Changes in Expression of the DNA Repair Protein Complex DNA-Dependent Protein Kinase after Ischemia and Reperfusion

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Reperfusion of ischemic tissue causes an immediate increase in DNA damage, including base lesions and strand breaks. Damage is reversible in surviving regions indicating that repair mechanisms are operable. DNA strand breaks are repaired by nonhomologous end joining in mammalian cells. This process requires DNA-dependent protein kinase (DNA-PK), composed of heterodimeric Ku antigen and a 460,000 Da catalytic subunit (DNA-PKcs). In this study, a rabbit spinal cord model of reversible ischemia was used to demonstrate the effect of acute CNS injury on the activity and expression of DNA-dependent protein kinase. The DNA-binding activity of Ku antigen, analyzed by an electrophoretic mobility shift assay, increased during reperfusion after a short ischemic insult (15 min of occlusion), from which the animals recover neurological function. After severe ischemic injury (60 min of occlusion) and reperfusion that results in permanent paraplegia, Ku DNA binding was reduced. Protein levels of the DNA-PK components—Ku70, Ku80, and

DNA-PKcs—were monitored by immunoblotting. After 60 min of occlusion, the amount of DNA-PKcs and the enzyme poly(ADP-ribose) polymerase (PARP) decreased with the same time course during reperfusion. Concurrently 150 and 120 kDa fragments were immunostained by an anti-DNA-PKcs monoclonal antibody. This antibody was shown to cross-react with α -fodrin breakdown products. The 120 kDa fodrin peptide is associated with caspase-3 activation during apoptosis. Both DNA-PKcs and PARP are also substrates for caspase-3-like activities. The results are consistent with a model in which after a short ischemic insult, DNA repair proteins such as DNA-PK are activated. After severe ischemic injury, DNA damage overwhelms repair capabilities, and cell death programs are initiated.

Key words: DNA damage; DNA repair; DNA-dependent protein kinase; Ku antigen; stroke; ischemia; reperfusion; spinal cord; fodrin

CNS injury from ischemia and reperfusion is proposed to occur via multiple interrelated mechanisms including excessive extracellular accumulation of the excitotoxin glutamate, an increase in intracellular Ca^{2+} , and oxidative stress. These processes contribute to the generation of reactive oxygen species that damage protein, lipid, and DNA (Chan, 1996; Simonian and Coyle, 1996). Depending on the severity of the cellular damage, the cell may activate repair or protective mechanisms or undergo cell death by necrosis or apoptosis.

DNA damage has been shown to occur early during reperfusion of ischemic tissue before the detection of internucleosomal fragmentation characteristic of programmed cell death. DNA lesions in nuclear genes were reported to increase within 10 min of reperfusion after 30 min of ischemia in a mouse forebrain model but decreased by 6 hr, suggesting that DNA excision repair mechanisms had reversed the damage (Liu et al., 1996). Reactive oxygen species also cause DNA strand breaks (Simonian and Coyle, 1996). Evidence of a reversible increase in single-strand DNA breaks after reperfusion of ischemic tissue has been reported in gerbil (Tobita et al., 1995) and rat (Chen et al., 1997) models of transient cerebral ischemia. Cells with single-strand

breaks are observed immediately after recirculation and increase over 3 d in the ischemic core but decrease after 30 min of reperfusion in the penumbral region (Chen et al., 1997). This suggests that cells in the surviving region are able to repair DNA damage.

In mammalian cells, DNA double-strand breaks are repaired by nonhomologous end joining. This process requires DNA-dependent protein kinase (DNA-PK), composed of the Ku autoantigen and a ~460,000 Da catalytic subunit (DNA-PKcs). Ku is formed by two proteins of ~70,000 Da (Ku70) and 80,000 Da (Ku80 or Ku86) (Anderson and Lees-Miller, 1992; Anderson and Carter, 1996) and was identified as an autoantigen in human patients with certain autoimmune diseases (Mimori et al., 1981). Ku heterodimer binds to free ends of double-stranded DNA and to single-strand nicks and gaps. DNA-PKcs is a serine/threonine kinase with homology to the phosphoinositol-3-phosphate kinase family (Hartley et al., 1995). The importance of DNA-PK in DNA repair and recombination in mammalian cells has been established by a series of somatic cell mutants and animals that are defective in DNA repair and V(D)J recombination, a process by which B or T cells rearrange noncontiguous segments of genomic DNA to form functional immunoglobulin or T-cell receptor genes, respectively (Jackson and Jeggo, 1995; McConnell and Dynan, 1996; Lieber et al., 1997).

