Endonuclease G does not play an obligatory role in poly(ADP-ribose) polymerase-dependent cell death after transient focal cerebral ischemia

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Abstract

Activation of poly(ADP-ribose) polymerase (PARP) and subsequent translocation of apoptosis-inducing factor contribute to caspase-independent neuronal injury from N-methyl-D-aspartate, oxygen-glucose deprivation, and ischemic stroke. Some studies have implicated endonuclease G in the DNA fragmentation associated with caspase-independent cell death. Here, we compared wild-type and endonuclease G null mice to investigate whether endonuclease G plays a role in the PARP-dependent injury that results from transient focal cerebral ischemia. Latex casts did not reveal differences in the cerebral arterial distribution territory or posterior communicating arterial diameter, and the decrease in laser-Doppler flux during middle cerebral artery occlusion was similar in wild-type and endonuclease G null mice. After 90 min of occlusion and 1 day of reperfusion, similar degrees of nuclear translocation of apoptosis-inducing factor and DNA degradation were evident in male wild-type and null mice. At 3 days of reperfusion, infarct volume and neurologic deficit scores were not different between male wild-type and endonuclease G null mice or between female wild-type and endonuclease G null mice. These data demonstrate that endonuclease G is not required for the pathogenesis of transient focal ischemia in either male or female mice. Treatment with a PARP inhibitor decreased infarct volume and deficit scores equivalently in male wild-type and endonuclease G null mice, indicating that the injury in endonuclease G null mice remains dependent on PARP. Thus, endonuclease G is not obligatory for executing PARP-dependent injury during ischemic stroke.

Key words: apoptosis, DR2313, ischemic stroke, mouse, middle cerebral artery
DNA repair is essential for maintaining genomic integrity. Endonucleases can participate in DNA repair. For example, the apurinic/apyrimidinic endonuclease/redox factor-1 can serve in base excision repair and protect the brain from oxidative stress (5). However, excessive cellular stress can produce apoptosis mediated by caspase cleavage of DNA fragmentation factor, thereby producing active nucleosomal DNA fragmentation and cell death. Interestingly, in the absence of this nuclease, residual DNA fragmentation can still occur (31). Endonuclease G (EndoG) is an alternative nuclease that is thought to be involved in caspase-independent apoptosis (12, 23). EndoG is normally localized in the mitochondria, but it can translocate to the nucleus and produce DNA degradation in cell death induced by oxidative stress (8) in various cells, including neurons (6) and astrocytes (24). Nuclear translocation of EndoG has been reported after oxygen-glucose deprivation (22, 34), focal cerebral ischemia (11, 17, 33), global cerebral ischemia (18), and spinal cord injury (26). Whether EndoG is required for cell death after cerebral ischemia has not been determined. 

We (4) and others (7) have generated lines of EndoG null (EndoG–/–) mice that develop to adulthood with no obvious abnormalities and that avoid the embryonic lethality associated with knocking out a portion of an exon that overlaps with another gene of unknown function (30). Unexpectedly, fibroblasts and splenocytes from EndoG–/– mice remain fully vulnerable to chemical activation of extrinsic and intrinsic caspase-dependent and caspase-independent pathways (4, 7). Moreover, the striatum of EndoG–/– mice is not protected from NMDA excitotoxicity (4). Thus, the functional role of EndoG in neuronal ischemic cell death remains unclear. Using a model of transient focal cerebral ischemia, we tested the hypothesis that infarct volume is reduced in EndoG–/– mice. Because the mechanisms of neuronal ischemic cell death can differ between males and females (29, 35), we studied both genders. 

