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The Antibiotic Erythromycin Induces Tolerance against Transient Global Cerebral Ischemia in Rats (Pharmacologic Preconditioning)

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Background: **Cerebral ischemic tolerance can be induced by a variety of noxious stimuli, but no clinically applicable regimen for preconditioning has been described. Therefore, the authors tested the ability of a pharmacologic preconditioning strategy using the well-known macrolide antibiotic erythromycin to induce tolerance against transient global cerebral ischemia *in vivo*. They also investigated whether tolerance induction by erythromycin involves transcriptional and translational changes of cerebral B-cell leukemia/lymphoma-2 (bcl-2) expression.**

Methods: **Male Wistar rats were treated with erythromycin (25 mg/kg intramuscularly) or vehicle and subjected to 15 min of transient global cerebral ischemia 6, 12, or 24 h after pretreatment. Neurologic deficit was evaluated once daily, and neuronal cell survival was assessed after 7 days of reperfusion. Additional animals were similarly pretreated, and cerebral bcl-2 messenger RNA (mRNA) and protein expression was analyzed 6 and 24 h later.**

Results: **Erythromycin improved postischemic neuronal survival in hippocampal CA1 and CA3 sectors and reduced functional deficit, with 12 h being the most efficient pretreatment interval. Bcl-2 mRNA in hippocampus was transiently up-regulated 6 h after erythromycin, but neuronal Bcl-2 protein remained unchanged.**

Conclusions: **Erythromycin can induce cerebral ischemic tolerance *in vivo* (pharmacologic preconditioning), suggesting a potential clinical strategy of preemptive neuroprotection. Changes in bcl-2 expression after erythromycin were small and transient. The induction of bcl-2–related pathways, although important for other preconditioning regimens, may therefore be less relevant for the neuroprotective effects of pharmacologic preconditioning using erythromycin.**

CELL death after ischemia can be decreased by preischemic conditioning that induces ischemic tolerance. Numerous preconditioning stimuli have been shown to be effective, among them brief episodes of ischemia,[1–5] hypoxia,[6] or hyperthermia[7,8] and low doses of the endotoxin lipopolysaccharide[9] or the mitochondrial toxin 3-nitropropionic acid.[10–12] Unfortunately, none of these regimens are suitable for clinical use, because of their serious side effects. A recent observation that pretreatment with a clinically used drug, the macrolide antibiotic erythromycin, can ameliorate tolerance against hypoxia *in vitro* [13] therefore prompted us to investigate whether erythromycin preconditioning may also exert long-term protective effects in an *in vivo* model of global cerebral ischemia. We also intended to identify the optimal time interval for such an intervention by comparing different pretreatment/injury intervals and their effects on outcome.

In addition, we wanted to elucidate whether up-regulation of B-cell leukemia/lymphoma-2 (bcl-2), which has been implicated in, for example, ischemic,[14] hypoxic,[15] or chemical[10] preconditioning, is also part of the mechanism of action of this novel pharmacologic method of preconditioning. Therefore, experiments were conducted in parallel to test for early and late effects of erythromycin treatment on cerebral expression of bcl-2 messenger RNA (mRNA) and protein.

This article is featured in "This Month in Anesthesiology." Please see this issue of ANESTHESIOLOGY, page 5A.

Materials and Methods

Experimental Protocol and Groups

The experimental protocol was approved by the local Animal Care Committee (Bezirksregierung Rheinhessen-Pfalz, Neustadt an der Weinstrasse, Germany). Male Wistar rats (270–370 g; Charles River, Kisslegg, Germany) were injected with 25 mg/kg erythromycin lactobionate[13] (a soluble salt of the macrolide antibiotic erythromycin suitable for intravenous and intramuscular application, Erythrocin; Abbott GmbH & Co. KG, Wiesbaden, Germany; estimated half life in rats approximately 2 h),[16,17] or with vehicle (normal saline, 0.9%) intramuscularly. One cohort of animals served to test the hypothesis that erythromycin pretreatment is able to induce neuronal tolerance against the effects of global cerebral ischemia. A second cohort was studied in parallel to assess the effects of erythromycin on cerebral bcl-2 mRNA and protein expression.

Cohort 1. Transient global cerebral ischemia was induced 6, 12, or 24 h after pretreatment with erythromycin or vehicle. After a power analysis, 56 animals were randomized to the respective groups, with n = 12 for

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the 6-h and n = 8 for the 12- and 24-h pretreatment paradigms (vehicle and erythromycin, respectively). Four animals (vehicle pretreated, n = 3; erythromycin pretreated, n = 1) had to be excluded during the recovery period, because of "no reflow" (n = 1), no electrophysiologic recovery (n = 1), or premature death during the first 24 h after the injury (n = 2). Animals recovered for 7 days after the injury, and neurologic deficit was assessed. On day 7, animals were killed and neuronal survival was assessed. Sham-operated (n = 6) and naive (n = 6) animals served as controls.

Cohort 2. In parallel, 18 animals were treated for the bcl-2 mRNA expression study and were killed 6 h (vehicle, n = 4; erythromycin, n = 4) or 24 h (n = 5, respectively) after treatment. Bcl-2 protein expression was assessed in three groups of animals killed 6 h after erythromycin (n = 7) or vehicle (n = 7) or 24 h after erythromycin (n = 7).

All animals were randomized to treatment groups, and investigators were blinded to group assignment throughout the experiments and until full completion of the data acquisition.

