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Induction of Cerebral Ischemic Tolerance by Erythromycin Preconditioning Reprograms the Transcriptional Response to Ischemia and Suppresses Inflammation

Ines P. Koerner, M.D., Matthias Gatting, M.D.,† Ruediger Noppens, M.D.,‡ Oliver Kempski, M.D., Ph.D.,§ Ansgar M. Brambrink, M.D., Ph.D.‖*

Background: **A single dose of the macrolide antibiotic erythromycin can induce tolerance against cerebral ischemia *in vivo* (pharmacologic preconditioning). This study identified potential mechanisms of tolerance induction by assessing effects of erythromycin preconditioning on the cerebral transcriptional response to transient global cerebral ischemia.**

Methods: **Preconditioned and nonpreconditioned rats were exposed to 15 min of global cerebral ischemia, and changes in cerebral gene expression were identified by complementary DNA expression array and quantified by real-time reverse-transcription polymerase chain reaction.**

Results: **Ischemia caused a widespread up-regulation of transcription in nonpreconditioned brains in this model. Tolerance induction by erythromycin preconditioning reversed this pattern and caused a net down-regulation of a majority of genes, effectively reprogramming the brain's response pattern to ischemia. The most striking change in transcriptional response found in preconditioned animals was an almost complete suppression of the otherwise profound induction of proinflammatory genes by global ischemia. In contrast, the same treatment had little effect on the expression of apoptosis-inducing genes after ischemia.**

Conclusions: **These findings present a new molecular correlate for the induction of ischemic tolerance achieved by erythromycin preconditioning and will further the understanding of this clinically important new regimen of preemptive neuroprotection.**

PREISCHEMIC conditioning has long been recognized as a powerful means to induce tolerance against cerebral ischemia and to reduce neuronal death and functional damage after an ischemic injury. Traditional preconditioning regimens using sublethal doses of otherwise damaging stressors, *e.g.*, brief episodes of ischemia,[1–5] hyperthermia,[6,7] or hypoxia,[8] as well as low doses of the endotoxin lipopolysaccharide[9] or the mitochondrial toxin 3-nitropropionic acid,[10–12] cannot be used in human patients. We have recently described a new pharmacologic method of preconditioning that uses a single dose of the macrolide antibiotic erythromycin to induce tolerance against cerebral ischemia *in vivo*.[13] A single injection of a clinically used dose of erythromycin 6–24 hours before the injury reduced ischemic neuronal death in hippocampus and parietal neocortex, and decreased neurologic deficit in rats after transient global cerebral ischemia.[13]

Erythromycin has long been in clinical practice as an antibiotic that has few side effects, which makes it an elegant representative for a novel regimen of ischemic-tolerance induction that is free of the potentially serious side effects of previous, harsher methods. We consider erythromycin to be a highly promising candidate for clinical application as a tolerance-inducing drug in patients at risk for cerebral ischemia (*e.g.*, during surgery).

Despite erythromycin's significant ability to affect cerebral ischemic tolerance, the molecular mechanisms that underlie this pharmacologic tolerance induction have yet to be identified. Induction of sustained, as opposed to acute, tolerance by traditional preconditioning regimens has been associated with *de novo* protein synthesis[14] *via* adapted gene transcription, suggesting that erythromycin also realizes its effect on a transcriptional level.

Accordingly, we decided to assess effects of erythromycin treatment on cerebral gene expression and, more specifically, of erythromycin preconditioning on the brain's genomic response to transient global cerebral ischemia. The first part of our study aimed to identify distinct transcriptional patterns evoked by preconditioning and by ischemia with a screening of several hundred genes using complementary DNA (cDNA) macroarray analysis of brain messenger RNA (mRNA) expression changes. We observed a profound change in the cerebral transcriptional response to ischemia after erythromycin preconditioning. Whereas ischemia alone induced an overall up-regulation of gene expression, the pattern in preconditioned brains was one of widespread down-regulation. Subsequently, we isolated functional groups of genes affected by ischemia and preconditioning, and analyzed individual target genes from these groups by real-time reverse-transcription polymerase chain reaction (RT-PCR) quantification. This showed a surprisingly specific effect of erythromycin on the induction of several proinflammatory mediators by ischemia, whereas regulators of programmed cell death, traditionally associated with neuronal death by global ischemia, were mostly unaffected.

Materials and Methods

Experimental Protocol and Groups

Male Wistar rats (311 ± 5 g; Charles River, Kisslegg, Germany) were treated in accordance with institutional and in-

* Resident, Department of Anesthesiology and Institute for Neurosurgical Pathophysiology, † Research Fellow, ‡ Resident, ‖ Associate Professor, Department of Anesthesiology, § Professor and Chair, Institute for Neurosurgical Pathophysiology, Johannes Gutenberg-University Mainz.

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Address correspondence to Dr. Brambrink: Department of Anesthesiology and Peri-Operative Medicine, Oregon Health and Science University, 3181 SW Sam Jackson Park Road, Mail-Code UHS-2, Portland, Oregon 97239-3098. brambrin@ohsu.edu. Individual article reprints may be purchased through the Journal Web site, www.anesthesiology.org.

ternational guidelines. We used a regimen of pharmacologic preconditioning that was previously shown to induce sustained tolerance against transient global ischemia in rats.[13] Fifty-three animals were randomly assigned to receive either 25 mg/kg erythromycin lactobionate[15] (Erythrocin; Abbott GmbH & Co. KG, Wiesbaden, Germany; estimated half-life in rats approximately 2 h[16,17]) or vehicle (0.9% normal saline) intramuscularly. One cohort of animals from each treatment group was exposed to transient global ischemia (erythromycin, n = 8; vehicle, n = 8) or sham operation (erythromycin, n = 6; vehicle, n = 6) 6 h after pretreatment and was killed for mRNA analysis at 24 h of reperfusion. Another cohort received erythromycin only without surgical intervention and was killed for mRNA analysis 6 h (n = 8) or 30 h (6 + 24 h; n = 8) later. Additional animals (n = 6) served as untreated naive controls. Three animals (1 vehicle, 2 erythromycin) that died during the 24-h recovery were excluded from further analysis and replaced according to the randomization protocol.

All animals were randomly assigned to treatment groups, and investigators were blinded for group assignment throughout the experiment.

