BNIP3 Upregulation and EndoG Translocation in Delayed Neuronal Death in Stroke and in Hypoxia

Zhengfeng Zhang, PhD; Xuefen Yang, MD; Surong Zhang, PhD; Xiuli Ma, MSc; Jiming Kong, PhD

Background and Purpose—Delayed neuronal death is a hallmark feature of stroke and the primary target of neuroprotective strategies. Caspase-independent apoptosis pathways are suggested as a mechanism for the delayed neuronal injury. Here we test the hypothesis that one of the caspase-independent apoptosis pathways is activated by BNIP3 and mediated by EndoG.

Methods—We performed immunohistochemistry, Western blotting, cell transfection, subcellular fractionation, and RNA interfering to analyze the expression and localization of BNIP3 and EndoG in degenerating neurons in models of stroke and hypoxia.

Results—BNIP3 was upregulated in brain neurons in a rat model of stroke and in cultured primary neurons exposed to hypoxia. The expressed BNIP3 was localized to mitochondria. Both forced expression of BNIP3 by plasmid transfection and induced expression of BNIP3 by hypoxia in neurons resulted in mitochondrial release and nuclear translocation of EndoG and neuronal cell death. Knockdown of BNIP3 by RNAi inhibited EndoG translocation and protected against hypoxia-induced neuronal death.

Conclusions—BNIP3 plays a role in delayed neuronal death in hypoxia and stroke and EndoG is a mediator of the BNIP3-activated neuronal death pathway. The results suggest that BNIP3 may be a new target for neuronal rescue strategies. (Stroke. 2007;38:1606-1613.)

Key Words: BNIP3 ■ EndoG ■ hypoxia ■ mitochondria ■ stroke

Stroke results in acute loss of neurons in the ischemic core region. The acute neuronal damage is followed by a second round of neuronal injury that occurs hours to days after brain ischemia, called delayed neuronal death, in the neighboring areas. Evidence suggests that the delayed cell death occurs primarily through an apoptosis mechanism. The caspase family of cysteine proteases plays an indispensable role in the signal transduction and execution of apoptosis. However, a growing number of studies supports that a large proportion of the delayed neuronal death in stroke is mediated by caspase-independent pathways.

BNIP3 is a member of a unique family of death-inducing mitochondrial proteins. Normally, BNIP3 is not expressed in neurons. Expression of BNIP3 is increased under prolonged hypoxic conditions primarily by the transcriptional factor hypoxia inducible factor 1 and causes cell death in a variety of cells. BNIP3-induced cell death is characterized by mitochondrial damage but is independent of caspase activation. BNIP3 is also implicated in autophagic cell death.

Recently, the mitochondrial protein EndoG has been shown to be involved in caspase-independent cell death pathways. This signal peptide is cleaved off on entering mitochondria and the mature EndoG can be released from mitochondria during apoptosis. Once released from mitochondria, EndoG is able to translocate to the nucleus and cleave chromatin DNA into nucleosomal fragments independently of caspases.

Here we have tested the hypothesis that BNIP3 activates a caspase-independent form of neuronal cell death in stroke and hypoxia and EndoG is a mediator of the BNIP3-activated cell death pathway. Our results suggest that BNIP3 may be a new target for neuronal rescue strategies.

Materials and Methods

Focal Brain Ischemia/Reperfusion

All animal protocols were approved by the University of Manitoba Animal Care Ethics Committee. Male Sprague-Dawley rats weighing 250 to 300 g (University of Manitoba Central Animal Care Breeding Facility, Winnipeg, Canada) were anesthetized with halothane. A 4–0 nylon suture with silicon-coated tip (Doccol Co) was inserted through the left external carotid artery to occlude the left middle cerebral artery (MCAO). The suture was withdrawn 90 minutes after MCAO to allow reperfusion. Sham-operated rats underwent only the dissection of the left carotid tree. At 12, 24, 48, and 72 hours after MCAO, the animals were examined by T2-weighted MRI using a Bruker Biospec MSL-X 7/21 spectrometer and then euthanized for further analyses.

Neuronal Culture and Hypoxia

Primary cortical neuronal cultures were prepared as described previously. Cells were plated in neurobasal medium (Invitrogen)
supplemented with 5 mmol/L HEPES, 1.2 mmol/L glutamine, 10% fetal bovine serum, 2% B27, and 25 μg/mL gentamicin at a density of 1×10^5 cells/cm² on plates or cover slips coated with poly-d-lysine. The medium was replaced with neurobasal without fetal bovine serum after 24 hours. After 7 days, glutamine was removed from the medium and neurons were then exposed to hypoxia for up to 72 hours in a hypoxia chamber (Billups-Rothenberg Inc) flushed with a preanalyzed gas mixture of 5% CO₂ and 95% N₂.