Transient Global Cerebral Ischemia

Animals were fasted overnight before surgery. Fifteen minutes of transient global ischemia was achieved using bilateral carotid artery occlusion and hypobaric hypotension, as previously described in detail.[10,18] In brief, rats were anesthetized (chloral hydrate, 360 mg/kg intraperitoneally), intubated, and mechanically ventilated. Both carotid arteries were exposed, and the left side was catheterized for blood pressure monitoring (complete vessel occlusion). For the brain injury, the right carotid artery was occluded by a thread wrapped around it and pulled by a 5-g weight. Fifteen minutes of global cerebral ischemia was achieved by the simultaneous reduction of mean arterial blood pressure to 35 mmHg using the hypobaric hypotension technique.[18,19] Ischemia was verified by laser-Doppler monitoring as previously described (repetitive regional cerebral blood flow measurements at 30 different locations [scanning technique] over the right hemisphere [closed cranial window, 24 mm^2, 1.3–5.3 mm lateral to the right and 1.5–7.5 mm occipital of the bregma]; stationary laser-Doppler flow probe [local cerebral blood flow] over the left hemisphere [closed cranial window, 4 mm^2, 3.3–5.3 mm lateral to the left and 5.0–7.0 mm occipital of the bregma][10,20,21]). After 15 min of ischemia, the nylon thread was removed and hypobaric hypotension was terminated to allow reperfusion of the brain. Rectal temperature was controlled to 37.5° ± 0.1°C using a thermostatically regulated warming blanket. Peribrain temperature was recorded at two different sites (right temporal muscle, left auricular tube) and controlled to 37°C using a near-infrared heating lamp throughout the experiment, as previously described.[22] After 90 min of reperfusion, the carotid artery catheter was removed, incisions were closed, and the animals were extubated and returned to their cage. Animals randomized to sham-operation were treated in the same way, except ischemia was not induced.

Neurologic Deficit

A neurologic evaluation was performed once daily (day −1, *i.e.*, the day before the injury [baseline] through day 7) at the same time (around 5:00 PM) by the same investigator, who was unaware of the group assignment. Consciousness, breathing, smell, vision and hearing, reflexes, motor function, overall activity, orientation, and presence of seizures were scored, according to a neurologic deficit score (0–100 scale; 0 = no deficit, 100 = most severe deficit; adapted from Katz *et al.*,[23] Neumar *et al.*,[24] and Nakase *et al.*[25]; table 1). Comparable scoring systems using the same or very similar items have previously been used in our laboratory,[10] as well as by others.[26–29] They allow the identification of neurobehavioral deficits after ischemic brain injury and were found to correlate well with other outcome end points, *e.g.*, electrophysiologic recovery or histopathologic damage.[10,26–29] All randomized animals were evaluated at baseline (day −1) to ensure a normal neurologic score.

Neurohistopathologic Evaluation

Neuronal cell density (number of viable neurons/mm^2) within parietal neocortex and hippocampus (CA1, CA2, CA3, CA4) was assessed in hematoxylin and eosin–stained coronal brain slices using a video microscope in conjunction with a computer system as previously described[18] and averaged for both hemispheres. The investigator was unaware of treatment assignment, and all brain slices evaluated were coded to ensure an unbiased cell counting and data evaluation.

Real-time Reverse-transcription Polymerase Chain Reaction Analysis

Anesthetized rats (chloral hydrate, 360 mg/kg intraperitoneally) were decapitated; hippocampus and neocortex were dissected and snap-frozen. Tissue samples were homogenized for each animal, and total RNA was extracted.[30] First-strand complementary DNA was synthesized after DNase I digestion (Invitrogen, Carlsbad, CA). Real-time quantitative polymerase chain reaction was performed for bcl-2 and glyceraldehyde-3-phosphate-dehydrogenase on a LightCycler® (Roche Molecular Biochemicals, Mannheim, Germany) thermocycler, using FastStart SYBR Green® PCR Reagents (Roche). Bcl-2 expression was normalized to glyceraldehyde-3-phosphate-dehydrogenase and analyzed with RelQuant®

Table 1. Neurologic Deficit Score

Function Studied	Grades and Points
Consciousness	Normal (0) Somnolent (5) Stuporose (10) Comatose (15)
Sense of smell	Normal (withdraws from strong smells, 0) Pathologic (2)
Vision	Normal (withdraws from strong light, 0) Pathologic (2)
Hearing	Normal (withdraws from loud noise, 0) Pathologic (2)
Breathing	Normal (0) Hypoventilation/hyperventilation (10)
Trigeminal nerve reflex	Normal (withdraws from touch to whiskers, 0) Pathologic (2)
Corneal reflex	Normal (0) Pathologic (2)
Ability to grab the edge of a bench	Normal (0) Absent (2)
Time animal holds on to a rope	21–30 s (0) 11–20 s (2) 0–10 s (4)
Ability to walk	Normal (0) Minimal ataxia (5) Moderate ataxia, tends to fall (10) Unable to walk (15) Unable to sit or stand upright (20)
Ability to walk on a wire grate	Normal (0) Weakness of one limb (2) Weakness of more than one limb (4)
Exploration of new environment	Normal (0) Moves in circles, unable to find way around objects (6) No spontaneous movement (12)
Feeding	Normal (0) Absent (4)
Water intake	Normal (0) Absent (4)
Overall activity	Normal exploration of environment (0) Hypoactive/hyperactive (2) No activity (4)
Interaction with environment	Normal (0) Anxious, not interested (2) Apathetic (4)
Epileptic seizures	Absent (0) Present (7)

0 = best to 100 = worst; neurologic deficit was scored daily by a blinded investigator.

software (Roche). Polymerase chain reaction experiments on duplicate samples were repeated twice.

Immunohistochemistry for Bcl-2

Anesthetized animals (chloral hydrate, 360 mg/kg intraperitoneally) were perfused with freshly prepared ice-cold buffered paraformaldehyde. Brains were processed for immunohistochemical analysis as previously described.[10] Briefly, coronal brain slices (3 μm) were incubated with rabbit polyclonal anti–bcl-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized with diaminobenzidine (DAKO, Hamburg, Germany). Antibody specificity was confirmed omitting the primary antibody.

The intensity of bcl-2 immunoreactivity was evaluated using a semiquantitative grading procedure.[10,31] Neuronal staining in parietal neocortex, hippocampus (CA1, CA3, CA4), and dentate gyrus was graded on a scale ranging from 0 for no staining to 4 for strong immunoreactivity.

