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Neuronal NOS Inhibitor That Reduces Oxidative DNA Lesions and Neuronal Sensitivity Increases the Expression of Intact c-fos Transcripts after Brain Injury

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Abstract

In response to oxidative stress, the ischemic brain induces immediate early genes when its nuclear genes contain gene damage. Antioxidant that reduces gene damage also reduces cell death. To study the mechanism of neuronal sensitivity, we investigated the transcription of the *c-fos* gene after brain injury of the ischemia-reperfusion type using focal cerebral ischemia-reperfusion in Long-Evans hooded rats. We observed a significant ($p < 0.01$) increase in *c-fos* mRNA in the ischemic cortex immediately after brain injury. However, the *c-fos* transcript was sensitive to RNase A protection assay (RPA) upon reperfusion. The transcript became significantly resistant to RPA (42%, $p < 0.03$) when 3-bromo-7-nitroindazole (25 mg/kg, i.p.), known to abolish nitric oxide, gene damage and neuronal sensitivity, was injected. Our data suggest that neuronal nitric oxide synthase and aberrant mRNA from genes with oxidative damage could be associated with neuronal sensitivity.

Keywords

Gene expression; Neuroregeneration; Oxidative stress; Stroke; Transcription

Introduction

Excessive oxidative stress may be associated with neural disorders [16,26,27,29,41]. Defining neurodegeneration pathways, whether in acute conditions [vascular disease in the brain (stroke) and traumatic brain injury] or progressive conditions, may facilitate intervention. Experimentally, cerebral oxidative stress can be induced in brain injury using forebrain ischemia-reperfusion, focal cerebral ischemia-reperfusion (FCIR) and weight-drop impact, which simulate, respectively, clinical cardiac attack, stroke and traumatic head injury in animals [19,22]. Oxidative stress in the brain induces reactive oxygen species. Reactive oxygen species cause cellular damage by attacking lipids, proteins and nucleic acids. Recent investigations have suggested that oxidative stress induces DNA fragmentation, which is observed during neuronal death (neurosensitivity) [7,10,20,28,33], and oxidative DNA lesions (ODLs), which are precursors of phenotype alteration and neuronal death [3,11,24]. Antioxidants [7-nitroindazole or 3-bromo-7-nitroindazole (3Br7NI)] that abolish neuronal death [2,15,17,30,41,47] have been shown to reduce ODLs during the early reperfusion period after brain injury of the ischemia-reperfusion type [4,14,21].

During the time of detecting ODLs, several immediate early genes are known to be expressed [42]. One of the IEG products, Fos, is expressed for the transcription activation of several genes in response to stress [25]. Fos protein, the product of the *c-fos* mRNA, combines with Jun protein to form activator protein-1 (AP-1), which activates the expression of the nerve growth factor (NGF) gene [5], metallothionein and DNA synthesis [40]. The expression of NGF appears to reduce free radical formation in certain cell populations [8]. The expression of the *c-fos* gene is important because it may be involved in cell viability [48]. The transcript of *c-fos* mRNA is an excellent reporter, because *c-fos* mRNA is generally below the detection level and its expression is induced by brain injury [1]. The newly transcribed *c-fos* mRNA after cerebral oxidative stress can be studied without the presence of previously transcribed mRNA [23]. We tested the effect of 3Br7NI on the expression of intact *c-fos* mRNA after brain injury using the FCIR model.

Materials and Methods

Brain Injury Model

Anesthesia was induced with pentobarbital sodium (Nembutal, 80 mg/kg, i.p.) 10 min before surgery [4,5]. The right middle cerebral artery and both common carotid arteries (the focal cerebral ischemia model) of male Long-Evans rats (weighing 200–225 g; Harlan, Indianapolis, Minn., USA) were occluded for 30–90 min to simulate stroke. The occlusion was then released to allow reperfusion of the affected area.