In investigating the effect of ischemia and reperfusion on nuclear factor (NF)- κ B activation in the rabbit spinal cord ischemia model, we noted a significant change in the DNA-binding activity of an unrelated complex in gel retardation assays that was identified as the Ku autoantigen (Zhang et al., 1998). Therefore

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we sought to determine how ischemia and reperfusion affected the components of DNA-dependent protein kinase. The DNA-binding activity of Ku antigen was analyzed after various times of ischemia and reperfusion. The protein levels of Ku70, Ku80, and DNA-PKcs were monitored by immunoblotting. The results suggest that after a short ischemic insult, from which the animals recover neurological function, Ku DNA binding increases and DNA repair proteins may be activated. After severe ischemic injury that results in permanent paraplegia, Ku DNA binding is reduced, and activation of proteases leads to degradation of the DNA-PKcs and other proteins.

MATERIALS AND METHODS

Reversible rabbit spinal cord ischemia model. Spinal cord ischemia was produced by occluding the aorta for up to 60 min with a snare ligature just caudal to the renal arteries in fully awake New Zealand White rabbits as described previously (Zivin et al., 1982). Paraplegia was evident within 2 min after the onset of occlusion. In control rabbits, a snare ligature was positioned around the aorta but was not clamped. After the ischemic period, the ligature was removed, and the animals were allowed to recover for 4 or 18 hr before being killed with BEUTHENASIA-D (Schering-Plough Animal Health Corporation, Kenilworth, NJ). All animal use procedures are in accordance with the *NIH Guide for Care and Use of Laboratory Animals* and are approved by the Animal Care Committee of the San Diego Veterans Administration Medical Center.

The spinal column from the costovertebral junction to the sacrum was removed en bloc, and the spinal cord was quickly pushed out, rapidly frozen, and stored at -70°C . In occluded rabbits, the segment from the lumbar enlargement to the caudal tip is ischemic, and the upper lumbar cord is normally perfused (Zivin and DeGirolami, 1986). The lower lumbar tissue from nonoccluded rabbits is not ischemic and is used as control tissue.

Preparation of nuclear and cytosolic extracts. Nuclear and cytosolic extracts from rabbit spinal cord were prepared as described by Dignam et al. (1983) with modifications (Zhang et al., 1998). Briefly, spinal cord segments (0.1–0.2 gm) were homogenized on ice in four volumes of homogenization buffer (20 mM HEPES, pH 7.9, 0.25 M sucrose, 15 mM KCl, 5 mM EDTA, 1 mM EGTA, 50 mM β -glycerophosphate, 0.5 mM dithiothreitol, and protease inhibitor cocktail). The final concentration of protease inhibitors used in the buffers was 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM benzamide, 50 $\mu\text{g}/\text{ml}$ leupeptin, 25 $\mu\text{g}/\text{ml}$ pepstatin A, and 50 $\mu\text{g}/\text{ml}$ aprotinin. Homogenates were centrifuged at $5000 \times g$ for 15 min at 4°C . The supernatant was the crude cytosolic fraction. The pellet was resuspended in one-half the packed volume of low-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl_2 , 20 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitor cocktail). An equal volume of high-salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM MgCl_2 , 1.5 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitor cocktail) was added, and nuclei were extracted for 30 min at 4°C with continuous gentle mixing. The extracted nuclear proteins were centrifuged at $48,000 \times g$ for 30 min at 4°C , and the supernatant was dialyzed against 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.2 mM PMSF. The dialyzed nuclear extract was again centrifuged at $48,000 \times g$ for 30 min at 4°C and frozen at -70°C . Crude cytosolic fractions were mixed with 1/10th volume of cytosolic extraction buffer (0.3 M HEPES, pH 7.9, 1.4 M KCl, and 0.03 M MgCl_2) and centrifuged at $100,000 \times g$ for 1 hr at 4°C , and the supernatants were dialyzed. Protein concentrations of the final nuclear and cytosolic fractions were determined by the BCA protein assay (Pierce, Rockford, IL) using bovine γ -globulin as the standard protein.

