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# Cerebral Blood Flow Thresholds for mRNA Synthesis After Focal Ischemia and the Effect of MK-801

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**Background and Purpose**—MK-801 is a noncompetitive antagonist of N-methyl-D-aspartate subtype glutamate receptors with protective efficacy in experimental stroke. This study examined the impact of MK-801 on cerebral blood flow (CBF) and its relationship to gene expression changes during focal ischemia.

**Methods**—Spontaneously hypertensive rats were subjected to surgical occlusion of the middle cerebral artery and ipsilateral common carotid artery after 30 minutes pretreatment with 5 mg/kg MK-801 or saline vehicle. After 2.5 hours of ischemia, regional CBF was evaluated by [<sup>14</sup>C]iodoantipyrine autoradiography and compared with distributions of gene expression changes evaluated by in situ hybridization detection of mRNAs encoding several immediate-early genes and the stress protein, hsp72.

**Results**—MK-801 increased CBF in contralateral cortex from  $93 \pm 15$  to  $187 \pm 37$  mL/100 g per minute and produced a significant 25% reduction in the volume of ischemic cortex ipsilateral to occlusion. The extent of cortex failing to express inducible mRNAs correspondingly decreased, but the CBF threshold for mRNA synthesis remained unchanged (25 to 30 mL/100 g per minute). Widespread immediate-early gene expression in the neocortex became restricted to periinfarct regions after MK-801 treatment, and hybridization patterns in the striatum and hippocampus reflected the altered topography of cortical activation after drug treatment.

**Conclusions**—MK-801 alters ischemia-induced gene expression by 2 distinct mechanisms. Generalized increases in CBF reduce the volume of cortex falling below ischemic injury thresholds, protecting tissue and facilitating transcription of inducible genes proximal to the ischemic focus. In addition, MK-801 attenuates the signals that induce expression of immediate-early genes in cortical and subcortical regions remote from the middle cerebral artery territory. (*Stroke*. 2005;36:2463-2467.)

**Key Words:** cerebral blood flow ■ focal ischemia ■ gene regulation ■ MK-801 ■ pharmacology

Numerous studies demonstrate protective effects of MK-801 in transient and permanent focal ischemia.<sup>1-5</sup> Although no longer a candidate for stroke therapy, it remains important to understand the mechanisms by which this agent reduces infarct volume in experimental models. One hypothesis is that MK-801 limits depolarization in the margin of the evolving infarct,<sup>5,6</sup> reducing metabolic stress in a region of compromised perfusion.<sup>7</sup> Studies in anesthetized animals suggested that increases in perfusion do not contribute to protection by MK-801,<sup>4,6,8,9</sup> but this agent significantly increases cerebral blood flow (CBF) in the absence of anesthesia.<sup>10</sup>

Changes in gene expression are sensitive indicators of the impact of ischemia in the brain. Immediate-early genes such as c-fos are induced by brief depolarizations and identify signal propagation to regions remote from an ischemic focus.<sup>11,12</sup> The stress protein, hsp72, is induced at higher stimulus thresholds, occurring predominantly at the margin of an evolving infarct.<sup>11-13</sup> Failure to accumulate inducible mRNAs or their

encoded proteins identifies regions falling below defined CBF levels required to support energy metabolism or the higher perfusion threshold for protein synthesis, respectively.<sup>14</sup>

Preliminary data in the spontaneously hypertensive rat indicated that a dose of MK-801 sufficient to reduce infarct volume acutely increased CBF in the margin of the ischemic territory (U. Dirnagl, M. Jacewicz, W.A. Pulsinelli, unpublished data). The present experiments were designed to verify this result and examine the functional consequences of increased perfusion using in situ hybridization to compare the distributions of CBF changes and mRNAs induced after focal ischemia in vehicle- and MK-801-treated rats.

