# MK 801 attenuates c-Fos and c-Jun expression after *in vitro* ischemia in rat neuronal cell cultures but not in PC 12 cells

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*Cellular homeostatic adaptation to cerebral ischemia is complex and contains changes in receptor mediated gene expression and signaling pathways. The proteins of the immediate early genes c-Fos and c-Jun are thought to be involved in coupling neuronal excitation to target gene expression, due to formation of heterodimers and binding to the AP1 promotor region. We used an* in vitro *model to compare ischemia induced c-Fos and c-Jun expression in rat neuronal cell cultures and nerve growth factor (NGF) differentiated PC 12 cells. Since activation of glutamate receptors is known to mediate ischemic injury we determined the effect of the noncompetitive N-methyl-D-aspartate(NMDA) receptor antagonist MK 801 on c-Fos and c-Jun expression in both cell culture systems during ischemia. Neuron rich cultures and NGF differentiated PC 12 cells were exposed to sublethal* in vitro *ischemia using an hypoxic chamber flushed with argon/$CO_2$ (95%/5%). C-Fos and c-Jun mRNA expression was analyzed by competitive reverse transcription–polymerase chain reaction using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as internal standard. One hour of* in vitro *ischemia significantly increased c-Fos and c-Jun mRNA levels in both cell culture systems. In neuron rich cultures a 10-fold (c-Fos) and 7-fold (c-Jun) mRNA increase was observed. The mRNA rise was less pronounced in PC 12 cells (5.5-fold and 2-fold) for c-Fos and c-Jun, respectively. The addition of MK 801 significantly reduced the expression of c-Fos and c-Jun mRNA in neuronal cultures, whereas no effect was detectable in PC 12 cells. Since MK 801 failed to reduce the c-Fos and c-Jun expression in NGF differentiated PC 12 cells different signaling pathways may initiate c-Fos and c-Jun expression in both cell culture systems.* [Neurol Res 2002; **24**: 725–729]

*Keywords: Ischemia; cell culture; gene expression; PC 12; MK 801; immediate early genes; c-Fos; c-Jun*

## INTRODUCTION

Although during the early post-ischemic state the protein synthesis in the brain is generally suppressed, specific genes such as the immediate early genes (IEG) and their corresponding proteins are being synthesised. IEG are a class of genes, many of which encode transcription factors, whose transcription is transiently activated within minutes of exposure to a wide range of stimuli. They are believed to control the physiological cellular response to the initial stimulation event by activating secondary programs of gene expression. The expression of c-Fos and c-Jun together with other IEG was demonstrated in numerous *in vivo* experiments[1–3], but only a few experiments showing the expression of c-Fos and c-Jun after *in vitro* ischemia in cell culture systems[4]. The role of c-Fos[5,6] and c-Jun in neurodegeneration or neuroprotection after cerebral ischemia is still controversial[7–9]. The comprehensive characterization of the rat pheochromocytoma PC12 cell line makes it well suited to compare the effect of sublethal ischemia on c-Fos and c-Jun expression with primary neuronal cell cultures. Nerve growth factor (NGF) has an initial mitogenic effect on PC 12 cells but then causes growth arrest and differentiation into sympathetic neuron like cells. Glutamate receptor stimulation causes the induction of transcription factors that belong to the class of immediate early genes and excitotoxic activation of glutamate receptors is also thought to be a key event for the molecular pathogenesis of post-ischemic delayed neuronal death. Therefore, we used a rat cell culture based model to study the effect of sublethal *in vitro* ischemia on c-Fos and c-Jun expression in neuron rich and NGF differentiated PC 12 cells to answer the question whether the noncompetitive receptor antagonist MK 801 effects the c-Fos and c-Jun expression.

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## MATERIALS AND METHODS

Neuron rich cell cultures were prepared from brains of fetal Lewis rats at gestation day 16[10]. Cells were maintained in neuronal growth medium (NGM), consisting of DMEM/HAM's F-12, containing 7.35 mg $l^{-1}$ glutamate, 4.5 g $l^{-1}$ glucose and additionally 30% conditioned medium (derived from rat astrocyte cultures, which were cultured for other reasons). PC 12 cells were purchased from the ATCC (Manassas, VA, USA) and were grown in RMPI 1640 medium containing 5% FCS and 5% horse serum (PGM). For neuronal differentiation of PC 12 cells the PGM was supplemen-


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ted with 50 ng $ml^{-1}$ nerve growth factor (NGF). All cells were cultured at 37°C in a humidified atmosphere with 95% air/5.0% $CO_2$. Media were purchased from Gibco BRL (Karlsruhe, Germany) and supplemented with an antibiotic mixture (PSN, Gibco BRL) containing 50 g penicillin, 100 g neomycin and 50 g streptomycin per ml medium.

