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Ischemic Preconditioning and Brain Tolerance

Temporal Histological and Functional Outcomes, Protein Synthesis Requirement, and Interleukin-1 Receptor Antagonist and Early Gene Expression

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Background and Purpose—A short duration of ischemia (ie, ischemic preconditioning [PC]) can provide significant brain protection to subsequent ischemic events (ie, ischemic tolerance [IT]). The present series of studies was conducted to characterize the temporal pattern of a PC paradigm, to systematically evaluate the importance of protein synthesis in PC-induced IT, and to explore candidate gene expression changes associated with IT.

Methods—Temporary middle cerebral artery occlusion (MCAO) (10 minutes) was used for PC. Various periods of reperfusion (ie, 2, 6, and 12 hours and 1, 2, 7, 14, and 21 days) were allowed after PC and before permanent MCAO (PMCAO) (n=7 to 9 per group) to establish IT compared with non-PC (sham-operated) rats (n=22). Infarct size, forelimb and hindlimb motor function, and cortical perfusion (laser-Doppler flowmetry; n=9 per group) were measured after PMCAO. The effects of the protein synthesis inhibitor cycloheximide administered just before PC (n=13 to 17) or administered long after PC but just before PMCAO (n=7 to 8) on IT were also determined. Interleukin-1 receptor antagonist mRNA (reverse transcriptase and polymerase chain reactions [n=20] and Northern analysis [n=50]) and protein expression (immunohistochemistry [n=16]) after PC and early response gene expression (Northern analysis [n=16]) after PMCAO in PC animals were determined.

Results—Hemispheric infarct was significantly ($P<0.01$) reduced only if PC was performed 1 day (decreased 58.4%), 2 days (decreased 58.1%), or 7 days (decreased 59.4%) before PMCAO. PC significantly ($P<0.01$) reduced neurological deficits (similar to reductions in infarct size). Cycloheximide eliminated ischemic PC-induced IT effects on both brain injury and neurological deficits if administered before PC ($P<0.05$) but not if administered long after PC but before PMCAO. PC did not produce any significant brain injury, alter cortical blood flow after PMCAO, or produce contralateral cortical neuroprotection. Interleukin-1 receptor antagonist mRNA and protein expression were increased significantly ($P<0.01$) only during the IT period. PC rats also exhibited a significant ($P<0.01$) reduction in *c-fos* and *zif268* mRNA expression after PMCAO.

Conclusions—PC is a powerful inducer of ischemic brain tolerance as reflected by preservation of brain tissue and motor function. PC induces IT that is dependent on de novo protein synthesis. New protein(s) that occurs at the PC brain site 1 to 7 days after PC contributes to the neuroprotection. Those proteins that are produced after the more severe PMCAO in PC animals apparently do not contribute to IT. The PC-induced IT is also associated with increased expression of the neuroprotective protein interleukin-1 receptor antagonist and a reduced postischemic expression of the early response genes *c-fos* and *zif268*. (*Stroke*. 1998;29:1937-1951.)

Key Words: gene expression ■ interleukin-1 receptor antagonist ■ middle cerebral artery occlusion ■ neurological deficits ■ neuroprotection ■ protein synthesis ■ stroke, ischemic

A short ischemic event (ie, ischemic preconditioning [PC]) can result in a subsequent resistance to severe ischemic tissue injury (ie, ischemic tolerance [IT]). This phenomenon has been described in several organs (especially brain and heart) and by various experimental paradigms and may represent a fundamental cell/organ response to certain types or levels of injury.¹⁻⁹ An early, short-lasting IT was

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observed initially in the heart.^{9,10} Myocardium protection was observed starting between 1 and 60 minutes of PC and lasting for <3 hours.^{9,11} A delayed tolerance (ie, a “second window of protection”) also was identified in the heart beginning ≈24 hours after ischemic preconditioning.^{9,12} No studies on brain

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tolerance have identified an acute, earlier PC protective effect as observed in the heart. For global brain ischemia, repetitive ischemic episodes of 2- to 5-minute durations (spaced at 1-hour intervals) are not protective and result in more severe neuronal damage than a single episode of the same duration.^{4,8} No information is available for early focal stroke effects after PC. In terms of PC and subsequent later protection from focal stroke injury, several reports have established this principle in the brain. For example, a mild global ischemic insult significantly decreased infarct size after permanent middle cerebral artery occlusion (PMCAO).¹³ The protective effect of short-term global ischemia preceding transient focal ischemia has also been demonstrated.¹⁴ In addition, brief, repetitive, transient MCAO decreased infarct size after a subsequent 100-minute transient MCAO followed by reperfusion for 1 to 2 days.¹⁵ However, no information is available regarding the protective effects of transient focal ischemia on permanent focal stroke and its consequences on neurological deficits. Furthermore, little is known of the molecular underpinning of PC-induced IT, although certain heat shock proteins (HSPs) have been implicated in IT.^{5,6,13} In addition, no data are available on the effects of protein synthesis inhibition on the tissue-protective effects of PC in focal stroke.

Interleukin-1 has been implicated as a mediator of neuronal injury after cerebral ischemia. Interleukin-1 β (IL-1 β) mRNA is elevated soon after focal ischemia,¹⁶⁻¹⁸ and IL-1 β protein has been demonstrated to increase in the ischemic brain.¹⁹ IL-1 β may be detrimental to neuronal survival after ischemic injury since IL-1 β administration markedly increases brain edema and the degree of infarct injury,²⁰ whereas neutralizing antibodies to IL-1 β delivered 1 hour after MCAO¹⁴ or inhibition of IL-1 β -converting enzyme by pharmacological or genetic manipulations reduces ischemic brain damage after MCAO.^{21,22} Furthermore, interleukin-1 receptor antagonist (IL-1ra), an endogenous antagonist of the IL-1 receptor^{23,24} that originally had been identified as a neuroprotective protein in the laboratory of Rothwell and Relton,^{25,26} has now also been demonstrated to be protective in several types of brain injury.²⁷⁻³² However, the role of the IL-1 system, the neuroprotective protein IL-1ra in particular, in PC-induced IT has not yet been studied.

The role of early response genes in regulating genetic responses by acting as transcription factors is critical to tissue changes induced by a variety of different stimuli.³³ After ischemia, immediate early gene transcription factors are markedly increased, apparently associated with their activation of diverse target genes.³⁴⁻³⁷ Although the broad implications of altered early response gene expression on cellular/tissue responses to stress are recognized, the role of PC in altering early response gene expression in PC-induced IT is unknown. More importantly, signals which might promote processes that can add to brain injury may be affected very early, as shown by changes in the response of these early response genes after severe focal stroke in previously PC animals.

The purpose of the present series of experiments was to establish and characterize a model of ischemic PC using transient focal MCAO followed by various periods of reperfusion that result in significant tolerance to subsequent

PMCAO. In addition to characterizing the extent and time course of brain protection and reduction in neurological deficits, the roles of blood flow and the importance and timing of protein synthesis in PC-induced IT were investigated. Finally, the effects of PC on IL-1ra expression and the changes of early response gene expression to PMCAO produced by previous PC were determined.

Materials and Methods

Focal Ischemic Preconditioning

Transient MCAO or sham surgery (SS) was performed in spontaneously hypertensive rats (SHR), 300 to 350 g in weight, under sodium pentobarbital anesthesia as described previously.³⁸⁻⁴⁰ SHR were used because of their increased sensitivity and decreased variability in responding to focal ischemia.³⁸ Animals were housed and cared for in accordance with the *Guide for the Care and Use of Laboratory Animals*, NIH publication 85-23, revised 1995. Procedures in which laboratory animals were used were approved by the Institutional Animal Care and Use Committee of SmithKline Beecham Pharmaceuticals.

All animals were allowed free access to food and water before and after surgery. Body temperature was maintained at 37°C with the use of a heating pad throughout the surgery procedure and during postsurgery recovery. Briefly, a craniotomy using stereotaxic procedures was made and the dura was opened over the right middle cerebral artery (MCA). The hooked tip of a platinum-iridium (0.0045-inch diameter) mounted on a micromanipulator was placed under the MCA (at the level of the inferior cerebral vein) and used to lift the artery away from the brain surface to temporarily occlude blood flow, as verified previously by monitoring cortical microvascular perfusion.³⁸ A period of 10-minute temporary MCAO was used for focal ischemic PC on the basis of previous occlusion time response data³⁸ and exploratory studies demonstrating that 10 minutes of temporary MCAO produced no brain injury but tended to reduce the response to ischemic injury 24 hours later. SS was conducted instead of PC (ie, the cranium was removed over the artery), and SS animals served as the control group for all subsequent comparisons.

Permanent Focal Ischemia

To systematically evaluate the effects of PC to induce IT, PMCAO was performed at various times (ie, 6 and 12 hours and 1, 2, 7, 14, and 21 days; n=7 to 9 per group) of reperfusion after PC. PMCAO was performed as described previously.³⁸⁻⁴⁰ The MCA was simultaneously occluded and cut dorsal to the lateral olfactory tract at the level of the inferior cerebral vein. SS was performed 24 hours after PC for comparative purposes (n=7). After SS and PC plus PMCAO surgeries, the temporalis muscle and skin were closed in two layers. The control groups of animals received SS instead of PC before PMCAO and received PMCAO at various times after SS (ie, in a counterbalanced manner to optimize the groups as comparative controls; n=22).