## Materials and Methods

### Ischemia Model and Treatment Groups

Rats (spontaneously hypertensive rat, male, 225 to 325 g; Harlan Laboratory Animals, Inc, Indianapolis, Ind) were subjected to occlusion of the right middle cerebral artery (MCA) and ipsilateral

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common carotid artery (CCA)<sup>15</sup> under a protocol approved by the Institutional Animal Care and Use Committee. Animals were anesthetized with 2% halothane in 30% O<sub>2</sub>, 70% N<sub>2</sub>. Rectal temperatures were monitored and maintained with a feedback-controlled heating blanket and lamp. The right CCA was accessed through a midline neck incision and looped with a Teflon/silastic occluding device for subsequent occlusion, and at the same time, the jugular vein was cannulated for later isotope injection. A femoral artery was cannulated for blood pressure monitoring and blood sampling. Rats then received an intraperitoneal injection of 5 mg/kg MK-801 or saline vehicle. Animals were intubated and ventilated with 1% halothane. The right MCA was exposed at the level of the rhinal fissure using a 37°C saline drip to maintain brain surface temperature during drilling. At 30 minutes after drug or vehicle injection, the CCA was occluded and the MCA was cauterized. Surgical incisions were sutured and animals were weaned from the respirator and returned to their cages with continued temperature monitoring and control.

### Cerebral Blood Flow Measurement

CBF was evaluated 2.5 hours after occlusion by an indicator fractionation method.<sup>16</sup> Rats were placed in a plastic restraining device and positioned in a guillotine. The arterial line was connected to a syringe pump programmed to withdraw at a rate of 1 mL/min. The pump was started and a bolus of 30  $\mu$ Ci [<sup>14</sup>C]iodoantipyrine in a volume of 0.4 mL was injected into the venous line. After approximately 6 sec, the animal was decapitated and the arterial line was simultaneously severed. The brain was rapidly dissected and frozen in hexane cooled to -40°C. Sets of cryostat sections (20  $\mu$ m) were collected at 0.5-mm intervals and air-dried onto glass slides. One set was imaged immediately on Kodak SB-5 film together with <sup>14</sup>C-plastic standards (RPA504; Amersham Biosciences), and the remaining slides were stored frozen and desiccated at -70°C for subsequent *in situ* hybridization. Aliquots of the sampled blood were decolorized with H<sub>2</sub>O<sub>2</sub> and radioactivity was determined by liquid scintillation counting. Autoradiographic images were digitized, normalized according to the total blood radioactivity, and saved as calibrated blood flow images (NIH image). The cortical area falling below identified flow thresholds was determined in each section and summed across all section intervals to yield a volume expressed in cubic millimeters.<sup>17</sup> For evaluation of flow thresholds, CBF was determined in defined regions of interest corresponding to the borders of gene expression changes identified subsequently.

### In Situ Hybridization

Sections contiguous with those used for CBF evaluation were subjected to *in situ* hybridization detection of mRNAs encoding the immediate-early genes *c-fos*, *junB*, *junD*, *zif/268*, and *nur77*, as well as the 70-kDa stress protein, *hsp72*. Oligonucleotide probes for the immediate-early genes<sup>18</sup> and *hsp72*<sup>19</sup> were <sup>35</sup>S-labeled and hybridized as previously described.<sup>19</sup> Iodoantipyrine diffused from sections during processing and did not confound hybridization signals.

### Statistical Analysis

All values are expressed as mean  $\pm$  standard deviation. Differences between saline and MK-801 groups were assessed using an unpaired *t* test, with *P* < 0.05 considered statistically significant.

## Results

### Cerebral Blood Flow

Physiological parameters did not differ between groups before treatment (Table 1) and MK-801 did not alter measured variables. Blood pressure was reduced during anesthesia, and PO<sub>2</sub> and PCO<sub>2</sub> were modestly elevated with accompanying mild acidosis under the ventilation conditions used, but changes were identical in the 2 groups. All parameters normalized prior to CBF measurement at 2.5 hr occlusion.