Transient Global Cerebral Ischemia

Animals were food restricted with free access to water overnight before surgery. Fifteen minutes of transient global ischemia was achieved by bilateral carotid artery occlusion plus hypobaric hypotension, as previously described in detail.[10,18] In brief, anesthetized (360 mg/kg intraperitoneal chloral hydrate) rats were intubated and mechanically ventilated. After both carotid arteries were exposed, the left side was catheterized for blood pressure monitoring (complete vessel occlusion). To achieve global ischemia, the right carotid artery was occluded by a thread that was looped around it, and mean arterial blood pressure was simultaneously reduced to 35 mmHg using the hypobaric hypotension technique.[18,19] After 15 min of ischemia, the thread was removed, and hypobaric hypotension was terminated to allow reperfusion of the brain. Ischemia was verified by laser-Doppler monitoring as previously described.[10,20,21] Rectal temperature was controlled to 37.4° ± 0.1°C using a thermostatically regulated warming blanket. Peribrain temperature was recorded (right auricular tube) and controlled to 37°C throughout the experiment using a near-infrared heating lamp, as previously described.[22] Arterial blood gases (pH, oxygen tension, carbon dioxide tension, hematocrit, lactate, glucose) were drawn before, during, and after ischemia and showed no differences between treatment groups. Blood pressure and temperature did not differ between treatment groups. 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 randomly assigned to sham operation were treated in the same way, except ischemia was not induced.

RNA Extraction

Rats were reanesthetized (360 mg/kg intraperitoneal chloral hydrate) and decapitated. Brains were rapidly removed from the skulls, subdissected, and snap-frozen. Tissue samples were homogenized for each animal, and total RNA was extracted according to established protocols.[23] A DNase I digestion was performed to eliminate residual genomic DNA contamination. RNA integrity was verified by denaturing agarose gel electrophoresis after DNase I digestion. The final RNA concentration ranged between 0.5 and 1.5 $\mu g/\mu l$.

cDNA Expression Array Analysis

Fifty micrograms pooled RNA per experimental group was converted to biotin-labeled first-strand cDNA (Atlas SpotLight Labeling Kit; BD Clontech, Heidelberg, Germany) and purified on a spin column. Efficacy of the biotin incorporation was assessed by dot-blotting. Freshly denatured biotinylated probe was hybridized to Atlas Rat Toxicology 1.2 Nylon Arrays (BD Clontech; 1185 individual genes) at 42°C overnight. Signal was detected with streptavidin-linked horseradish peroxidase and luminol (SpotLight Chemiluminescent Hybridization & Detection Kit; BD Clontech) after a series of stringency washes, and arrays were exposed to x-ray film (Eastman Kodak Company, New Haven, CT). Films were scanned on a densitometer. Digitalized data were imported into AtlasImage Software (BD Clontech). After manual fine-tune alignment of individual spots, arrays were normalized using the sum method provided by AtlasImage. External background calculation was used and signal-to-background threshold was set to 200%. Reports were exported into Excel® (Microsoft Corporation, Redmond, WA) spreadsheets for further annotation. Arrays from all treatment groups were compared to naive animals to identify differentially regulated genes. A stringent threshold of a twofold or higher change in expression was used before a gene was considered differentially expressed, to reduce the number of false-positive results that are known to hamper macroarray data analysis. Because the focus of this study was on identifying pathophysiologic pathways relevant for ischemic-tolerance induction rather than individual genes, we analyzed gene expression patterns of functional groups. Genes were annotated and grouped into functional groups according to functional annotations provided by the manufacturer of the cDNA expression arrays as well as annotations provided by The Gene Ontology Consortium.#

Real-time Reverse-transcription Polymerase Chain Reaction Analysis

We applied real-time quantitative RT-PCR to quantify the effects of cerebral ischemia and erythromycin preconditioning on candidate genes from relevant functional groups of genes that were identified by the cDNA

Resources used for functional grouping and gene annotating were the Atlas Array annotation tool, available at: http://www.clontech.com/clontech/atlas/genelists/index.shtml, and The Gene Ontology Consortium, available at: http://www.geneontology.org Web sites. Both accessed June 10, 2006.

Table 1. Gene Names and Primer Sequences Used for Real-time Reverse-transcription Polymerase Chain Reaction

Group	Gene Name	Primers Used
Inflammation	TNF-α	Sense 5′ATG AGC ACA GAA AGC ATG ATC3′ Antisense 5′CAG AGC AAT GAC TCC AAA GTA3′
	IL-6	Sense 5′ TCT CTC CGC AAG AGA CTT CC3′ Antisense 5′ TTC TGA CAG TGC ATC ATC GC3′
	RANTES	Sense 5′ATCCCTCACCGTCATCCTC3′ Antisense 5′GCACTTGCTGCTGGTGTAAA3′
	INOS	Sense 5′CACCTTGGAGTTCACCCAGT3′ Antisense 5′ACCACTCGTACTTGGGATGC3′
Cell adhesion	ICAM	Sense 5′GGAGGCCCTAAAACTCAAGG3′ Antisense 5′GGCTGTAGGTGTGTCCAGGT3′
Apoptosis	Fas	Sense 5′ACCTGGTGACCCTGAATCTG3′ Antisense 5′TGATACCAGCACTGGAGCAG3′
	FasLigand	Sense 5′GGAATGGGAAGACACATATGGAACTGC3′ Antisense 5′CATATCTGGCCAGTAGTGCAGTAATTC3′
	Caspase 3	Sense 5′AATTCAAGGGACGGGTCATG3′ Antisense 5′GCTTGTGCGCGTACAGTTTC3′
	Bcl-2	Sense 5′ATGGGGTGAACTGGGGGAGGATTG3′ Antisense 5′TTTCATATTTGTTTGGGGCAGGTC3′
Stress response	C-fos	Sense 5′CCCGTAGACCTAGGGAGGAC3′ Antisense 5′CAATACACTCCATGCGGTTG3′
	Homer1a	Sense 5′AAGTTTGGCCAATGGGC3′ Antisense 5′ACCAAATGGAATTCCATATTTATCC3′
	Cu/Zn-SOD	Sense 5′GAAGCATGGCGATGAAGGCCGTGTGCGTGC3′ Antisense 5′GACTCAGACCACATAGGGAATGTTTATTGG3′
	Mn-SOD	Sense 5′ACTGCTGGGGATTGATGTGT3′ Antisense 5′TCTTTGATGGCCTTATGATGAC3′
DNA synthesis and repair	APDG	Sense 5′ACAGTCAGAGCAGCAGCAGA3′ Antisense 5′TCTGTTCCATCAGCAAGTCG3′
Translation	Ribosomal protein S20	Sense 5′GAGGAACAAGTCGGTCAGG3′ Antisense 5′GTCCACTCCAGGCTCAATACT3′
Posttranslational modification	HSP90β	Sense 5′GATTGACATCATCCCCAACC3′ Antisense 5′CTGCTCATCATCGTTGTGCT3′
	Cyclophilin	Sense 5′ACCCCACCGTGTTCTTCGAC3′ Antisense 5′CATTTGCCATGGACAAGATG3′
Protein turnover	NGF	Sense 5′CTGGACTAAACTTCAGCATTC3′ Antisense 5′TGTTGTTAATGTTCACCTCGC3′
Cytoskeleton	β-Actin	Sense 5′TTGTAACCAACTGGGACGATATGG3′ Antisense 5′GATCTTGATCTTCATGGTGCTAGG3′
Metabolism	GAPDH	Sense 5′CCTGGCCAAGGTCATCCATGACAACTTTGG3′ Antisense 5′GCCATGAGGTCCACCACCCTGTTGCTGTAG3′