**Cell Transfection**

Plasmids pcDNA3-huBNIP3 and pcDNA3-huBNIP3ΔTM were gifts from the late Dr A.H. Greenberg (University of Manitoba, Winnipeg). The pcDNA3-huBNIP3 carrying the hamster BNIP3 was a generous gift from Dr R.K. Bruick (University of Texas Southwestern Medical Center, Dallas, Tex). For preparation of pEGFP-C2-rBNIP3, rat BNIP3 (rBNIP3) cDNA was isolated from primary neuronal cultures exposed to hypoxia for 36 hours by reverse-transcription polymerase chain reaction from pcDNA3-huBNIP3 and ligated to pGEM-T (Promega) by T-A cloning. After sequencing, the BNIP3 fragments were subcloned into pEGFP-C2 vector (Clontech). Neurons were transfected on day 4 in vitro using Lipfectamine 2000 (Invitrogen).

**shRNA Lentiviral Vectors**

The shRNA sequences were designed using Invitrogen’s BLOCK-iT RNAi Designer. The oligonucleotides were synthesized, annealed to generate double-stranded oligos, and cloned to pENTR/U6 vectors (Invitrogen). After cotransfection with BNIP3 plasmids into HEK293 cells, the inhibition efficiencies of the shRNAs were determined by immunofluorescence microscopy and quantitative Western blot analysis. Selected shRNA sequences were inserted into Invitrogen’s BLOCK-iT Lentiviral RNAi Expression system. Lentiviral stocks were produced using ViraPower Packaging Mix (Invitrogen). After cotransfection with BNIP3 plasmids into HEK293 cells, the inhibition efficiencies of the shRNAs were determined by immunofluorescence microscopy and quantitative Western blot analysis. Selected shRNA sequences were inserted into Invitrogen’s BLOCK-iT Lentiviral RNAi Expression system. Lentiviral stocks were produced using ViraPower Packaging Mix (Invitrogen). Transduction of neurons was performed by adding the viral vectors (multiplicity of infection >20) to medium 1 day before hypoxia.

**Western Blotting**

Protein samples were separated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride membranes. The primary antibodies included: polyclonal BNIP3 (1:1000), monoclonal BNIP3 (1:500), EndoG (1:800; Prosci Inc.), Cox IV (1:10 000; Abcam), Histone H1 (1:500; Prosci Inc.), and β-actin (1:2000; Sigma). The secondary antibodies were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1:10 000; Amersham Pharmacia Biotech). Immunoblotting was detected by ECL (Amer sham). Fluorescence pictures were taken on a Nikon TE2000-E microscope equipped with a RETIGA camera (QImaging).

**Immunohistochemistry**

Brain sections or cells on coverslips were labeled with primary antibodies (BNIP3, 1:200; NeuN, 1:500; or EndoG, 1:100) followed by appropriate secondary antibodies (1:200; Jackson). Nuclei were stained by Hoechst 33342 (Calbiocam). Degenerating neurons were labeled with Fluoro-Jade C (FJC; Chemicon). Fluorescence pictures were taken on a Nikon TE2000-E microscope equipped with a RETIGA camera (QImaging).

**Cell Death and Viability Assays**

Cell death was estimated using trypan blue exclusion. Neuronal viability was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay on a WallacVICTOR3 microplate reader (Perkin Elmer Life Sciences).

**Statistical Analysis**

One-way ANOVA was used to test for overall statistical significance. A difference was considered significant at P<0.05.

---

**Results**

**BNIP3 Expression Is Upregulated in Brain Ischemia**

Of 35 rats operated for MCAO, 5 died postoperatively and 6 were discarded for insufficient brain ischemia as determined by MRI. Each group consisted of 6 animals for further analysis (3 for immunohistochemistry and 3 for Western blot).