Neurological Deficits

Twenty-four hours after PMCAO, a neurological examination was performed as previously reported.³⁸⁻³⁹ Briefly, forelimb scores were 0 (no observable deficit), 1 (any contralateral forelimb flexion when suspended by the tail), and 2 (reduced resistance to lateral push toward the paretic, contralateral side). A hindlimb placement test consisted of pulling the contralateral hindlimb away from the rat over the edge of a table. A normal response is an immediate placement of the limb back onto the table, and an abnormal/deficit response is no limb placement/movement.

Brain Injury Analysis

After the neurological evaluation, rats were killed with an overdose of sodium pentobarbital, and the forebrain was sliced into 7 coronal slices (2 mm thick) that were immediately immersed in 1% triph-

nyltetrazolium chloride (TTC) as described previously.³⁸ Stained tissues then were fixed by infiltration, photographed, and quantified for ischemic damage with an image analysis system,^{38,40} and the degree of brain damage was corrected for the contribution made by brain edema/swelling as described previously.^{41,42} Briefly, infarct size was expressed as the percent infarcted tissue in reference to the contralateral hemisphere, and infarct volume (mm³) was calculated from the infarct areas on forebrain sections.

Effects of Preconditioning on Potential Early Ischemic Tolerance

To determine whether PC produced an earlier IT-protective effect in this focal ischemia model, a separate experiment was conducted in which SS or PC was performed only 2 hours before PMCAO (n=7 to 8 per group). Animals were then evaluated as described above for neurological deficits and brain injury 24 hours after PMCAO.

Preconditioning and Cell Death

To examine whether the PC procedure per se produced any significant brain injury that could not be detected by gross TTC histological evaluation, forebrain tissue was also prepared as histological sections (6 μ m) and stained with hematoxylin and eosin and then evaluated microscopically for injury. In addition, apparent apoptotic or DNA reparative cell changes were evaluated with in situ end labeling 1 and 2 days and 2 and 4 weeks after PC, as described previously (43; n=3 to 4 per time point). After fixation, the brains were cryoprotected in 30% sucrose and frozen, and sections were cut at 10 μ m on a cryotome. Sections on slides were stored at -70°C. Before they were stained, sections were dried onto the slides at 37°C for 30 minutes, and then endogenous peroxidase activity was quenched by 10 minutes of incubation in 1% H₂O₂ in methanol. After rehydration, sections were incubated for 15 minutes in 10 μ g/mL proteinase K in 50 mmol/L EDTA, 100 mmol/L Tris (pH=8.0) buffer. Reaction was terminated by washing sections for 5 minutes in 100 mmol/L Tris buffer at pH 8. After they were washed in Tris buffer, sections were incubated for 90 minutes with a solution containing 400 pmol biotinylated dATP (biotin-14-dATP; GIBCO/BRL), 0.1 μ mol/L CoCl₂ (Boehringer Mannheim), and 25 U of terminal transferase (TdT, Boehringer Mannheim) in TdT buffer (Boehringer Mannheim). The end-labeling reaction was terminated by washing sections twice in Tris buffer. Sections were then incubated 1 hour at room temperature in avidin-biotin-horseradish peroxidase complex (1:100; Vector Labs). After three 5-minute washes in Tris buffer, diaminobenzidine (0.02%) and H₂O₂ (0.02%) were used to visualize the catalyzed reaction product. After multiple rinses in Tris buffer, sections were counterstained with hematoxylin, dehydrated, cleared, and coverslipped.

Cross-Hemispheric Preconditioning

The effects of focal ischemic PC in one hemisphere on susceptibility to focal ischemic injury in the contralateral hemisphere were determined as follows. PC or SS was performed with right MCAO as described above, and 24 hours later PMCAO was performed on the left MCA. Rats were then killed, and their forebrains were analyzed as described above (n=3 to 4 per group).

Cerebral Blood Flow

Laser-Doppler flowmetry was used to verify occlusion and reperfusion of the MCA by monitoring local cortical microvascular perfusion in the ischemic cortex receiving blood supply from the artery, as described previously.³⁸ A 2- to 3-mm diameter hole was drilled through the skull above the cortical area receiving blood supply from the artery (ie, centered at anteroposterior=0 mm, lateral=4 mm from bregma with level skull). The probe (1 mm in diameter) of a laser-Doppler perfusion monitor (Periflux PF3) then was positioned on the surface of the dura, and the local cortical perfusion was monitored before and after MCAO and during MCA reperfusion on day 1 (for 10-minute temporary MCAO PC or SS groups; n=9 per group); before and after PMCAO on day 2 for both groups; and on day 3 (24 hours after PMCAO) for both groups. Extreme care was

TABLE 1. Oligonucleotide Primers of IL-1ra and rpL32 Used for PCR*

Gene	Sequence	S/A	Position
IL-1ra	5'-TCTGCAGGGACCTTACAGT-3'	S	8-27
	5'-GGTCTTCTCGGAAGTAGAAC-3'	A	513-532
rpL32	5'-GTGAAGCCCAAGATCGTC-3'	S	28-45
	5'-GAACACAAAACAGGCACAC-3'	A	422-440

S indicates sense; A, antisense.

*Base pair positions are those given in the published cDNA sequences for rat IL-1ra²³ and rpL32.⁴⁷

taken to position the perfusion monitor probe in exactly the same cortical location on each day. Animals were anesthetized with pentobarbital and positioned in the stereotaxic unit as described above, and the cortex was closed with suture between recordings. The calibrated output of the perfusion monitor was connected to a Beckman R711 polygraph and averaged in 5-minute periods for comparison between groups. Basal perfusion was recorded before PC or SS on day 1, and all data on day 1 and subsequent days were normalized as a percentage of that value.

Preconditioning and Protein Synthesis

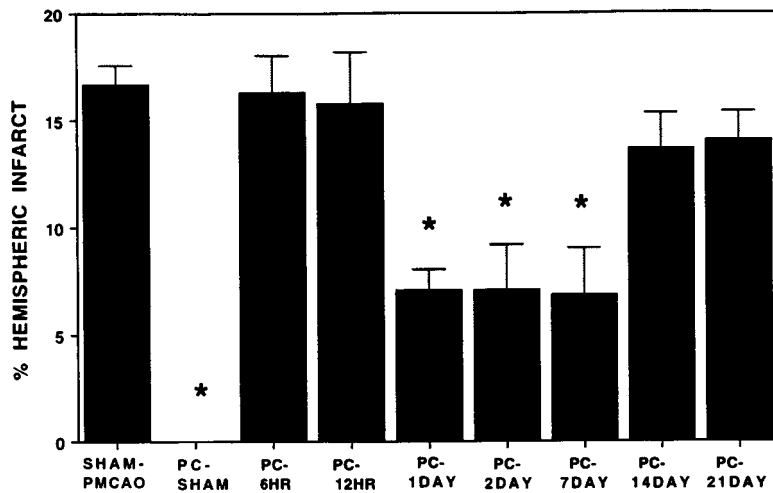
Cycloheximide, a protein synthesis inhibitor,^{44,45} was used to study the role of protein synthesis in the PC induction of IT in 2 separate studies. In the first study, cycloheximide, used at a dose demonstrated previously to block protein synthesis in vivo for \approx 24 hours⁴⁵ (1 mg/kg IP), or vehicle (distilled water) was administered to SHR at a dose of 2.5 mL/kg, 30 minutes before PC or SS, as described above (ie, protein synthesis was inhibited primarily for the 24-hour period after PC and before PMCAO; n=13 to 17 per group). Twenty-four hours after either PC or SS, all animals underwent PMCAO as described above. Twenty-four hours after PMCAO, a neurological examination was performed as described above, and animals were then killed, the brain was removed and stained with TTC, and infarct size and infarct volume were quantified as described above. In the second study, cycloheximide (at the same dose) or vehicle was administered much later (30 minutes before PMCAO) in SHR that had received PC or SS 24 hours earlier (ie, the completely counterbalanced experimental design was identical to the first experiment except that protein synthesis was primarily inhibited for the 24-hour period after PMCAO; n=7 to 8 per group). A neurological examination was performed 24 hours after PMCAO; animals were then killed, and infarct size and infarct volume were quantified as described above.

IL-1ra mRNA Expression

PC was performed as described above, and then forebrains were removed for cortical dissection at 6, 12, 24, or 48 hours after PC (n=4 per group) or 24 and 48 hours after SS (n=4). The PC frontoparietal cortex was dissected from the ipsilateral hemisphere. The contralateral cortex was dissected as the nonischemic control from the same rat.⁴⁰ The cortical samples were immediately frozen in liquid nitrogen and stored at -80°C. Total cellular RNA was prepared from cortical samples and processed as previously described⁴⁶ and initially subjected to quantitative reverse transcriptase and polymerase chain reactions (RT/PCR) analysis. Briefly, the cellular RNA (5 μ g) isolated from the cortical samples at the indicated time points after PC was reverse transcribed with 200 U of RNase H⁻ SuperScript II reverse transcriptase (GIBCO/BRL) for 60 minutes at 37°C primed with 1 μ g of oligo(dT)₁₂₋₁₈ (GIBCO/BRL) at conditions recommended by the manufacturer. The RT products were extracted with phenol/chloroform, ethanol was precipitated, and the products were resuspended in 200 μ L of 10 mmol/L Tris plus 1 mmol/L EDTA, pH 7.5, and stored at -20°C.