APDG = N-methylpurine-DNA-glycosylase; Cu/Zn-SOD = copper/zinc superoxide dismutase; GAPDH = glyceraldehyde-3-phosphate dehydrogenase; HSP90β = heat shock protein 90β; IL-6 = interleukin 6; INOS = inducible nitric oxide synthase; Mn-SOD = manganese superoxide dismutase; NGF = nerve growth factor; RANTES = regulated upon activation, normal T cell expressed and secreted; TNF-α = tumor necrosis factor α.

expression array analysis. RT-PCR analysis was performed on two brain regions that are sensitive to global cerebral ischemia in this model, hippocampus and neocortex. First-strand cDNA was synthesized from 2 μg RNA according to the manufacturer's protocol (Roche Molecular Biochemicals, Mannheim, Germany). Real-time quantitative RT-PCR was performed for the candidate genes and for glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as internal control on a LightCycler® (Roche) thermocycler, using FastStart SYBR Green® PCR Reagents (Roche). Primer sequences specific for rat were designed using Primer3 software.** Sequences for individual primers are shown in table 1. Specificity of PCR products was verified by melting curve analysis and subsequent agarose gel electrophoresis. Expression levels were normalized to GAPDH and analyzed with RelQuant® software (Roche). PCR experiments on triplicate samples were repeated twice.

Statistical Analyses

Data are shown as mean ± SD. One-way analysis of variance and *post hoc* Student-Newman-Keuls test were used to identify differences between experimental groups (SigmaStat® Software; SPSS Inc., Chicago, IL). Significance was set at $P < 0.05$.

** Free Primer 3 software. Available at: http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi. Accessed May 8, 2004.

Results

Global Ischemia Alters Cerebral Gene Expression in Adult Rats. Using a stringent threshold for our cDNA array analysis (≥ 2-fold change; for details, see Materials and Methods), we found that 15 min of global cerebral ischemia elicited a distinctive change in cerebral gene expression. Fifteen percent (176 of 1,185) of the analyzed genes were differentially expressed 24 h after ischemia in vehicle-pretreated animals (fig. 1). We saw an overall pattern of increased transcription after ischemia: 62% (109 of 176) of the differentially regulated genes were up-regulated, compared with naive animals, whereas down-regulation was seen for 38% (67 of 176). Up-regulation was most pronounced among DNA binding and chromatin proteins, genes associated with transcription or RNA processing, and oncogenes and tumor suppressors. More than 20% (range, 21–40%) of the genes present on the array were up-regulated in each of these four functional groups (fig. 1).

Erythromycin Preconditioning Alters Postischemic Transcriptional Response

Cerebral gene expression 24 h after ischemia in animals that had received a single tolerance-inducing dose of erythromycin 6 h before ischemia was markedly different from the pattern seen in nonpreconditioned (vehicle) postischemic animals. A similar number of genes

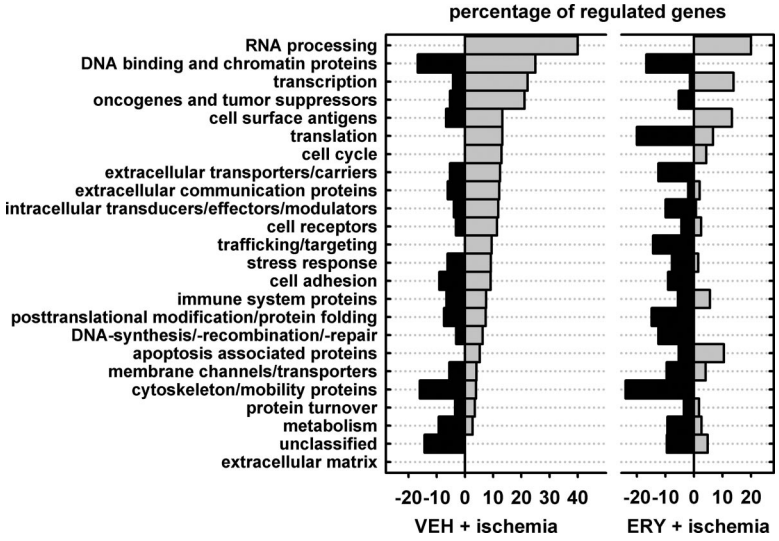

Fig. 1. Erythromycin preconditioning alters postischemic cerebral transcriptional response in adult rats. Percentage of genes of different functional classes that were differentially regulated by global cerebral ischemia in brains of nonpreconditioned (VEH + ischemia) or erythromycin preconditioned (ERY + ischemia) rats 24 h after ischemia, assessed by complementary DNA expression array. *Black bars* represent genes that were down-regulated, compared with naive controls; *gray bars* represent up-regulated genes. A majority of genes that were differentially expressed after 15 min of global ischemia in nonpreconditioned brains were up-regulated, whereas most genes were down-regulated after erythromycin preconditioning and ischemia.

were differentially expressed after preconditioning plus ischemia (11%; 130 of 1,185) as after vehicle plus ischemia (15%; 176 of 1,185). However, the overall pattern in erythromycin-pretreated brains was one of reduced expression, contrasting with the overall increased transcription after nonpreconditioned (vehicle) ischemia alone (fig. 1). Expression of two thirds (69%; 90 of 130) of the regulated genes after preconditioning plus ischemia was reduced, compared with naive animals, most pronounced among genes associated with translation, or cytoskeleton/mobility proteins (> 20% [range, 20–24%] down-regulated genes). Expression of genes associated with RNA processing was still increased, but less pronounced than after ischemia only (fig. 1).