Statistical Analyses

Parametric data, *i.e.*, physiologic variables, neuronal cell densities, and bcl-2 mRNA expression are shown as mean ± SD and were found to be normally distributed.

Table 2. Physiologic Variables of Ischemia–Reperfusion Subgroups

	Baseline			15 min Ischemia			3 min Reperfusion			60 min Reperfusion		
	Vehicle (n = 25)	Erythro (n = 27)	Sham (n = 6)	Vehicle (n = 25)	Erythro (n = 27)	Sham (n = 6)	Vehicle (n = 25)	Erythro (n = 27)	Sham (n = 6)	Vehicle (n = 25)	Erythro (n = 27)	Sham (n = 6)
MABP, mmHg	75 ± 9	75 ± 8	75 ± 6	35 ± 1*	35 ± 1*	71 ± 6	71 ± 14	74 ± 14	78 ± 10	79 ± 8	80 ± 11	74 ± 5
Regional CBF, LD units	97 ± 24	88 ± 31	90 ± 26	2 ± 1*	2 ± 1*	85 ± 33	48 ± 38	44 ± 43	83 ± 29	43 ± 13*	41 ± 25*	91 ± 38
Arterial blood analysis												
pH	7.31 ± 0.03	7.34 ± 0.03	7.33 ± 0.04				7.29 ± 0.06	7.32 ± 0.04†	7.31 ± 0.04	7.28 ± 0.03	7.32 ± 0.03*†	7.25 ± 0.03
$Paco_2$, mmHg	40 ± 3	39 ± 4	41 ± 2				37 ± 5	33 ± 5*†	41 ± 5	39 ± 4	36 ± 4*	44 ± 7
Pao_2, mmHg	106 ± 11	104 ± 10	107 ± 10				141 ± 19*	139 ± 25*	109 ± 11	111 ± 11	120 ± 13*†	106 ± 10
Base excess, mEq/l	−5 ± 2	−4 ± 2	−4 ± 2				−8 ± 2*	−8 ± 3*	−6 ± 2	−7 ± 2	−7 ± 2	−7 ± 2
Serum glucose, mg/dl	79 ± 18	94 ± 33	91 ± 41				86 ± 26	96 ± 39	97 ± 21	109 ± 28	108 ± 40	94 ± 29
Serum lactate, mmol/dl	0.6 ± 0.4	0.7 ± 0.3	0.4 ± 0.2				1.8 ± 0.7*	1.9 ± 0.7*	0.4 ± 0.1	0.6 ± 0.3	0.8 ± 0.5*	0.3 ± 0.1
Temperature												
Rectal, °C	37.0 ± 0.1	36.9 ± 0.1	37.0 ± 0.1	35.3 ± 1.1*	35.6 ± 1.1*	37.0 ± 0.1	35.5 ± 1.0*	35.5 ± 0.9*	36.9 ± 0.1	37.0 ± 0.1	37.0 ± 0.1	37.0 ± 0.1
Temporal muscle, °C	36.8 ± 0.3	37.0 ± 0.1	37.0 ± 0.1	34.6 ± 1.3*	34.7 ± 0.9*	36.9 ± 0.1	35.0 ± 1.2*	35.2 ± 0.8*	36.8 ± 0.2	36.7 ± 0.3	36.8 ± 0.3	36.9 ± 0.2
Tympanic membrane, °C	36.7 ± 0.3	36.5 ± 0.5	36.8 ± 0.1	35.0 ± 0.7*	35.0 ± 0.6*	36.9 ± 0.1	35.1 ± 0.8*	35.2 ± 0.6*	36.9 ± 0.1	36.7 ± 0.3	36.4 ± 0.5*	36.9 ± 0.2

Between-group analysis (analysis of variance) did not detect significant differences; physiologic data from animals with the same treatment at different time points were therefore pooled. Values are mean ± SD.

* $P < 0.05$ *vs.* sham. † $P < 0.05$ *vs.* vehicle.

CBF = cerebral blood flow; LD = laser-Doppler; MABP = mean arterial blood pressure; $Paco_2$ = arterial carbon dioxide tension; Pao_2 = arterial oxygen tension.

For these data sets, one-way analysis of variance and *post hoc* Student-Newman-Keuls test was used to identify differences between experimental groups. Nonparametric data (*i.e.*, neurologic deficit score) were expressed as median ± 25th/75th percentiles, and groups were compared using the rank sum test. Significance was set at $P < 0.05$. Because of the semiquantitative character of the grading procedure for Bcl-2 protein expression, immunohistochemistry data were not statistically evaluated for differences between groups.

Results

Physiologic Variables during Ischemia–Reperfusion

At baseline, vital signs of all animals from the cohort were within normal limits, independent of treatment assignment. Upon initiation of global cerebral ischemia, physiologic variables changed as expected for all animals randomized to the injury, but normalized during early reperfusion. Cerebral blood flow ceased immediately after induction of bilateral carotid artery occlusion and hypobaric hypotension, and laser-Doppler flow measurements showed values reflective of biologic zero (5 ± 1 laser-Doppler units during the first minute and 2 ± 1 during the remaining 14 min). No apparent differences were found between treatment groups besides a lower partial pressure of carbon dioxide and corresponding pH during reperfusion in the erythromycin group (see table 2 for details).

Postischemic Neurologic Deficit with and without Erythromycin Pretreatment

All animals exhibited a moderate neurologic deficit on the first days after the ischemic injury. Deficit improved in all animals over time, although baseline (no neuronal deficit on the day before the brain injury; day −1) was not reached again during the postischemic observation period. Deficits were consistently less severe in animals that had been pretreated with erythromycin (fig. 1; $P < 0.05$ on days 4–7).

