Gene Expressions After Thrombolytic Treatment of Middle Cerebral Artery Clot Embolism in Mice

Takayuki Hara, MD; Günter Mies, MD; Ryuji Hata, MD; Konstantin-Alexander Hossmann, MD, PhD

Background and Purpose—Thrombolytic treatment of stroke may result in reperfusion injury. To investigate the role of selective gene expressions, C57Bl/6J mice were subjected to middle cerebral artery (MCA) clot embolism, followed after 1 hour by intracarotid infusion of 10 mg/kg recombinant tissue plasminogen activator (rtPA) or vehicle.

Methods—Before the onset of treatment and at 1, 3, 6, and 24 hours of recirculation, animals were frozen in situ and hsp70, c-fos, junB, and NSE mRNAs were imaged on cryostat sections using in situ hybridization autoradiography. Cerebral protein synthesis (CPS) and ATP content were measured on adjacent brain sections.

Results—hsp70 mRNA was upregulated in the penumbral cortex of untreated animals and in the MCA core region of animals receiving rtPA (ie, regions characterized by a mismatch between high ATP levels and suppressed CPS). c-fos and junB mRNAs were transiently expressed mainly in the peri-infarct intact cortex for up to 3 to 6 hours in the treated and up to 24 hours in the untreated animals. In both groups, NSE mRNA declined in the central parts of the MCA territory together with a loss of silver impregnation, but this decline was more pronounced in the untreated animals.

Conclusions—The genomic expression pattern after thrombolytic recanalization of clot embolism resembles that of other types of transient ischemia such as reversible thread occlusion, although the outcome is markedly different. The investigated gene expressions, notably hsp70 mRNA, reflect the kind and severity of the ischemic stress, but they do not predict reversibility of the ischemic injury. (Stroke. 2001;32:1912-1919.)

Key Words: cerebral ischemia, focal ■ gene expression ■ stress genes ■ thrombolysis

Cerebral ischemia induces a complex pattern of genomic responses, of which the pathogenic importance may vary for different kinds of circulatory impairment.1,2 In permanent focal ischemia, the contribution of genetically controlled mechanisms to brain injury is only marginal because the dominating change is the primary failure of energy metabolism.3 In transient ischemia, however, restoration of blood flow and energy metabolism results in a multitude of gene expressions that may interfere in different ways with the postischemic recovery process.2,4

With the advent of thrombolytic therapy, genetically controlled injury mechanisms may become of clinical importance, because they may critically interfere with the postischemic recovery process. Experiments in animals have revealed that combination strategies for the treatment of both hemodynamic and molecular disturbances may improve the outcome of ischemic injury.5 However, before proceeding to clinical applications, possible pathophysiological differences between experimental and clinical stroke have to be considered. The experimental model most frequently used for this kind of research is the reversible intraluminal thread occlusion of the middle cerebral artery (MCA).6,7 In this model, an intraluminal thread is advanced from the carotid into the MCA to produce focal ischemia, and after some time, the thread is retracted to restore blood circulation. In most of the clinical conditions, however, the MCA is obstructed by thromboembolic occlusion, and reperfusion gradually returns either spontaneously or through drug-induced clot lysis. Moreover, neurotoxic side effects of thrombolytic agents on the brain have to be considered.8 It is, therefore, rather unlikely that reversible thread occlusion replicates the pathophysiology of spontaneous or drug-induced reperfusion after thromboembolic occlusion.

In fact, transient thread occlusion resulted in the marked dissociation between rapid recovery of energy metabolism and persisting inhibition of protein synthesis, followed by secondary ATP breakdown, whereas thrombolysis of clot embolism led to a slower but gradually improving recovery of both ATP and protein synthesis without secondary deterioration.9,10

To explore these differences in more detail, we investigated the mRNA expression of the immediate-early genes c-fos and junB and of the heat shock protein 70 (hsp70) after thrombolytic reperfusion after MCA clot embolism. These pathophysiologically important genes have been studied extensively in various models of cerebral ischemia and there-
fore are particularly suited for revealing differences in injury evolution. To further facilitate direct comparisons, we combined genomic with metabolic imaging and used the same battery of imaging techniques as previously applied to transient MCA clot occlusion.9

Materials and Methods
Experiments were carried out in accordance with European Community Council Directive 86/609/EEC and approved by the local governmental authorities. Animals were housed under diurnal lighting conditions and allowed food and water ad libitum until the day of the experiment. Anesthesia was induced with 1.5% halothane and maintained with 1% halothane in 70% N₂O and 30% O₂.