For one set of experiments, four spinal cord segments (0.5 cm each), ~2–3 cm apart, spanning the caudal-to-rostral lumbar regions were excised from each rabbit subjected to 0, 15, or 60 min of occlusion and 18 hr of reperfusion. Segment 1 was excised from the lumbar enlargement. Segments 2, 3, and 4 were taken at ~2–3 cm intervals rostral to the previous segment. Nuclear extracts were prepared as described above.

Preparation of total soluble extracts. Spinal cord segments from the lumbar enlargement were homogenized (0.1 gm/ml) in buffer (0.32 M

sucrose, 5 mM HEPES, pH 8, 5 mM benzamide, 2 mM 2-mercaptoethanol, 3 mM EGTA, 0.5 mM MgSO_4 , 5 mM potassium fluoride, 20 mM sodium pyrophosphate, 25 mM β -glycerophosphate, 0.1 mM Na_2VO_4 , 0.1 mM PMSF, 50 $\mu\text{g}/\text{ml}$ leupeptin, 25 $\mu\text{g}/\text{ml}$ pepstatin A, and 50 $\mu\text{g}/\text{ml}$ aprotinin). Homogenates were centrifuged for 1 hr at $100,000 \times g$ at 4°C to separate the particulate ($100,000 \times g$ pellet) from the soluble (supernatant) fraction. Protein concentrations were determined as described (Lowry et al., 1951) with bovine γ -globulin as the standard protein.

Electrophoretic mobility shift assay. Double-stranded oligonucleotide (5'-AGTTGAGGGGACTTCCAGGC-3') containing the NF- κ B-binding motif from the mouse κ light chain enhancer (Promega, Madison, WI) was end-labeled using [γ - ^{32}P]ATP (6000 Ci/mmol; Dupont NEN, Boston, MA) and T4 polynucleotide kinase and was purified by centrifugation through a size-exclusion column.

DNA binding was performed in 20 μl reactions containing 25 mM Tris-Cl, pH 7.5, 50 mM NaCl, 3 mM MgCl_2 , 1 mM dithiothreitol, 0.01% Nonidet P-40 (NP-40), 2 mM EDTA, 5% glycerol, 50 $\mu\text{g}/\text{ml}$ bovine serum albumin, 1 μg of double-stranded poly(dI-dC) (Pharmacia, Piscataway, NJ), and 4×10^5 cpm (Cerenkov) of labeled probe. Six micrograms of either nuclear or cytosolic extract were added to the binding mix and incubated at room temperature for 30 min. The reaction mixtures were resolved by electrophoresis through 6% native polyacrylamide gels in buffer containing 50 mM Tris, 0.38 M glycine, and 2 mM EDTA, pH 8.5. Gels were dried and subjected to autoradiography with an intensifying screen for 8–24 hr. For supershift assays, 6 μg of nuclear or cytosolic extract was incubated on ice for 60 min with 0.6 μg of a goat anti-Ku80 or goat anti-Ku70 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or with 3 μg of a monoclonal anti-Ku80 antibody (Sigma, St. Louis, MO) before addition of the DNA-binding mix and incubation for 30 min at room temperature.

Immunoblotting. Proteins in the nuclear, cytosolic, or total soluble fractions were separated by SDS-PAGE on 8.5% acrylamide and 0.17% bisacrylamide gels (Laemmli, 1970) and transferred to Immobilon-P (Millipore, Bedford, MA) using a semidry graphite electroblotter. The gel and Immobilon-P membrane were sandwiched between filter papers soaked in anode buffer 1 (0.3 M Tris-Cl, pH 10.4, and 10% methanol), anode buffer 2 (25 mM Tris-Cl, pH 10.4, and 10% methanol), and cathode buffer (25 mM Tris-Cl, 40 mM glycine, pH 9.4, and 10% methanol). A typical transfer of a 15×15 cm gel was performed for 1.5 hr each at 200 and 300 mA.