MK-801 increased mean cortical CBF contralateral to the occlusion from 93  $\pm$  15 to 187  $\pm$  37 mL/100 g per min, evident in the relative signal intensities of representative autoradiograms (Figure 1). MK-801 reduced volumes of cortex below each of several ischemic flow thresholds (Figure 2).

### Gene Expression

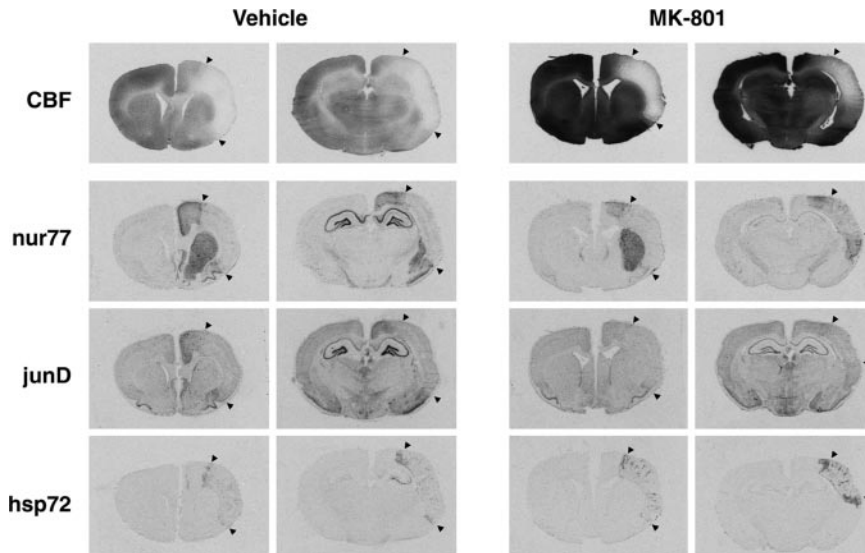
Immediate-early genes were induced throughout ipsilateral cortex of vehicle-treated animals, except within the severely ischemic MCA territory (Figure 1). The pattern illustrated for *nur77* was identical to that of *c-fos*, *junB*, and *zif/268*, showing expression in the entire ipsilateral striatum as well as bilateral hippocampus. *Jun D* showed higher basal expression and no striatal induction. *Hsp72* expression was restricted to the margin of the ischemic territory, occasional signal within the evolving infarct, and rare involvement of ipsilateral hippocampus.

For all mRNAs, MK-801 shifted the proximal margin of induced cortical mRNA accumulation laterally into the MCA territory (arrowheads in Figure 1), decreasing the cortical area failing to support mRNA synthesis (Figure 2). The CBF threshold for mRNA expression did not differ among transcripts and MK-801 did not change this parameter, averaging 27 mL/100 g per minute (Table 2). CBF thresholds for *Fos* protein expression (not shown) also did not differ between saline and MK-801-treated animals (37  $\pm$  2 and 42  $\pm$  8 mL/100 g per minute, respectively) but were higher than the threshold for mRNA synthesis.

MK-801 blocked expression of immediate-early genes in cortex remote from the ischemic territory, as well as in the hippocampus, and expression became restricted to the lateral part of the dorsal striatum, where signal intensity also tended to increase (Figure 1). With the exception of weak, persistent periinfarct induction, *junD* expression decreased to baseline. Interactions between cortical and striatal gene expression are further documented for *zif/268* expression (Figure 3). MK-801 reduced mRNA induction in anterior and posterior