Erythromycin Treatment Alone Has Little Effect on Cerebral Gene Expression

We found that the tolerance-inducing stimulus used in this study, a single injection of the antibiotic erythromycin, alone caused only limited changes in cerebral gene expression patterns in rats not subjected to subsequent ischemia. Six hours after erythromycin treatment, approximately 1% of the genes analyzed by cDNA array were differentially regulated, mostly cytoskeleton proteins, extracellular transporters/carriers, and cell adhesion genes (fig. 2). Almost all of these genes were down-regulated, and this pattern was persistent 1 day (30 h [6 + 24]) after treatment.

Erythromycin Preconditioning Reprograms the Cerebral Transcriptional Response to Ischemia

In addition to the overall shift in the response pattern to global cerebral ischemia that was elicited by erythromycin preconditioning, we found that there was little overlap of genes affected by either condition (fig. 3). A minority of genes (43; 20 up-regulated, 23 down-regulated) were differentially regulated after ischemia in both nonpreconditioned and erythromycin-preconditioned brains, whereas 133 (89 up-regulated, 44 down-regu-

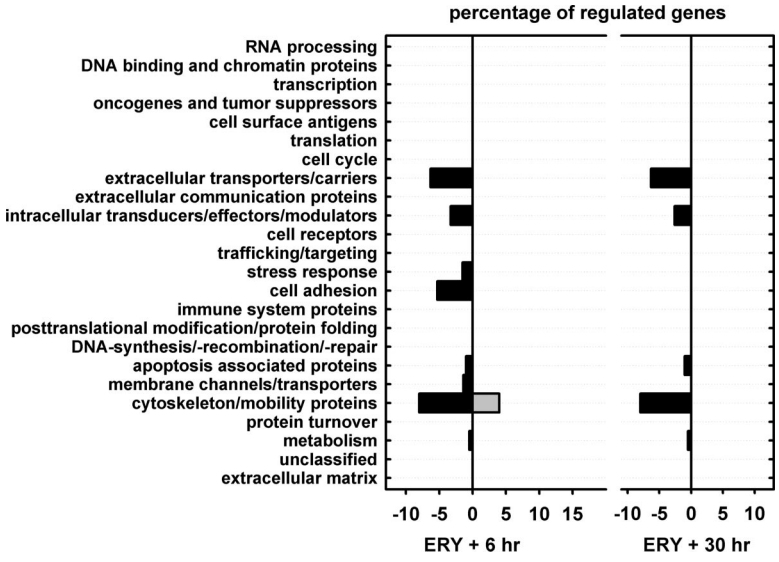

Fig. 2. Erythromycin treatment alone has little effect on cerebral gene expression. Percentages of genes of different functional classes, as assessed by complementary DNA expression array, that were differentially regulated 6 h (ERY + 6 h) or 30 h (6 + 24 h; ERY + 30 h) after a tolerance-inducing single dose of erythromycin (25 mg/kg). *Black bars* represent genes that were down-regulated, compared with naive controls; *gray bars* represent up-regulated genes. Few genes were affected by erythromycin treatment only in nonischemic rats, mostly cytoskeleton proteins, extracellular transporters/carriers, and cell adhesion genes.

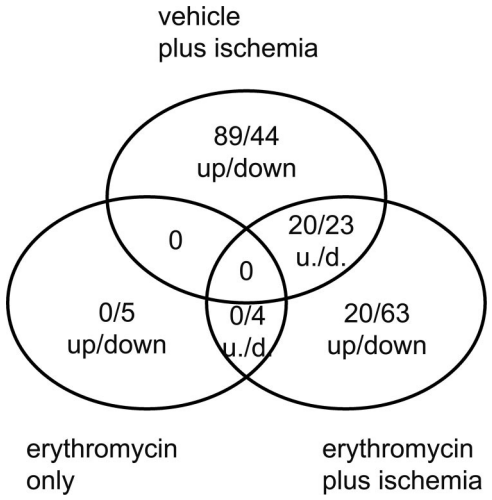

Fig. 3. Erythromycin preconditioning reprograms cerebral response to ischemia. Number of genes that were differentially expressed (up- or down-regulated) 24 h after 15 min of global ischemia in brains of nonpreconditioned (vehicle plus ischemia) or erythromycin-preconditioned (erythromycin plus ischemia) rats, compared with genes differentially expressed 30 h after erythromycin treatment only (erythromycin only). Little overlap was seen between genes differentially regulated in each treatment group.

lated) genes (vehicle plus ischemia) and 83 (20 up-regulated, 63 down-regulated) genes (erythromycin plus ischemia), respectively, were exclusively affected in only one group. The extent of change observed after preconditioning suggest a profound reprogramming of the transcriptional response by erythromycin.

Quantification of Gene Expression by Real-time RT-PCR

To further evaluate and quantify gene expression changes elicited by erythromycin preconditioning, we performed quantitative RT-PCR analyses of representative genes from functional groups suggested by the cDNA array data and known or presumed to be relevant for cerebral ischemic damage or cell survival.

Stress Response Genes

We chose the immediate-early genes c-fos and Homer1a as well as the free radical scavengers Cu/Zn–superoxide dismutase (Cu/Zn-SOD) and manganese superoxide dismutase (Mn-SOD) as candidate genes of stress response (fig. 4). C-fos expression increased after ischemia in neocortex and in hippocampus, and this was reduced (in hippocampus) or abolished (neocortex) in erythromycin preconditioned brains. In contrast, expression of homer1a, an immediate early gene implicated in neuroprotection, was decreased 24 h after ischemia. Erythromycin blunted this response in the hippocampus. Erythromycin preconditioning also diminished the increase of Cu/Zn-SOD (in hippocampus) as well as Mn-SOD expression (in both regions) after ischemia.