Animal Preparation
Fifty-three adult male C57Black/6J mice weighing 20 to 30 g had MCA clot embolism induced.11 One group (n=25) was treated at 1 hour after embolism with recombinant tissue plasminogen activator (rtPA); the untreated control group (n=23) received an infusion of rtPA Treatment 1 hour after embolism with recombinant tissue plasminogen activator (rtPA); the untreated control group (n=23) received an infusion of recombinant tissue plasminogen activator (rtPA) and was allowed to reperfuse 1 hour after embolism (reperfusion group). The remaining untreated animals were excluded because of spontaneous reperfusion, and 5 rtPA-treated animals were excluded because of unsuccessful recanalization, reducing the number of treated and untreated animals to 20 for each. Five animals were killed before treatment at 1 hour after embolization. Survival times after rtPA or vehicle infusion were 1, 3, 6, and 24 hours, respectively (n=5 per time point).

Clot Preparation
To produce blood clots, 0.4 mL of fresh heterologous arterial blood was mixed with 0.08 mL of thrombin (1 mg/mL; Sigma Chemical Co) and quickly injected into a polyethylene PE10 catheter. The blood was allowed to clot in the catheter at 37°C and ambient pressure for 2 hours, followed by 22 hours of stabilization at 4°C. Then, the clots were ejected from the catheter and inspected under an operating microscope for selection of fibrin-rich segments. These segments were cut into cylindrical emboli of 150-μm diameter and 4-mm length and drawn into a PE10 catheter for subsequent embolization.

Surgical Procedures
Rectal temperature was maintained between 36.5°C and 37°C using a feedback-controlled heating system. Laser-Doppler flow was recorded with a flexible probe attached to the skull over the territory of the MCA, 2 mm caudal to the bregma and 6 mm lateral to the midline. After midline neck incision, the right common carotid artery was isolated, and the ipsilateral external carotid, thyroid, and occipital arteries were ligated and cut. After further ligation of the right pterygopalatine artery, 2 microvascular clips (FE691; Aesculap) were placed close to the carotid bifurcation. After removal of the clip from the internal carotid artery, the clots were injected into the internal carotid artery during a period of 5 seconds.

rtPA Treatment
Thrombolysis was carried out with an intracarotid infusion of 10 mg/kg human single-chain rtPA (Activlyse; Boehringer Ingelheim) dissolved in 0.1 mL distilled water (vehicle). The dose was about 10 times higher than in clinical studies to account for the lower sensitivity of murine clots to human rtPA.12,13 Infusion of rtPA started 1 hour after clot embolism and lasted for 30 minutes. Untreated animals received the vehicle without rtPA.

Forty-five minutes before the animals were killed, L-[4,5-3H]leucine (300 μCi per animal, specific activity 151 Ci/mmol; Amersham) was administered intraperitoneally for autoradiographic evaluation of the cerebral protein synthesis (CPS) rate.14 Experience indicated that this treatment at least 60 minutes before killing was required to achieve the desired level of labeling of cortical and subcortical brain areas. Animals were killed by cervical dislocation, and selected brain areas were fixed for 15 minutes in 4% paraformaldehyde/PBS, pH 7.4. After fixation for 10 minutes with 0.25% acetic anhydride/triethanolamine, sections were dehydrated and incubated in 10 μL hybridization buffer containing 35S-labeled oligonucleotide probe (10 pg/μL), 2× concentrated SSC buffer (300 mmol/L sodium chloride and 30 mmol/L sodium citrate, pH 7.0), 50% formamide, 10% dextran sulfate, 100 μg/mL poly(A)⁺, 120 μg/mL heparin, 1 mg/mL herring sperm DNA, 5 μmol/L dithiothreitol, and 1 mg/mL bovine serum albumin and covered with a coverslip. After overnight hybridization at 42°C, sections were washed twice at 50°C in 2× SSC/50% formamide for 30 minutes and exposed together with [14C] standards to x-ray film (Hyperfilm β-max; Amersham) for 21 days. The autoradiograms were digitized and measured with an image analyzer (SIAS, Siemens).Biochemical imaging was performed on a Multispectral Imaging System (from Molecular Devices). Excitation light with a wavelength of 300 nm was used to determine the levels of bioluminescence on cryostat sections at −20°C, an optimal temperature for detection of luciferase activity. ATP bioluminescence was recorded with a luciferase-specific optical probe (Biomon, Amersham) for 2 minutes. The bioluminescence signals were converted to a dose of 1000 luciferase units and corrected for background (400 luciferase units).

