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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Xu Z, Zhang J, David KK, Yang ZJ, Li X, Dawson TM, Dawson VL, Koehler RC. Endonuclease G does not play an obligatory role in poly(ADP-ribose) polymerase-dependent cell death after transient focal cerebral ischemia. Am J Physiol Regul Integr Comp Physiol 299: R215–R221, 2010. First published April 28, 2010; doi:10.1152/ajpregu.00747.2009.—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 neurological 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.

apoptosis; DR2313; mouse; middle cerebral artery

DNA repair is essential for maintaining genomic integrity. Endonucleases can participate in DNA repair. For example, the apurinic/pyrimidinid 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.

Our laboratory (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 N-methyl-D-aspartate 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 sexes.

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 treated with 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 transloca-

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tion 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 facemask 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.

MCAO. 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 ~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 neurological 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 neurological 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-tetrahydrothiopyran-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, 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 wt 5 min before the onset of MCAO and again 5 min before reperfusion (85 min of MCAO); continuous infusion of 1 ml·kg−1·h−1 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 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 was rapidly harvested 24 h after sham surgery or transient focal ischemia. Genomic DNA was isolated from ipsilateral hemisphere (approximately +2.2 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 milliabsorbance units/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 transillumination.

Statistical analysis. Infarct volume, LDF, distance to the line of anastomoses, and diameter of the posterior communicating arteries were presented as means ± 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
Female WT and EndoG 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 mice. Histones and MnSOD served as nuclear and mitochondrial protein markers, respectively.

**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, neurological 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, and 3 for 25th, 50th, and 75th percentile, respectively) and EndoG mice (2.6, 3, and 3, respectively). 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, and 2 for 25th, 50th, and 75th percentile, respectively) and EndoG mice (2, 3, and 3, respectively). 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 mitochondrial fraction 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 with 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 with saline infusion in either genotype (Fig. 6). However, by 3 days of reperfusion, NDS was significantly less in both WT and EndoG mice than in saline-infused mice (Fig. 7).

**Fig. 4.** Western immunoblots for poly(ADP-ribose) polymerase-1 (PARP-1), apoptosis-inducing factor (AIF), histones, and manganese superoxide dismutase (MnSOD) on nuclear and mitochondrial fractions from brains 1 day after sham surgery or 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 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 mice. Lane 1 shows molecular weight markers (kilobase pairs). The pattern of DNA fragments after MCAO was similar in WT and EndoG tissue.

**Fig. 6.** Laser-Doppler flow over lateral cortex (means ± SD), expressed as a percentage of preischemic baseline, at 5 and 10 min of MCAO in 8 male WT mice infused with saline, 8 male WT mice infused with (2-methyl-3,5,7,8-tetrahydrothiopyrano[4,3-\textit{d}]pyrimidine-4-one) (DR2313), 6 male EndoG mice infused with saline, and 5 male EndoG mice infused with DR2313. No significant differences existed between saline and DR2313 groups.

**Fig. 7.** Neurological 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 mice treated with saline (n = 6) or DR2313 (n = 5). Box plots present medians and 25% and 75% percentiles. *P < 0.05 vs. saline treatment.
receiving DR2313 compared with the corresponding WT and EndoG<sup>−/−</sup> 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<sup>−/−</sup> mice treated with DR2313 than in EndoG<sup>−/−</sup> mice treated with saline (Fig. 8D). The decreases in infarct volume were significant in each of the midcoronal sections, where the infarct was most prominent in WT (Fig. 8A) and EndoG<sup>−/−</sup> mice (Fig. 8C). DR2313-induced reduction in infarct volume was uniform between WT and EndoG<sup>−/−</sup> 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<sup>−/−</sup> 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 (11, 17, 33). Moreover, knockdown of EndoG by short interfering RNA 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 infarct 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 EndoG 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 intracranial 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 N-methyl-D-aspartate-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 decreases 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 one-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 overactivation 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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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