**TABLE 1. Physiological Variables Are Unaltered by MK-801 Pretreatment**

	Before Treatment		Preocclusion		2.5 hr Occlusion	
	Vehicle	MK-801	Vehicle	MK-801	Vehicle	MK-801
Blood pressure	161 $\pm$ 13	170 $\pm$ 8	128 $\pm$ 12	144 $\pm$ 23	163 $\pm$ 13	165 $\pm$ 20
pH	7.35 $\pm$ 0.03	7.37 $\pm$ 0.02	7.29 $\pm$ 0.03	7.27 $\pm$ 0.03	7.39 $\pm$ 0.02	7.39 $\pm$ 0.01
PO <sub>2</sub> , mm Hg	87 $\pm$ 8	86 $\pm$ 6	113 $\pm$ 15	111 $\pm$ 12	89 $\pm$ 4	89 $\pm$ 4
PCO <sub>2</sub> , mm Hg	37 $\pm$ 3	38 $\pm$ 3	49 $\pm$ 4	53 $\pm$ 5	35 $\pm$ 2	36 $\pm$ 2
Glucose, mmol/L	6.2 $\pm$ 0.9	6.1 $\pm$ 1.1	6.9 $\pm$ 0.7	6.8 $\pm$ 1.0	...	...
Hematocrit	0.52 $\pm$ 0.01	0.51 $\pm$ 0.02	0.49 $\pm$ 0.02	0.50 $\pm$ 0.02	...	...
Temperature, °C	37.8 $\pm$ 0.3	37.9 $\pm$ 0.3	37.4 $\pm$ 0.3	37.5 $\pm$ 0.2	37.7 $\pm$ 0.2	37.6 $\pm$ 0.3



**Figure 1.** CBF and mRNA expression and the effect of MK-801. Autoradiograms illustrate the distribution of CBF and inducible mRNAs. Arrowheads identify corresponding anatomical locations in each column, demarcating the proximal margins of cortical gene expression. Most immediate-early genes, like *nur77*, were induced in the cortex, striatum, and bilateral hippocampus, whereas *junD* failed to show striatal expression and *hsp72* was restricted to the periinfarct cortex. MK-801 decreased the ischemic territory, with a corresponding shift in the rim of mRNA expression, and attenuated mRNA induction in remote cortical and subcortical regions.

cingulate cortex but increased activation of motor cortex. Corresponding changes were seen in corticostriate projection fields, decreasing in medial limbic regions but increasing in lateral dorsal sensorimotor striatum. MK-801 consistently decreased the cross-sectional area of striatal signal for all mRNAs (Figure 3).

### Discussion

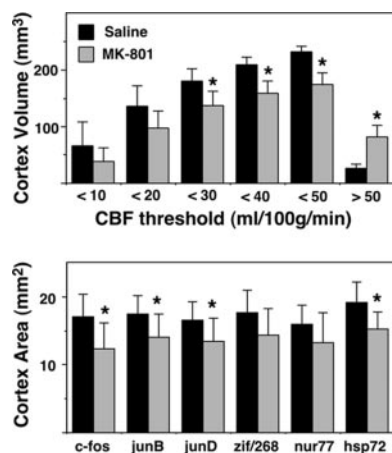
MK-801 markedly increased CBF in the rat brain after recovery from halothane anesthesia and reduced the volume of ischemic territory during MCA occlusion. The region of cortex failing to support expression of inducible mRNAs was correspondingly decreased with no change in the CBF threshold for mRNA synthesis. These results indicate that increased perfusion is a significant mediator of cerebroprotective effects of MK-801. Changes in expression of immediate-early

genes remote from the site of tissue injury appear to reflect drug effects on signal propagation.

### Cerebral Blood Flow Effects of MK-801 and Their Pathophysiological Relevance

This study replicated the design of typical treatment studies involving recovery from initial anesthesia.<sup>2,3</sup> The CBF results agree with previous evaluations of MK-801 and other NMDA receptor antagonists in awake animals.<sup>10,20–22</sup> The preponderance of evidence therefore indicates that such agents increase CBF in experimental stroke. Such effects are unrelated to functional activity, because MK-801 generally decreases cortical glucose utilization in awake rats.<sup>23</sup> However, MK-801 specifically increases glucose utilization in limbic circuitry, an effect largely attenuated by halothane anesthesia.<sup>23</sup> Stimulation at discrete sites such as the fastigial nucleus and rostral ventrolateral medulla can globally increase perfusion.<sup>24,25</sup> Conceivably, MK-801 could act through such loci to produce anesthesia-sensitive CBF increases.