Regional Measurement of ATP and Protein Synthesis
Pictorial measurements of ATP were performed on coronal cryostat sections using ATP-specific bioluminescence.15,16 Adjacent brain sections were incubated in 10% trichloroacetic acid to remove labeled free leucine and metabolites that were not incorporated into proteins for the measurement of CPS. Subsequently, slices were exposed for 14 days together with [3H] standards to tritium-sensitive radiographic film (Hyperfilm β-max; Amersham) for autoradiographic visualization of 3H-labeled proteins.14

Probes for mRNAs
The probe sequences of c-fos (45 mer), junB (45 mer), hsp70 (30 mer), and NSE (40 mer) corresponded to the mouse c-fos gene (bases 290 to 334; accession No. J00370), the mouse junB gene (bases 1307 to 1351; accession No. J03236), mouse inducible hsp70 gene (bases 1401 to 1430; accession No. M76613), and mouse NSE gene (bases 80 to 119; accession No. NM013509), respectively (accession numbers correspond to GenBank designation (Table). Each probe was 3′-end-labeled using terminal deoxynucleotidyl transferase (GIBCO BRL) and a 30:1 molar ratio of [35S]dATP (1200 Ci/mmol). Specific activity was >0.5×10⁷ dpm/μg.

In Situ Hybridization
In situ hybridization was carried out as described previously.3,9 Briefly, coronal brain sections (20 μm) were fixed for 15 minutes in 4% paraformaldehyde/PBS, pH 7.4. After treatment for 10 minutes with 0.25% acetic anhydride/triethanolamine, sections were dehydrated and incubated in 10 μL hybridization buffer containing 35S-labeled oligonucleotide probe (10 pg/μL), 2× concentrated SSC buffer (300 mmol/L sodium chloride and 30 mmol/L sodium citrate, pH 7.0), 50% formamide, 10% dextran sulfate, 100 pg/mL heparin, 1 mg/mL herring sperm DNA, 5 μmol/L dithiothreitol, and 1 mg/mL bovine serum albumin and covered with a coverslip. After overnight hybridization at 42°C, sections were washed twice at 50°C in 2× SSC/50% formamide for 30 minutes and exposed together with [3H] standards to an x-ray film (Hyperfilm β-max; Amersham).

Morphometric Analysis of Ischemia-Induced Metabolic Disturbances and Gene Expression
Bioluminescence and autoradiographic images were digitized with a CCD camera system and analyzed using NIH Image software (version 1.55). ATP depletion was defined as the decline to <30% of the mean value of the contralateral side. The threshold for CPS inhibition was set to the lowest CPS value of the nonischemic hemisphere excluding fiber tracts. The areas of preserved ATP and protein synthesis were superimposed to demarcate the metabolically dissociated area in which protein synthesis was suppressed but ATP was preserved. In situ hybridization autoradiograms were prepared with [3H] standards containing different radioactivities as indices of mRNA levels. Optical densities were measured at the level of caudate-putamen in areas of cerebral cortex, representing the ische-
mic core, the CPS/ATP mismatch region, and normal tissue, as defined by CPS and ATP imaging. Signals were normalized to the homotopic areas of the contralateral hemisphere. Thresholds for the upregulation of c-fos, junB, and hsp70 mRNA expressions were set at $2\times$ the contralateral value, and that for the downregulation of NSE mRNA was set at $0.7\times$.

### Histology
Histological injury was evaluated according to a silver impregnation method. After autoradiography, sections were incubated in the impregnation solution for 2 minutes, washed 6 times for 1 minute in distilled water, and transferred for 3 minutes to a developer solution. After 3 additional washes for 1 minute in

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### Table: Untreated MCA clot embolism

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>CPS</th>
<th>ATP</th>
<th>c-fos</th>
<th>junB</th>
<th>hsp70</th>
<th>NSE</th>
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### Table: rt-PA treated MCA clot embolism

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<th>ATP</th>
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### Figure 1. Multiparametric imaging of biochemical and morphological injury after untreated MCA clot embolism of mice. Images of CPS, tissue ATP content, various genomic expressions (c-fos, junB, hsp70, and NSE mRNAs), and histological changes (silver impregnation) at 1 hour after clot embolism and at various times after the onset of vehicle infusion. Areas of preserved ATP and CPS were outlined to demarcate the metabolically impaired regions from the normal brain tissue (core, depletion of ATP and suppression of CPS; CPS/ATP mismatch, penumbral dissociation between suppressed CPS and preserved ATP). Note gradual disappearance of the peri-infarct penumbra and differential expression of immediate-early genes (c-fos, junB) and hsp70 in core and penumbra.