Antioxidant that specifically inhibits neuronal nitric oxide synthase, 3Br7NI, was injected intraperitoneally in oil at a dose of 25 mg/kg 5 min after vessel occlusion [4,14]. Body temperature was maintained at 37 ± 0.5 °C during surgery and the postoperative period until the animals recovered fully from anesthesia. Housing and anesthesia complied with guidelines established by the institutional animal welfare committee in accordance with the NIH Guide for the Care and Use of Laboratory Animals, USDA Regulations and with the American Veterinary Medical Association Panel on Euthanasia guidelines. All animals were kept in well-ventilated incubators at 24 ± 0.5 °C during the reperfusion period.

Tissue Preparation

At the designated reperfusion time points, animals were anesthetized and sacrificed. Brain tissue was removed and was flash frozen for RNA isolation for reverse transcription (RT) and polymerase chain reaction (PCR) amplification [5,23].

For in situ hybridization analyses, the animals were perfused with 200 ml each of saline followed by two fixatives in sequence (fixative A: 0.8 g of NaOH, 8 g of paraformaldehyde and 1.64 g of sodium acetate in 200 ml of distilled H₂O, adjusted with 50% glacial acetic acid to pH 6.5; fixative B: 1.4 g of NaOH, 14 g of paraformaldehyde and 13.35 g of borax, adjusted to pH 9.5 with 50% HC1) and cryoprotected in 20% sucrose in fixative B overnight at 4°C as previously described [3–5]. Brain slices were prepared within 24 h and stored at -20°C for no longer than 4 weeks before analysis. The fixativetreated brain tissue was cut into 20-µm coronal sections, posterior to the bregma (1–3 mm), within 24 h after the sucrose treatment. Two brain tissue samples were mounted on a poly-lysine-treated glass slide at 100-micron intervals and stored at -70 °C (referred to as frozen tissue sections). The frozen tissue sections were used within 12 months. All experiments were repeated in a minimum of 3 animals by at least two investigators.

In situ Hybridization Assay

The presence of *c-fos* or actin mRNA in the brain sections was detected in 24 animals (n = 4 per time point or treatment). The ³²P-cRNA was transcribed from the *c-fos* cDNA clones in

the presence of ^{32}P -UTP at 37°C for 1 h as previously described [5,21]. The actin ^{32}P -cRNA probe was transcribed from a cDNA clone NGF and used as a control. The ^{32}P -probe transcription was treated with RNase-free DNase (1 u/ μl) and then G-25 Sephadex resin in a spin column to remove free nucleotides. RNA probes (10^9 cpm/ μg) in the antisense (cRNA) and sense (mRNA) orientations of both the *c-fos* and NGF genes were hybridized to the brain tissue in a hybridization buffer [62.5% formamide, 12.5% dextran sulfate, 375 mM NaCl, 12.5 mM Tris (pH 8.0) and 1.25 mM EDTA (pH 8.0) in $1.25 \times$ Denhardt's solution, RNA probe = $5 \times 10^6/\text{ml}$]. After hybridization, the brain slices were digested using DNase-free RNase A (20 $\mu\text{g}/\text{ml}$) at 37°C for 30 min to remove nonspecific hybrids. The brain sections were placed in an autoradiography cassette and developed at the same time. The radiolabeled [^{32}P]-mRNA probe did not produce any signal, but the cRNA probe did; therefore, results using antisense probes were presented. An autoradiograph was developed and the signal intensity was quantitatively measured using Alphamager 3.2 (Alphs Innotech, San Leandro, Calif., USA). The pixel values of intact mRNA per brain were statistically analyzed using GraphPad Prism 2.0 (GraphPad Software Inc., San Diego, Calif., USA).

Results and Discussion

An enhanced transcription of the immediate early genes as a response to stress can occur following injury (ischemia, seizures and trauma) and in neurodegenerative conditions [38,39, 46]. Unbalanced production or activation of the peptides, such as *bax* and *bcl2*, may alter the cascade toward cell viability or aging. We have considered one potential consequence of ODLs in the brain, i.e. signal transduction via the expression of immediate early genes. Signal transduction is an important mechanism that ensures the normal functioning of brain cells [6, 9,12,13,18,32,43-45]. To study the relationship between gene expression in the signal transduction pathway, several investigators have studied the expression of the *c-fos* gene (the reporter gene). In the present study, *c-fos* mRNA transcription occurred as early as 30 min after the start of reperfusion (fig. 1), when each copy of the nuclear *c-fos* gene contained at least one ODL [4,21]. The *c-fos* mRNA detected during early reperfusion represented newly transcribed mRNA from its nuclear genes (fig. 1), because very little *c-fos* mRNA was being transcribed in nonischemic contralateral cortices.