The blots were blocked for ~18 hr at 4°C in 10% nonfat dry milk in Tris-buffered saline (0.14 M NaCl and 10 mM Tris-HCl, pH 7.4) with 0.1% Tween 20 (TBST) and incubated with antibody at room temperature for 1 hr. The blots were washed with TBST, incubated for 1 hr with goat anti-rabbit IgG (1:500,000; Life Technologies, Gaithersburg, MD) or rabbit anti-mouse IgG (1:10,000; Zymed, San Francisco, CA) coupled to horseradish peroxidase, and then washed again with TBST. The bound antibody was detected by enhanced chemiluminescence (SuperSignal Ultra; Pierce).

The mouse anti-DNA-PK (p350) monoclonal (PharMingen, San Diego, CA) recognizes the catalytic subunit and was used at a dilution of 1:500. Rabbit anti-Ku70 (1:3000) and anti-Ku80 (1:3000) were purchased from Serotec (Indianapolis, IN). Monoclonal anti-poly(ADP-ribose) polymerase (anti-PARP; C-2-10) antibody (1:1000) was obtained from Calbiochem (La Jolla, CA), and monoclonal anti- α -fodrin (nonerythroid spectrin; MAB1622; 1:10,000) was from Chemicon (Temecula, CA). Molecular weights were estimated using prestained SDS electrophoresis molecular weight markers from Bio-Rad (Hercules, CA).

DNA-cellulose binding. Spinal cord or cell extracts were incubated with a 60 μl packed volume of preswollen double-stranded DNA (dsDNA)-cellulose (Sigma) for 4 hr on ice in binding buffer (25 mM HEPES-KOH, pH 7.9, 50 mM KCl, 10 mM MgCl_2 , 20% glycerol, 0.1% NP-40, and 1 mM dithiothreitol) (Finnie et al., 1995). The unbound fraction was removed by centrifugation, and the dsDNA-cellulose was washed three times with binding buffer. The bound proteins were eluted with Laemmli gel sample buffer and analyzed by SDS-PAGE and immunoblotting as described above.

Data analysis. All radioactive gels were quantified using a Molecular Dynamics PhosphorImager and ImageQuant software (Sunnyvale, CA). Data were analyzed by ANOVA, and tests of the statistical significance of differences among cell means were found using the Newman-Keuls multiple comparisons method.

Figure 1. Identification of Ku antigen–DNA complex. Equal amounts (6 μ g) of nuclear or cytosolic extracts were incubated with 0.6 μ g of a goat (g) anti-Ku70 or anti-Ku80 antibody or 3 μ g of a monoclonal (m) anti-Ku80 antibody as indicated before the binding reaction and were analyzed by EMSA. The nuclear and cytosolic fractions of extracts from a rabbit subjected to 15 min of ischemia and 18 hr of reperfusion contain Ku antigen in complex III. The analogous fractions from a rabbit subjected to 60 min of ischemia and 18 hr of reperfusion contain reduced amounts of complex III and supershifted complex. *Nuclear, Cytosol*, The left lane contains free probe. Complexes supershifted by Ku antibodies are denoted by an asterisk. The retarded band labeled O represents variable nonspecific binding. Most of the uncomplexed oligonucleotide probe was electrophoresed off the bottom of the gel. *Ab*, Antibody.

RESULTS

Identification of the Ku antigen as an NF- κ B-binding complex

Nuclear and cytosolic extracts from the low-lumbar region of rabbit spinal cords were analyzed for DNA-binding activity to an oligonucleotide containing the NF- κ B motif using an electrophoretic mobility shift assay (EMSA). Three major DNA–protein complexes were observed (Fig. 1). Complex I was competed by an excess of unlabeled probe and was determined to contain NF- κ B subunits RelA(p65) and p50 by supershift assays with specific antibodies (Zhang et al., 1998). Complex II binding was not sequence specific and was detected using either the wild-type probe or a mutant probe containing a single-base substitution that abolishes NF- κ B binding (Zhang et al., 1998).