Quantitative PCR was performed in a manner similar to that described in detail previously.⁴⁶ A reference gene (ribosomal protein L32 [rpL32]) previously demonstrated to exhibit constant expression

PRECONDITIONING REDUCES HEMISPHERIC INFARCT



PROFILE OF HEMISPHERIC INFARCTION

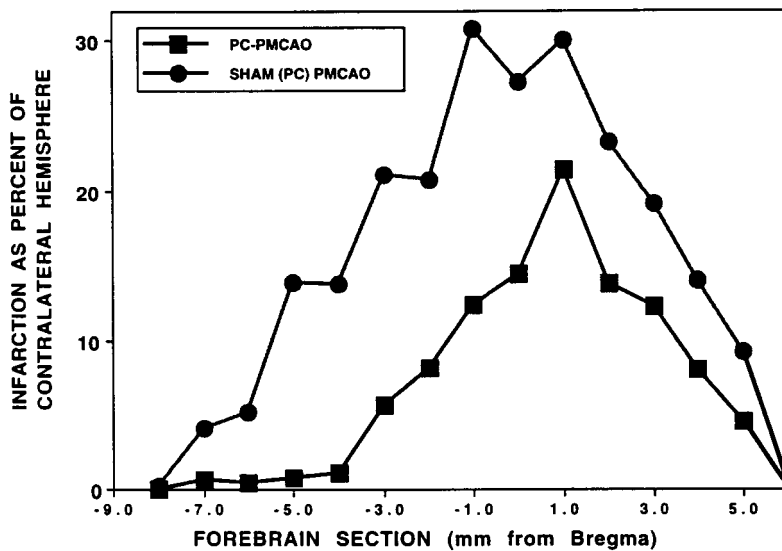


Figure 1. Ischemic PC reduced percent hemispheric infarcts produced by PMCAO. A short (10-minute) period of MCAO (ischemic PC) significantly reduced the degree of percent hemispheric infarction (ie, infarct normalized to the normal contralateral control hemisphere) produced by a later 24 hours of PMCAO compared with SS followed by PMCAO (SHAM-PMCAO). Top, Percent hemispheric infarct was significantly reduced when PMCAO was performed at 1, 2, and 7 days after PC. In the SHAM-PMCAO group (n=22), SS was conducted at various times before PMCAO, and times were grouped together as the appropriate control condition. In the PC-SHAM group (n=7), PC was conducted 1 day before SS, and results demonstrated that PC did not damage the brain. All other groups had PMCAO performed at the times indicated after PC (n=7 to 9 per group). **P*<0.01 compared with SHAM-PMCAO; ANOVA with Dunnett follow-up test. Bottom, Profile of percent hemispheric infarcts over fore-brain slices at various distances from the skull landmark bregma corresponding to data for SHAM-PMCAO and PC-1DAY groups in top panel, demonstrating the significant neuroprotection/brain tolerance exhibited throughout the forebrain as a result of PC.

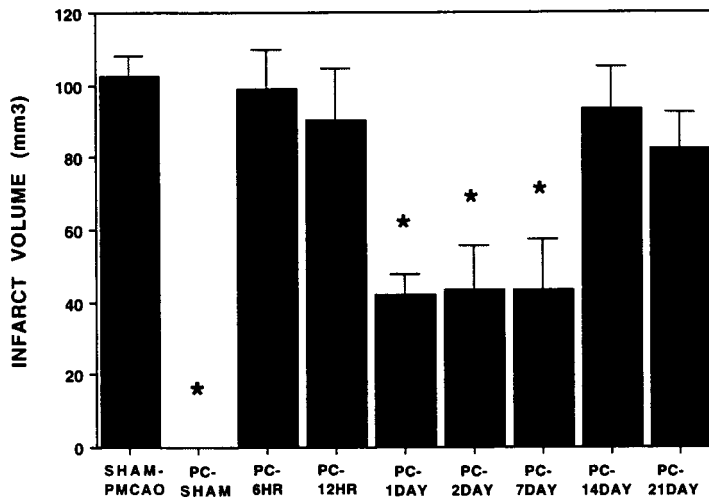
throughout the time course after MCAO⁴⁶ was used as an internal control for coamplification with the targeted gene, IL-1ra. PCR primers used for amplification of IL-1ra and rpl32 were synthesized according to published sequences (Table 1).^{23,47} The optimal amplification conditions were determined as described previously,⁴⁶ and the linear portions of the amplification for both IL-1ra and rpl32 were used for PCR reactions in a total of 50 μ L of reaction mixture (ie, RT products from 0.1 μ g RNA, 28 cycles of amplification, containing 1×10^6 cpm ³²P-labeled antisense primer for IL-1ra and 5×10^4 cpm for rpl32, together with 100 ng each of nonradioactive sense and antisense primers) (Table 1). The amplification was performed with the use of 2.5 U of TaqAmpli polymerase (Perkin-Elmer Cetus) in a thermocycler (Perkin-Elmer Cetus) according to the conditions described previously⁴⁶: initial denaturation, 3 minutes at 94°C; initial annealing, 1 minute at 54°C; and initial extension, 3 minutes at 72°C. The subsequent cycles were as follows: denaturation, 15 seconds at 94°C; annealing, 20 seconds at 54°C; and extension, 1 minute at 72°C. Ten microliters of the PCR product was electrophoresed through a 6% polyacrylamide gel. The gel was dried and subjected to autoradiography at room temperature. The signal intensity was quantified with PhosphorImager (Molecular Dynamics) analysis, and the relative mRNA levels were determined by calculating the ratio of IL-1ra to rpl32 in each coamplified sample.

To confirm the quantitative RT/PCR data, we applied Northern blot analysis using poly(A) RNA isolated from PC and contralateral control cortex of 50 rats 24 hours after PC. Ten micrograms per lane of poly(A) RNA was electrophoresed through formaldehyde agarose gel and transferred to a GeneScreen Plus membrane (Du Pont–New England Nuclear). For Northern analysis, the cDNA fragments for IL-1ra and rpl32 were gel purified after PCR amplification (as described above) and were uniformly labeled with [α -³²P]dATP (3000 Ci/mmol, Amersham Corp) with a random-priming DNA labeling kit (Boehringer Mannheim). Hybridization and washing were performed as described in detail previously.^{18,46}

IL-1ra Protein Expression

After PC or SS, rats were allowed to recover for 6, 12, 24, or 48 hours (n=3 to 4 per group). Rats were overdosed with sodium pentobarbital and perfused through the aorta with 100 mmol/L phosphate-buffered saline for 5 minutes followed by 100 mmol/L phosphate-buffered saline containing 2% paraformaldehyde and 0.2% glutaraldehyde (4°C) for 15 minutes. The brain was then removed and postfixed in 100 mmol/L phosphate buffer containing 2% paraformaldehyde for 6 hours. Brains were stored at 4°C in 120 mmol/L sodium phosphate buffer containing 0.06% sodium azide. Each brain was sectioned (50 μ m) on a vibratome in ice-cold

PRECONDITIONING REDUCES INFARCT VOLUME



PROFILE OF INFARCT VOLUME

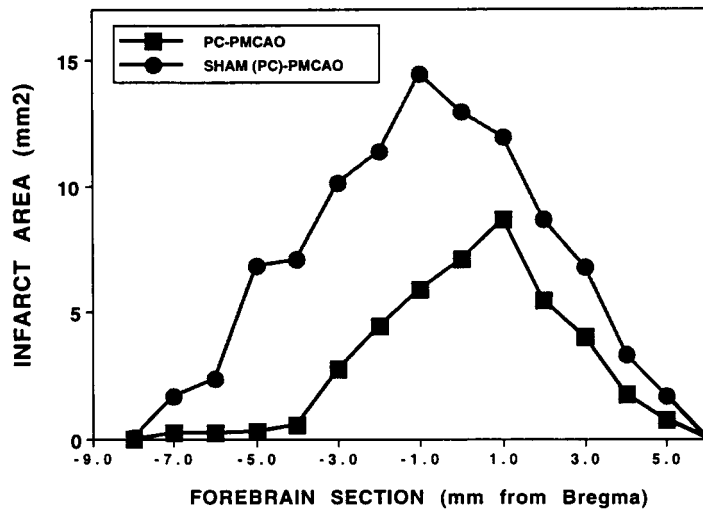


Figure 2. Ischemic PC reduced absolute infarct volume produced by PMCAO. Data are similar to those in Figure 1 except for depiction of absolute measure of infarct volume. Top, Infarct volume was significantly reduced when PMCAO was performed at 1, 2, and 7 days after PC. In the SHAM-PMCAO group (n=22), SS was conducted at various times before PMCAO, and times were grouped together as the appropriate control condition. In the PC-SHAM group (n=7), PC was conducted 1 day before SS, and results demonstrated that PC did not damage the brain. All other groups had PMCAO performed at the times indicated after PC (n=7 to 9 per group). *P<0.01 compared with SHAM-PMCAO; ANOVA with Dunnett follow-up test. Bottom, Profile of infarct volume over forebrain slices at various distances from the skull landmark bregma corresponding to SHAM-PMCAO and PC-1DAY groups in top panel, demonstrating the significant neuroprotection/brain tolerance exhibited throughout the forebrain as a result of PC.