Gene transcripts from 2 of 3 animals contained *c-fos* transcript with shorter fragments than that from normal brain (fig. 2). Aberrant mRNA probably are degraded and not translated at all. In the case of *c-fos* mRNA, aberrant *c-fos* mRNA suggests a shortage of functional Fos/AP-1 immediately following reperfusion [5]. The consequences of lacking a functional *c-fos* mRNA are not clear, but the results suggest a delay in the activation of certain genes that could be crucial for the restoration of neuronal function after injury [8,40,48].

We have reported that functional Fos/AP-1, the product of *c-fos* mRNA, transactivates the transcription of FCIR-induced NGF mRNA [5]. It suggests that NGF mRNA transcription occurs downstream of Fos/AP-1. We have used a full-length wild-type cRNA probe to detect cRNA-mRNA hybrids in the brain, followed by treatment with RNase A [23]. The result of this assay detects mRNA that is protected by the cRNA probe, and is similar to that of the RNase A protection assay (RPA). Indeed, expression of *c-fos* mRNA was induced, but very little NGF mRNA was observed before 90 min of reperfusion (results not shown). The increase in NGF mRNA was noted in the hippocampus and the penumbra of the ipsilateral cortex after 90 min of reperfusion (fig. 3). We showed here that the expression of FCIR-induced NGF mRNA is temporally different from that of *c-fos* mRNA (fig. 3b), while very little NGF mRNA was expressed in the non-FCIR brain (fig. 3a). No NGF mRNA was observed in the core, where cell death would eventually develop.

To determine whether an intact mRNA transcript is transcribed in the ischemic brain, we used *in situ* RPA to detect c-fos mRNA expression. We noted that the ischemic core did not express a significant amount of intact c-fos mRNA after FCIR of 60/15 (fig. 4a). This is consistent with the results shown using 90-min vessel occlusion [4].

We have shown that the antioxidant 3Br7NI inhibits the formation of ODLs in ischemic brains during the early reperfusion period [4,14], while others have shown that 3Br7NI reduces infarct volume in experimental stroke using various animal models [17,30,41,47]. We examined the expression of intact c-fos mRNA in animals that received 3Br7NI after FCIR. Figure 4b shows the expression of intact c-fos mRNA in a group of 6 animals that had been treated with FCIR (60/15). The increase with 3Br7NI treatments was noted in the dentate gyrus of the hippocampus and the penumbra of the ipsilateral cortex. We especially noted that intact c-fos mRNA was increased in the core of ischemic animals treated with 3Br7NI. Figure 4c shows that the expression of intact c-fos mRNA in the animals with FCIR and 3Br7NI was significantly increased by 42% ($p < 0.03$). These data suggest that FCIR-induced c-fos mRNA survived the RPA in the presence of 3Br7NI. The data also suggest that there was no inhibition of gene transcription following brain injury of the ischemia-reperfusion type [49]. The protected mRNA was distributed also in the ipsilateral cortex, where a reduction in necrosis has been reported. Taken together, 3Br7NI, at a dose and with a treatment regime that has been shown to reduce nitric oxide formation and ODLs in nuclear genes [4,14,21], reduces the amount of aberrant c-fos mRNA transcripts.