The purity of neuronal cultures was about 85%–90% as demonstrated after immunohistochemical staining with an antibody to the pan neuronal marker PGP 9.5 (Paesel & Lorei, Hanau, Germany).

To apply ischemia to cells DMEM without serum and glucose was used (IM). Growth medium was removed and stored at 37°C (95% air/5.0% $CO_2$), the cells were washed with phosphate buffered saline (PBS) and IM was added. Culture dishes were placed in the *in vitro* chamber which was flushed with argon/$CO_2$ (95%/5%) at 37°C. The argon/$CO_2$ (95%/5%) gas mixture was chosen, because oxygen replacement in the medium was found to be much faster compared to nitrogen[11]. Oxygen reduction in the medium was monitored by measuring the partial tissue oxygen pressure ($ptiO_2$) using a modified Clarke electrode. The $ptiO_2$ declined from about 126 mmHg to 2 mmHg within 10 min and remained at this level during all experiments. Medium temperature was measured using a temperature probe (both Licox GMS, Kiel, Germany). After 1 h the IM was removed and the cell specific growth medium was replaced. Cell cultures were stored at 37°C (95% air/5.0% $CO_2$) for 1 h of reoxygenation.

Controls were randomly selected and treated in parallel with the experimental cell cultures. Instead of IM growth medium according to the specific cell type was added and cultures were kept at 37°C under normoxic conditions for 1 h.

In certain experiments 10 $\mu$M of MK 801, a non-competitive N-methyl-D-aspartate (NMDA) receptor antagonist was added to the IM (during the course of ischemia) and the NGM or PGM, respectively (during the time of reoxygenation).

Cell viability was assessed by measurement of lactate dehydrogenase (LDH) activity in the culture medium using the cytotoxicity detection kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

RNA was prepared using RNAclean (Hybaid AGS, Heidelberg, Germany) according to the manufacturer, quality checked by denaturing agarose gel electrophoresis and subsequently transcribed into cDNA using oligo-$(dT)_{12-18}$ as primer and Superscript RNase $H^-$ reverse transcriptase (both Gibco BRL). Two $\mu$g of total RNA were used according to the protocol of the manufacturer.

To allow comparison of samples a competitive PCR assay was utilised to determine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA content which was then used as an internal standard. The competitor was constructed from a cloned fragment of rat GAPDH (cDNA position 301 797 [12]) in pT3T7[13] by deleting a 35 bp Nsi I/EcoN I fragment. Experimental cDNAs and defined amounts of DNA standard were co-amplified using GAP2/GAP5 primers (CCA GTG AGC TTC CCG TTC AGC and CTT CAC CAC CAT GGA GAA GGC, respectively) using the following thermal protocol: 5′ of initial denaturation at 95°C, followed by 18 cycles, each consisting of 30′′ at 94°C, 30′′ at 55°C, and 25′′ at 65°C, and a final elongation step of 7′ at 72°C.

C-Fos and c-Jun cDNA was analysed using the primer pairs according to the published sequences[14,15], applying the following thermal protocol: 5′ of initial denaturation at 95°C, followed by 24 cycles, each consisting of 30′′ at 94°C, 30′′ at 55°C, and 30′′ at 65°C, and a final elongation step of 7′ at 72°C. All amplifications were performed in a total volume of 25 $\mu$l using 1U of AmpliTaq DNA polymerase (Perkin Elmer), 5 pmole of each primer, 25 nmol dNTPs using the PE2400 PCR system (Perkin Elmer, Wellesley, MA, USA). To allow sensitive detection of PCR products all sense primers were labeled with $^{33}P$ using T4 polynucleotidkinase (NEB) according to the manufacturers protocol using $^{33}P$-c-ATP (NEN).

In preliminary experiments the validity of the RT–PCR assay was confirmed. It was proven that under the PCR conditions chosen amplification of c-Fos and c-Jun is still in the exponential phase of the PCR and therefore the log amount of PCR product is proportional to the initial concentration of c-Fos or c-Jun cDNA (data not shown). The expression of GAPDH mRNA remained unaltered by ischemia treatment as proven by competitive RT–PCR. Therefore it was chosen as internal standard to normalize for sample variations.