Unilateral MCAO induced severe ischemia of brain areas (supplemental Figure 1, available online at http://stroke.ahajournals.org). Immunohistochemical staining with a polyclonal antibody to BNIP3 did not reveal positive staining in brains of animals euthanized at 0, 12, and 24 hours after MCAO. In the 48-hour and 72-hour groups, intensive BNIP3 staining was found exclusively in the ischemic regions of brain, especially in the penumbral region of ischemia (Figure 1A). The expressed BNIP3 was primarily found in large cells and predominately localized to the cytoplasm (Figure 1B). In 19.7% of BNIP3-positive cells, BNIP3 was found in both the cytoplasm and the nuclei. Exclusive nuclear localization of BNIP3 was rarely found. Double labeling with antibodies to BNIP3 and NeuN revealed that most of the BNIP3-positive cells were neurons (Figure 1C). Preincubation of the BNIP3 antibody with a BNIP3–GST protein completely blocked the immunostaining for BNIP3 (Figure 1D). Double labeling with the BNIP3 antibody and the dying neuron marker FJC showed that almost all BNIP3 expressing cells were FJC-positive (Figure 1E through 1H). Using a monoclonal antibody recognizing rat BNIP3, we further determined by Western blotting the BNIP3 levels in brain extracts. Levels of BNIP3 were low in ischemic brains for up to 24 hours after MCAO. BNIP3 accumulated at 48 hours after brain ischemia (Figure 1I). High levels of BNIP3 were detected in the ipsilateral brain as compared with the contralateral and normal brains at 72 hours after MCAO (Figure 1K). Quantification of the immunoblotting bands showed that the levels of BNIP3 were increased by 12-fold and 11-fold, respectively, at 48 hours and 72 hours after MCAO (Figure 1J and 1L). To determine whether the expressed BNIP3 was membrane-bound, brain extracts were separated into heavy membrane pellets (P100) and supernatants (S100) by ultracentrifugation. The cleanliness of the subcellular preparations was confirmed by the absence of the mitochondrial membrane protein cytochrome c oxidase (Cox IV; Figure 1M) in the S100 fraction. BNIP3 was primarily found in the membrane fraction samples of the ipsilateral brains, indicating that the expressed BNIP3 was active because inactive BNIP3 does not integrate with cell membranes.

**Hypoxia Induces BNIP3 Expression in Primary Neuronal Cultures**

Primary rat cortical neurons at day 8 in vitro were subjected to normoxia or hypoxia for 6, 12, 24, 36, 48, 60, and 72 hours, respectively. Levels of BNIP3 expression were determined by Western blotting using a polyclonal BNIP3 antibody described previously. In addition to a nonspecific band, the antibody recognized two BNIP3 bands with molecular weights of 30 kD and 60 kD, respectively (Figure 1N). For control, immunoblotting
with a monoclonal β-actin antibody was performed and the β-actin bands were used as standards for calculation of BNIP3 expression. Levels of BNIP3 were low in neurons exposed to hypoxia for <24 hours and the BNIP3 protein consisted of almost entirely the 30-kDa form. After exposure to hypoxia for 36 hours, both forms of the BNIP3 protein started to accumulate (4-fold). After 72 hours of hypoxic exposure, the levels of BNIP3 increased to ~10-fold (Figure 1O). BNIP3 was not observed to accumulate in neurons under normoxic conditions.

**Figure 1.** Upregulation of BNIP3 in hypoxia and stroke. A, Representative immunohistochemical image of brain section cut from a rat euthanized at 48 hours after MCAO. The dotted line separates the ischemic area from the normal. BNIP3 was not detectable in nonischemic regions but upregulated in the ischemic regions (blue, nuclei; green, BNIP3; bar=250 μm). B, The expressed BNIP3, as determined by immunohistochemistry with an antibody to BNIP3, was predominately localized to neuronal cytoplasm (bar=50 μm). C, Double labeling with antibodies to NeuN and BNIP3 (red, NeuN; green, BNIP3; bar=50 μm). D, Preincubation of the BNIP3 antibody with a BNIP3–GST protein completely blocked the immunostaining for BNIP3 (bar=50 μm). E through H, Representative image showing colocalization of BNIP3 and FJC in ischemic neurons 48 hours after MCAO. Brain sections were stained for BNIP3 immunohistochemically and further labeled with FJC and Hoechst 33342 (bar=50 μm). I and J, Time course of BNIP3 expression after MCAO by Western blot analysis. K and L, Expression levels of BNIP3 in the ipsilateral brain (lane 1) and the contralateral (lane 2) and normal brains (lane 3) 72 hours after MCAO. M, Protein samples prepared from the ipsilateral brain (lane 1) and the contralateral (lane 2) and normal brains (lane 3) were separated into membrane (P100) and supernatant (S100) fractions and then Western-blotted. The amount of proteins loaded in each S100 lane was 30 μg, and in each P100 lane was 9 μg. N and O, Hypoxia-induced BNIP3 expression in primary cortical neurons. Protein samples were Western-blotted. A sample from BNIP3-transfected 293T cells was used as control. Results shown in (O) represent the mean±SD of both the 30-kD and 60-kD BNIP3 bands from 4 independent experiments. **P<0.01.
Inhibition of BNIP3 by RNAi Protects Neurons From Hypoxia-Induced Cell Death