In Caenorhabditis elegans, homologs of EndoG and apoptosis-inducing factor (AIF), another mitochondrial protein that can translocate to the nucleus, appear to cooperate in producing DNA degradation (19, 25). Human EndoG can also form complexes with AIF (9).
Activation of poly(ADP-ribose) polymerase (PARP) produces caspase-independent cell death by triggering nuclear translocation of AIF (27, 28). In focal cerebral ischemia, inhibition of PARP or gene deletion of PARP-1 decreases AIF translocation and infarct size (2, 29). Because EndoG is postulated to participate in caspase-independent cell death, it may participate in PARP-mediated ischemic injury, as suggested by in vitro work with oxygen-glucose deprivation (22) and oxidative stress (20). Accordingly, the protection afforded by PARP inhibition may be diminished in the absence of functional EndoG. Therefore, we also tested a second hypothesis that the decrease in infarct volume that is expected to be observed in wild-type (WT) mice receiving a PARP inhibitor will be diminished in EndoG−/− mice treated with a PARP inhibitor. Because protection by a PARP inhibitor is seen in male mice but not in female mice subjected to middle cerebral artery (MCA) occlusion (MCAO) (15), this second hypothesis was tested only in male mice. We also determined whether translocation of AIF to the nucleus occurs after MCAO in EndoG−/− mice and whether the pattern of DNA degradation is altered in EndoG−/− mice.

MATERIALS AND METHODS

All experiments were conducted in accordance with the guidelines of the National Institutes of Health for the care and use of animals in research and were approved by the institutional animal care and use committee. The generation of EndoG−/− mice has been reported previously (4). Anesthesia was induced in WT and EndoG−/− mice (25–30 g) with 5% isoflurane. Once the mice were insentient, the anesthetic was administered at a nominal concentration of 2% in 25%–30% O₂ via face mask with spontaneous ventilation for a brief period during incision of the skin and isolation of the carotid artery. Anesthesia was then maintained with a nominal inspired concentration of 1.2% isoflurane for the remainder of the procedure. The concentration was increased if spontaneous movement occurred or the
frequency of ventilation increased. Rectal temperature was maintained at ~37°C during the surgery.

*Middle cerebral artery occlusion.* The intraluminal filament model of MCAO was used to produce transient focal cerebral ischemia (32). A laser-Doppler flow (LDF) probe was secured on the skull over the lateral parietal cortex to monitor perfusion in the ischemic cortical core. The right common carotid artery (CCA) was exposed through a 15-mm submandibular midline incision. The external carotid artery was carefully separated from the adjacent vagus nerve and muscle to prepare a site for coagulation and division located 3–5 mm distal to the CCA bifurcation. The proximal end of the CCA was temporarily ligated with a 6-0 suture. A 7-0 nylon monofilament with a silicone-coated tip was inserted from the distal end of the isolated external carotid artery, gently introduced into the internal carotid artery, and advanced approximately 6–8 mm past the CCA bifurcation to the origin of the MCA to produce 90 min of MCAO. After ensuring an adequate decrease in LDF during the first 10 min of MCAO, the incision was closed, anesthesia was discontinued, and a neurologic deficit score (NDS) was determined on a 0–4 scale (0 = no deficit; 1 = forelimb weakness; 2 = circling to affected side; 3 = unable to bear weight on affected side; 4 = no spontaneous motor activity). To establish reperfusion, the mouse was briefly anesthetized, the monofilament was withdrawn, and the CCA ligature was removed. The neck incision was closed with a suture, and anesthesia was discontinued. Mice with no neurologic deficit during ischemia were excluded. NDS and infarct volume were evaluated 3 days after the MCAO. Infarct volume was measured by standard volumetric analysis of anterior and posterior views of five coronal slabs stained with triphenyltetrazolium chloride, with correction for swelling.

*PARP inhibitor infusion protocol.* Intravenous infusion of the PARP inhibitor DR2313 (2-methyl-3,5,7,8-tetrahydrothiopyrano[4,3-d]pyrimidine-4-one) has been reported to decrease infarct volume after MCAO (16). In subsets of male WT and EndoG−/− mice, a catheter was placed into the right jugular vein and exited through a small incision in the back. DR2313 (Alexis
Corporation, Lausen, Switzerland) was dissolved in sterile saline at a final concentration of 10 mg/ml. The dosing regimen was based on that used by others (16) to achieve a pharmacologically active brain concentration: bolus injection of 1 ml/kg body weight 5 min before the onset of MCAO and again 5 min before reperfusion (85 min of MCAO); continuous infusion of 1 ml/kg/h throughout the 90 min of MCAO and the first 4.5 h of reperfusion. Control cohorts received the same volume of saline as the drug-treated cohorts.