Following amplification PCR-products were separated on 5% polyacrylamide gels containing 7M urea (Mini-Protean gel system; Biorad, Munich, Germany). Gels were dried under vacuum (gel dryer 543, Biorad) and analysed using a phosphoimager device (BAS 3000; Fuji, Tokyo, Japan) after exposure overnight. Scans were analysed using TINA software package version 2.09 (Raytest, Straubenhardt, Germany) calculating relative amounts of the specific PCR products, as well as the GAPDH wildtype/competitor ratio used for normalization between samples.

For all statistical calculations Prophet version 5.0 (BBN Systems and Technology, Cambridge, MA, USA) was used and statistical significance was calculated using the two-sample equal-variances *t* test for neuronal and PC 12 samples. Numbers represent the optical density (mean ± SD) if not otherwise indicated. Probability values $< 0.05$ were considered to be significant.

## RESULTS

One hour of *in vitro* ischemia followed by 1 h of reoxygenation caused a significant increase (10-fold) in c-Fos ($17.93 \pm 6.66$ compared to $1.73 < 0.32$; $p < 0.05$) and c-Jun (7-fold) mRNA level ($35.58 \pm 4.16$ compared to $4.99 \pm 1.0$; $p < 0.05$), respectively in neuron rich cultures (*Figure 1*). In NGF differentiated PC 12 cells the increase was 5.5-fold for c-Fos mRNA ($8.27 \pm 1.94$ compared to $1.49 \pm 0.31$; $p < 0.01$) and 2-fold for c-Jun mRNA ($13.86 \pm 5.63$ compared to $6.34 \pm 1.95$ $p < 0.05$) (*Figure 2*).