### Figure 2. Effect of rtPA-induced thrombolysis on biochemical and morphological injury after MCA clot embolism in mice. Images of CPS, tissue ATP content, various gene expressions (c-fos, junB, hsp70, and NSE mRNAs), and histological changes (silver impregnation) at 1 hour after clot embolism and at various times after onset of rtPA treatment. Areas of preserved ATP and CPS were outlined to demarcate the metabolically impaired region from the normal brain tissue. Note gradual reversal of metabolic changes after infusion of rtPA and differential expression of hsp70 and immediate-early genes (c-fos, junB) inside and outside areas of the suppressed CPS.
distilled water, sections were air dried and scanned with a CCD camera. The compositions of the silver impregnation and the developer solutions were described previously.\textsuperscript{17}

**Incidence Maps of Regional Alterations**

To evaluate the regional reproducibility of gene expression in respect to the metabolic alterations, regional incidence maps were constructed. The areas of altered gene expressions and biochemical disturbances were outlined in each individual experiment and stacked onto representative cross sections of the brain. With the Image analysis software, the incidence of alterations was calculated pixelwise at the level of caudate-putamen and expressed in percent of the number of animals in each group.

**Statistical Analysis**

All values are given as mean±SD. Differences of regional measurements are compared using 1-way ANOVA followed by 2-tailed Bonferroni’s multiple comparison test. $P<0.05$ was considered to indicate statistical significance.

**Results**

Representative in situ hybridization autoradiograms of \textit{hsp70}, \textit{c-fos}, \textit{junB}, and \textit{NSE} mRNAs are shown in Figures 1 and 2. The outlines of preserved ATP and protein synthesis (CPS) were superimposed onto adjacent cryosections to facilitate the regional allocation of hybridization signals; that is, the infarct core was defined as the region of ATP depletion, and the area at risk of infarction as the region of preserved ATP in which protein synthesis was suppressed (CPS/ATP mismatch).\textsuperscript{18}

**Heat Shock Protein**

In untreated animals, \textit{hsp70} mRNA was expressed mainly in the cortex of CPS/ATP mismatch but not in the ischemic core. Signal intensity of \textit{hsp70} mRNA reached its peak at 6 hours after vehicle infusion and then declined in parallel with the disappearance of CPS/ATP mismatch (Figures 1, 3, and 4). In the absence of ATP, \textit{hsp70} mRNA was not upregulated and after 24 hours declined below constitutive level (Figure 4).

After rtPA-induced thrombolysis, \textit{hsp70} mRNA was sharply upregulated in the area of CPS/ATP mismatch. This pattern was particularly striking at 1 and 3 hours after the onset of treatment, where a sharp demarcation existed between ATP-depleted and ATP-repleted parts of the MCA territory (Figures 2 and 5). With longer recirculation times, cortex and basal ganglia exhibited a different expression pattern. In the caudate-putamen, \textit{hsp70} mRNA level started to decline at 6 hours and returned to near control level at 24 hours regardless of CPS inhibition. In the cortex, upregula-
tion of hsp70 mRNA persisted throughout the observation time of 24 hours, even in those regions in which CPS recovered to normal (Figures 5 and 6).

Immediate-Early Genes

One hour after clot embolism, expression of c-fos and junB mRNAs slightly increased in the peripheral part of the CPS/ATP mismatch area, and even more so in the ipsilateral nonischemic cortex up to the midline (Figures 1 and 2). This pattern corresponds to that of peri-infarct depolarizations, which spread over the whole ipsilateral hemisphere but not to the contralateral side.

In the untreated group, the signal intensity of c-fos and junB mRNAs increased with ongoing ischemia time, reaching the peak at 3 hours after the onset of vehicle infusion. Subsequently, expression gradually declined, but in some animals, increased signal intensity could still be detected as late as 24 hours after embolism (Figures 3 and 4).

In the rtPA-treated group, the expression of c-fos and junB mRNA did not continue to rise after the onset of treatment at 1 hour after embolism. At 6 hours, hybridization signals gradually declined and, in contrast to the untreated group, disappeared in all animals within 24 hours (Figures 5 and 6).

Neuron-Specific Enolase and Silver Impregnation

NSE mRNA levels gradually declined after clot embolism. In the untreated animals, NSE mRNA fell to <50% of control in the infarct core at 24 hours (Figure 4). In the rtPA-treated animals, mRNA levels also declined despite successful restoration of ATP (Figure 6), but the changes were less pronounced. The pattern of reduced NSE mRNA resembled that of reduced silver impregnation, which in untreated animals was also more pronounced and widespread than that after rtPA treatment.