100 mmol/L Tris buffer (pH 7.6). Immunohistochemistry was performed on free-floating sections. Sections were incubated in 1% H₂O₂/Tris for 1 hour, rinsed in Tris, then rinsed in Tris containing 0.1% Triton X-100 and in Tris containing 0.1% Triton X-100 and 0.005% bovine serum albumin. Sections were incubated 1 hour in 10% horse serum in Tris containing 0.1% Triton X-100 and 0.005% bovine serum albumin to block nonspecific immunoreactivity. After 2 Tris buffer washes, sections were incubated overnight at 4°C in Tris buffer containing a sheep anti-rat IL-1ra affinity-purified antibody (1:10 000; National Institute for Biological Standards and Control). After 2 Tris buffer washes, sections were incubated in Tris buffer containing a biotinylated horse polyclonal antibody raised against sheep IgG (1:500; Vector Laboratories Inc). After 2 Tris buffer washes, sections were incubated 1 hour in avidin-biotin-horseradish peroxidase complex (1:1 000; Vector Laboratories Inc). After 3 Tris buffer washes, sections were immersed for 20 minutes in diaminobenzidine-tetrachloride (0.02%; Sigma Chemical Co) made up in Tris buffer containing 0.15 mg/100 mL glucose oxidase, 40 mg/mL ammonium chloride, and 200 mg/100 mL β-D(+)-glucose (Sigma Chemical Co). Appropriate control experiments were used in these studies (eg, optimal antibody dilutions were determined by serial dilutions on control and experimental sections, and additions of IL-1ra [R&D Systems] were administered to demonstrate adsorption out of the immunoreactivity signal observed in the PC cortex).

Incubation of sections in solution was conducted with a shaker bath. Immunohistochemically stained sections were mounted onto coated slides, air dried overnight, dehydrated, and coverslipped. Analysis was performed with the use of an Olympus microscope, and representative sections were photographed for illustration.

Early Response Gene Expression

In 4 separate groups of SHR, SS or PC surgery was followed 24 hours later with PMCAO. After 2 hours of PMCAO, ipsilateral (ischemic) and contralateral (control) cortex samples (corresponding to the control infarction region) were dissected as described previously^{37,46} and immediately frozen in liquid nitrogen and stored at -80°C for evaluation for early response gene expression (n=4 per group). The 2-hour time point was selected on the basis of previous data demonstrating optimum early response gene expression in this model.³⁷ Total cellular RNA was extracted and evaluated as described previously.^{37,46} Briefly, RNA samples (20 μg per lane) were electrophoresed through formaldehyde-agarose slab gels and transferred to membranes. Northern hybridizations and stripping of the cDNA probes for *c-fos*, *zif268*, and *rpL32* were performed as previously described.³⁷ PhosphorImager was used to quantify the band intensities of the Northern blots, which then underwent computer image analysis. Relative mRNA levels (percentage) for *c-fos*

and zif268 early response genes were normalized to the rpL32 mRNA signals in each sample as described previously.^{18,37,46}

Statistical Analysis

All data are presented as mean±SEM. Comparisons between multiple groups or time periods were made with ANOVA; follow-up analyses were done with the least significant difference or Dunnett test. Nonparametric data (ie, hindlimb neurological deficits/scores) were analyzed by the χ^2 test. Differences were considered significant at $P<0.05$.

Results

Ischemic Tolerance to Brain Injury

Brain injury in this model of focal stroke involves an infarction restricted primarily to the cortex.³⁸ PC produced significant tolerance to subsequent focal ischemia, as reflected by reduced cortical infarct size when performed at certain times before PMCAO. Percent hemispheric infarct produced by 24 hours of PMCAO was significantly ($P<0.01$) reduced if PC was performed 1 day (58.4%), 2 days (58.1%), or 7 days (59.4%) previously (Figure 1, top panel). However, percent hemispheric infarct was not affected if PC was performed 6 hours, 12 hours, 14 days, or 21 days before PMCAO. PC alone produced no tissue injury that could be detected by TTC staining (data not shown; $n=7$), and PC performed 24 hours before SS (ie, the PC-SS group) produced absolutely no infarction. The spatial profile of percent hemispheric infarct illustrates that protection was extended throughout all cortical sections when PMCAO was performed 1 day after PC (Figure 1, bottom panel). Identical results were observed for infarct volume, which was significantly reduced if PC was performed 1 day (57.5%), 2 days (56.4%), or 7 days (56.2%) but not 6 hours, 12 hours, 14 days, or 21 days before PMCAO (Figure 2, top panel) and for the profile of infarct areas throughout cortical sections (Figure 2, bottom panel). Cortical infarct protection due to PC-induced IT was greater in the more posterior than in the anterior forebrain (Figures 1 and 2, bottom panels).

No Early Ischemic Tolerance After Preconditioning

Table 2 lists the effects of 24 hours of PMCAO in rats receiving SS or PC only 2 hours previously. There was no neuroprotective effect of PC on brain injury or neurological function when PMCAO was initiated this soon after PC.

No Contralateral Effects of Preconditioning

The ability of PC to induce IT was restricted to the area made ischemic by PC, and no significant neuroprotection was

TABLE 2. Effects of 24-Hour PMCAO Initiated Only 2 Hours After PC

Measure	SS	PC
Hemispheric infarct, %	14.4±2.2	13.5±1.6
Infarct volume, mm ³	115.5±17.6	108.9±13.7
Forelimb deficit, grade	1.86±0.14	1.88±0.13
Hindlimb deficit, grade	0.86±0.14	0.75±0.16

No significant differences were observed (ie, no early, more immediate neuroprotective effects of PC were observed); $n=7-8$ per group.

TABLE 3. Effects of PC Using Right Transient (10-Minute MCAO) on Left Permanent MCAO-Induced Brain Injury and Neurological Deficits

Measure	Right MCAO SS (n=3)	PC (Right MCAO for 10 min) (n=4)
Hemispheric infarct, %	17.0±3.6	14.9±1.5
Infarct volume, mm ³	106.3±23.3	95.3±9.6
Forelimb deficit, Grade	2.0±0.0	2.0±0.0
Hindlimb deficit, Grade	1.0±0.0	0.75±0.2

Left PMCAO was always performed 24 hours after right MCAO sham surgery (SS) or PC. No significant differences were observed (ie, no protective effects of PC were observed in the contralateral cortex).

observed in the contralateral cortex (Table 3). PC (compared with SS) of the right MCA did not significantly modify the degree of infarction 24 hours after left PMCAO (ie, did not affect the degree of brain injury to the left hemisphere due to PMCAO when performed 24 hours later).

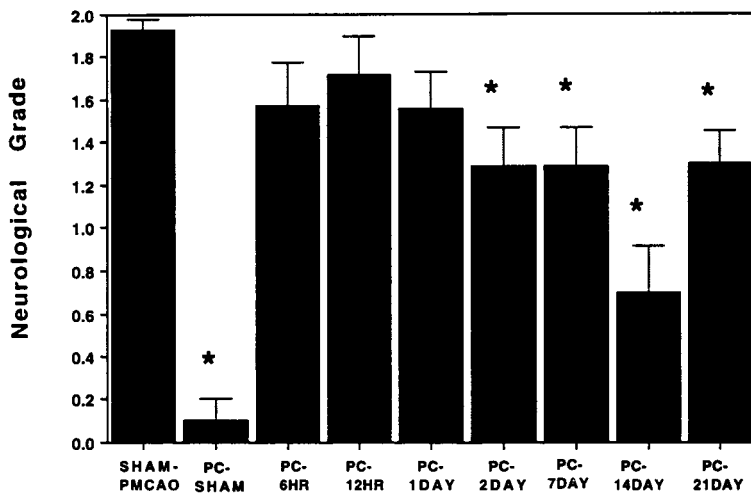
Ischemic Tolerance to Neurological Deficits

PC, when performed at certain times before PMCAO, produced significant reductions in neurological deficits that paralleled and extended beyond reductions in infarct size. The forelimb deficit grade quantifies the contralateral hemiparesis and hemiparesis that can be a consequence of focal ischemia due to ipsilateral cortical infarction. Forelimb deficit was significantly reduced if PC was performed 2 days (31.0%), 7 days (31.0%), 14 days (64%), or 21 days (31.0%) but not 6 hours, 12 hours, or 1 day before PMCAO (Figure 3, top panel). PC alone produced no forelimb deficit (data not shown; $n=7$), and PC performed 24 hours before SS (ie, the PC-SS group) produced no significant forelimb deficits. Hindlimb deficit grade quantifies a deficit in proprioception associated with loss of ipsilateral cortical function. Hindlimb deficit after 24 hours of PMCAO was significantly reduced if PC was performed 1 day (52%), 2 days (100%), 7 days (100%), or 14 days (62%) previously (Figure 3, bottom panel). However, hindlimb deficit was not affected if PC was performed 6 hours, 12 hours, or 21 days before PMCAO. PC alone produced no hindlimb deficit (data not shown; $n=7$), and PC performed 24 hours before SS (ie, the PC-SS group) produced absolutely no hindlimb deficit.