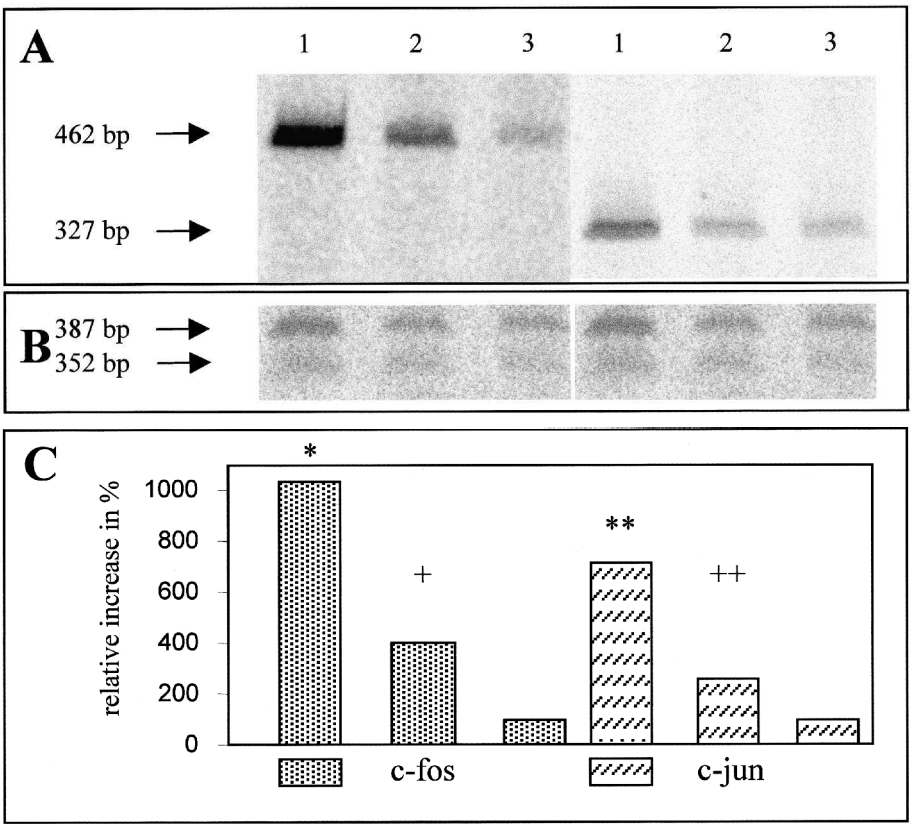


**Figure 1:** Analysis of c-fos and c-jun mRNA expression after *in vitro* ischemia in neuron rich cultures. **A:** Representative samples of c-Fos (462bp) and c-Jun (327bp) RT–PCR products after electrophoretic separation (5% polyacrylamide gel). 1, ischemia treated cultures; 2, ischemia treated cultures with MK 801 added to the cultures during ischemia and reoxygenation; 3, control cultures. **B:** Competitive RT–PCR analysis of GAPDH mRNA content, which was used for normalisation, depicting the products derived from endogenous cDNA (387bp) as well as from the added competitor (352bp). **C:** Relative increase of mRNA levels compared to controls in % as the mean of at least three independent experiments in each group. Left: 1, 10-fold increase of c-Fos mRNA, * significant compared to control ($p < 0.05$); 2, + significant ($p < 0.05$) reduction of c-Fos mRNA after addition of MK 801 to the medium compared to samples without MK 801; 3, control. Right: 1, 7-fold increase of c-Jun mRNA, ** significant compared to control ($p < 0.05$); 2, ++ significant ($p < 0.05$) reduction of c-Jun mRNA after addition of MK 801 compared to samples without MK 801; 3, control

The noncompetetive NMDA receptor antagonist MK 801, which is known to reduce the infarct volume during *in vivo* ischemia experiments, was examined on its ability to attenuate ischemia induced increase in c-Fos and c-Jun mRNA levels. To determine the influence of MK 801 on c-Fos or c-Jun expression during *in vitro* ischemia 10 $\mu$M MK 801 were added to the ischemic medium (IM) at the beginning of *in vitro* ischemia and to the growth medium during the reoxygenation period. This treatment significantly (2.6-fold) reduced the expression of c-Fos ($6.98 \pm 2.33$ compared to $17.93 \pm 6.66$; $p < 0.05$) and c-Jun (2.8-fold) mRNA ($12.81 \pm 8.63$ compared to $35.58 \pm 4.16$; $p < 0.05$) in neuron rich cultures (*Figure 1*). In contrast, no significant influence on mRNA expression was detected in PC 12 cells, neither for c-Fos ($8.13 \pm 2.91$ compared to $8.27 \pm 1.94$) nor for c-Jun ($15.3 \pm 5.28$ compared to $13.86 \pm 5.63$, respectively).

No difference was found in cell viability between *in vitro* ischemia treated cultures and controls, because LDH release into the medium during and after *in vitro* ischemia did not differ significantly compared to controls. These data confirm that *in vitro* ischemia in the applied time window was not accompanied by significant cell death.

## DISCUSSION

Focal cerebral ischemia has been shown to increase the expression of IEG, especially within the injured hemisphere, which may be caused by spreading depression. This induction includes the transcription factors c-Fos and c-Jun[16–18]. Since excitotoxic activation of glutamate receptors is thought to be a key event in the molecular pathogenesis of post-ischemic delayed neuronal death, the interference with glutamate receptors was an approved experimental neuroprotective strategy *in vivo*[19] and *in vitro*[20–22]. Glutamate receptor stimulation also causes the induction of immediate early genes, which are thought to be involved in coupling neuronal excitation to target gene expression[23–25].