Genes Associated with Inflammatory Response

Ischemia caused a profound increase (up to 95-fold) in transcription of all inflammatory mediators evaluated in this study, two cytokines (tumor necrosis factor α [TNF-α] and interleukin 6 [IL-6]), a chemokine (regulated upon activation, normal T cell expressed and secreted [RANTES]), and an adhesion molecule (intercellular adhesion molecule [ICAM]) as well as the inflammatory marker inducible nitric oxide synthase (iNOS) (fig. 5). This group of genes exhibited the greatest increases in

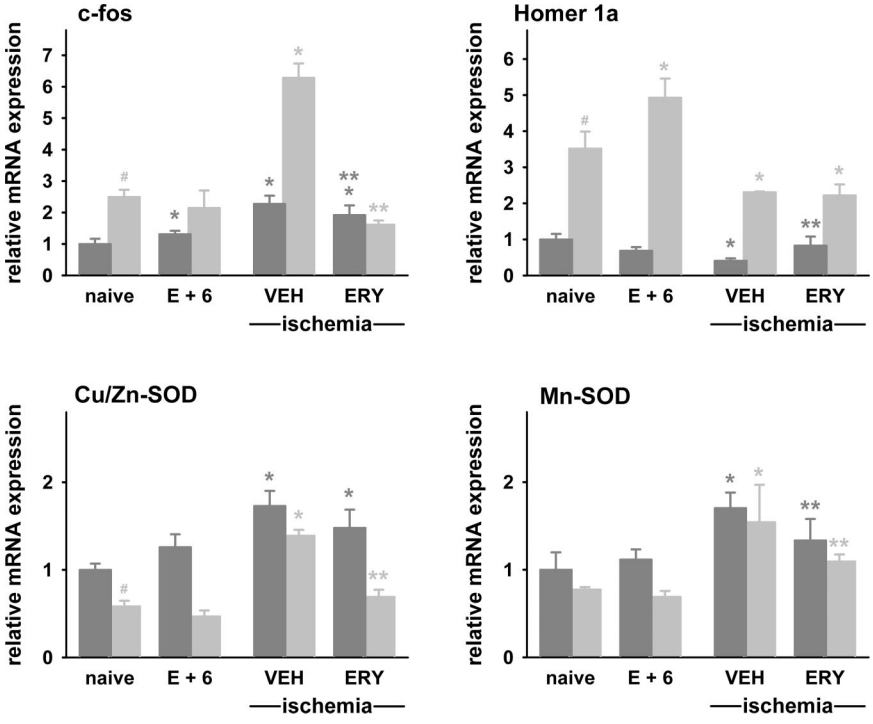

Fig. 4. Relative messenger RNA (mRNA) expression levels of stress response genes in hippocampus and neocortex. Global cerebral ischemia induced expression of c-fos, Cu/Zn-SOD and Mn-SOD, and reduced Homer1a mRNA. Erythromycin diminished the postischemic increase of c-fos, Cu/Zn-SOD, and Mn-SOD and prevented down-regulation of Homer1a in the hippocampus. For easy comparison, expression of each gene is shown relative to the hippocampus of naive animals. *Dark gray bars* are expression levels in the hippocampus; *light gray bars* are expression levels in the neocortex. Groups: naive: untreated animals, n = 6; E + 6: animals injected 6 h earlier with 25 mg/kg erythromycin, n = 8; VEH ischemia: nonpreconditioned animals 24 h after 15 min of global cerebral ischemia, n = 8; ERY ischemia: animals preconditioned with 25 mg/kg erythromycin 6 h before 15 min of ischemia, analyzed 24 h after ischemia, n = 8. * $P <$ 0.05 compared with naive. ** $P < 0.05$ compared with vehicle plus ischemia. # $P < 0.05$ compared with hippocampus.