Preconditioning Does Not Induce Cell Death

No significant injury was identified that was due to PC. On hematoxylin and eosin-stained histological sections, only a localized injury was identified at the surgical MCA site. In addition, no difference in the incidence of end-labeled neurons was observed between the ipsilateral and contralateral cortices (ie, rarely, in situ end-labeled neurons and/or glial cells were observed similarly in both PC and contralateral control cortex). A few in situ end-labeled neurons could sometimes be identified at the surgical site 1 to 2 days after PC. Similar results were also observed in SS rats killed 1 day after PC. No end-labeled or abnormal cells were detected at 2 or 4 weeks after PC. Therefore, no significant tissue injury or cell death was identified that could be attributed to the PC procedure (data not shown; $n=3$ to 4 rats per time point).

PRECONDITIONING REDUCES FORELIMB DEFICIT



PRECONDITIONING REDUCES HINDLIMB DEFICIT

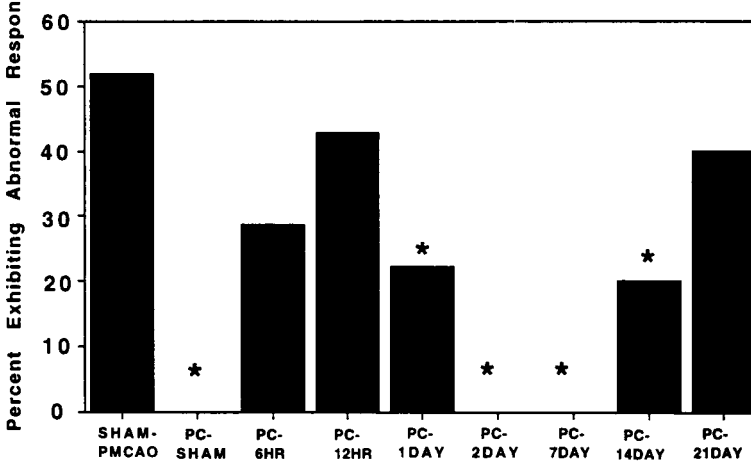


Figure 3. Ischemic PC reduced neurological deficits produced by PMCAO. A short (10-minute) period of MCAO (ischemic PC) significantly reduced neurological deficits produced by a later 24 hours of PMCAO compared with SS followed by MCAO (SHAM-MCAO). Top, Forelimb neurological deficit was significantly reduced when PMCAO was performed at 2, 7, 14, and 21 days after PC. * $P < 0.05$ compared with SHAM-PMCAO; ANOVA with Dunnett follow-up test. Bottom, Hindlimb neurological deficit was significantly reduced when PMCAO was performed at 1, 2, 7, and 14 days after PC. Animals per group and group labeling are exactly as described in Figures 1 and 2 (ie, the same rats were used for all measures). * $P < 0.05$ compared with SHAM-PMCAO; χ^2 test.

Cerebral Blood Flow Unaffected by Preconditioning

On day 1, SS produced no change from 100% basal cortical flow, and PC produced an immediate decrease in flow to $< 30\%$ of basal flow, which recovered to 100% immediately on reperfusion and persisted for ≥ 60 minutes of continuous monitoring, as described previously in this model³⁸ (data not shown). On day 2, similar basal flow between groups before PMCAO was observed, and a similar decrease in blood flow was observed for both PC and SS groups after PMCAO, as illustrated in Figure 4. This lack of any significant difference between groups was observed for ≥ 60 minutes of post-PMCAO monitoring. Although blood flow was reduced on day 3 in both groups, no significant difference in cortical perfusion was observed between SS or PC groups at this time (ie, 24 hours after PMCAO; data not shown).

Preconditioning Requires Protein Synthesis

In the first study, administration of cycloheximide 30 minutes before PC blocked its protective effects, as demonstrated by infarct size, infarct volume, and neurological deficits (Figure 5). Vehicle-treated PC animals demonstrated a reduced

($P < 0.05$) percent hemispheric infarct compared with vehicle-treated SS animals, whereas cycloheximide-treated PC animals did not exhibit reduced hemispheric infarct compared with cycloheximide-treated SS animals (Figure 5, top left panel). Animals that underwent SS all had significantly greater ($P < 0.05$) and similarly sized infarcts compared with vehicle-treated PC animals. These relationships were also evident for infarct volume (Figure 5, top right panel). PC animals treated with cycloheximide did not exhibit reduced infarct volume (ie, they did not exhibit IT/were not protected). In addition, cycloheximide also blocked the protective effect of PC on neurological outcome. Both forelimb and hindlimb deficits (Figure 5, bottom left and bottom right panels, respectively) were significantly reduced ($P < 0.05$) in vehicle-treated PC animals compared with vehicle-treated SS rats. No protective effects of PC were observed in animals treated with cycloheximide. Blocking protein synthesis early after PC before PMCAO abolished PC-induced IT.

In the second study, the results were completely different. The administration of cycloheximide 30 minutes before PMCAO did not block PC-induced IT-protective effects (Figure 6). Both vehicle- and cycloheximide-treated PC

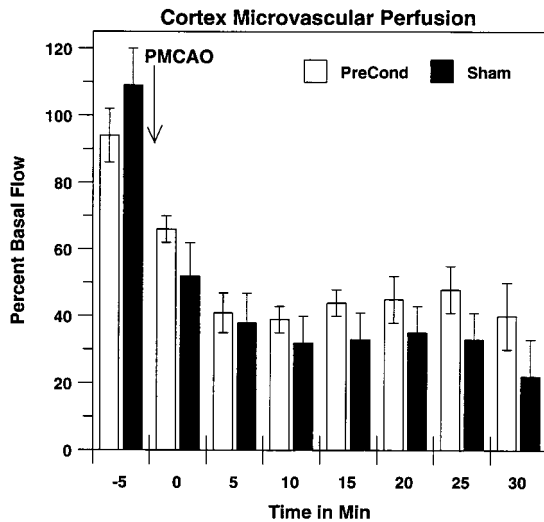


Figure 4. Ischemic PC does not alter cortical microvascular perfusion. Cortex microvascular perfusion (ie, MCA territory) was measured with laser-Doppler flowmetry before and after PMCAO for animals receiving PC (PreCon; n=9) or SS (n=9) 1 day previously. No significant differences were observed for cortical blood flow that could explain the PC-induced neuroprotection.

animals demonstrated a reduced ($P<0.05$) percent hemispheric infarct compared with SS animals (Figure 6, top left panel). Animals that underwent SS all had significantly greater ($P<0.05$) and similarly sized infarcts compared with PC-treated animals. These relationships were also evident for infarct volume (Figure 6, top right panel). In addition, cycloheximide did not block the protective effect of PC on neurological outcome when administered later before PMCAO. Both forelimb and hindlimb deficits (Figure 6, bottom left and bottom right panels, respectively) were significantly reduced ($P<0.05$) in both vehicle- and cycloheximide-treated PC animals compared with SS rats. Blocking protein synthesis much later after PC (ie, essentially after PMCAO) did not block PC-induced IT. The health of animals after cycloheximide treatment in both studies was good. However, it should be noted that they did require $\approx 20\%$ less pentobarbital anesthesia for PMCAO surgery after this treatment.

Preconditioning Induces IL-1ra mRNA Expression

Figure 7 (top panel) illustrates the quantitative analysis of IL-1ra mRNA (n=4) normalized to the internal standard. Sham-operated samples were taken at 24 and 48 hours. Only very low levels of IL-1ra mRNA were detected in the sham-operated animals or in the contralateral (control) cortex, as well as in the early time points of the ipsilateral (PC) cortical samples. The level of IL-1ra mRNA was markedly increased in the ischemic cortex at 24 hours ($P<0.01$) and 48 hours ($P<0.01$) after PC. The expression of IL-1ra in the ipsilateral and contralateral cortices 24 hours after PC was confirmed by Northern analysis (ie, was in close agreement with the data generated by quantitative RT/PCR) (presented as an inset to the graph describing those data [Figure 7, top panel]).⁴⁶