To identify a shRNA sequence that is of high-inhibition efficiency for BNIP3, 12 pairs of oligonucleotides were initially designed, synthesized, and cloned into Invitrogen pENTR/U6 vectors. The vectors were cotransfected with the BNIP3-expressing plasmid pcDNA3-haBNIP3 into HEK 293 cells. The inhibition efficiencies of BNIP3 varied from none to almost complete inhibition as determined by immunofluorescence microscopy and quantitative Western blot analysis (data not shown). One pair of oligonucleotides (N167, forward, 5'-CACC-GCTTCCGTCTCTATTTATAAGAGAATAGAGACGGAAGC-3'; backward, 5'-AAAA-GCTTCCGTCTCTATTTATAATCTTGAAATAATAGAGACGGAAGC-3' (bold, sense and antisense strands; underlined, loop) targeting the nucleotides 167 to 188 in the BNIP3 mRNA sequence (GenBank accession number NM_053420) showed the most potent inhibition (Figure 2). Quantification of the Western blot bands revealed that the inhibition efficiency of the N167 for BNIP3 expression was 98.1% as compared with the nontransfected controls. In addition to its inhibition on hamster BNIP3, the N167 was able to inhibit the expression of mouse and rat BNIP3 genes with similar efficiencies (data not shown). To inhibit BNIP3 expression in neurons, we developed a lentiviral vector carrying the N167 sequence. Transfection of primary cortical neurons with the N167 lentiviral vector resulted in complete inhibition of BNIP3 in neurons exposed to hypoxia for 48 hours (Figure 2C), whereas no inhibition of BNIP3 was observed with a lentiviral vector carrying the LacZ sequence and a vector carrying a scrambled sequence (S167) that contained the same nucleotide composition as N167.

We next tested the effects of inhibition of BNIP3 on hypoxia-induced neuronal death. Primary cortical neuron cultures were infected with the N167 or the LacZ lentiviral vectors 24 hours before hypoxic exposure. As shown in Figure 3A, hypoxia induced neuronal cell death in a time-dependent manner. Inhibition of BNIP3 expression by the N167 vector reduced hypoxia-induced neuronal death by 15% to 23% and the protection was statistically significant at 36 and 48 hours of hypoxic exposure. Analysis of cell viability by MTT measurement revealed significant protection of the N167 for neurons exposed to hypoxia for 24, 36, 48, and 60 hours. No protective effects of the LacZ vector were observed.
Hypoxia-Induced BNIP3 Is Localized to Mitochondria

To determine how the expression of BNIP3 leads to neuronal death, we examined the subcellular localization of hypoxia-induced BNIP3. By differential centrifugation, mitochondrial, cytosolic, and nuclear fractionations were prepared and Western blotted with a BNIP3 antibody. Controls were performed using a β-actin antibody for cytosolic, a Cox IV antibody for mitochondrial, and a histone H1 antibody for nuclear fractions. Hypoxia-induced BNIP3 was primarily localized to mitochondria, particularly at prolonged hypoxia (60 hours and 72 hours). A small amount of BNIP3 was present in the cytosol and only a trace amount of BNIP3 was observed in the nuclear fractionation at late stages of hypoxic exposure (Figure 4A and 4B).

EndoG Is a Mediator of the BNIP3-Activated Cell Death Pathway

We next tested the involvement of EndoG in hypoxia-induced neuronal death. As shown in Figure 5, total amount of EndoG did not vary with exposure times to hypoxia. EndoG in the mitochondrial fraction decreased with exposure times to hypoxia; it started to decrease significantly after hypoxic exposure for 36 hours and disappeared from mitochondria after 72 hours of hypoxia. Meanwhile, EndoG was detected in nuclear fraction after hypoxia for 36 hours and accumulated with increased times of hypoxia, suggesting that the released EndoG from mitochondria was translocated to nuclei. This time course correlates well with that of hypoxia-induced BNIP3 expression (Figure 1).