Complex III showed a preference for binding to the wild-type versus the mutant oligonucleotide but was not supershifted by antibodies to NF- κ B subunits. Numerous unidentified DNA–protein complexes have been detected using the EMSA. A complex that has been identified repeatedly as a novel transcription factor is formed by Ku autoantigen, which binds to the free ends of double-stranded DNA independent of sequence (Anderson and Carter, 1996; Klug, 1997). Therefore antibodies to the subunits Ku70 and Ku80 were used to determine whether Ku contributed to one of the DNA–protein complexes. As shown in Figure 1, a monoclonal and a polyclonal antibody to Ku80 completely supershifted complex III, and a goat anti-Ku70 antibody partly shifted the complex.

Complex III containing Ku antigen was observed in both nuclear and cytosolic extracts of rabbit spinal cord (Fig. 1). In analyzing the effect of ischemia and reperfusion on NF- κ B bind-

Figure 3. Effect of ischemia and reperfusion on Ku antigen DNA-binding activity in spinal cord cytosolic extracts. Rabbits were subjected to 0, 15, or 60 min of ischemia as indicated and reperused for 4 hr (*A*) or 18 hr (*B*). Equal amounts (6 μ g) of cytosolic extracts from the low-lumbar spinal cord were analyzed for DNA-binding activity by EMSA. Each lane contains an extract from an individual animal; lane 1 is on the left. Complex III containing Ku antigen is reduced in samples from animals occluded for 60 min.

also observed in the nuclear fractions of animals subjected to 60 min of ischemia and 18 hr of reperfusion that differed significantly from that of those animals subjected to 15 min of ischemia and 4 hr ($p < 0.05$) or 18 hr ($p < 0.001$) of reperfusion. In contrast, animals occluded for 15 min displayed a 1.41-fold increase in DNA binding in the nuclear fractions after 18 hr of reperfusion and, at the same time, a 23% decrease in complex III in the cytosols.

The reduction in DNA binding of the Ku antigen after ischemia and reperfusion was limited to the lower lumbar region. In Figure 5 nuclear extracts were prepared from sections along the

spinal cord of three animals and analyzed by EMSA. Section 1 was from the lumbar enlargement, and sections 2, 3, and 4 were ~2, 4, and 6 cm, respectively, rostral to section 1. Sections 1 and 2 are from an area of the cord subjected to reduced blood flow during occlusion, whereas section 4 should have normal blood flow. Section 3 is a transition area that might have reduced flow. Figure 5 shows that the Ku–DNA complex III was greatly reduced in sections 1–3, but not in section 4, after 60 min of ischemia and 18 hr of reperfusion. In contrast, the amount of complex III was increased in one animal occluded for 15 min. The lower amount of complex III in section 1 of the control rabbit was not observed in other rabbits. A separate set of animals analyzed as in Figure 5 showed similar changes in the Ku–DNA complex after ischemia and reperfusion (data not shown).

It has been shown that the order in which components are added in the EMSA assay affects the amount of Ku antigen detected (Klug, 1997). In Figures 1–3 and 5, the EMSA was performed by adding a cocktail containing poly(dI–dC) and labeled probe to the nuclear or cytosolic extract. Figure 6 demonstrates the difference in the amount of complex III detected when poly(dI–dC) is added before (*C*) or after (*B*) addition of the labeled oligonucleotide. Preincubation of the spinal cord cytosolic or nuclear extract with poly(dI–dC) reduces the amount of Ku antigen observed, whereas adding it after the probe greatly increases Ku binding. The relative difference in Ku DNA binding between samples, however, was still preserved. For example, the amount of complex III in animals occluded for 15 min and reperused for 4 hr ($n = 5$) was $1.14 (\pm 0.26)$ relative to that in the controls ($n = 4$) when poly(dI–dC) was added last compared with $0.93 (\pm 0.37)$ when assayed as in Figures 1–3. The corresponding values for animals occluded for 60 min and reperused for 4 hr ($n = 6$) were $0.34 (\pm 0.40)$ and $0.35 (\pm 0.41)$, respectively.