Available evidence indicates complex MK-801 effects on the interactions among CBF, periinfarct depolarization, and subsequent pathology. Although CBF increases after MK-801 treatment are blunted by anesthesia, reductions in depolarization frequency remain evident,<sup>5</sup> and acute ischemic lesions are

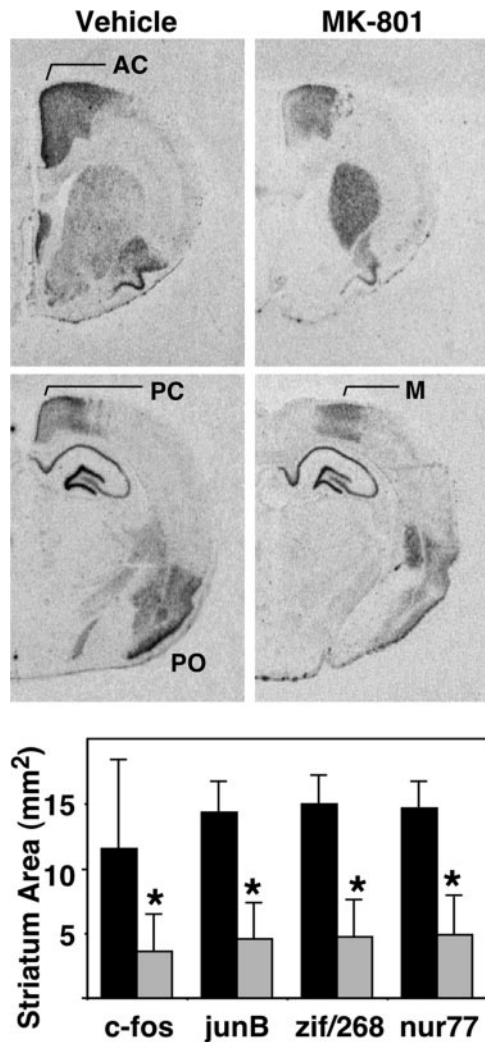


**Figure 2.** Quantitative analysis of CBF and cortical gene expression and the effect of MK-801. (Upper) Tissue volumes below or above the indicated flow thresholds 2.5 hr after occlusion, demonstrating a reduced volume of ischemic cortex after MK-801 treatment. (Lower) The decrease in severely ischemic cortex at the level of striatum defined by failure to support induced mRNA expression (between arrowheads in Figure 1), reaching statistical significance for 4 transcripts. \*Significantly different from the saline group.

**TABLE 2. CBF Thresholds for mRNA Synthesis Unchanged by MK-801**

	CBF Threshold (mL/100 g per min)	
	Vehicle	MK-801
c-fos	30.5±2.9	26.1±3.5
junB	27.0±3.8	26.5±4.1
junD	28.4±2.8	26.9±4.8
zif/268	27.8±2.8	28.6±6.2
nur77	25.4±3.4	24.5±5.2
hsp72	24.5±3.6	25.5±5.5
Mean	27.3±4.6	26.4±1.9





**Figure 3.** Striatal gene expression and the effect of MK-801. (Upper) Moderate postischemic zif/268 mRNA expression throughout striatum in a vehicle-injected rat, but expression is restricted to lateral dorsal striatum after MK-801 injection. This was associated with shifts in cortical gene expression, with attenuated signal in anterior cingulate (AC) and more prominently posterior cingulate (PC) and primary olfactory cortex (PO), but increased involvement of the frontal parietal motor cortex (M). (Lower) Quantitative comparison of the distribution of induced gene expression. Areas of striatal expression were determined for the 4 immediate-early genes induced during focal ischemia. In all cases, MK-801 decreased the territory exhibiting detectable expression. \*Significantly different from the saline group.