Postischemic Neuronal Death with and without Erythromycin Pretreatment

Hippocampal CA1 Region. With vehicle pretreatment (n = 25, no difference between treatment intervals; pooled), neuronal cell density was reduced by 75% seven days after transient global cerebral ischemia, as compared with sham-operated controls (n = 6). More neurons survived in animals treated with erythromycin before the injury. Erythromycin pretreatment 12 h be-

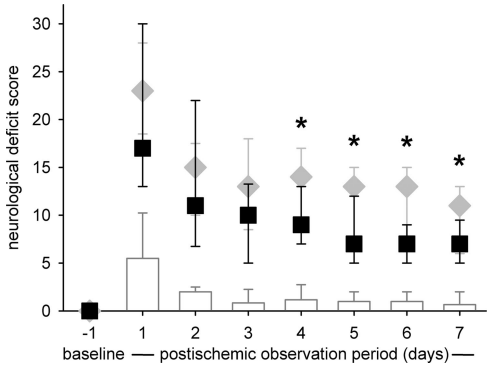

Fig. 1. Postischemic neurologic deficit of animals pretreated with erythromycin (25 mg/kg intramuscularly) 6, 12, or 24 h before the injury (*black squares*, n = 27, pooled) or with vehicle only (*gray diamonds*, n = 25, pooled). Erythromycin-pretreated animals had less neurologic deficit after global ischemia beyond the immediate postoperative recovery (days 4–7, $P < 0.05$). Data for sham-operated animals (*vertical bars*, n = 6) are provided for comparison. Data shown are median and 25th/75th percentiles. * $P < 0.05$ *versus* vehicle-treated animals; rank sum test.

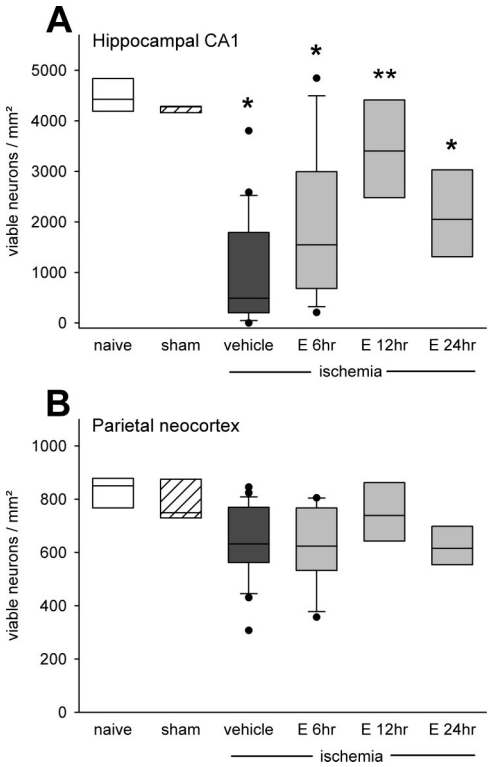

Fig. 2. Neuronal density (viable neurons/mm²) in hippocampal CA1 region (*A*) and parietal neocortex (*B*) of naive or sham-operated rats compared with neuronal density 7 days after 15 min of global cerebral ischemia with vehicle (n = 25, pooled) or erythromycin pretreatment (25 mg/kg intramuscularly) 6 h (n = 12), 12 h (n = 8), or 24 h (n = 7) before the injury. Erythromycin pretreatment increased the density of viable neurons after ischemia. * $P < 0.05$ *versus* sham operation; ** $P < 0.05$ *versus* vehicle pretreatment, analysis of variance and *post hoc* Student-Newman-Keuls test. E = erythromycin pretreatment.

fore ischemia increased the number of surviving neurons in CA1 by threefold, as compared with vehicle pretreatment ($P < 0.05$; fig. 2).

Parietal Neocortex. With vehicle pretreatment, approximately 20% of viable neurons in this brain region were lost 7 days after transient global cerebral ischemia (not significant, $P = 0.052$). In animals pretreated with erythromycin 12 h before the injury, 95% of neurons survived ($P = 0.066$ *vs.* vehicle; fig. 2).

Other Hippocampal Regions. Transient global cerebral ischemia with vehicle pretreatment caused a significant reduction of neuronal cell counts in CA2 and CA3 ($P < 0.05$, as compared with sham operation) but not CA4 subregions. Animals pretreated with erythromycin 12 h before ischemia had cell counts similar to sham-operated controls (table 3; $P < 0.05$ *vs.* vehicle in CA3).

Expression of Bcl-2 mRNA after Treatment with Erythromycin

Erythromycin injection was followed by an increased expression of bcl-2 mRNA in hippocampus (1.6-fold compared with vehicle; $P < 0.05$) and neocortex (1.9-fold; not significant) at 6 h but not at 24 h (fig. 3). We observed a spatial difference in bcl-2 mRNA expression between neocortex and hippocampus at 6 h but not at 24 h, independent of treatment assignment.

Table 3. Neuronal Cell Densities in Hippocampal CA2, CA3, and CA4 Regions

Group	n	CA2	CA3	CA4
Sham	6	2,271 ± 393	2,323 ± 199	797 ± 49
Vehicle	25	1,298 ± 728*	1,733 ± 385*	717 ± 209
E 6 h	12	1,445 ± 355	1,577 ± 345*	673 ± 141
E 12 h	8	2,003 ± 600	2,117 ± 354†	859 ± 148
E 24 h	7	1,619 ± 444	1,825 ± 260	703 ± 99

Values are mean ± SD, viable neurons/mm², postischemic day 7.
* $P < 0.05$ *vs.* sham. † $P < 0.05$ *vs.* vehicle.
E = erythromycin pretreatment.

Bcl-2 Protein Expression after Treatment with Erythromycin

Bcl-2 protein staining was strictly localized to the cytoplasm of the neurons evaluated (fig. 4). At baseline (6 h after vehicle treatment, n = 7), staining was virtually absent (grade 0) in dentate gyrus, faint (1) in CA1, mild (2) in CA4, and mild to moderate (2–3) in CA3 and the parietal neocortex. However, Bcl-2 immunoreactivity did not change at either 6 or 24 h after erythromycin treatment compared with vehicle (n = 7 each; fig. 5).

