmHg in the treated group, respectively. After 15 min the right carotid ligature was removed, and MABP allowed to recover. MABP returned to the control level immediately and maintained that level thereafter in both groups. MABP was not significantly different between both groups throughout the experiment. Physiological variables likewise showed no significant changes of blood gases and temporal muscle temperature before and after induced hypotension and between groups (Table 1).

Local cerebral blood flow (lCBF) was measured by a Vasomedics laser flow blood perfusion monitor (model BPM 403a; Vasomedics) using a 0.8 mm needle probe. lCBF is expressed in LD units. Hb SO_2 was determined by microlightguide spectrophotometry (EMPHO II, Bodenseewerk Geratetechnik GmbH) [10]. lCBF and Hb SO_2 were measured at 25 (5 × 5) locations in a scanning procedure by means of a stepping-motor driven and computer-controlled micromanipulator [7]. The calculation of median rCBF values from these locations demonstrated a sudden drop during ischemia to 13.2 ± 7.9 LD-units from the control 39.4 ± 9.8 LD-units in the non-treated group, and to 11.1 ± 5.5 LD-units from 36.4 ± 12.3 LD-units in the treated group. rCBF remained low for 15 min during induced hypotension without statistical difference between both groups. rCBF then recovered above the original level in both groups. rCBF in the non-treated group increased to a maximum 72.5 ± 23.3 LD-units 30 min after induced hypotension ($P < 0.05$ vs. flow at that time point in treated group) and then normalized, whereas in the treated group it had an earlier maximum during the initial 15 min (52.2 ± 25.7 LD-units, not significantly different from maximal flow in the untreated group) and then returned to the control level (40.5 ± 26.0 LD-units). Hence, postischemic hyperperfusion occurred earlier in the treated group. In a previous study in gerbils we could show that animals with a better outcome also had earlier and more extensive hyperperfusion than animals with a bad histological outcome [16]. In those experiments hyperperfusion lasted longer in animals with a bad outcome as an indication that with protection the reperfusion began earlier and was more efficient [16]. In the current study, the hyperperfusion phase again was completed earlier in 3-NPA treated animals, suggesting that chemical preconditioning enabled the cerebral parenchyma to cope with cerebral ischemia more easily. The calculation of median tissue $HbSO_2$ values from the 25 locations in each animal revealed a sudden drop in accordance with rCBF to 24.4 ± 14.2% from a baseline at 52.8 ± 6.1% in non-treated animals, and to 28.1 ± 11.9% from baseline 54.1 ± 16.0% in treated animals. Tissue $HbSO_2$ in both groups returned to the original level with reperfusion where it remained till the end of the experiment. There were no significant differences between the groups during the experiment.

After this acute phase of the experiments the rats were observed for 4 days. Two non-treated rats died, one at day 1

Table 1
Data from arterial blood gas analyses and temporal muscle temperature (TMT), sampled before (= baseline) and after (= recirculation) carotid occlusion with hypotension[a]

Parameter	Treated ($n = 13$)	Non-treated ($n = 12$)
Baseline		
TMT (°C)	35.9 ± 0.36	35.7 ± 0.47
PO_2 (mmHg)	79.1 ± 5.5	84.7 ± 9.8
PCO_2 (mmHg)	46.2 ± 3.2	45.8 ± 4.4
pH	7.34 ± 0.04	7.33 ± 0.06
Recirculation		
TMT (°C)	35.3 ± 0.48	35.9 ± 0.36
PO_2 (mmHg)	83.4 ± 4.6	79.5 ± 12.8
PCO_2 (mmHg)	45.5 ± 5.2	45.9 ± 4.5
pH	7.31 ± 0.06	7.34 ± 0.05

[a] Values are expressed as means ± SD. There are no statistical differences between the groups and between baseline and recirculation.

and another at day 2 after the operation, whereas all treated rats survived. Non-treated animals continued to loose body weight reaching 76.2 ± 2.8% on day 4, when body weight in the treated group began to normalize and had increased to 84.7 ± 4.6%. Accordingly, differences of body weight between the groups became significant on the final day ($P < 0.001$).

Motor performance of the animals was examined daily with the inclined screen test, the balance beam test, and the prehensile-traction test [3]. Each test was carried out three times, and results averaged. In the prehensile-traction test a nylon rope, 700 mm long with a diameter of 5 mm, was stretched horizontally over a case with a sponge pad on its bottom. The rat was permitted to grab the rope with its forepads and was then released. The time on the rope was measured. The score of the prehensile traction test was significantly different between groups on day 3 ($P < 0.029$) and day 4 ($P < 0.041$), and so was that of the inclined screen test on day 3 after the operation ($P < 0.019$) (Table 2). In the inclined screen test a 300 × 300 mm board covered with cork was mounted on a pole. The trial started after placing the rat on the horizontal board. By rotating the pole slowly the plane was inclined to a maximum angle of 60°. The angle at which the rat fell off the board was recorded.

The balance beam test, where the time the rats could remain on a wooden rod (25 mm wide) was recorded, showed only a moderate worsening after global ischemia in both groups, without significant differences between the groups (data not shown).