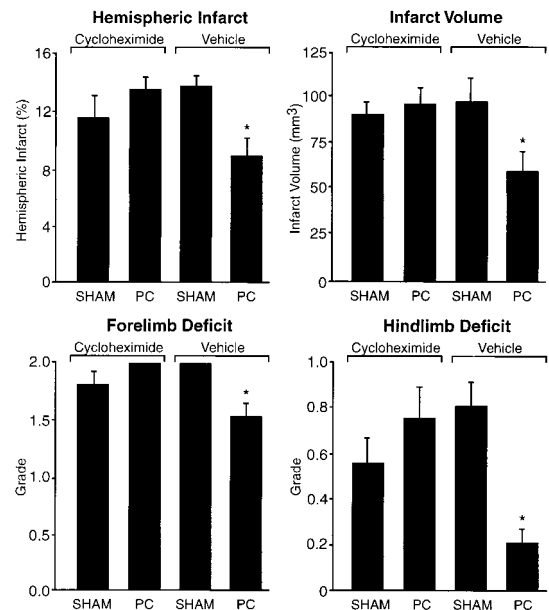


Figure 5. Blocking protein synthesis after PC eliminated PC-induced IT effects of reduced brain injury and neurological deficits. Completely counterbalanced study was conducted to evaluate effects of protein synthesis inhibition with cycloheximide treatment. Cycloheximide administered 30 minutes before PC (as described in Materials and Methods) demonstrated that new protein(s) synthesized during the period after PC was essential to PC-induced IT-neuroprotective effects on later PMCAO. Top left, Significant neuroprotective effect of reduced percent hemispheric infarct in PC compared with SS performed 1 day before PMCAO in animals treated with vehicle was not observed in animals treated with cycloheximide. Cycloheximide treatment did not alter the degree of ischemic injury due to PMCAO (ie, in SS animals) but blocked the ability of PC to reduce injury (ie, blocked PC-induced IT when administered before PC). Top right, Similar results for infarct volume. Bottom left, Significant neuroprotective effect of reduced forelimb deficit in PC compared with SS performed 1 day before PMCAO in animals treated with vehicle was not observed in animals treated with cycloheximide. Cycloheximide treatment did not alter the degree of forelimb deficit due to PMCAO (ie, in SS animals) but blocked the ability of PC to reduce deficits (ie, blocked PC-induced IT when administered before PC). Bottom right, Similar results for hindlimb deficit. n=13 to 17 per group. * $P<0.05$ different from all other groups; ANOVA with least significant difference follow-up test.

Preconditioning Induces IL-1ra Protein Expression

IL-1ra protein was not observed in the PC cortex at 6 and 12 hours after PC or in the contralateral control cortex at any time after PC (Figure 7, bottom left panel). However, at 24 and 48 hours after PC, significant, specific IL-1ra labeling was observed in cells scattered throughout the PC cortex, as illustrated in Figure 7 (bottom right panel; n=3 to 4 per time point). The significant IL-1ra protein expression at 24 and 48 hours appeared to be restricted within cells having histologically identified neuronal morphology in the PC cortex and was not detected at all or was only lightly present in a few cells immediately adjacent to the surgical wound in SS rats (n=3).

Ischemic Tolerance to Early Response Gene Expression

PC 24 hours before PMCAO produced a significant reduction in the expression of early response genes 2 hours after

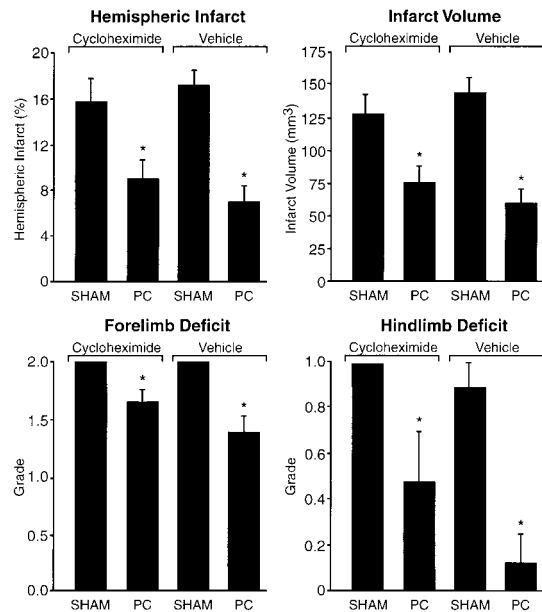


Figure 6. Blocking protein synthesis after PMCAO (but not during the period after PC preceding PMCAO) did not affect PC-induced IT effects of reduced brain injury and neurological deficits. Completely counterbalanced study was conducted to evaluate effects of protein synthesis inhibition with cycloheximide treatment. Cycloheximide administered later, 30 minutes before PMCAO (as described in Materials and Methods), demonstrated that new protein synthesized later after PMCAO in PC animals was not important for PC-induced IT. Top left, Significant neuroprotective effect of reduced percent hemispheric infarct in PC compared with SS performed 1 day before PMCAO was observed in animals treated with cycloheximide or vehicle. Cycloheximide treatment did not alter the degree of ischemic injury due to PMCAO (ie, in SS animals) and did not affect the ability of PC to reduce deficits (ie, did not block PC-induced IT when administered much later after PC, 30 minutes before PMCAO). Top right, Similar results for infarct volume. Bottom left, Significant neuroprotective effect of reduced forelimb deficit in PC compared with SS performed 1 day before PMCAO was observed in animals treated with cycloheximide or vehicle. Cycloheximide treatment did not alter the degree of forelimb deficit due to MCAO (ie, in SS animals) and did not affect the ability of PC to reduce deficits (ie, did not block PC-induced IT when administered much later after PC, 30 minutes before PMCAO). Bottom right, Similar results for hindlimb deficit. $n=7$ to 8 per group. * $P<0.05$ different from both SS groups; ANOVA with least significant difference follow-up test.

PMCAO. We previously reported that *c-fos* mRNA expression is increased in the ischemic cortex compared with the control cortex 2 hours after MCAO; however, previous PC treatment significantly reduced this response (Figure 8, top panel). Similar results were also observed for *zif268*, the expression of which was significantly reduced as a result of previous PC at 2 hours after MCAO (Figure 8, bottom panel). Northern blots were similar to those presented previously from this laboratory³⁷ (data not shown). Basal levels of *c-fos* and *zif268* mRNA in the control cortex were not altered by PC compared with SS and were similar to basal levels described previously in the control cortex or after SS.³⁷

Discussion

It has been previously demonstrated that brief periods of global ischemia having no untoward consequences per se

resulted in smaller infarctions much later after PMCAO.¹³ The protective effect of short bouts of global ischemia preceding transient focal ischemia has also been demonstrated.¹⁴ A 20-minute period of transient MCAO (which produced focal injury) reduced neuronal necrosis after subsequent global ischemia in the rat.⁴⁸ Only 1 report has demonstrated that previous transient focal ischemia reduces subsequent transient focal ischemic injury.¹⁵ In this later study, three 10-minute periods of transient ischemia separated by 45 minutes of reperfusion (which by itself produced brain injury) reduced the degree of infarction due to 100 minutes of transient MCAO if applied 2, 3, and 5 days (but not 1 or 7 days) before this more severe transient focal ischemia.¹⁵ The present report is the first demonstration of tolerance to permanent focal stroke due to PC with transient focal ischemia. Although it has been demonstrated previously that reperfusion accelerates the tissue response to ischemia,⁴⁹ the present technique of PC (which was selected from a pilot study of duration of ischemia-injury response relationships) produced no significant brain injury, as shown by gross histology and by evaluating histological sections and in situ end-labeled neurons after PC. In this respect, our PC paradigm differs from all previous focal models. In addition, unlike IT in the heart, this series of studies contains the first data suggesting that as for cerebral global ischemia, no early (ie, neuroprotection within 2 hours after PC) protection can be demonstrated for PC on PMCAO injury. Finally, this is the first demonstration of IT in SHR. Additional studies should determine whether there are strain differences in PC-induced IT, as have been identified for strain differences in ischemic sensitivity.³⁸

The degree of tissue protection in this model is remarkable, exceeding 50% reduction in infarct size from 1 to 7 days after PC. In this distal PMCAO model, brain infarction is restricted to the cortex. The brain protection associated with PC-induced IT was distributed across the entire forebrain cortical infarction, with a larger reduction observed in the more posterior forebrain cortex (see Figures 1 and 2, bottom panels). A reduction of neurological deficits due to IT is also demonstrated for the first time, which is in accord with the degree of tissue protection. Interestingly, the 14- and 21-day prior PC measures of forelimb deficit and the 14-day prior PC measure of hindlimb deficit are still significantly reduced even though brain protection due to the prior PC had already subsided (ie, the infarct extended back to the larger, non-PC, nonprotected size), suggesting a greater PC protective effect on neurological functions. The timing of protection from neurological deficits might be related to the different profile of protection from infarction observed across the forebrain. For example, hindlimb deficit protection correlated more closely with PC-induced brain protection and involved the more posterior, parietal cortical area (ie, that area protected to a greater degree by PC, as shown in Figures 1 and 2, bottom panels), a cortical area more important for somatosensory integration more critical to a normal limb placement-proprioceptive response. Forelimb placement, however, correlated less well with PC-induced brain protection and involved both the parietal and the more anterior, frontal cortical area (ie, that area protected to a lesser degree by PC),