**Vascular anatomy evaluation.** To visualize the arterial anatomy, mice were perfused with a black latex suspension (14). Briefly, mice were anesthetized with pentobarbital (50 mg/kg, ip), papaverine hydrochloride (50 mg/kg) was injected into the liver to produce maximal vasodilation, and a 20-gauge intracatheter was inserted through the left ventricular wall with the tip placed into the aorta. A solution of warmed 0.9% NaCl was infused followed by infusion of a warmed black latex suspension until the suspension was observed to flow freely from the incised right atrium. The mouse was placed in ice for 15 min, and then the brain was harvested, fixed in 10% formalin, and photographed. The line of anastomoses between the MCA and anterior cerebral artery (ACA) territories was delineated, and the distance of this line from the midline was measured at 1-mm intervals from the frontal pole. The diameter of the posterior communicating arteries was also measured.

**Nuclear AIF translocation.** Brains were rapidly harvested after decapitation, and ischemic hemispheres were isolated after removing the anterior and posterior poles at approximately +2.2 mm and –4.9 mm from bregma. Tissue was homogenized and nuclear subfractonation was performed by ultracentrifugation with a sucrose gradient technique as previously described (13). Western immunoblotting was performed by standard techniques on 4-12% Tris-glycine gels using primary antibodies for 62 kDa AIF. Histones were used as a protein loading control for nuclear proteins, and manganese superoxide dismutase was used as a mitochondrial protein marker to assess contamination of the nuclear fraction with mitochondrial protein. In order to obtain sufficient protein in a highly purified nuclear pellet
without detectable mitochondrial protein, tissue samples were pooled from two hemispheres for each lane on Western blots.

DNA fragmentation. To evaluate DNA fragmentation, DNA was isolated and separated by agarose gel electrophoresis in separate sets of mice than those used for nuclear AIF measurements. Brains were rapidly harvested 24 h after sham surgery or transient focal ischemia. Genomic DNA was isolated from ipsilateral hemisphere (approximately +2.2 mm and −4.9 mm from bregma) with DNeasy blood and tissue kit (QIAGEN, Valencia, CA). In brief, brain samples were incubated with lysis buffer containing proteinase K (60 mAU/ml) and DNAase-free RNase A (2 mg/ml), applied into a mini spin column, and centrifuged. The DNA was finally collected from the mini spin column with elute buffer. DNA samples (2 µg) were fractionated by agarose gel (1.2%) electrophoresis containing 0.5 µg/ml ethidium bromide and photographed under UV transilumination.

Statistical analysis. Infarct volume, LDF, distance to the line of anastomoses, and diameter of the posterior communicating arteries are presented as mean ± SD and were compared between WT and EndoG−/− mice by t-test. Within each genotype, comparisons of outcome from saline and DR2313 infusion were made by t-test. For infarct volume in individual brain slices, two-way analysis of variance was performed, and results from each of the five coronal levels were compared between groups by the Newman-Keuls multiple range test. Comparisons of NDS between groups were made the Mann-Whitney rank sum test. Differences of $P < 0.05$ were considered to be statistically significant for all tests.

RESULTS

Cerebral arteries. The location of the MCA-ACA border region, as assessed by latex casts, was similar in WT and EndoG−/+ mice (Fig. 1A). The distance from the midline to the line of anastomoses was not significantly different between WT and EndoG−/− mice, indicating a
similar volume at risk in the two genotypes (Fig. 1B). Infarct volume during MCAO also depends on blood flow supplied to the Circle of Willis through the posterior communicating arteries. The diameters of the right and left posterior communicating arteries were not different between WT and EndoG\(^{-/-}\) mice (Fig. 1C).

**MCAO in male mice.** Induction of MCAO produced similar decreases in LDF in male WT (28 ± 11% of baseline) and EndoG\(^{-/-}\) mice (27 ± 7%). After 90 min MCAO and 3 days of reperfusion, neurologic deficits were present in both groups. The range of NDS on a 0–4 scale was not significantly different between male WT (2, 2.5, 3 for 25\(^{th}\), 50\(^{th}\), 75\(^{th}\) percentile, respectively) and EndoG\(^{-/-}\) mice (2.6, 3, 3). Infarct volume was not different between the groups at any coronal level (Fig. 2A). Furthermore, infarct volume in cerebral cortex, striatum, and the entire hemisphere were not significantly different between male WT and EndoG\(^{-/-}\) mice (Fig. 2B).