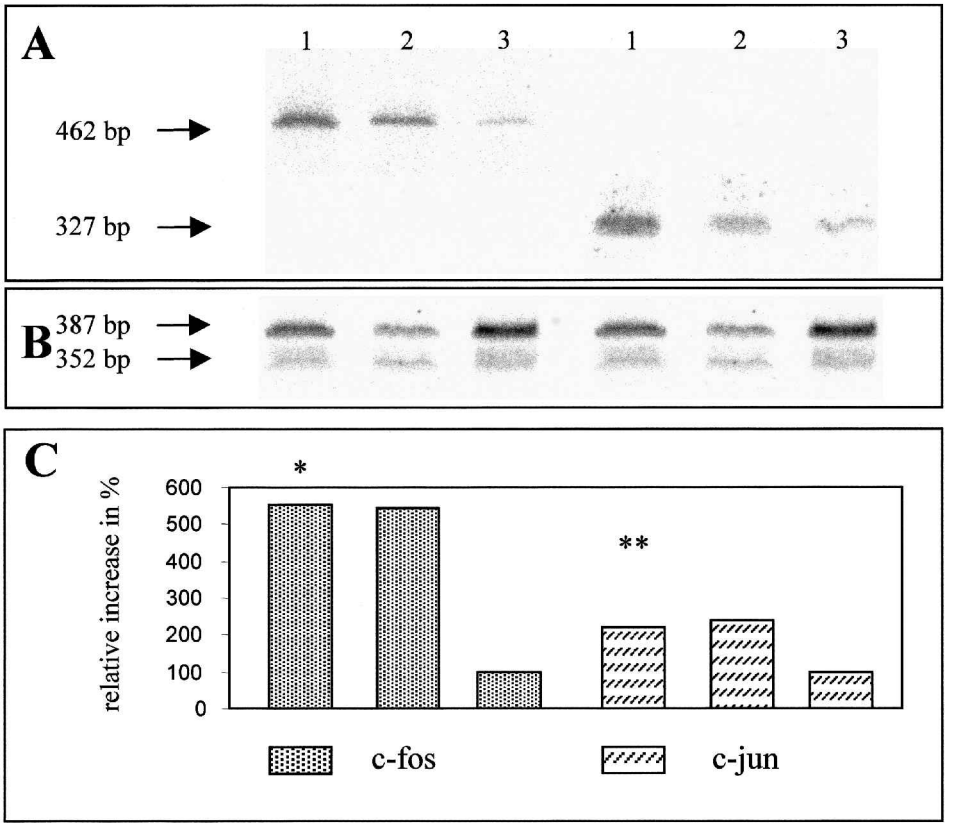


**Figure 2:** Analysis of c-Fos and c-Jun mRNA expression after *in vitro* ischemia in PC12 cell cultures. **A:** Representative samples of c-Fos (462bp) and c-Jun (327bp) RT–PCR products after electrophoretic separation (5% polyacrylamide gel). 1, ischemia treated cultures; 2, ischemia treated cultures with MK 801 added to the cultures during ischemia and reoxygenation; 3, control cultures. **B:** Competitive RT–PCR analysis of GAPDH mRNA content, which was used for normalisation, depicting the products derived from endogenous cDNA (387bp) as well as from the added competitor (352bp). **C:** Relative increase of mRNA levels compared to controls in % as the mean of at least three independent experiments in each group. Left: 1, 5.5-fold increase of c-Fos mRNA, * significant compared to control ($p < 0.05$); 2, The addition of MK 801 to the medium did not alter c-Fos mRNA compared to samples without MK 801; 3, control. Right: 1, 2-fold increase of c-Jun mRNA, ** significant compared to control ($p < 0.05$); 2, The addition of MK 801 to the medium did not alter c-Jun mRNA compared to samples without MK 801; 3, control

To determine whether N-methyl-D-aspartate (NMDA) receptors are functionally involved in signaling cascades resulting in c-Fos and c-Jun expression in neuron rich cultures and PC12 cells we compared the effect of MK 801 during 1 h of sublethal *in vitro* ischemia and 1 h of re-oxygenation in both cell culture systems. In contrast to PC-12 cells[4] ischemia induced c-Fos and c-Jun expression has not been demonstrated in neuronal cultures yet. The addition of 10 $\mu$M MK 801 during ischemia and reoxygenation significantly reduced the induction of c-Fos and c-Jun mRNA only in neuron rich cultures, while PC 12 cell cultures remained uneffected. These results are consistent with *in vivo* data from a rat focal ischemia model regarding the sensitivity of c-Fos and c-Jun expression to MK 801[26]. C-Fos expression could also be blocked by other NMDA receptor antagonist, as it was shown for ketamine[27]. MK 801 also blocked spreading depression as well as the induction of IEG outside the infarcted and peri-infarcted area[17].

In this series MK 801 failed to reduce the c-Fos and c-Jun expression in NGF differentiated PC 12 cell cultures. Because it is known that NGF increases both the NMDAR1 protein and the total amount of functional receptors[28,29] we extended the time frame for NGF induced differentiation of PC 12 cells up to 120 h, but no effect of MK 801 on c-Fos and c-Jun mRNA expression was observed (data not shown). Kobayashi and Millhorn[30] demonstrated that NGF differentiated PC12 cells express functional NMDA receptors and that chronic exposure to hypoxia attenuates the N-methyl-D-aspartate-induced $Ca(^{2+})$ accumulation in these cells via down-regulation of N-methyl-D-aspartate receptor subunit 1[30]. NGF also up-regulates the NMDA receptor subunit 1 promoter in PC12 cells[29]. Therefore the initial stimulating event which activates a signaling cascade resulting in upregulation of c-Fos and c-Jun mRNA in PC12 cells seems to be mediated independently of NMDA receptor activation.

Whether the attenuation of c-Fos and c-Jun expression by MK 801 in neuron rich cell cultures after *in vitro* ischemia is neuroprotective or not, needs to be clarified in further experiments extending the time of ischemia. In

this experimental series neither a significant LDH release into the cell medium nor cellular morphological changes were observed.

## CONCLUSION

Different receptor coupled signaling pathways are likely to mediate the c-Fos and c-Jun expression in neuronal and PC 12 cells after sublethal *in vitro* ischemia since the addition of MK 801 had no effect in PC 12 cells.

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