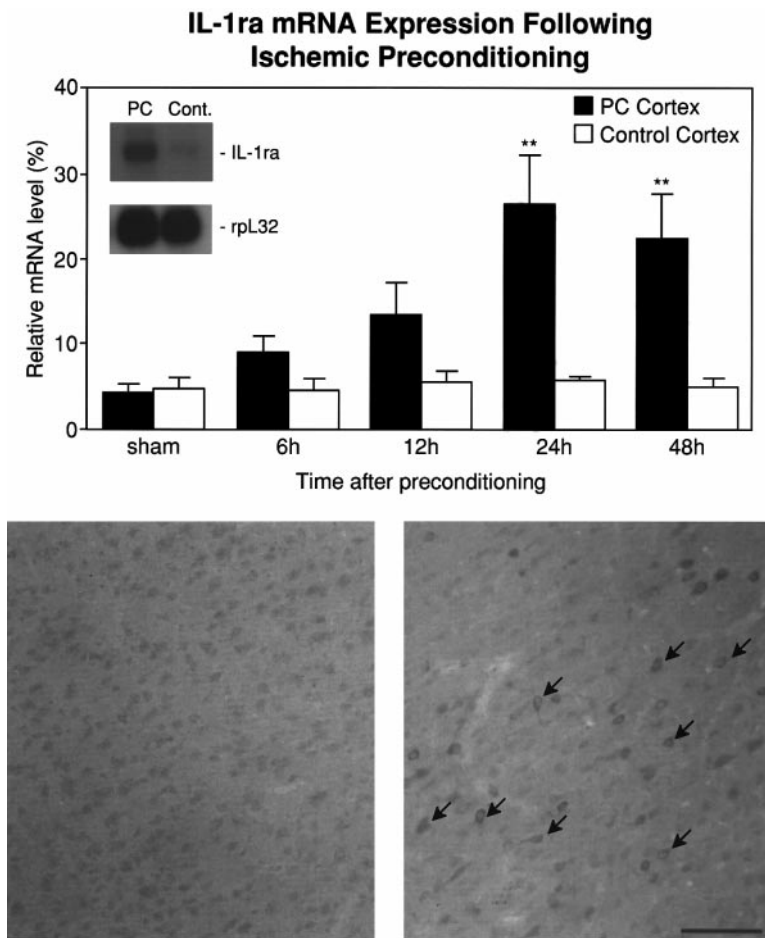


Figure 7. Ischemic PC increases the expression of the neuroprotective protein IL-1ra. PC increased the expression of IL-1ra in PC but not the control cortex. Expression of IL-1ra expression occurred when neuroprotection due to PC was observed (see Figures 1 and 2). Top, Increased IL-1ra mRNA after PC as demonstrated by quantitative RT-PCR (see Materials and Methods). Quantitative data depict the time course of relative IL-1ra mRNA levels after PC. The coamplified DNA bands of IL-1ra and rpl32 were quantified by PhosphorImager analysis. The ratio of IL-1ra/rpl32 in each coamplified sample was calculated, and the relative levels (ie, that normalized to rpl32 mRNA with the sum of all cortical samples set to 100%) of IL-1ra mRNA are presented. Data are presented as the mean \pm SE of 4 separate experiments (ie, $n=4$ different pairs of rat cortical samples used for each time point). ** $P<0.01$ compared with SS (24 hours) cortex samples (ie, Control Cortex); ANOVA with Dunnett follow-up test. Top inset, Results also were verified by Northern blot analysis. Northern analysis showed that expression of IL-1ra and rpl32 mRNA was increased 24 hours after PC in PC but not in control (Cont.) rat cortex. Poly (A) RNA (10 μ g per lane) was extracted from the PC and contralateral control cortex of 50 rats, resolved by electrophoresis, transferred to a nylon membrane, and hybridized to the cDNA probes as indicated in text and previously.⁴⁶ Bottom, Photomicrographs of IL-1ra immunoreactivity in control and PC cortex. No significant IL-1ra immunoreactivity was observed in the control cortex after SS (left photomicrograph), in 6- or 12-hour post-PC cortex, or in the contralateral cortex of animals at any time after PC. IL-1ra immunoreactivity was observed in cells exhibiting neuronal morphology (only several of many labeled cells are indicated by arrows) throughout the PC cortex (ie, throughout the MCA territory that would become infarcted if longer periods of ischemia were performed) at 1 and 2 days after PC (right photomicrograph). $n=3$ to 4 per time point/condition; bar=100 μ m.

a cortical area important for normal limb function and control.⁵⁰ Perhaps other factors, such as increased nervous system plasticity after injury,⁵¹ are also involved in protection from neurological deficits in PC-induced IT. Growth factors have been demonstrated to preserve function and increase recovery from injury,⁵² and their expression and involvement in IT need to be determined. It is important to note that the neurobehavioral measures tend to be more variable than brain injury measurements. However, the 1-day PC-induced IT effects on neurological deficits were similar and quite consistent for both neurological measures, as depicted in Figures 5 and 6. In any event, although reduction in hindlimb deficit sometimes more closely correlated with histological protection than forelimb function preservation by PC, both reflect the significant IT effects of PC.

No significant difference was observed in cortical blood flow that could explain the PC-induced neuroprotection. Lack of regional cerebral blood flow changes in brain areas due to PC-induced tolerance has also been reported in other models.^{14,15} Furthermore, no significant contribution of plasma glucose, blood gases, or blood pressure changes could be associated with brain PC and the induction of IT.¹³⁻¹⁵ In addition, the present study demonstrates that IT is not associated with systemic release of neuroprotective factor(s) since the protection was localized to the ipsilateral preconditioned cortex and was not observed in the contralateral hemisphere.

The molecular mechanisms associated with PC have not yet been elucidated. However, significant changes in gene transcription/translation have been documented after focal stroke that consist of well-defined sequential expression of genes with diverse functions that may bear on tissue remodeling and resolution of the ischemic brain.^{53,54} The present data are the first demonstration that newly synthesized proteins are critical to PC-induced brain tolerance. It has also been demonstrated that the PC-induced IT in the heart is associated with new protein synthesis that occurs within 60 minutes after PC⁵⁵ and is important for the later, delayed induction (ie, "second window") of protection.^{56,57} Understanding specific changes in gene expression and the identification of newly translated proteins can be critical to understanding the mechanism of tissue protection in this model of IT.

Ischemic injury has been shown to induce the expression of HSPs.^{35,58-61} HSPs are believed to contribute to cellular repair processes by refolding denatured proteins and acting as molecular chaperones in normal processes such as protein translocation and folding (for review, see Reference 62). The highly inducible member of the family of HSPs, HSP70, has been cited in association with tolerance to ischemic injury in the brain.^{5,13,48,61,63,64} Although available data indicate the strong relationship between HSPs and IT, other data also suggest that HSP expression is not required for IT (ie, HSP

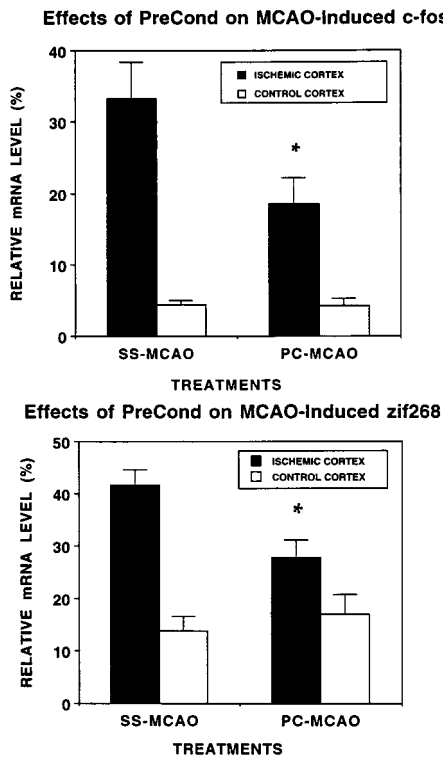


Figure 8. Ischemic PC reduced PMCAO-induced increases in early response gene expression. A short (10-minute) period of MCAO (ischemic PC) significantly reduced ischemic cortex early response gene expression produced by 2 hours of PMCAO (PC-MCAO) compared with SS followed by 2 hours of MCAO (SS-MCAO). Top, Results for *c-fos* mRNA ($n=4$ per group). Bottom, Results for *zif268* mRNA ($n=4$ per group). Northern blots were quantified by PhosphorImager analysis and normalized to rpl32 mRNA bands for samples loaded in each lane as described in text and previously.³⁷ The relative mRNA level for each probe is displayed graphically, with a sum of 100% for all cortical samples. * $P<0.05$ compared with SS-MCAO ischemic cortex; ANOVA with Dunnett follow-up test.

expression is not observed to be associated with neuroprotection induced by PC).^{15,65} We recently demonstrated increased HSP70 and HSP27 expression after PC and currently are trying to understand the relationship of this protein expression to IT (data not shown).