We have reported ODLs in several nuclear genes after cerebral oxidative stress. It is probable that excessive ODLs are present in most of the nuclear genes after FCIR despite of a functioning repair mechanism in normal animals. The conditions resemble the presence of excessive ODLs, due to disease conditions [29], or repair deficit in humans [31,34-37]. Therefore, transcription of immediately early genes with ODLs immediately during cerebral oxidative stress gives rise to aberrant or variant mRNA. The fact that neuronal nitric oxide synthase inhibitor increases neuronal viability after cerebral oxidative stress induced by several models of brain injury suggests that the aberrant expression of immediate early genes may be responsible, at least in part, for neuronal death. The data suggest that (1) brain nitric oxide may partially inhibit the transcription of c-fos mRNA, and (2) there is a possible link between an increase in the intact fos mRNA and the reduction in ODLs in the nuclear gene. The presence of intact c-fos mRNA in the ischemic core using 3Br7NI in this study may support the theory that Fos/AP-1 reduces necrosis.

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References

1. Akins PT, Liu PK, Hsu CY. Immediate early gene expression in response to cerebral ischemia. Friend or foe? *Stroke* 1996;27:1682-1687. [PubMed: 8784149]
2. Ayata C, Ayata G, Hara H, Matthews RT, Beal MF, Ferrante RJ, Endres M, Kim A, Christie RH, Waeber C, Huang PL, Hyman BT, Moskowitz MA. Mechanisms of reduced striatal NMDA excitotoxicity in type I nitric oxide synthase knock-out mice. *J Neurosci* 1997;17:6908-6917. [PubMed: 9278526]
3. Cui JK, Holmes EH, Cao ST, Greene TG, Liu PK. Oxidative DNA damage precedes DNA fragmentation after experimental stroke in rat brain. *FASEB J* 2000;14:955-967. [PubMed: 10783150]
4. Cui JK, Holmes EH, Liu PK. Oxidative damage to the c-fos gene and reduction of its transcription after focal cerebral ischemia. *J Neurochem* 1999;73:1164-1174. [PubMed: 10461908]