Discussion

The presented data show for the first time that a single dose of the antibiotic erythromycin, applied 6–24 h before transient global cerebral ischemia, induces tolerance and improves postischemic outcome, as measured by neurologic deficit and neuronal survival, in a well-

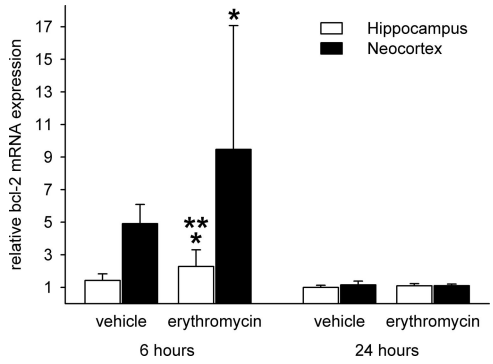

Fig. 3. Quantification of bcl-2 messenger RNA (mRNA) expression in nonischemic neocortex and hippocampus early (6 h) and late (24 h) after erythromycin (25 mg/kg intramuscularly, n = 4; n = 5) or vehicle (n = 4; n = 5), respectively. For easy comparison, bcl-2 mRNA levels are shown relative to hippocampal expression of vehicle-treated animals. Bcl-2 mRNA was increased 6 h but not 24 h after erythromycin treatment. * $P < 0.05$ *versus* 24 h. ** $P < 0.05$ *versus* vehicle-treated control. Data are mean ± SD; analysis of variance and *post hoc* Student-Newman-Keuls test.

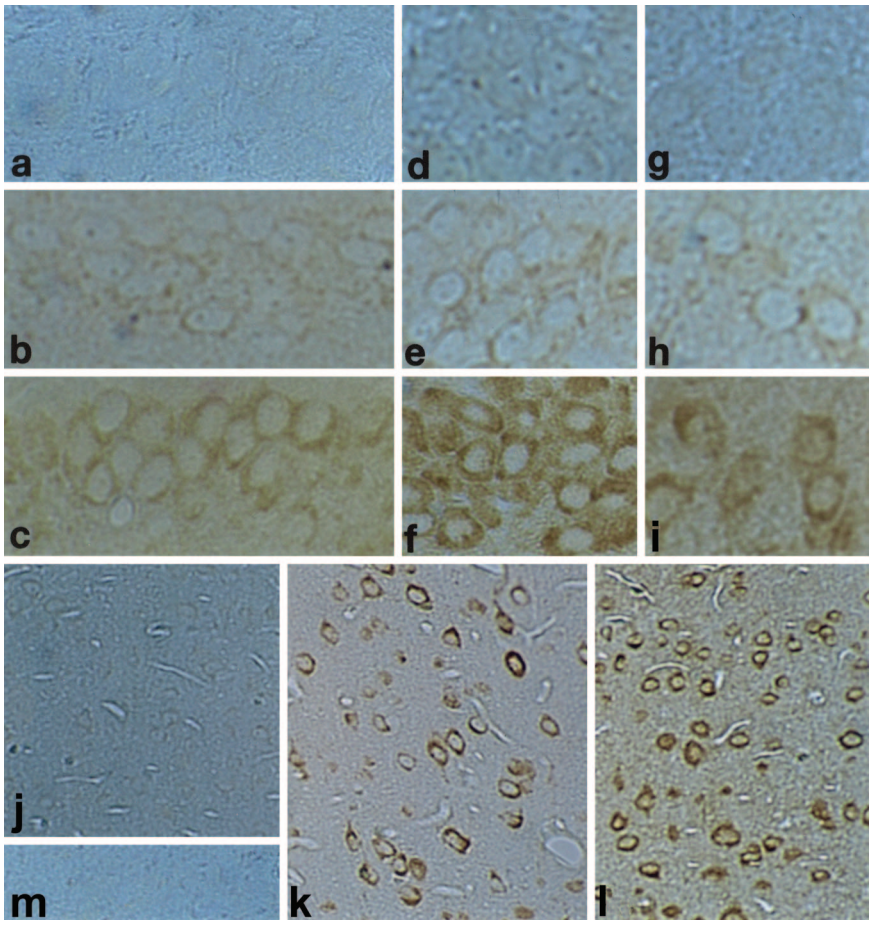

Fig. 4. Bcl-2 protein was localized in the cytoplasm of neurons in all brain regions evaluated (nonischemic tissue). CA1 neurons (magnification at 40×) at baseline (after vehicle treatment, *A*) and 6 h (*B*) and 24 h (*C*) after erythromycin treatment (25 mg/kg intramuscularly). Neurons in hippocampal CA3 (*D–F*) and CA4 (*G–I*) regions (40×) at baseline (*D*, *G*) and 6 h (*E*, *H*) and 24 h (*F*, *I*) after erythromycin. Neocortical neurons (20×) at baseline (*J*) and 6 h (*K*) and 24 h (*L*) after erythromycin. Negative controls, omitting the primary anti–Bcl-2 antibody, were included in each staining series (*M*).

controlled chronic rat model (pharmacologic preconditioning).

Histopathologic and Functional Outcome

The applied injury resulted in the significant histopathologic damage in ischemia susceptible brain regions, *i.e.*, hippocampal CA1, CA2, and CA3, 7 days after global ischemia, that is expected in this model.[19] The CA1 and CA3 regions were well protected by erythromycin pretreatment 12 h before the injury. In contrast, the same treatment did not render a statistical significant improvement in hippocampal CA2 and parietal neocortex. This may be due to the relatively smaller damage in these regions, compared with CA1. However, differences were close to statistical significance ($P = 0.052$–0.086), so small sample sizes may have prevented us from detecting protection in these regions. Alternatively, these findings could suggest region-specific differences in the neuroprotective potential of erythromycin preconditioning. Neuronal damage in hippocampal CA4 usually is small with the applied injury, limiting the potential to detect a significant neuroprotection in this region.