To evaluate the role of BNIP3 in hypoxia-induced EndoG translocation in neurons, we transfected neurons with our N167 lentiviral vector and then exposed neurons to hypoxia for up to 72 hours. Western blotting analysis revealed that inhibition of BNIP3 delayed mitochondrial release and nuclear translocation of EndoG by 24 hours (Figure 5B and 5C). In the absence of BNIP3, EndoG was present in mitochondria even after 72 hours of hypoxic exposure and did not accumulate in nuclei until after 60 hours of hypoxia. The result was also confirmed by double-labeling immunohistochemistry with antibodies to BNIP3 and EndoG (Figure 5D); inhibition of BNIP3 by RNAi with the N167 lentiviral vector prevented EndoG translocation in neurons exposed to hypoxia for 48 hours. The control LacZ vector showed no inhibition on BNIP3 expression and no effect on hypoxia-induced EndoG translocation. As a result, DNA condensation was obvious in BNIP3-expressing neurons.
Figure 5. EndoG translocation in hypoxic neurons. A, Western blot analysis of EndoG in cortical neuronal cultures exposed to hypoxia. B, Western blot analysis of EndoG in cortical neuron cultures transfected with the N167 lentiviral vector 24 hours before hypoxia. C, Inhibition of BNIP3 by RNAi delayed hypoxia-induced EndoG release and translocation for 24 hours. D, Representative images of neurons transfected with indicated vectors for 24 hours and then exposed to hypoxia for 48 hours. Green, BNIP3; red, EndoG; blue, DNA.
Delayed neuronal death is a hallmark feature of brain ischemia. This is evidenced by the fact that even if cerebral blood flow is re-established quickly enough to prevent immediate cell death after brain ischemia, many of the initially surviving neurons still die hours to days after reperfusion. Of the many pathophysiological events that may contribute to this delayed injury, apoptotic signaling pathways are, among others, an established feature. The present study has revealed the delayed feature of BNIP3 expression in the MCAO model of stroke and in neurons exposed to hypoxia and stroke. BNIP3 is upregulated in the MCAO model of stroke and in hypoxia but also plays a role in neuronal death independent of Apaf-1, caspase activation, cytochrome c release, and nuclear translocation of apoptosis-inducing factor.

### Discussion

Delayed neuronal death is a hallmark feature of brain ischemia. This is evidenced by the fact that even if cerebral blood flow is re-established quickly enough to prevent immediate cell death after brain ischemia, many of the initially surviving neurons still die hours to days after reperfusion. Of the many pathophysiological events that may contribute to this delayed injury, apoptotic signaling pathways are, among others, an established feature. The present study has revealed the delayed feature of BNIP3 expression in the MCAO model of stroke and in neurons exposed to hypoxia and stroke. BNIP3 is upregulated in the MCAO model of stroke and in hypoxia but also plays a role in neuronal death independent of Apaf-1, caspase activation, cytochrome c release, and nuclear translocation of apoptosis-inducing factor.

**Figure 6.** Expression of BNIP3 results in EndoG translocation. Results shown represent the mean ± SD from 5 independent experiments. **P < 0.01. A. Percentage of BNIP3-positive cells with EndoG nuclear translocation in primary cortical neurons transfected with the indicated plasmids at 48 hours. B. Cell death rates determined by trypan blue exclusion in transfected neurons 48 hours after transient transfection with the indicated plasmids.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Neuronal death (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BNIP3</td>
<td>73%</td>
</tr>
<tr>
<td>BNIP3ΔTM</td>
<td>22%</td>
</tr>
</tbody>
</table>

pcDNA3-huBNIP3 plasmid encoding the full length of human BNIP3. Immunohistochemical analysis showed that the majority (62%) of BNIP3 expressing neurons had nuclear presence of EndoG. Neurons transfected with a truncated form of BNIP3 (pcDNA3-huBNIP3ΔTM) that did not contain the C-terminal transmembrane domain did not induce EndoG translocation (Figure 6A). Cell death rates in BNIP3 and BNIP3ΔTM transfected neurons were 73% and 22%, respectively (Figure 6B).