5. Cui JK, Hsu CY, Liu PK. Suppression of post-ischemic hippocampal nerve growth factor expression by a c-fos antisense oligodeoxynucleotide. *J Neurosci* 1999;19:2784–2893.
6. Deng X, Ladenheim B, Tsao LI, Cadet JL. Null mutation of c-fos causes exacerbation of methamphetamine-induced neurotoxicity. *J Neurosci* 1999;19:10107–10115. [PubMed: 10559418]
7. Du C, Hu R, Csernansky CA, Hsu CY, Choi DW. Very delayed infarction after mild focal cerebral ischemia: A role for apoptosis? *J Cereb Blood Flow Metab* 1996;16:195–201. [PubMed: 8594050]
8. Dugan LL, Creedon DJ, Johnson EM Jr, Holtzman DM. Rapid suppression of free radical formation by nerve growth factor involves the mitogen-activated protein kinase pathway. *Proc Natl Acad Sci USA* 1997;94:4086–4091.
9. Estus S, Zaks WJ, Freeman RS, Gruda M, Bravo R, Johnson EM Jr. Altered gene expression in neurons during programmed cell death: Identification of c-jun as necessary for neuronal apoptosis. *J Cell Biol* 1994;127:1717–1727. [PubMed: 7798322]
10. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 1992;119:493–501. [PubMed: 1400587]
11. Hall ED, Braughler JM. Central nervous system trauma and stroke. II. Physiological and pharmacological evidence for involvement of oxygen radicals and lipid peroxidation. *Free Radic Biol Med* 1989;6:303–313. [PubMed: 2663663]
12. Hengerer B, Lindholm D, Heumann R, Ruther UU, Wagner EF, Thoenen H. Lesion-induced increase in nerve growth factor mRNA is mediated by c-fos. *Proc Natl Acad Sci USA* 1990;87:3899–3903.
13. Horigome K, Bullock ED, Johnson EM Jr. Effects of nerve growth factor on rat peritoneal mast cells. Survival promotion and immediate-early gene induction. *J Biol Chem* 1994;269:2695–2702. [PubMed: 8300599]
14. Huang D, Shenoy A, Huang W, Cao S, Cui J, Liu PK. In situ detection of AP sites and DNA single stranded breaks with 3'-phosphate terminus in cerebral ischemia-reperfusion. *FASEB J* 2000;14:407–417. [PubMed: 10657997]
15. Iadecola C, Zhang F, Casey R, Nagayama M, Ross ME. Delayed reduction of ischemia brain injury and neurological deficits in mice lacking the inducible nitric oxide synthase gene. *J Neurosci* 1997;23:9157–9164. [PubMed: 9364062]
16. Jenner P, Dexter DT, Sian J, Schapira AH, Marsden CD. Oxidative stress as a cause of nigral cell death in Parkinson's disease and incidental Lewy body disease. The Royal Kings and Queens Parkinson's Disease Research Group. *Ann Neurol* 1992;32(suppl):S82–S87. [PubMed: 1510385]
17. Kamii H, Mikawa S, Murakami K, Kinouchi H, Yoshimoto T, Reola L, Carlson E, Epstein CJ, Chan PH. Effects of nitric oxide synthase inhibition on brain infarction in SOD-1-transgenic mice following transient focal cerebral ischemia. *J Cereb Blood Flow Metab* 1994;16:1153–1157. [PubMed: 8898687]
18. Lerant A, Freeman ME. Dopaminergic neurons in periventricular and arcuate nuclei of proestrous and ovariectomized rats: Endogenous diurnal rhythm of Fos-related antigens expression. *Neuroendocrinology* 1997;65:436–445. [PubMed: 9208406]
19. Lewen A, Matz P, Chan P. Free radical pathways in CNS injury. *J Neurotrauma* 2000;17:871–890. [PubMed: 11063054]
20. Li Y, Chopp M, Jiang N, Zaloga C. In situ detection of DNA fragmentation after focal cerebral ischemia in mice. *Brain Res Mol Brain Res* 1995;28:164–168. [PubMed: 7707871]
21. Lin L, Cao S, Yu L, Cui J, Hamilton WJ, Liu PK. Up-regulation of base excision repair activity for 8-hydroxy-2'-deoxyguanosine in the mouse brain after forebrain ischemia-reperfusion. *J Neurochem* 2000;74:1098–1105. [PubMed: 10693941]
22. Lipton P. Ischemic cell death in brain neurons. *Physiol Rev* 1999;79:1431–1568. [PubMed: 10508238]
23. Liu, PK.; Cui, JK. Expression of aberrant messenger RNA during oxidative stress in the mouse brain: Transcription activators from nuclear genes that are damaged. submitted
24. Liu PK, Hsu CY, Dizdaroglu M, Floyd RA, Kow YW, Karakaya A, Rabow LE, Cui JK. Damage, repair and mutagenesis in nuclear genes after mouse forebrain ischemia-reperfusion. *J Neurosci* 1996;16:6795–6806. [PubMed: 8824320]