reduced.<sup>6</sup> However, cortical glucose utilization, already reduced by anesthesia, is further lowered by MK-801,<sup>23</sup> which would be protective in the absence of flow effects. One early study indicated that MK-801 preferentially reduced the number of longer depolarizations,<sup>5</sup> and depolarization duration is well correlated with the magnitude of local perfusion deficit in cortex.<sup>26</sup> Superimposed KCl-induced depolarizations increase infarct volume,<sup>27,28</sup> consistent with the view that substrate availability limits metabolic recovery during periinfarct depolarizations.<sup>7,29,30</sup> Therefore, the combination of increased CBF and reduced metabolic rate could constitute a composite mechanism by which MK-801 shortens depolarizations and limits lesion expansion in rodent focal ischemia models.

### Gene Expression Thresholds

To the extent that the capacity for mRNA synthesis primarily reflects nucleotide availability, the present results are consistent with previous studies indicating a low CBF threshold for energy failure unaffected by MK-801.<sup>14,31</sup> The precise level of 25 to 30 mL/100 g per minute required to support mRNA expression determined here (Table 2) is slightly higher than the 15 to 20 mL/100 g per minute noted previously for ATP depletion.<sup>14,31</sup> Slight differences in blood flow methodologies or in the relative sensitivities of hybridization detection versus ATP imaging could contribute to this small systematic variation. A direct comparison of these parameters in a mouse focal ischemia model indicated close anatomic correspondence between regions displaying induced mRNA synthesis and ATP preservation.<sup>32</sup> The observation of unchanged flow thresholds after MK-801 treatment, under conditions of markedly altered absolute perfusion, strongly indicates that energy failure is the primary determinant of the proximal margin of inducible mRNA expression in cortex during focal ischemia. The incidental observation of unchanged CBF threshold for Fos protein accumulation after MK-801 treatment contrasts with a reported decrease in threshold for overall protein synthesis,<sup>31</sup> again perhaps reflecting both differing detection sensitivities and the influence of anesthesia.

### Neuronal Activation and Expression of Immediate-Early Genes

It has long been recognized that there are MK-801-sensitive mechanisms by which immediate-early genes are induced in remote neocortical regions after focal insults, often attributed to spreading depression. Recurrent depolarizations during focal ischemia are largely restricted to the periinfarct territory.<sup>5,33</sup> Nevertheless, immediate-early genes exhibit widespread cortical expression after focal ischemia,<sup>11,12,34</sup> which can be blocked by MK-801 except in the immediate periinfarct region.<sup>35,36</sup> Subneocortical structures outside the ischemic territory, eg, ipsilateral and even contralateral hippocampus, can also show gene expression changes after focal ischemia in the absence of overt depolarization.<sup>12</sup> These responses are substantially attenuated by MK-801 (Figures 1 and 3). A particularly striking pattern is seen in the striatum, which shows widespread induction of immediate-early genes in vehicle-injected animals, becoming restricted to the lateral part of the dorsal striatum after MK-801 treatment. Striatal immediate-early gene induction occurs through stimulation of corticostriate projections, reflecting the anatomical topography.<sup>37,38</sup> MK-801 eliminated mRNA induction in striatal regions receiving input from the limbic and, to some extent, association the cortex, with preserved response in the somatic sensory/motor region, reflecting the more limited extent of cortical activation. Intensification of this residual signal in striatum may result from an increased representation of motor cortex in the region of activation at the margin of the ischemic territory. MK-801 can directly block striatal immediate-early gene induction in response to some types of cortical stimulation.<sup>37</sup> However, persisting gene expression in the periinfarct cortex and its projection areas in striatum seen here after MK-801 treatment would apparently involve signaling through non-NMDA receptors.

## Conclusions

These results indicate that increased CBF contributes to MK-801 effects in focal ischemia, identifying a mechanism by which it can attenuate recurrent depolarizations, reduce infarct volume, and facilitate gene expression at the margin of an evolving lesion. Far-reaching effects occur through altered input to remote subcortical structures secondary to modified patterns of cortical activation involving combined effects on perfusion and signal propagation.

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