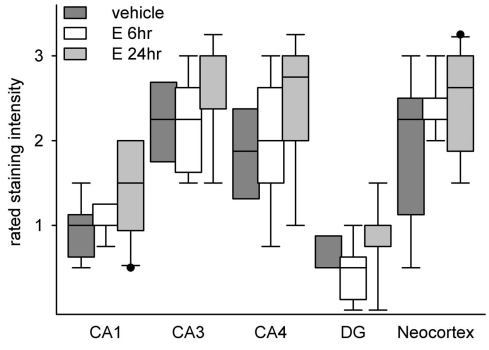

Fig. 5. Semiquantitative rating of immunohistochemical staining for Bcl-2 protein expression in different regions of hippocampus, dentate gyrus (DG), and parietal neocortex after vehicle (n = 7) or erythromycin treatment (25 mg/kg intramuscularly) 6 h (n = 7) or 24 h (n = 7) earlier. Bcl-2 protein expression increased only slightly after erythromycin, compared with vehicle injection. E = erythromycin pretreatment; vehicle = vehicle pretreatment (baseline).

Although neuronal density is a readily available tool to assess ischemic damage, neurologic deficit and function are even more relevant for clinical applications. The erythromycin-pretreated animals fared better throughout the entire 7-day observation period. This was significant in the second half of the follow-up period, at a time when animals have recovered from immediate surgical stress and residual anesthetic effects, and cerebral edema has vanished.

Our *in vivo* findings confirm previous *ex vivo* work on hippocampal slices, demonstrating tolerance against

hypoxia after erythromycin pretreatment.[13] The extent of hippocampal CA1 protection from ischemic damage that could be achieved by erythromycin pretreatment in our model (*i.e.*, tripling of the number of surviving neurons) was similar to, or even more pronounced than, the effects we observed earlier after a chemical preconditioning regimen using a single dose of 3-nitropropionic acid[10] (50% increase in surviving CA1 neurons). In animals pretreated with erythromycin 12 h before ischemia, 75% of CA1 neurons survived 7 days after ischemia, a survival rate that is comparable to the 70–80% surviving CA1 neurons found after classic ischemic preconditioning in rat models of global cerebral ischemia using four-vessel occlusion[32] or bilateral carotid artery occlusion with hemorrhagic hypotension.[33,34] Tolerance induction by the clinically used drug erythromycin may therefore be similarly effective as these earlier, more invasive regimens, but contrary to its predecessors, this new strategy lacks significant side effects and could therefore potentially be applied to human patients.

Previous work suggests that preconditioning has two windows of opportunity, one early (at approximately 3 h) and one late (24 h and beyond).[1,10,25,35–37] The effect of erythromycin preconditioning in our hands was maximal after a pretreatment interval of 12 h before the injury, somewhat earlier than the classic delayed preconditioning window. This is in accordance with *ex vivo* work that found brief pretreatment intervals to be most effective for tolerance induction by erythromycin.[13]

Bcl-2 Differential Expression

A small and transient increase of bcl-2 mRNA was noted early after erythromycin treatment. Expression of bcl-2 mRNA was increased 6 h but not 24 h after erythromycin in both neocortical and hippocampal specimens, but Bcl-2 protein did not increase in the same regions at either time point.

Baseline expression of both bcl-2 mRNA and Bcl-2 protein was higher in neocortical than in hippocampal (CA1) neurons. This correlates well with previous reports[10,31] and may in part explain the relatively higher intrinsic tolerance of cortical neurons against the applied ischemic injury in this model.

Potential Mechanisms of Pharmacologic Preconditioning Using Erythromycin

Erythromycin is known to inhibit mitochondrial protein synthesis[38–40] and allegedly reduces cell respiration by reducing the production of enzymes necessary for optimal function of the respiratory chain.[40,41] A functional impairment of mitochondrial oxidative phosphorylation after the application of a single dose of erythromycin might therefore cause a mild burst of reactive oxygen species in neuronal cells, which subsequently may induce gene expression of, for example, apoptosis-inhibiting bcl-2, similar to the mechanism that has been described for 3-nitropropionic acid.[10,42]

However, differential bcl-2 expression after erythromycin injection was transient and less pronounced than what we saw after 3-nitropropionic acid. It may therefore only partially explain the observed neuroprotection provided by the antibiotic.

On the other hand, erythromycin may possibly suppress the immunologic response that mediates damage after cerebral ischemia,[43–45] because this macrolide has been reported to influence several inflammatory mechanisms in other organ systems.[46,47] This concept is supported by recent reports on the reduction of postischemic cytokine expression and inflammation after cerebral preconditioning[48,49] and the beneficial effects of antibiotic treatment after experimental ischemia,[50] and certainly warrants further experimental work.

Limitations

Sample size is a potential limitation of our study. We had performed a power analysis to determine sample size before we started our study. However, besides profound protection by erythromycin in hippocampal CA1 and CA3, histopathologic analysis found differences between treatment groups in parietal neocortex and hippocampal CA2 that were very close to but did not reach statistical significance. Interpretation of these data are difficult because, by convention, there are no differences, but one is tempted to state a trend. Future studies in other models may help to decide whether tolerance induction by erythromycin is indeed region specific, and less pronounced in CA2 compared with CA1.

We assessed bcl-2 expression after a 6-h and a 24-h but not after a 12-h pretreatment interval. However, our previous work using a model of chemical preconditioning showed an early and sustained increase of bcl-2 expression starting at 3 h, most pronounced at 24 h after pretreatment.[10] We found only small and transient changes of bcl-2 mRNA, but not protein, expression early after erythromycin treatment. Therefore, we do not expect results to be different after 12 h. We therefore conclude that bcl-2 is not as relevant for tolerance induction by erythromycin as it is for other preconditioning regimens.