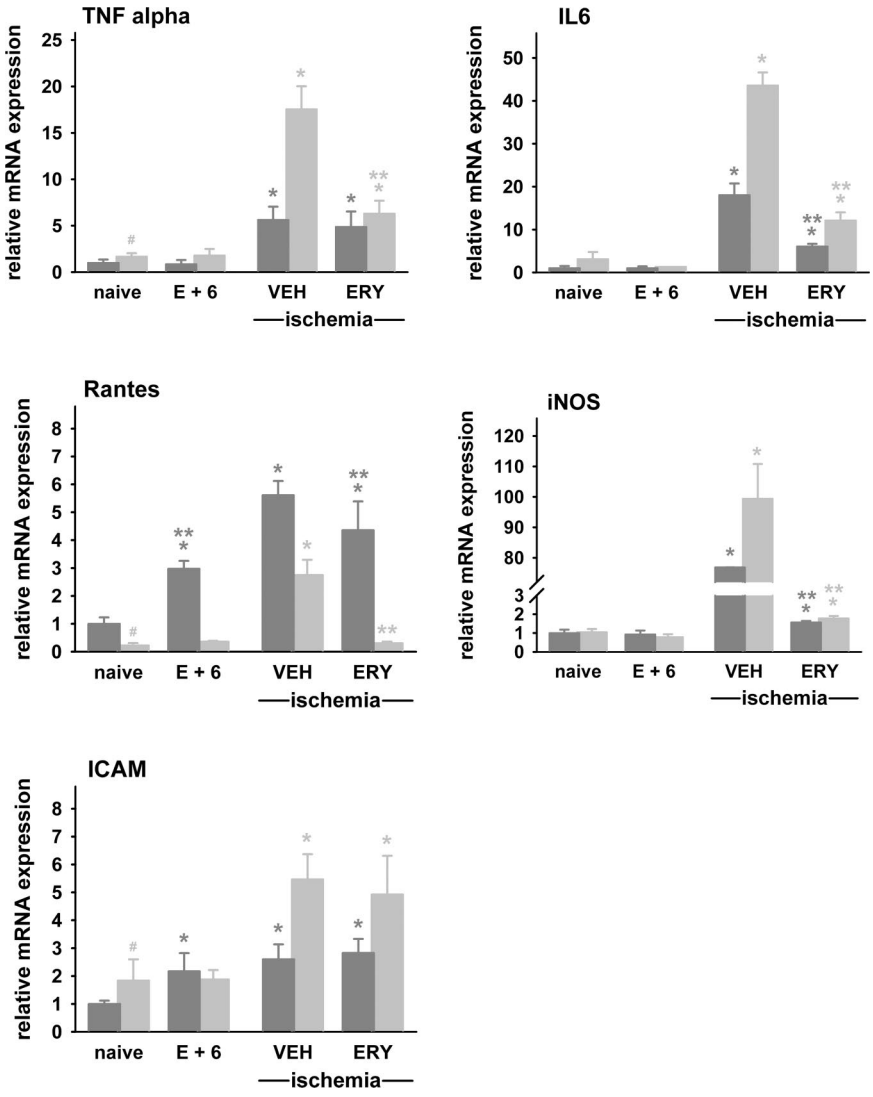

Fig. 5. Relative messenger RNA (mRNA) expression levels of inflammatory genes in hippocampus and neocortex. Expression of inflammatory genes was strongly increased 24 h after global ischemia. Erythromycin preconditioning attenuated the induction of interleukin 6 (IL-6), Rantes, inducible nitric oxide synthase (iNOS), and neocortical tumor necrosis factor α (TNF-α), but not of intracellular adhesion molecule (ICAM). Expression of each gene is shown relative to the hippocampus of naive animals. *Dark gray bars* are expression levels in the hippocampus; *light gray bars* are expression levels in the neocortex. For group details, see figure 4. * $P < 0.05$ compared with naive. ** $P < 0.05$ compared with vehicle plus ischemia. # $P < 0.05$ compared with hippocampus.

mRNA levels by ischemia found in this study. Erythromycin preconditioning, however, abolished or substantially reduced most of these changes (IL-6, RANTES, iNOS, and neocortical TNF-α). ICAM expression, in contrast, was not different in preconditioned *versus* nonpreconditioned animals.

Apoptosis-associated Genes

Apoptosis-associated genes were little affected by erythromycin preconditioning. Expression of apoptosis-inducing Fas receptor and FasLigand was substantially increased after ischemia (2- to 5-fold; fig. 6), and preconditioned animals had almost identical mRNA levels of both genes as nonpreconditioned ones. mRNA of apoptosis effector caspase 3 was slightly (1.5-fold) increased by ischemia and not influenced by erythromycin in our model. Similarly, preconditioning had only small effects on antiapoptotic genes. Bcl-2 expression increased 1.5-fold after ischemia, which was reduced by erythromycin preconditioning in hippocampus only.

Other Genes

We studied one target gene of each of the functional groups DNA synthesis/recombination/repair (N-alkylpurine-DNA-glycosylase [APDG]), translation (S20), protein turnover (nerve growth factor [NGF]), and cytoskeleton/mobility (β-actin) and two genes involved in posttranslational modification, cyclophilin and the heat-shock protein 90β (HSP90β) (fig. 7). Expression of the heat shock protein after ischemia increased in the neocortex only, and was unaffected by preconditioning. Likewise, APDG mRNA increased after ischemia in the neocortex only, which was inhibited by erythromycin. Surprisingly, expression of S20 and β-actin, which are commonly used as internal controls for gene expression quantification, increased in response to ischemia. Erythromycin diminished this response for β-actin in neocortex and hippocampus and reduced hippocampal S20. Changes in NGF expression were inconsistent in both brain regions studied, increasing after ischemia in the neocortex and decreasing in the hippocampus, but erythromycin damp-

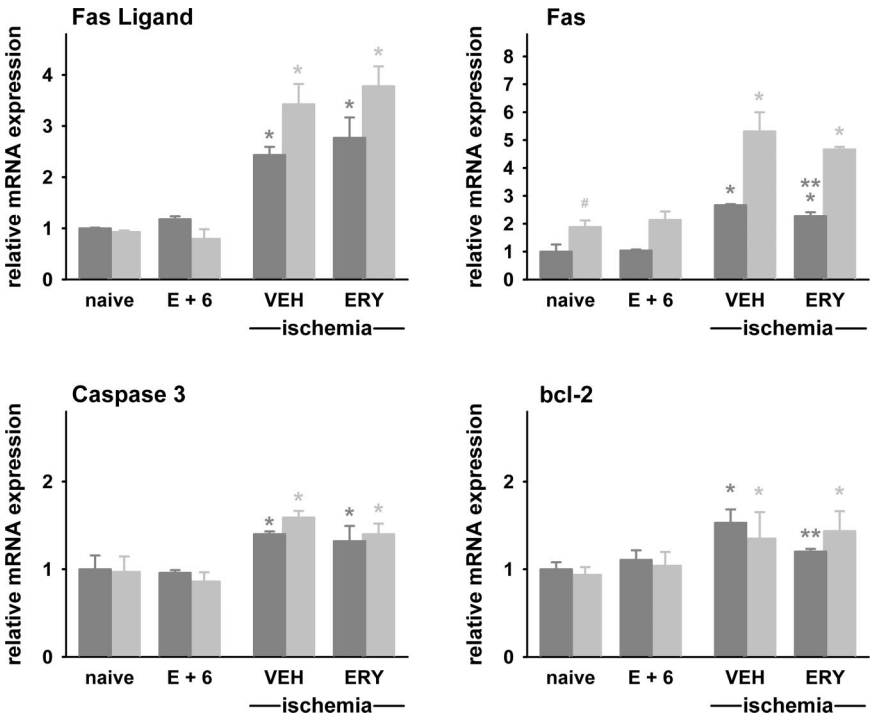

Fig. 6. Relative messenger RNA (mRNA) expression levels of apoptosis-associated genes in hippocampus and neocortex. Expression of apoptosis-inducing Fas and FasLigand was increased after global ischemia in neocortex and in hippocampus. Similarly, mRNA levels of apoptosis-mediating caspase 3 and antiapoptotic bcl-2 were elevated after ischemia. Erythromycin preconditioning did not affect FasLigand or caspase 3 expression, and attenuated Fas and bcl-2 levels in hippocampus only. Expression of each gene is shown relative to the hippocampus of naive animals. *Dark gray bars* are expression levels in the hippocampus; *light gray bars* are expression levels in the neocortex. For group details, see figure 4. * $P < 0.05$ compared with naive. ** $P < 0.05$ compared with vehicle plus ischemia. # $P < 0.05$ compared with hippocampus.

ened both changes. Cyclophilin, another commonly used internal control gene, was not affected by experimental treatments.