The presence of low levels of IL-1ra in the normal brain and the marked upregulation of IL-1ra mRNA and protein after ischemic injury⁴⁶ suggest that IL-1ra can serve as a defense system to attenuate inflammatory reactions elicited by brain injury. Neuroprotection produced by IL-1ra, first demonstrated in the laboratory of Rothwell, has now been demonstrated in many animal models of brain injury,²⁵⁻³² and the temporal induction profile of IL-1ra after MCAO⁴⁶ virtually parallels that of IL-1 β ,¹⁷ suggesting that IL-1ra may counteract IL-1 β effects after ischemic stroke. Therefore, increased IL-1ra expression induced by PC could interfere with the development of injury associated with ischemia-induced IL-1 β production. The present study is the first demonstration that PC can induce expression of this neuroprotective protein and that this increased expression is apparently in neurons, although it is at this time based only on cell morphology. The increased expres-

sion of both IL-1ra message and protein correlates in time with PC-induced neuroprotection. Recent data from the laboratory of Rothwell and Stroemer³¹ suggest that blocking IL-1 in the striatum with IL-1ra reduces focal stroke cortical injury (ie, stimulation of IL-1 receptors in the striatum contributes to cortical ischemic injury, and blocking those receptors contributes to the neuroprotective effects of IL-1ra IL-1ra's neuroprotective effects through a polysynaptic pathway or release of a specific substance or substances).³¹ Although we did not study the striatum in the present study, it is interesting to speculate that cortical PC-induced IL-1ra-expressing neurons project to the striatum to mediate the cortical protection observed in the present study. The IL-1ra message and expression data are really only circumstantially linked to IT, and these data are primarily "hypothesis generating." To test such a hypothesis, future studies should evaluate whether blocking IL-1ra can reduce IT under these conditions.

The mRNA encoded by the *c-fos* gene and its protein product, Fos, provide an index of cell activation.⁶⁶ Together with Fos, Jun, Zif264, and other related proteins, these transcription-regulating factors can couple diverse stimuli to widespread expression of other genes.³³ For example, the dimerization of Fos and Jun forms a functional transcription factor complex (eg, AP-1) that binds to regulatory DNA sequences located in the upstream regions of target genes and regulates gene transcription.⁶⁷ The *zif268* sequence encodes a "zinc finger" protein that acts at another class of transcription regulatory sites.⁶⁸ The increased expression of *c-fos* has been demonstrated after ischemia,⁶⁹⁻⁷¹ and Fos- and Jun-like immunoreactivity has been identified in neurons and astrocytes in ischemia-tolerant brain tissue.^{72,73} Early increased expression of *zif268* has been demonstrated after ischemia.^{37,69,74} The present study is the first demonstration of altered ischemia-induced early response gene expression in a model of PC. We do not have evidence of cellular or topographical localization of early response genes after focal stroke in the present study. Poststroke protein product expression for early response genes has been demonstrated previously by others, as noted above. However, the present data demonstrating reduced early response gene message expression after focal stroke indicate that even this very early cellular response to ischemia was reduced by previous PC, thus demonstrating the increased brain tolerance to insult early after injury. One can expect that this reduced response to more severe ischemia produced by PC can alter downstream gene expression effects, which might contribute to a reduced degree of ultimate tissue injury. Others have also shown that even brief periods of MCAO-induced ischemia, which causes only mild cortical damage, increase *c-fos* and *c-jun* mRNA exclusively in the ipsilateral cortex with a later increase in the DNA-binding activity of AP-1.⁷⁵ Recently, we have demonstrated that PC produces a small but significant early increase in *c-fos* and *zif268* (F.C. Barone, PhD, et al, unpublished data, 1997) that might be responsible for the PC-induced IL-1ra response and the attenuated early response gene response after PMCAO in the previously PC cortex. However, the present

data do not delineate the relationship of early response genes to IL-1ra, and changes in early gene expression might even be correlated with increased HSP expression.

Brain resistance to ischemic injury is not limited to ischemic PC but has also been observed after other tissue stresses such as heat shock treatment⁷⁶ and chemical metabolic stress.⁷⁷ In addition, IT can be produced by spreading depression (a common ischemia-related phenomenon). Indeed, spreading depression can induce ischemic tolerance,^{78,79} and this appears to activate glial cells,⁸⁰ suggesting that increased support by these cells may be involved in subsequent neuroprotection. Available data suggest that threshold depolarizations required to induce tolerance are comparable to those that induce transcription factor mRNAs (eg, *c-fos*), while that inducing HSP72 approaches closer to the threshold for neuronal injury.^{65,78} The coordinated expression of protective antioxidant enzymes⁸¹ and nerve growth factors^{69,82} may also be involved in IT. In addition, PC might alter tissue metabolism in a manner that sustains IT tissue by providing an increased penumbra and a reduced ischemic core zone, without altering blood flow. Finally, PC might attenuate later postischemic leukocyte adhesion and emigration.⁸³

In summary, the present experiments describe a model of PC that results in substantial and prolonged IT. The hallmarks of this model of focal ischemia are as follows: (1) a remarkable delayed yet prolonged neuroprotection to permanent focal stroke; (2) a PC-induced IT that is unrelated to changes in cerebral blood flow; (3) elimination of the possibility of blood-borne factors in the phenomenon since cross-hemispheric tolerance cannot be produced; (4) a neuroprotection/IT that is associated with changes in gene expression and is dependant on newly synthesized protein(s); and (5) a PC-induced resistance/tolerance to focal stroke brain injury that is associated with the increased prestroke expression of the neuroprotective protein IL-1ra and the reduced poststroke expression of early response genes. It has been suggested that transient ischemic attacks might provide the preconditioning necessary to protect the brain from later, more severe insults,⁸⁴ and recent data suggest that this indeed might be the case.⁸⁵ Certainly, the use of controlled transient ischemic attacks as a potential strategy bears the danger and risk of significant brain injury and is an unrealistic approach to therapy. However, it can be expected that as the mechanism(s) responsible for PC become more fully understood, we will increase our ability to identify novel targets for the posttreatment protection from focal stroke brain injury, and/or we will be more likely to discover a safe pharmacological preconditioning agent (ie, to produce a chemical preconditioning) that can protect the brain in high-risk individuals or before invasive cerebral surgical procedures.

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85. Alteri M, Melle GV, Bogousslavsky J. Do transient ischemic attacks protect from severe subsequent stroke? *Stroke*. 1998;29:320. Abstract.

Editorial Comment

Several studies have reported that in the brain, as in the heart, brief periods of mild focal or global ischemia can protect against a subsequent major ischemic episode.^{1–5} The article by Barone et al has identified a specific period when mild, transient focal cerebral ischemia in SHR reduces damage resulting from subsequent permanent focal ischemia (MCAO). Animals exposed to a brief 10-minute period of MCAO followed by reperfusion exhibited reduced damage when subjected to permanent MCAO 1, 2, or 7 days later. However, the protective effects of temporary MCAO were not seen when permanent MCAO was performed after 24 hours or earlier than 14 days. These data are consistent with the findings of others who have reported a similar specific period during which preconditioning is protective.⁴

The mechanisms underlying the protective effects of mild ischemia are unknown. However, several mediators have been proposed, including specific immediate early genes, heat shock proteins, neutrophins, and cytokines.^{6,7} Hallenbeck's group (Tasaki et al^{7,8}) have reported that pretreatment of rats with bacterial lipopolysaccharide, which induces cytokines, or with IL-1 or tumor necrosis factor (TNF- α), 2 to 4 days before cerebral ischemia, inhibits subsequent damage. Ohtsuki et al⁹ further suggested that induction of IL-1 before ischemia contributes to the protective effect of mild preconditioning because administration of IL-1ra prevents tolerance in gerbil hippocampal neurons. Thus, although IL-1 has been proposed as a mediator of ischemic brain damage,¹⁰ its expression before ischemia may lead to protective mechanisms. The work of Barone et al extends those earlier findings by demonstrating that IL-1ra mRNA and protein is upregulated 24 and 48 hours after mild temporary ischemia at a time when preconditioning protection is seen. From these observations, it is proposed that upregulation of endogenous IL-1ra contributes to preconditioning protection by blocking actions of IL-1.

IL-1ra does appear to act as an endogenous as well as an exogenous inhibitor of ischemic brain damage. An earlier

study showed that inhibition of the actions of endogenous IL-1ra by passive immunoneutralization enhances the damage caused by permanent MCAO.¹¹ However, it is not yet possible to determine the functional importance of IL-1ra in preconditioning protection, since intervention studies were not performed in the present study. Furthermore, it is likely that a number of other cytokines or neuroprotective factors (eg, tumor growth factor- β , neutrophins, heat shock proteins) may also contribute. For instance, Nawashiro et al¹² demonstrated that TNF- α injected intracisternally into mice before distal MCAO produced a significant decrease in infarct volume, supporting a broader role for certain cytokines in preconditioning. Similarly, it is not known whether IL-1ra is upregulated as a direct response to cerebral ischemia or to other factors such as IL-1, itself an important regulator of IL-1ra expression. However, one preliminary report has suggested that the protection offered by pretreatment with IL-1 in rats exposed to MCAO cannot be ascribed to increased production of IL-1ra.¹³

The generation of cytokines during experimental focal cerebral ischemia and the protective effects of IL-1ra raise the hypothetical possibility that preconditioning with cytokine exposure or by minor ischemic events within the stricken territory may be beneficial in stroke patients. However, as yet there is no clinical evidence to suggest that this is feasible or occurs naturally in patients. Nonetheless, the relative production of IL-1ra or IL-1 in response to either mild or severe ischemia appears to be quite important in determining outcome. On this basis, a better understanding of the elements that contribute to preconditioning protection in ischemic stroke may lead to new targets for pharmacological intervention.

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