Conclusion

Erythromycin preconditioning improves neurologic function and neuronal survival after ischemia and is associated with a transiently increased cerebral expression of the antiapoptotic gene bcl-2 in rats. This neuroprotective effect of erythromycin suggests a potential clinical strategy for preischemic conditioning, which could be beneficial for patients scheduled to undergo surgical procedures associated with an increased risk of

perioperative brain ischemia, *e.g.*, in cardiovascular surgery or neurosurgery. Additional research is needed to determine the clinical role for this new method of preemptive neuroprotection and to further clarify the molecular mechanisms involved.

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References

1. Kitagawa K, Matsumoto M, Tagaya M: "Ischemic tolerance" phenomenon found in the brain. Brain Res 1990; 528:21–4
2. Chen J, Graham SH, Zhu RL, Simon RP: Stress proteins and tolerance to focal cerebral ischemia. J Cereb Blood Flow Metab 1996; 16:566–77
3. Simon RP, Niiro M, Gwinn R: Prior ischemic stress protects against experimental stroke. Neurosci Lett 1993; 163:135–7
4. Glazier SS, O'Rourke DM, Graham DI, Welsh FA: Induction of ischemic tolerance following brief focal ischemia in rat brain. J Cereb Blood Flow Metab 1994; 14:545–53
5. Miyashita K, Abe H, Nakajima T, Ishikawa A, Nishiura M, Sawada T, Naritomi H: Induction of ischaemic tolerance in gerbil hippocampus by pretreatment with focal ischaemia. Neuroreport 1994; 6:46–8
6. Gidday JM, Fitzgibbons JC, Shah AR, Park TS: Neuroprotection from ischemic brain injury by hypoxic preconditioning in the neonatal rat. Neurosci Lett 1994; 168:221–4
7. Chopp M, Chen H, Ho KL, Dereski MO, Brown E, Hetzel FW, Welch KM: Transient hyperthermia protects against subsequent forebrain ischemic cell damage in the rat. Neurology 1989; 39:1396–8
8. Ikeda T, Xia XY, Xia YX, Ikenoue T: Hyperthermic preconditioning prevents blood-brain barrier disruption produced by hypoxia-ischemia in newborn rat. Brain Res Dev Brain Res 1999; 117:53–8
9. Tasaki K, Ruetzler CA, Ohtsuki T, Martin D, Nawashiro H, Hallenbeck JM: Lipopolysaccharide pre-treatment induces resistance against subsequent focal cerebral ischemic damage in spontaneously hypertensive rats. Brain Res 1997; 748:267–70
10. Brambrink AM, Schneider A, Noga H, Astheimer A, Gotz B, Korner I, Heimann A, Welschof M, Kempski O: Tolerance-inducing dose of 3-nitropropionic acid modulates bcl-2 and bax balance in the rat brain: A potential mechanism of chemical preconditioning. J Cereb Blood Flow Metab 2000; 20:1425–36
11. Dawson DA, Furuya K, Gotoh J, Nakao Y, Hallenbeck JM: Cerebrovascular hemodynamics and ischemic tolerance: Lipopolysaccharide-induced resistance to focal cerebral ischemia is not due to changes in severity of the initial ischemic insult, but is associated with preservation of microvascular perfusion. J Cereb Blood Flow Metab 1999; 19:616–23
12. Deplanque D, Bordet R: Pharmacological preconditioning with lipopolysaccharide in the brain. Stroke 2000; 31:1465–6
13. Huber R, Kasischke K, Ludolph AC, Riepe MW: Increase of cellular hypoxic tolerance by erythromycin and other antibiotics. Neuroreport 1999; 10: 1543–6
14. Maulik N, Engelman RM, Rousou JA, Flack JE III, Deaton D, Das DK: Ischemic preconditioning reduces apoptosis by upregulating anti-death gene Bcl-2. Circulation 1999; 100:II369–75
15. Bossenmeyer-Pourie C, Koziel V, Daval JL: Effects of hypothermia on hypoxia-induced apoptosis in cultured neurons from developing rat forebrain: Comparison with preconditioning. Pediatr Res 2000; 47:385–91
16. Girard AE, Girard D, English AR, Gootz TD, Cimochowski CR, Faiella JA, Haskell SL, Retsema JA: Pharmacokinetic and *in vivo* studies with azithromycin (CP-62,993), a new macrolide with an extended half-life and excellent tissue distribution. Antimicrob Agents Chemother 1987; 31:1948–54
17. Duthu GS: Interspecies correlation of the pharmacokinetics of erythromycin, oleandomycin, and tylosin. J Pharm Sci 1985; 74:943–6
18. Brambrink AM, Kopacz L, Astheimer A, Noga H, Heimann A, Kempski O: Control of brain temperature during experimental global ischemia in rats. J Neurosci Methods 1999; 92:111–22
19. Soehle M, Heimann A, Kempski O: Postischemic application of lipid peroxidase inhibitor U-101033E reduces neuronal damage after global ischemia in rats. Stroke 1998; 29:1240–6
20. Heimann A, Kroppenstedt S, Ulrich P, Kempski OS: Cerebral blood flow autoregulation during hypobaric hypotension assessed by laser Doppler scanning. J Cereb Blood Flow Metab 1994; 14:1100–5
21. Soehle M, Heimann A, Kempski O: On the number of measurement sites required to assess regional cerebral blood flow by laser-Doppler scanning during cerebral ischemia and reperfusion. J Neurosci Methods 2001; 110:91–4
22. Brambrink AM, Kopacz L, Astheimer A, Noga H, Heimann A, Kempski O: Control of brain temperature during experimental global ischemia in rats. J Neurosci Methods 1999; 92:111–22