25. Liu PK, Salminen A, He YY, Jiang MH, Xue JJ, Liu JS, Hsu CY. Suppression of ischemia-induced Fos expression and AP-1 activity by an antisense oligodeoxynucleotide to c-fos mRNA. *Ann Neurol* 1994;36:566–576. [PubMed: 7944289]
26. Mecocci P, MacGarvey U, Beal MF. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol* 1994;36:747–751. [PubMed: 7979220]
27. Mecocci P, MacGarvey U, Kaufman AE, Koontz D, Shoffner JM, Wallace DC, Beal MF. Oxidative damage to mitochondrial DNA shows marked age-dependent increases in human brain. *Ann Neurol* 1993;34:609–616. [PubMed: 8215249]
28. Namura S, Zhu J, Fink K, Endres M, Srinivasan A, Tomaselli KJ, Yuan J, Moskowitz MA. Activation and cleavage of caspase-3 in apoptosis induced by experimental cerebral ischemia. *J Neurosci* 1998;18:3659–3668. [PubMed: 9570797]
29. Nunomura A, Perry G, Pappolla MA, Wade R, Hirai K, Chiba S, Smith M. RNA oxidation is a prominent feature of vulnerable neurons in Alzheimer's disease. *J Neurosci* 1999;19:1959–1964. [PubMed: 10066249]
30. O'Neill MJ, Hicks C, Ward M. Neuroprotective effects of 7-nitroindazole in the gerbil model of global cerebral ischemia. *Eur J Pharmacol* 1996;310:115–122. [PubMed: 8884206]
31. Parshad R, Sanford KK, Price FM. Fluorescence light-induced chromatid breaks distinguish Alzheimer disease cells from normal cells in tissue culture. *Proc Nati Acad Sci USA* 1996;93:5146–5150.
32. Pennypacker KR, Thai L, Hong JS, McMillian MK. Prolonged expression of AP-1 transcription factors in the rat hippocampus after systemic kainate treatment. *J Neurosci* 1994;14:3998–4006. [PubMed: 8027758]
33. Petito CK, Torres-Munoz J, Roberts B, Olarte JP, Nowak TS Jr, Pulsinelli WA. DNA fragmentation follows delayed neuronal death in CA1 neurons exposed to transient global ischemia in the rat. *J Cereb Blood Flow Metab* 1997;17:967–976. [PubMed: 9307610]
34. Reardon JT, Bessho T, Kung HC, Bolton PH, Sanear A. In vitro repair of oxidative DNA damage by human nucleotide excision repair system: Possible explanation for neurodegeneration in xeroderma pigmentosum patients. *Proc Nati Acad Sci USA* 1997;94:9463–9468.
35. Robbins JH, Brumback RA, Polinsky RJ, Wirtschafter JD, Tarone RE, Scudiero DA, Otsuka F. Hypersensitivity to DNA-damaging agents in abiotrophies: A new explanation for degeneration of neurons, photoreceptors, and muscle in Alzheimer, Parkinson, and Huntington diseases, retinitis pigmentosa, and Duchenne muscular dystrophy. *Basic Life Sci* 1985;35:315–344. [PubMed: 2933027]
36. Robbins JH, Otsuka F, Tarone RE, Polinsky RJ, Brumback RA, Moshell AN, Nee LE, Ganges MB, Cayeux SJ. Radiosensitivity in Alzheimer disease and Parkinson disease. *Lancet* 1983;i:468–469. [PubMed: 6131182]
37. Robbins JH, Scudiero DA, Otsuka F, Tarone RE, Brumback RA, Wirtschafter JD, Polinsky RJ, Barrett SF, Moshell AN, Scarpinat RG. Hypersensitivity to DNA-damaging agents in cultured cells from patients with Usher's syndrome and Duchenne muscular dystrophy. *J Neurol Neurosurg Psychiatry* 1984;47:391–398. [PubMed: 6726265]
38. Sagar SM, Sharp FR, Curran T. Expression of c-fos protein in brain: Metabolic mapping at the cellular level. *Science* 1988;240:1328–1331. [PubMed: 3131879]
39. Salminen A, Liu PK, Hsu CY. Alteration of transcription factor binding activities in the ischemic rat brain. *Biochem Biophys Res Commun* 1995;212:939–944. [PubMed: 7626134]
40. Scanlon KJ, Jiao L, Funato T, Wang W, Tine T, Rossi JJ, Kashani-Sabet M. Ribozyme-mediated cleavage of c-fos mRNA reduces gene expression of DNA synthesis enzymes and metallothionein. *Proc Nati Acad Sci USA* 1991;88:10591–10595.
41. Schulz JB, Mathews RT, Jenkins BG, Ferrante RJ, Siwek D, Henshaw DR, Cipolloni PB, Mecocci P, Kowall NW, Rosen BR, Beal MF. Blockade of neuronal nitric oxide synthase protects against excitotoxicity in vivo. *J Neurosci* 1995;15:8419–8429. [PubMed: 8613773]
42. Sharp FR, Massa SM, Swanson RA. Heatshock protein protection. *Trends Neurosci* 1999;22:97–99. [PubMed: 10199631]
43. Shioda S, Funahashi H, Nakajo S, Yada T, Maruta O, Nakai Y. Immunohistochemical localization of leptin receptor in the rat brain. *Neurosci Lett* 1998;243:41–44. [PubMed: 9535108]