Discussion

In the present study, we combine for the first time metabolic imaging with in situ hybridization of gene expressions after thrombolysis of MCA clot embolism. The pattern of metabolic alterations confirmed our previous investigation on the evolution and reversal of ischemic injury after thrombolysis of clot embolism. According to these observations, 2 different degrees of ischemic injury could be differentiated: ie, inhibition of protein synthesis with and without additional suppression of energy metabolism. Suppression of both CPS and ATP occurred in the core of the MCA territory of untreated animals, and a mismatch between suppressed CPS and preserved ATP content occurred in the peri-infarct surrounding of untreated and in the recirculated regions of treated animals, respectively. The CPS/ATP mismatch in the surrounding of the permanent ischemic territory corresponds to the peri-infarct penumbra, which in untreated animals is gradually recruited into the infarct core.
The expression pattern of \textit{hsp70} mRNA closely reflected this difference in metabolism. In areas with suppression of both CPS and ATP, \textit{hsp70} mRNA declined below the already low level of constitutive expression. In contrast, in regions of CPS/ATP mismatch, \textit{hsp70} mRNA was clearly upregulated after ischemia. This upregulation lasted only as long as the mismatch persisted and disappeared when CPS recovered (as in the recirculated regions of treated animals) or ATP secondarily declined (as in the penumbra of untreated animals). In only the caudate-putamen did \textit{hsp70} mRNA expression return to control level at 24 hours after rtPA treatment, although CPS was not fully restored. The significance of this downregulation is not clear at the moment, but it would be of interest to find out whether there is a relationship to the previously documented higher sensitivity of this nucleus to ischemic injury.\(^{19}\)

Expression of \textit{hsp70} mRNA in association with CPS inhibition has also been observed in other models of brain injury, such as permanent or reversible MCA thread occlusion,\(^{3,9,20,21}\) global brain ischemia,\(^{22}\) or kainic acid–induced seizures.\(^{21,23}\) Because heat shock protein 70, along with other molecular chaperones, facilitates the structural and functional restitution of misfolded proteins, disturbances of protein folding may be responsible for the observed upregulation of \textit{hsp70} mRNA.\(^{24,25}\) However, the association of \textit{hsp70} mRNA upregulation with the global inhibition of protein synthesis prevents the adequate translation of the message. Expression of \textit{hsp70} mRNA therefore does not predict whether the tissue is able to survive. In fact, \textit{hsp70} mRNA is expressed after both transient thread occlusion and thrombolysis of clot embolism although in the latter situation only, tissue progressively recovers.\(^{9,10}\)

In contrast to \textit{hsp70}, upregulation of immediate-early gene mRNAs was most marked in untreated animals and in areas outside the ischemic territory. This is similar to permanent MCA thread occlusion\(^ {3}\) and has been related to the generation of peri-infarct depolarizations.\(^ {4}\) Immediate-early genes such as \textit{c-fos} and \textit{junB} are expressed within minutes in response to a wide range of stimuli and are known to orchestrate the transcription of target genes involved in adaptational processes.\(^ {26}\) Interestingly, immediate-early gene expression ceased with the reperfusion of the tissue, probably as a consequence of tissue repolarization and the normalization of extracellular potassium. Previous studies revealed that peri-infarct depolarizations contribute to the progression of injury in the penumbral zone of the permanently occluded MCA territo-
The cessation of such depolarizations after induction of thrombolysis explains that contrary to the nontreated group, infarcts do not expand after thrombolysis even in animals in which treatment is not able to reverse the initial injury.

Our findings on the close relationship between downregulation of NSE mRNA and delayed ischemic injury is in line with similar observations after different injurious stimuli such as focal and global brain ischemia or status epilepticus. Obviously, the reduction in NSE mRNA is associated with a loss of cell integrity because the neuron-specific enolase protein product, the γ isoform of which is found exclusively in neurons and neuroendocrine cells, increases in the serum and cerebrospinal fluid of patients who have neuronal damage after brain ischemia. Neuron-specific enolase is a glycolytic enzyme that is widely used as a marker of functional neuronal integrity. In the present study, reduced NSE mRNA expression may therefore reflect not only the established neuronal loss but also the preceding disturbance of neuronal function.

In conclusion, 1-hour clot embolism of the MCA followed by rtPA thrombolysis induces c-fos and junB mRNAs mainly in the intact peri-infarct region and hsp70 mRNA in the areas of CPS/ATP mismatch. This expression pattern is the same as that after transient intraluminal thread occlusion, although metabolic recovery markedly differs. The genes investigated in the present study are markers of ischemic stress, but they do not colocalize with the final region of injury. Further studies will be carried out to explore the pathogenetic importance of other gene families.

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