Effect of ischemia and reperfusion on protein levels of Ku antigen

Mechanisms of regulating Ku antigen binding to DNA have not been described. The reciprocal change in the nuclear and cytosolic fractions of animals occluded for 15 min suggests a possible nuclear translocation of Ku. The protein levels of Ku70 and Ku80 were analyzed by immunoblotting to determine whether they were altered by injury. Detection of the Ku subunits in the rabbit samples was difficult compared with detection in human tissue because of the 50-fold lower expression of Ku in rodents and rabbits compared with primates (Anderson and Lees-Miller, 1992; Finnie et al., 1995) and also because of the reported poor antibody cross-reactivity between species (Porges et al., 1990; Wang et al., 1993). Nevertheless, a band comigrating with human Ku80 was detected in the rabbit spinal cord nuclear and cytosolic samples. The anti-Ku70 antibody detected two bands in the rabbit, whereas a single major band was observed in human HeLa cell extracts. To determine whether both bands were Ku70, we bound cytosolic extracts to DNA-cellulose and compared them with human and rat cell extracts (Fig. 7). Ku70 from the rat cell line pheochromocytoma 12 (PC12) migrated slower than did the human form (Fig. 7, lane 7 vs lane 8). The lower M_r band of the doublet from rabbit samples was preferentially bound to DNA-cellulose, indicating that this band was Ku70 (Fig. 7, lanes 5, 6). Ku80 was also bound to DNA-cellulose as shown by sequential immunostaining of the blot in Figure 7 (data not shown).

Cytosolic samples from animals reperused for 18 hr were

Figure 4. Quantification of the Ku antigen–DNA complex. Nuclear and cytosolic extracts were prepared from the low-lumbar spinal cord of rabbits subjected to 0, 15, or 60 min of ischemia and 4 or 18 hr of reperfusion and assayed by EMSA as shown in Figures 2 and 3. The amount of Ku-bound DNA in complex III was quantified using a Molecular Dynamics PhosphorImager, and the values were normalized to that of control animals. Data represent the mean $\pm$ SD values. Statistical differences among means were determined using the Newman–Keuls multiple comparisons method. The n values for the experimental conditions are the following: for 0 min, $n = 4$; for 15 min/4 hr, $n = 5$; for 60 min/4 hr, $n = 6$; for 15 min/18 hr, $n = 6$; and for 60 min/18 hr, $n = 6$. In cytosolic extracts, the amount of Ku–DNA complex in animals occluded for 60 min was significantly decreased compared with that in the controls and with the amount in animals occluded for 15 min. In nuclear extracts, the amount of Ku–DNA complex in animals subjected to 15 min of ischemia and 18 hr of reperfusion was significantly increased compared with the amount in animals occluded for 60 min. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

immunoblotted (Fig. 8*A*). Animals subjected to 60 min of ischemia had a decreased amount of Ku70 and Ku80 protein in five out of the six animals. This correlated with the five samples that showed reduced Ku DNA binding by EMSA. In general, the lower band of the Ku70-immunoreactive doublet decreased more than did the upper band. Animals occluded for 60 min and reperused for 4 hr also had a reduced amount of Ku80 protein compared with that in the controls or in animals occluded for 15 min (Fig. 8*B*). The levels of Ku protein and DNA binding, however, did not correlate as well in cytosolic samples from animals reperused for 4 hr as in those reperused for 18 hr. For example, the samples in *lanes 11* and *12* of Figure 8*B* show a comparable decrease in Ku80 but a 7.5-fold difference in DNA binding (Fig. 3*A*, *lanes 10*, *11*). These results suggest that modification of the Ku70/Ku80 dimer or other interacting factors may regulate binding.