44. Strauss S, Otten U, Joggerst B, Pluss K, Volk B. Increased levels of nerve growth factor (NGF) protein and mRNA and reactive gliosis following kainic acid injection into the rat striatum. *Neurosci Lett* 1994;168:193–196. [PubMed: 8028775]
45. Tammariello S, Quinn MT, Estus S. NADPH oxidase contributes directly to oxidative stress and apoptosis in nerve growth factor-deprived sympathetic neurons. *J Neurosci* 2000;20:RC53. [PubMed: 10627630]
46. Yang K, Mu S, Xue JJ, Whitson J, Salminen A, Dixon CE, Liu PK, Hayes RL. Increased expression of c-fos mRNA and AP-1 transcription factor after cortical impact injury in rats. *Brain Res* 1994;664:141–147. [PubMed: 7895023]
47. Yoshida T, Limmroth V, Irikura K, Moskowitz MA. The NOS inhibitor, 7-nitroindazole, decreases focal infarct volume but not the response to topical acetylcholine in pial vessels. *J Cereb Blood Flow Metab* 1994;14:924–929. [PubMed: 7523430]
48. Zhang YJ, Widmayer MA, Zhang B, Cui JK, Baskin DS. Suppression of post-ischemic-induced fos protein expression by an antisense oligonucleotide to c-fos mRNA leads to increased tissue damage. *Brain Res* 1999;19:112–117.
49. Zhou W, Doetsch PW. Effects of abasic sites and DNA single-strand breaks on prokaryotic RNA polymerases. *Proc Natl Acad Sci USA* 1993;90:6601–6605.

Fig. 1.

Transcription of c-fos mRNA after 60 min of vessel occlusion in an FCIR model of brain injury using RT-PCR (18 cycles). The primers for c-fos cDNA amplification were: 5'-GCATGGGCTCCCCTGTCAAC-3' (upstream) and 5'-GCCCAGGTCATTGGGGATC-3' (downstream). One of the c-fos primers was ^{32}P labeled as previously described [5,23]. The left (L or contralateral) and right (R or ipsilateral) cortical RNA was amplified from animals ($n = 4$ in each lane) with a reperfusion time of 30 min (lane 1), 60 min (lane 2), 90 min (lane 3), 120 min (lane 4), 3 days (lane 5), 2 weeks (lane 6) and no ischemia (lane 7). M = Marker c-fos from a cloned cDNA that was used for in situ RPA.

Fig. 2.

Transcription of *c-fos* mRNA (arrows) after FCIR (**a**: 60 min of ischemia followed by 30 min of reperfusion, or 60/30, singlegene amplification; **b**: 60/15, coamplification) using RT-PCR (30 cycles). N = Normal (no FCIR); I = FCIR treated; M = KB DNA size marker.

Fig. 3. Topographical presentation of NGF mRNA expression using in situ RPA. **a** Expression of FCIR-induced NGF mRNA. **b** Expression of NGF mRNA in the non-FCIR brain. The expression of intact NGF genes in the ischemic brains was determined in 8 animals. The tissue sections were hybridized to the ^{32}P -cRNA probe [5], followed by treatments with RNase A. The autoradiogram was developed in the same cassette at -70°C for 2 days. The intensity of mRNA from each sample (resistant to RNase A treatment) was determined and statistically analyzed as described in the text.

Fig. 4.

Increased intact c-fos mRNA after treatment with 3Br7NI. The expression of intact c-fos mRNA after FCIR (60/15), using in situ RPA, increased significantly ($p < 0.03$) in animals treated with 3Br7NI ($n = 6$). Oil alone (a) or the drug in oil (b) was injected 5 min after vessel occlusion. Four brain sections (20 μ m thick, each separated by 100 μ m) per animal were tested. An autoradiogram was developed at the same time at -70°C for 7 h. The image on the autoradiogram from each animal was quantitatively measured using Alphamager 3.2, and the data were analyzed using GraphPad Prism. No signal was detected using the sense mRNA probe. A small effect was observed in the contralateral cortex. The intensity of ischemia-induced intact (RNase A-resistant) c-fos mRNA in the brain using in situ RPA (in pixel values)

was 599 ± 49 in animals treated with oil ($n = 4$) and 843 ± 67 in animals treated with 3Br7NI (c). * $p < 0.03$ (t test).