**MCAO in female mice.** Similar results were obtained in female WT and EndoG\(^{-/-}\) mice. During the onset of MCAO, LDF decreased to the same extent in female WT (16 ± 3% of baseline) and EndoG\(^{-/-}\) mice (13 ± 3%). At 3 days of reperfusion, the range of NDS did not significantly differ between female WT (2, 2, 2 for 25\(^{th}\), 50\(^{th}\), 75\(^{th}\) percentile, respectively) and EndoG\(^{-/-}\) mice (2, 3, 3). Moreover, infarct volume was not significantly different between female WT and EndoG\(^{-/-}\) mice at any coronal level (Fig. 3A) or in cerebral cortex, striatum, or the entire hemisphere (Fig. 3B).

**AIF translocation and DNA degradation.** Focal cerebral ischemia produces AIF nuclear translocation, which leads to DNA degradation and cell death. Expression of AIF in the mitochondria was equivalent in male WT and EndoG\(^{-/-}\) mice. Measurements of AIF in the nuclear fraction of ischemic hemispheres at 1 day of reperfusion indicated a similar increase in nuclear AIF in WT and EndoG\(^{-/-}\) mice compared to sham-operated mice (Fig. 4). Degradation of DNA was prominent at 1 day of reperfusion, and qualitative differences in fragment size were not evident between WT and EndoG\(^{-/-}\) mice (Fig. 5).
Effect of PARP inhibitor. PARP-1 was expressed to a similar extent in WT and EndoG−/− mice (Fig. 6). To test whether a PARP inhibitor could rescue tissue in EndoG−/− mice to the same extent as in WT, cohorts of male WT and EndoG−/− mice were infused intravenously with either the saline vehicle or the PARP inhibitor DR2313. The decrease in LDF over lateral parietal cortex during the onset of MCAO was not affected by infusion of DR2313 compared to saline infusion in either genotype (Fig. 6). However, by 3 days of reperfusion NDS was significantly less in both WT and EndoG−/− mice receiving DR2313 compared to the corresponding WT and EndoG−/− mice receiving saline (Fig. 7).

Infarct volumes in cortex, striatum, and hemisphere were significantly smaller in WT mice treated with DR2313 than in WT mice treated with saline (Fig. 8B). Likewise, infarct volume in each structure was significantly smaller in EndoG−/− mice treated with DR2313 than in EndoG−/− mice treated with saline (Fig. 8D). The decreases in infarct volume were significant in each of the mid-coronal sections where the infarct was most prominent in WT (Fig. 8A) and EndoG−/− mice (Fig. 8C). DR2313-induced reduction in infarct volume was uniform between WT and EndoG−/− mice at each coronal level (Fig. 8E). Infarct volume in cortex, striatum, and hemisphere was decreased to a similar extent by DR2313 in WT and EndoG−/− mice (Fig. 8F).

DISCUSSION

The first major finding of this study is that gene deletion of EndoG does not affect the size of the infarct obtained from transient focal cerebral ischemia in either male or female mice. The second major finding is that inhibition of PARP continues to provide robust protection from ischemic stroke in male mice in the absence of EndoG. These findings indicate that EndoG is not required for the PARP-dependent cell death that is prominent in ischemic stroke.