The Ku heterodimer binds to DNA strand breaks and appears to stabilize binding of the DNA-PK catalytic subunit to DNA. To determine the fate of the DNA-PK catalytic subunit after ischemia and reperfusion, we immunoblotted samples (Fig. 8*B*) with a monoclonal antibody to DNA-PKcs. DNA-PKcs was routinely detected in samples from human cell lines using polyclonal or monoclonal antibodies, but the rabbit or rat homolog was not consistently detected. This is probably caused by the lower expression of DNA-PKcs in rodents and rabbits compared with primates as noted for Ku. The immunoreactive band of $M_r \sim 250,000$ has been observed previously in rat cell lines and was reported to be unrelated to DNA-PKcs (Casciola-Rosen et al., 1995; Peterson et al., 1997). In Figure 8*B*, the intact DNA-PKcs was barely detectable in some of the rabbit samples. A doublet of M_r 150,000, however, was observed in the samples from rabbits subjected to 60 min of ischemia and 4 hr of reperfusion. It was shown previously that cleavage of DNA-PKcs by caspase-3 generates several large fragments (Ajmani et al., 1995; Casciola-Rosen et al., 1995; Han et al., 1996; Song et al., 1996; Teraoka et al., 1996; McConnell et al., 1997).

Comparison of Ku, DNA-PKcs, PARP, and fodrin protein expression after ischemia and reperfusion: evidence of caspase-3 activation

Total soluble fractions from low-lumbar spinal cord homogenates were used to compare the expression of Ku antigen and DNA-PKcs after 0, 15, or 60 min of ischemia and 18 hr of reperfusion. The blot was also used to monitor the DNA damage-activated enzyme PARP, another well characterized substrate for caspase-3-like activity. Figure 9, *panel 1*, shows that intact DNA-PKcs was detectable in the spinal cord samples of control rabbits and those occluded for 15 min. Expression was lost in those occluded for 60 min and reperused for 18 hr, and concurrently, a 150 kDa fragment and a faint 120 kDa band were observed. The expression of the Ku70 and Ku80 subunits was also reduced in the spinal cords of rabbits occluded for 60 min, although the decrease in protein levels was not as dramatic as the loss of Ku antigen DNA-binding activity detected by EMSA (Fig. 9, *panels 3*, *4*). A decrease in the amount of PARP protein was noted in the samples from the 60 min-occluded animals (Fig. 9, *panel 2*). The characteristic 85 kDa caspase-3-generated fragment of PARP (Nicholson et al., 1995; Tewari et al., 1995) was not readily detected.

The molecular weights of the 250 kDa band and the 150 kDa doublet immunostained by the anti-DNA-PKcs monoclonal antibody were similar to those expected for α -fodrin and its major proteolytic breakdown product (Harris and Morrow, 1988; Seubert et al., 1989; Saido et al., 1993). To address the possibility of cross-reaction, a blot containing the same samples analyzed in Figure 9, *panels 1–4*, was immunoblotted with a monoclonal antibody to α -fodrin (*panel 5*). Figure 9 demonstrates that the fodrin antibody recognized an ~ 250 kDa protein and a small amount of the 150 kDa doublet in control samples and those from rabbits subjected to 15 min of ischemia and 18 hr of reperfusion. In animals occluded for 60 min and reperused, a large increase in the 150 kDa doublet was observed as well as the appearance of an additional 120 kDa fragment, although only a slight reduction in the amount of intact 250 kDa fodrin was noted. Cleavage of

Figure 5. Changes in the DNA binding of Ku antigen in the lumbar spinal cord after reperfusion are confined to ischemic regions. Nuclear extracts were prepared from four segments spanning the caudal-to-rostral lumbar spinal cord of rabbits subjected to 0, 15, or 60 min of ischemia and 18 hr of reperfusion as indicated. Aliquots (6 μ g) of the nuclear extracts were analyzed by EMSA. *Section 1* was from the lumbar enlargement, and *sections 2, 3, and 4* were $\sim$ 2, 4, and 6 cm, respectively, rostral to section 1. *Sections 1 and 2* are from an area of the cord subjected to reduced blood flow during occlusion, whereas *section 4* should have normal blood flow. *Section 3* is a transition area that might have reduced flow. *Lanes* are labeled with *section numbers*. Conventions are as described in Figure 1.