23. Katz L, Ebmeyer U, Safar P, Radovsky A, Neumar R: Outcome model of asphyxial cardiac arrest in rats. J Cereb Blood Flow Metab 1995; 15:1032–9
24. Neumar RW, Bircher NG, Sim KM, Xiao F, Zadach KS, Radovsky A, Katz L, Ebmeyer E, Safar P: Epinephrine and sodium bicarbonate during CPR following asphyxial cardiac arrest in rats. Resuscitation 1995; 29:249–63
25. Nakase H, Heimann A, Uranishi R, Riepe MW, Kempski O: Early-onset tolerance in rat global cerebral ischemia induced by a mitochondrial inhibitor. Neurosci Lett 2000; 290:105–8
26. Schmitz B, Bottiger BW, Hossmann KA: Functional activation of cerebral blood flow after cardiac arrest in rat. J Cereb Blood Flow Metab 1997; 17:1202–9
27. Geocadin RG, Ghodadra R, Kimura T, Lei H, Sherman DL, Hanley DF, Thakor NV: A novel quantitative EEG injury measure of global cerebral ischemia. Clin Neurophysiol 2000; 111:1779–87
28. Geocadin RG, Muthuswamy J, Sherman DL, Thakor NV, Hanley DF: Early electrophysiological and histologic changes after global cerebral ischemia in rats. Mov Disord 2000; 15 (suppl 1):14–21
29. Vogel P, Putten H, Popp E, Krumnikl JJ, Teschendorf P, Galmbacher R, Kisielow M, Wiessner C, Schmitz A, Tomaselli KJ, Schmitz B, Martin E, Bottiger BW: Improved resuscitation after cardiac arrest in rats expressing the baculovirus caspase inhibitor protein p35 in central neurons. ANESTHESIOLOGY 2003; 99:112–21
30. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162:156–9
31. Krajewski S, Mai JK, Krajewska M, Sikorska M, Mossakowski MJ, Reed JC: Upregulation of bax protein levels in neurons following cerebral ischemia. J Neurosci 1995; 15:6364–76
32. Miao B, Yin XH, Pei DS, Zhang QG, Zhang GY: Neuroprotective effects of preconditioning ischemia on ischemic brain injury through down-regulating activation of JNK1/2 *via* N-methyl-D-aspartate receptor-mediated Akt1 activation. J Biol Chem 2005; 280:21693–9
33. Zhan RZ, Fujihara H, Baba H, Yamakura T, Shimoji K: Ischemic preconditioning is capable of inducing mitochondrial tolerance in the rat brain. ANESTHESIOLOGY 2002; 97:896–901
34. Sugawara T, Noshita N, Lewen A, Kim GW, Chan PH: Neuronal expression of the DNA repair protein Ku 70 after ischemic preconditioning corresponds to tolerance to global cerebral ischemia. Stroke 2001; 32:2388–93
35. Kato H, Liu Y, Araki T: Temporal profile of the effects of pretreatment with brief cerebral ischemia on the neuronal damage following secondary ischemic insult in the gerbil: Cumulative damage and protective effects. Brain Res 1991; 553:238–42
36. Wiegand F, Liao W, Busch C, Castell S, Knapp F, Lindauer U, Megow D, Meisel A, Redetzky A, Ruscher K, Trendelenburg G, Victorov I, Riepe M, Diener HC, Dirnagl U: Respiratory chain inhibition induces tolerance to focal cerebral ischemia. J Cereb Blood Flow Metab 1999; 19:1229–37
37. Sugino T, Nozaki K, Takagi Y, Hashimoto N: 3-Nitropropionic acid induces ischemic tolerance in gerbil hippocampus *in vivo*. Neurosci Lett 1999; 259:9–12
38. Ibrahim NG, Beattie DS: Protein synthesis on ribosomes isolated from rat liver mitochondria: Sensitivity to erythromycin. FEBS Lett 1973; 36:102–4
39. Doersen CJ, Stanbridge EJ: Erythromycin inhibition of cell proliferation and *in vitro* mitochondrial protein synthesis in human HeLa cells is pH dependent. Biochim Biophys Acta 1982; 698:62–9
40. Karbowski M, Kurono C, Nishizawa Y, Horie Y, Soji T, Wakabayashi T: Induction of megamitochondria by some chemicals inducing oxidative stress in primary cultured rat hepatocytes. Biochim Biophys Acta 1997; 1349:242–50
41. Pious DA, Hawley P: Effect of antibiotics on respiration in human cells. Pediatr Res 1972; 6:687–92
42. Riepe MW, Ludolph AC: Chemical preconditioning: A cytoprotective strategy. Mol Cell Biochem 1997; 174:249–54
43. Iadecola C, Alexander M: Cerebral ischemia and inflammation. Curr Opin Neurol 2001; 14:89–94
44. Boutin H, LeFeuvre RA, Horai R, Asano M, Iwakura Y, Rothwell NJ: Role of IL-1alpha and IL-1beta in ischemic brain damage. J Neurosci 2001; 21:5528–34
45. Danton GH, Dietrich WD: Inflammatory mechanisms after ischemia and stroke. J Neuropathol Exp Neurol 2003; 62:127–36
46. Labro MT, Abdelghaffar H: Immunomodulation by macrolide antibiotics. J Chemother 2001; 13:3–8
47. Morikawa K, Zhang J, Nonaka M, Morikawa S: Modulatory effect of macrolide antibiotics on the Th1- and Th2-type cytokine production. Int J Antimicrob Agents 2002; 19:53–9
48. Pera J, Zawadzka M, Kaminska B, Szczudlik A: Influence of chemical and ischemic preconditioning on cytokine expression after focal brain ischemia. J Neurosci Res 2004; 78:132–40
49. Rosenzweig HL, Lessov NS, Henshall DC, Minami M, Simon RP, Stenzel-Poore MP: Endotoxin preconditioning prevents cellular inflammatory response during ischemic neuroprotection in mice. Stroke 2004; 35:2576–81
50. Meisel C, Prass K, Braun J, Victorov I, Wolf T, Megow D, Halle E, Volk HD, Dirnagl U, Meisel A: Preventive antibacterial treatment improves the general medical and neurological outcome in a mouse model of stroke. Stroke 2004; 35:2–6