Several studies have detected increased EndoG in the nucleus in vitro after oxidative stress (6, 24) or oxygen-glucose deprivation (22, 34) and in vivo after focal cerebral ischemia
Moreover, knockdown of EndoG by siRNA in neurons has been reported to protect against oxidative stress (6). In contrast, the lack of effect on infarct volume of EngoG gene deletion in our study indicates that the observed translocation of EndoG is not essential for the infarction process in vivo. Possible explanations for the lack of effect are that other nucleases normally are already executing cell death in most neurons before EndoG translocation takes place or that other nucleases compensate for the life-long loss of EndoG. In particular there are evolutionarily conserved EndoG-like endo/exonucleases in higher eukaryotes that may substitute for the absence of EndoG or play more important roles in DNA fragmentation initiated by PARP-dependent cell death pathways (3). Future investigations are required to identify these endo/exonucleases. In the present study, the pattern of DNA fragments at 1 day of reperfusion was not substantially altered after stroke by Endo G gene deletion, suggesting that any compensation by another endonuclease would have had to result in DNA fragments similar to those produced by EndoG in the WT mice. Thus, the possibility that other nucleases normally are already degrading DNA before EndoG becomes involved appears to be a more likely alternative. Interestingly, an EndoG paralog possessing both exonuclease and endonuclease activity has been described (3), but its ability to translocate from the inner mitochondrial membrane in pathological conditions is uncertain. Alternatively, the translocated EndoG recognized by antibodies might not be in a fully active form. For example, EndoG functions as a homodimer, and its DNase activity is modulated by multiple nuclear factors and heat shock protein-70 (9). One also has to consider the possibility that ischemia-induced necrosis in some neurons disrupts the mitochondrial membrane integrity sufficiently to release EndoG, causing its appearance in the nucleus after cell death becomes irreversible.

On a regional basis, infarct volume was similar between WT and EndoG−− mice in cerebral cortex and striatum and at each coronal level. Because the line of anastomoses between the MCA and ACA territories had a similar location in WT and EndoG−− mice, the volume at risk of infarction apparently was similar between the mouse genotype groups.
Moreover, the decrease in LDF during MCAO and the diameter of the posterior communicating arteries, which can influence intracerebral collateral blood flow, were not affected by EndoG gene deletion. Thus, it is unlikely that differences in the volume at risk or the ischemic severity masked a potential contribution of EndoG to the infarction process. Furthermore, the lack of differences in cerebral arterial architecture between WT and EndoG−/− mice is consistent with the lack of other obvious phenotypes in EndoG−/− mice (4, 7).

Nuclear translocation of EndoG after oxygen-glucose deprivation in cortical neurons can be inhibited by a PARP inhibitor (22). Excessive activation of PARP leads to nuclear translocation of AIF and cell death (27, 28). Based on work in C. elegans, EndoG has been implicated in AIF-dependent DNA degradation (19, 25). However, EndoG−/− mouse fibroblasts are still susceptible to PARP-dependent cell death, and EndoG−/− mice are still vulnerable to NMDA-induced neuronal injury, which is known to be dependent on PARP activation and AIF translocation (4). The present results demonstrate that EndoG gene deletion does not disturb expression of PARP-1 in the nucleus or AIF in the mitochondria and that translocation of AIF to the nucleus still occurs in EndoG−/− mice after stroke. Moreover, the effectiveness of PARP inhibition in reducing infarct volume after MCAO is unchanged in EndoG−/− mice. Thus, a substantial portion of cell death after stroke in EndoG−/− mice remains dependent on PARP activity, and EndoG is not obligatory for PARP-dependent infarction.

Several studies have shown that administration of various PARP inhibitors decrease infarct volume at 1 day after MCAO (1, 15, 16, 21). However, continued inflammatory processes beyond 1 day could negate the short-term benefit. Our results show that infarct volume measured at 3 days of reperfusion was reduced by more than half with DR2313 administration. Thus, the efficacy of this PARP inhibitor is robust. PARP inhibitors can exert anti-inflammatory effects after global ischemia (10), and this property may contribute to sustained protection in experimental stroke.
In summary, the present study demonstrates that EndoG does not play an obligatory role in infarct development after transient focal cerebral ischemia in either male or female mice. The injury in EndoG−/− mice remains dependent on PARP, as demonstrated by tissue rescue with a PARP inhibitor. These results do not support the notion that EndoG participates in ischemic neuronal injury known to rely on PARP-dependent stimulation of AIF translocation.