By coupling rational design of shRNAs with their validation in cell transfection, we have identified the N167 shRNA sequence specific for BNIP3 and achieved an inhibition efficiency of 98.1%. This is so far the most powerful tool for BNIP3 inhibition. With this tool, we found that knockdown of BNIP3 protected 15% to 23% of neurons from hypoxia-induced cell death (Figure 4). It is noticeable that the protective rates are much lower than the rate of neuronal death (73%) caused by forced expression of BNIP3 (Figure 6B). This may suggest that other members of the BNIP3 family, eg, Nix, may compensate for the loss of BNIP3. Also, the BNIP3 pathway may exist in tandem with other cell death pathways, inhibition of one pathway may likely cause shift of others. Further research is required to determine the neuroprotective effects by inhibiting simultaneously multiple pathways.

Another finding of the present study is the identification of EndoG as a downstream event of the BNIP3-activated cell death pathway. Previous extensive studies have established that BNIP3-induced cell death is mitochondria-mediated, involving opening of the mitochondrial permeability transition pores, accumulation of reactive oxygen species, and loss of mitochondrial membrane potential. The BNIP3 pathway differs from conventional apoptosis in that it appears to be independent of Apaf-1, caspase activation, cytochrome c release, and nuclear translocation of apoptosis-inducing factor. Although a previous study with primary cardiac myocytes supported the involvement of caspase activity in BNIP3-induced cell death and suggested the involvement might be cell type-specific, in primary neuron cultures we failed to observe any protective effects of caspase inhibitors, alone or in combination, on cell death caused by transient experiments, in which MRI was used to verify the extent of brain ischemia after MCAO, we show clearly that BNIP3-positive neurons are found only in the ischemic brain regions and concentrated in the penumbra, a region where programmed cell death usually occurs after brain ischemia. The expressed BNIP3 is primarily in the cytoplasm, as shown in both immunohistochemistry and Western blot analyses. We have observed nuclear localization of BNIP3 in <20% of the BNIP3-expressing neurons. This is in disagreement with 2 recent reports on the distribution of BNIP3 in brain ischemia based on their immunohistochemistry data. This discrepancy may be resulted from different antibodies used, cell types examined, and the time course of BNIP3 expression observed. However, the functional role of nuclear localization of BNIP3 in neurons is unclear. In tumor cells, nuclear localization of BNIP3 has been suggested as a mechanism of cell’s escaping of apoptosis.
transfection of BNIP3. In consistent with a previous report, we did not detect any apoptosis-inducing factor translocation in BNIP3-expressing neurons. Previous studies support that BNIP3-induced cell death involves chromatin condensation and DNA fragmentation, implicating a caspase-independent DNase in the BNIP3 pathway. The present study provides evidence that expression of BNIP3 results in mitochondrial release and nuclear translocation of EndoG, and inhibition of BNIP3 delayed hypoxia-induced EndoG translocation by 24 hours (Figure 5). The 24-hour delay in EndoG translocation should be considered very significant, taking into consideration the presence of many other inducers of EndoG translocation in the cells such as caspases and other members of the BNIP3 gene family that are activated in hypoxia. Once in the nucleus, EndoG first induces large-scale DNA fragmentation, followed by subsequent oligonucleosomal DNA fragmentation. Recently, Lee et al reported early (4 hours after MCAO) EndoG translocation in a mouse model of brain ischemia. This early EndoG release/translocation is not observed in our experiments and is certainly not a BNIP3-induced event, because BNIP3 is not activated within 24 hours after MCAO. Nevertheless, the role of EndoG in mediating BNIP3-activated cell death needs to be further established using EndoG-deficient cells and animals.

Sources of Funding
This work was supported by grants from Canadian Institutes of Health Research, Heart and Stroke Foundation of Canada and Manitoba Health Research Council (to J.K.). Dr Zhengfeng Zhang received a Manitoba Health Research Council postdoctoral fellowship. Dr Surong Zhang received a postdoctoral fellowship from Manitoba Health Research Council (to J.K.). Dr Zhengfeng Zhang received a New Investigator Award from Heart and Stroke Foundation of Canada.

Disclosures
None.

References
BNIP3 Upregulation and EndoG Translocation in Delayed Neuronal Death in Stroke and in Hypoxia
Zhengfeng Zhang, Xuefen Yang, Surong Zhang, Xiuli Ma and Jiming Kong

Stroke. 2007;38:1606-1613; originally published online March 22, 2007;
doi: 10.1161/STROKEAHA.106.475129
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://stroke.ahajournals.org/content/38/5/1606

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published
in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office.
Once the online version of the published article for which permission is being requested is located, click
Request Permissions in the middle column of the Web page under Services. Further information about this
process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/