Effects of Sham Operation on Cerebral Gene Expression

Cerebral expression of the majority of genes in this study was unchanged after sham operation, independent of pretreatment, with few exceptions (data not shown). Noteworthy exceptions were the inflammatory mediators IL-6, TNF-α, RANTES, iNOS, and ICAM, whose expression increased after sham operation, although to a much lesser extend than after ischemia. This increase after sham operation was also reduced by erythromycin pretreatment, similar to the changes after ischemia.

Discussion

This study demonstrates three important findings. First, erythromycin preconditioning characteristically down-regulates cerebral gene expression after transient global cerebral ischemia. Second, erythromycin preconditioning blunts the otherwise pronounced induction of inflammatory mediators after ischemia. Third, in contrast to classic ischemic preconditioning, erythromycin preconditioning in our hands does not induce protective genes, but rather alters the brain's disposition to express damaging genes in response to ischemia. Taken together, our findings present a molecular correlate for ischemic tolerance induced by erythromycin preconditioning, *i.e.*, a reprogrammed genomic response to transient cerebral ischemia.

Our cDNA array–based screening of cerebral mRNA expression showed a profound change of gene transcription after ischemia in erythromycin-preconditioned animals, which may be critical for the increased tolerance toward ischemia in preconditioned brains. Previous studies investigating mechanisms of preconditioning were mostly focused on the transcriptional response to the preconditioning stimulus itself. A few studies that examined postischemic gene expression in preconditioned brains showed an attenuated postischemic induction of injurious genes in the ischemia-tolerant state, *e.g.*, immediate-early genes,[14] cyclooxygenase 2,[24] or proapoptotic bax.[10] A genome-wide characterization of postischemic transcription in the tolerant brain was attempted only recently.[25–27] Two groups reported widespread down-regulation of gene expression after transient focal cerebral ischemia in ischemia-preconditioned brains.[25,26] We found similar changes after transient global ischemia in erythromycin-preconditioned animals, suggesting that a general pattern of down-regulation of genes after cerebral ischemia may be a common characteristic response of the tolerant brain to an ischemic stimulus, independent of the model of ischemia used.

Quantitative RT-PCR analysis revealed a pronounced suppression of the postischemic transcriptional up-regulation of inflammatory mediators by erythromycin preconditioning. Our data from nonpreconditioned animals show a prominent transcriptional up-regulation of proinflammatory cytokines and chemokines after ischemia (*e.g.*, IL-6 up to 40-fold), suggesting that inflammation may indeed be a relevant pathomechanism in our model

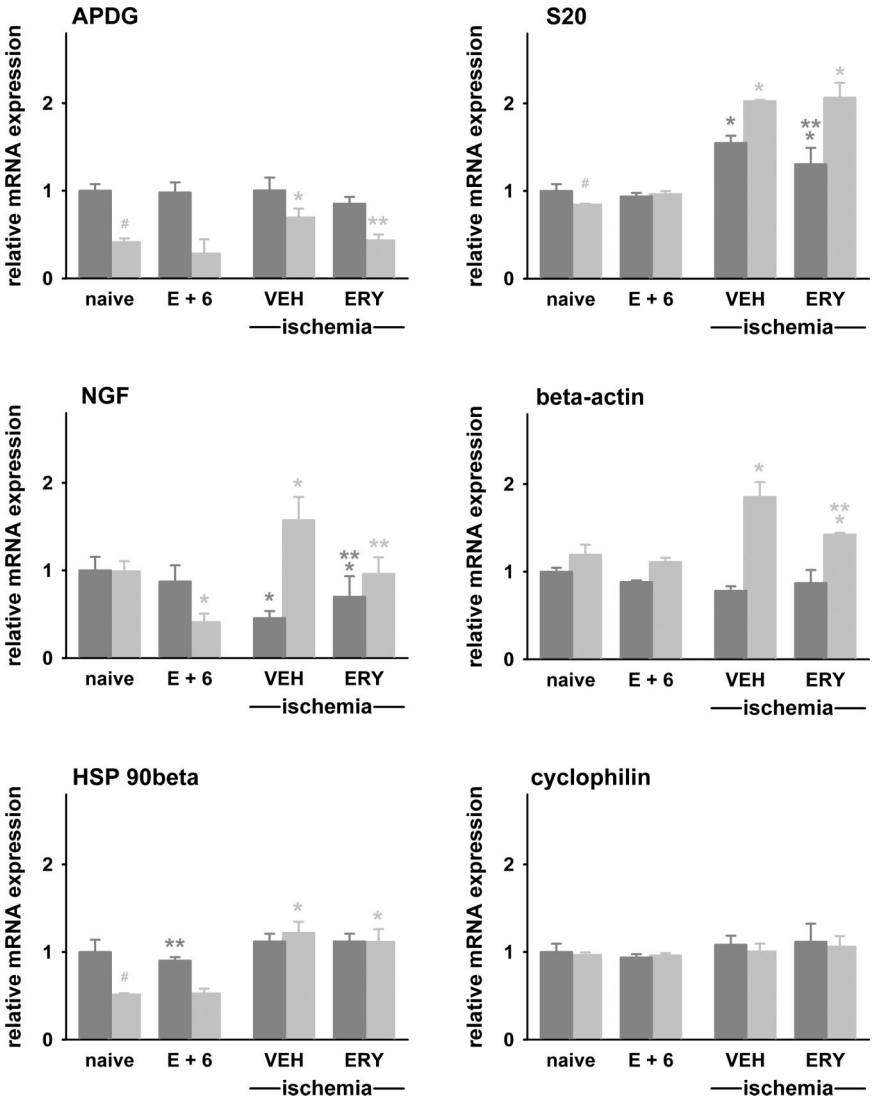

Fig. 7. Relative messenger RNA (mRNA) expression levels of other genes in hippocampus and neocortex. N-alkylpurine-DNA-glycosylase (APDG) mRNA increased after ischemia in the neocortex only, which was inhibited by erythromycin. Expression of S20 and β-actin increased in response to ischemia. Erythromycin diminished this response for β-actin and reduced hippocampal S20. Inconsistent changes in nerve growth factor (NGF) expression were seen in both brain regions studied, with increased expression after ischemia in the neocortex and decreased in the hippocampus. Erythromycin dampened both changes. Expression of HSP90β increased after ischemia in the neocortex only, which was unaffected by preconditioning. Cyclophilin mRNA was not affected by experimental treatments. Expression of each gene is shown relative to the hippocampus of naive animals. *Dark gray bars* are expression levels in the hippocampus; *light gray bars* are expression levels in the neocortex. For group details, see figure 4. * $P < 0.05$ compared with naive. ** $P < 0.05$ compared with vehicle plus ischemia. # $P < 0.05$ compared with hippocampus.