PERSPECTIVES AND SIGNIFICANCE

Neuronal cell death during ischemia has traditionally been assumed to occur by necrotic mechanisms. Classical apoptosis with apoptotic bodies is not a prominent morphological feature in experimental ischemic stroke. However, inhibitors of PARP generally provide greater protection in MCAO models than broad spectrum caspase inhibitors. Because over-activation of PARP by oxidative stress leads to cell death that is dependent on nuclear translocation of AIF, this caspase-independent pathway appears to be a major mechanism of cell death in stroke. Although some reports have assumed that EndoG executes cell death initiated by AIF translocation in mammals, our work indicates that EndoG is not essential for cell death in ischemic stroke. Although the results from this study on the role of EndoG in ischemia are negative, they are significant in raising the question of which endonuclease is responsible for executing neuronal death in ischemic stroke.
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Figure Legends

Fig. 1. Wild-type (WT) and endonuclease G knockout (KO) mice show no differences in cerebral artery anatomy. A: Examples of brains perfused with black latex suspension to visualize the cerebral artery distribution regions in male WT and endonuclease G KO mice. B: The distance from the midline to anastomoses of the middle and anterior cerebral arteries on the pial surface was measured at 1-mm increments from the frontal pole. This distance from the midline was not significantly different between WT \( (n = 5) \) and endonuclease KO (EndoG\(^{-/-}\); \( n = 4 \)) mice. C: The diameter of the left and right posterior (P) communicating artery (art) was not significantly different between WT and EndoG\(^{-/-}\) mice. Values are means ± SD.

Fig. 2. Infarct volumes were not significantly different between male WT and EndoG\(^{-/-}\) mice. A: Infarct volume (mm\(^3\); ±SD) in each of five coronal sections (section 1 is anterior) in 8 male WT and 11 male EndoG\(^{-/-}\) mice. B: Infarct volume (percent of ipsilateral structure volume) in cerebral cortex, striatum, and entire hemisphere.

Fig. 3. Infarct volumes were not significantly different between female WT and EndoG\(^{-/-}\) mice. A: Infarct volume (mm\(^3\); ±SD) in each of five coronal sections (section 1 is anterior) in 6 female WT and 7 female EndoG\(^{-/-}\) mice. B: Infarct volume (percent of ipsilateral structure volume) in cerebral cortex, striatum, and entire hemisphere.

Fig. 4. Western immunoblots for PARP-1, AIF, histones, and manganese superoxide dismutase (MnSOD) on nuclear and mitochondrial fractions from brains one day after sham surgery or 90 min of MCAO in WT and EndoG\(^{-/-}\) mice. Results indicate that PARP-1 expression in nucleus and AIF expression in mitochondria are similar in WT and EndoG\(^{-/-}\) mice and that the increase
in nuclear AIF after MCAO is similar in WT and EndoG<sup>−/−</sup> mice. Histones and MnSOD served as nuclear and mitochondrial protein markers, respectively.

Fig. 5. DNA fractionation by agarose gel electrophoresis from brains 1 day after sham surgery or MCAO in WT and EndoG<sup>−/−</sup> mice. Lane 1 shows molecular weight markers (kilobase pairs). The pattern of DNA fragments after MCAO was similar in WT and EndoG<sup>−/−</sup> tissue.

Fig. 6. Laser-Doppler flow over lateral cortex (±SD), expressed as a percent of pre-ischemic baseline, at 5 and 10 min of MCAO in 8 male WT mice infused with saline, 8 male WT mice infused with DR2313, 6 male EndoG<sup>−/−</sup> mice infused with saline, and 5 male EndoG<sup>−/−</sup> mice infused with DR2313. No significant differences existed between saline and DR2313 groups.

Fig. 7. Neurologic deficit scores on a 0–4 scale at 3 days of reperfusion in male WT mice treated with saline (n=8) or DR2313 (n=8) and in male EndoG<sup>−/−</sup> mice treated with saline (n=6) or DR2313 (n=5). Box plots present medians and 25<sup>th</sup> and 75<sup>th</sup> percentiles. * P < 0.05 vs. saline treatment.

Fig. 8. PARP inhibition reduced MCAO-induced infarct volume equally in WT and EndoG<sup>−/−</sup> mice. Infarct volume (±SD) was compared in: male WT mice treated with saline (n=8) or DR2313 (n=8) (A, B); male EndoG<sup>−/−</sup> mice treated with saline (n=6) or DR2313 (n=5) (C, D); and WT and EndoG<sup>−/−</sup> mice treated with DR2313 (E, F) for each coronal section (A, C, E; mm<sup>3</sup>) and for cerebral cortex, striatum, and the entire hemisphere (B, D, F; % of structure). * P < 0.05 vs. saline treatment.