Four days after operation, the animals were perfusion-fixed with 4% paraformaldehyde, brains embedded in paraffin, and sections stained with hematoxylin-eosin and cresyl violet. Light microscopic images were projected onto the screen of an Amiga 2000 computer (Commodore) using a color CCD-IRIS camera (Sony) and a genlock interface. Calibrated grids were superimposed over the video image for histomorphometry [14]. The number of intact neurons in the hippocampal CA1, CA2, CA3 and CA4 subsectors and the parietal cortex were counted at 2.0–3.0 mm posterior from bregma, and the density of neurons per mm^2 of the pyramidal cell layer was determined in continued blinded fashion [14]. Average neuron counts of the left and right hippocampi were determined for each animal. Ischemia caused significant neuronal losses in all examined brain regions of both groups as compared to the sham-operated group ($P < 0.05$, Table 3). Neuronal density in CA2, CA3 and CA4 sectors of the hippocampus was significantly higher in the treated than in the non-treated group. For quantification of surviving neurons in parietal cortex, four adjoining frames (0.28 × 0.16 mm) were located on the extension of the landmark of the CA1 sector. Significant differences were found in the superficial frames 1 and 2, which included cortical lamina I–IV. Hence, maximal neuroprotection was achieved in the upper cortical layers and in the hippocampal CA2 sector. In the hippocampal CA1 sector a similar trend was observed, however, without reaching statistical significance. This might correspond to the well-known selective vulnerability of hippocampal region CA1 which makes this sector sensitive to much shorter episodes of ischemia than used in the current protocol. After 5 min of ischemia in the gerbil, Sugino et al. [17] found neuroprotection after 3-NPA particularly in the CA1 sector. Fifteen minutes of global cerebral ischemia might be too long to be tolerated by vulnerable CA1 neurons even

Table 2
Motor scores performed daily before (day 0) and after ischemia (days 1–4)[a]

	Treated ($n = 13$)	Non-treated ($n = 12$)	Significance
Prehensile traction test (s)			
Day 0	18.0 ± 4.9	19.1 ± 5.6	N.S.
Day 1	15.2 ± 6.0	14.8 ± 6.1	N.S.
Day 2	15.6 ± 6.1	15.2 ± 7.4	N.S.
Day 3	18.5 ± 6.2	11.2 ± 5.8	$P < 0.029$
Day 4	19.2 ± 7.4	12.4 ± 5.6	$P < 0.041$
Inclined screen test (° from horizontal)			
Day 0	59.2 ± 6.1	61.6 ± 5.8	N.S.
Day 1	50.8 ± 7.3	49.8 ± 11.4	N.S.
Day 2	51.1 ± 8.5	50.5 ± 6.9	N.S.
Day 3	54.9 ± 7.0	45.3 ± 8.0	$P < 0.019$
Day 4	53.9 ± 9.3	46.9 ± 17.5	N.S.

[a] Values are expressed as means ± SD. N.S., not significant.

Table 3
Neuronal density (/mm^2) in the hippocampal CA1, CA2, CA3, and CA4 sector and in cerebral cortex (Frames 1–4)[a]

	Sham-operated	Treated	Non-treated
Hippocampus			
CA1	4326.3 ± 406.4	3042.1 ± 531.2† (70.3 ± 12.3%)	2573.7 ± 705.0† (59.5 ± 16.3%)
CA2	1956.6 ± 242.0	1397.5 ± 251.6*† (71.4 ± 12.9%*)	1001.8 ± 363.3† (51.2 ± 18.6%)
CA3	2041.8 ± 152.7	1495.1 ± 304.8*† (73.2 ± 14.9%*)	1181.4 ± 376.8† (57.9 ± 18.5%)
CA4	1290.1 ± 169.8	1031.1 ± 206.3*† (79.8 ± 16.0%*)	798.9 ± 244.7† (61.9 ± 18.9%)
Cortex			
Frame1	1469.2 ± 129.0	978.8 ± 133.1*† (66.6 ± 9.1%*)	689.0 ± 181.6† (46.9 ± 12.3%)
Frame2	1252.4 ± 195.1	855.7 ± 212.4*† (68.3 ± 16.9%*)	620.7 ± 213.5† (49.6 ± 17.0%)
Frame3	714.2 ± 27.2	542.0 ± 85.2† (75.8 ± 11.9%)	521.4 ± 128.0† (73.0 ± 17.9%)
Frame4	1041.9 ± 183.2	835.9 ± 170.9† (80.2 ± 16.4%)	763.0 ± 150.8† (73.2 ± 14.5%)

[a] Numbers in brackets: surviving neurons (%) as compared to sham-operated animals. Values are expressed as means ± SD. *$P < 0.05$ vs. non-treated animals, $^{\dagger}P < 0.05$ vs. sham-operated animal.

after chemical preconditioning with 3-NPA in the current dosage and time frame. Alternatively the time course for neuroprotection has to be taken into account. Kato et al. [6] reported that a 5 min, 1 h and 6 h interval between a 2 min preconditioning ischemia and 3 min occlusion of bilateral common carotid artery in gerbils could not protect the CA1, but even aggravated neuronal damage. Protection was effective 1–7 days after the first preconditioning, and disappeared 14 days thereafter. Very recently, however, rapid tolerance induction within 30 min by brief focal ischemia has been reported which did not persist longer than 2 h [15]. In the present study 3-NPA induced ischemic tolerance also with an early onset, with a 3 h interval between pretreatment and global cerebral ischemia, which confirms results from the earlier ex-vivo studies [12,13]. However, our own data indicate that in the rat 15 min ischemia model 3-NPA induces sustained tolerance also after 1 day pretreatment [1]. Therefore we have to assume that 3-NPA induces long lasting tolerance with an early onset. Future studies will have to investigate in more detail the time pattern and the molecular mechanisms of protection by 3-NPA. Moreover it remains to be shown whether early-onset protection persists chronically since Perez-Pinzon et al. [11] recently have shown a loss of the protective effect of preconditioning with postischemic observation time.

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