of transient global ischemia. Inflammation is widely recognized as a major contributor to neuronal damage and death after cerebral ischemia,[28–30] and inhibition of inflammatory response ameliorates outcome after stroke as well as global ischemia.[31–33] Recently, ischemic as well as chemical preconditioning was reported to reduce induction of two inflammatory cytokines, IL-1β and IL-6, after focal cerebral ischemia.[34] The prevention of postischemic inflammation may therefore be a specific theme of tolerance induction that erythromycin shares with other preconditioning regimens.

The inhibitory effect of erythromycin preconditioning was specific for cytokines, chemokines, and iNOS only, whereas the postischemic transcriptional up-regulation of other genes, *e.g.*, the adhesion molecule ICAM, was unaffected. We therefore believe that the suppression of inflammatory genes after ischemia is causal for tolerance induction by erythromycin, rather than secondary to reduced damage in preconditioned animals.

The lack of effect of erythromycin on ICAM transcription may be related to a selective action of this drug on the transcription factor nuclear factor κB (NF-κB).[35,36] Whereas cytokine transcription seems to rely mostly on NF-κB, ICAM is also regulated by activator protein 1 (AP1),[37,38] leaving an alternative pathway for ICAM induction by ischemia, if NF-κB activation is attenuated after erythromycin preconditioning.

In contrast to its profound effect on inflammatory mediators, erythromycin preconditioning did not affect the postischemic mRNA expression of apoptosis-regulating genes in our model. Ischemic preconditioning, however, attenuates the induction of apoptosis-mediating FasLigand by focal ischemia,[34] and chemical preconditioning using 3-NPA induces antiapoptotic bcl-2, shifting the bcl-2/bax balance toward antiapoptosis[10] in the same model of transient global ischemia we used in our current study. The apparent lack of this effect in erythromycin-preconditioned animals suggests that the inhibition of programmed cell death may be less relevant for tolerance induction by erythromycin than it is for other preconditioning regimens. Similarly, we found only moderate and region-specific effects of erythromycin on

other genes previously implicated in tolerance induction by other means, *e.g.*, on Cu/Zn- or Mn-SOD,[39,40] suggesting that erythromycin may elicit a distinct genomic response that overlaps only partially with other preconditioning protocols.

To our surprise, we found that global cerebral ischemia in our hands induced transcription of β-actin and S20, both housekeeping genes that are widely used as internal controls for mRNA and protein quantification. These findings reinforce that controls for quantification must be verified for each individual ischemia model. Expression levels of cyclophilin and of GAPDH (which we used for quantification) were not affected by our interventions and therefore are useful internal controls for our specific model of preconditioning and ischemia.

Interestingly, in our study, the preconditioning stimulus itself, a single injection of erythromycin in the absence of subsequent cerebral ischemia, elicited only a limited change of cerebral gene expression. More traditional preconditioning stimuli, *e.g.*, brief periods of ischemia, are associated with induced expression of, among others, immediate-early genes,[41] heat-shock proteins,[42] or free radical scavengers.[43] The resulting neuroprotection after ischemic preconditioning has traditionally been attributed to processes downstream of these mediators. In contrast, erythromycin preconditioning by itself does not induce gene expression, but it alters the transcriptional response to subsequent ischemia. This suggests that erythromycin may act *via* a change in disposition toward subsequent gene induction by a stressful stimulus, rather than an altered baseline gene expression status. A similar effect of erythromycin has previously been shown in immune cells: Erythromycin affects gene expression only after the cells are stimulated (for review, see Labro and Abdelghaffar[44]) but does not cause expression changes in baseline cells. Potential mechanisms responsible for this effect might include changes in phosphorylation status or ubiquitination of transcription factors elicited by erythromycin,[35,36,45] as well as changes in the cellular redox state[46] or cyclic AMP content,[47] that subsequently alter transcription factor activity. In addition, erythromycin's recently described ability to interfere with nuclear mRNA splicing[48] might also contribute to the changed disposition. Taken together, these data indicate that erythromycin may represent a new class of preconditioning agents that use a unique mechanism of action by reprogramming the response to ischemia rather than by providing beneficial products.

Our study revealed changes in the brain's transcriptional response to ischemia that may represent potential mechanisms for the induction of ischemic tolerance by pharmacologic preconditioning. However, we did not investigate further mechanisms upstream and downstream of these transcriptional changes. Future experiments blocking individual transcription factors may help to further elucidate the mechanisms involved in reprogramming by erythromycin and to determine the specificity of these mechanisms for the induction of ischemic tolerance. Moreover, we have analyzed mRNA expression only, which may not translate into protein expression in all cases. Although we investigated transcriptional effects of erythromycin at two time points (6 and 30 h), we studied only one time point (24 h) after ischemia. We therefore may have missed transient postischemic transcriptional changes that were present only very early or very late after ischemia. Future studies are needed to define the exact temporal pattern of cerebral transcriptional changes in response to erythromycin preconditioning.

We conclude that induction of ischemic tolerance by the antibiotic erythromycin distinctively alters the brain's transcriptional response to ischemia. An altered disposition to elicit a damaging, specifically inflammatory, reaction after ischemia seems to be a major tier of a complex reprogramming of the cerebral genomic response by this macrolide. Further study is warranted to fully unravel the tolerance-inducing effects of the antibiotic erythromycin and to understand the clinical potential of antibiotic-induced tolerance in patients at risk for cerebral ischemia.

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