α -fodrin at a hypersensitive site in the middle of the molecule by numerous proteases, including calpain I and caspases, generates a 150 kDa C-terminal fragment (Harris and Morrow, 1988; Martin et al., 1995; Wang et al., 1998). Proteolysis by caspase-3-like activity only, however, further processes the peptide to 120 kDa (Cryns et al., 1996; Nath et al., 1996; Jänicke et al., 1998). It has been shown that the antibody used in this study recognizes both of these fragments (Martin et al., 1995; Cryns et al., 1996; Jänicke et al., 1998; Wang et al., 1998). Thus besides recognizing the 460 kDa DNA-PKcs protein, the monoclonal anti-DNA-PKcs used in this study appears to cross-react with fodrin and its breakdown products.

In Figure 10 the amount of total soluble extract analyzed was increased twofold compared with that used in Figure 9 to improve detection of PARP. Samples from rabbits subjected to 60 min of ischemia and reperfusion displayed a reduced amount of intact 110 kDa PARP protein and a faint band of $\sim$ 85 kDa. The 85 kDa PARP fragment was readily observed in the human neuroblastoma cell line SH-SY5Y induced to undergo apoptosis (Fig. 10, lanes 1, 5). It was found, however, that mixing a spinal cord extract with the apoptotic human cell extract obliterated most of the signal from the 85 kDa fragment but not from intact PARP (lane 2). This was not caused by additional proteolysis because boiling the extracts before or after mixing gave the same result. The interference in immunoblotting was probably caused by migration of an abundant protein detectable by protein staining at

the molecular weight of the PARP fragment (data not shown). Detection of Ku80, which migrated just below 85 kDa, was not affected (data not shown).

The same blot was probed with the anti- α -fodrin antibody to monitor the time course of fodrin proteolysis (Fig. 10, bottom). An increase in the 150 kDa breakdown products was observed by 4 hr of reperfusion in animals occluded for 60 min. This was expected because the anti-DNA-PKcs antibody recognized similar bands at that time point (Fig. 8B). The anti-fodrin antibody in addition reacted with a 120 kDa band in the ischemic samples that increased in amount from 4 to 18 hr of reperfusion.

DISCUSSION

Several recent studies demonstrated that reperfusion after ischemic injury leads to DNA damage consistent with that mediated by reactive oxygen species. Single-strand DNA breaks are generated immediately after reperfusion and precede the internucleosomal cleavage characteristic of later stages of programmed cell death (Tobita et al., 1995; Chen et al., 1997). DNA repair of double-strand breaks in mammalian cells is accomplished by nonhomologous end joining. Four essential components of this system have been defined genetically, including the p80 and p70 subunits of Ku antigen, DNA-dependent protein kinase catalytic subunit, and a protein that interacts with DNA ligase IV. The present study demonstrates that ischemia and reperfusion in the rabbit spinal cord ischemia model (RSCIM) affect three of these components, Ku antigen and the catalytic subunit that form the trimeric DNA-PK complex. Durations of ischemia that lead to permanent paraplegia were associated with decreased DNA-binding activity of Ku antigen and loss of DNA-PKcs. In contrast, a short ischemic insult, from which the animals recover neurological function after reperfusion, led to increased Ku DNA-binding activity and no loss of DNA-PKcs.

Ku heterodimer in the absence of the DNA-PK catalytic subunit binds to double- and single-stranded oligodeoxynucleotides, single-stranded nicks and gaps in DNA, and single- to double-

Figure 6. EMSA assay conditions affect Ku antigen DNA binding. Spinal cord cytosolic extracts from rabbits subjected to 15 min (lane 1) or 60 min (lane 2) of ischemia and 18 hr of reperfusion were compared. *A*, EMSA components, including poly(dI-dC) and labeled oligonucleotide probe, were added as a cocktail to the aliquot (6 μ g) of cytosolic extract. *B*, Poly(dI-dC) was added after the other EMSA components were mixed with the extract. *C*, The extracts were incubated with poly(dI-dC) before the addition of the other EMSA components including the labeled probe.

Figure 7. Identification of Ku70 protein by binding to DNA-cellulose. Aliquots of cytosolic extracts from lumbar spinal cord or lysates of PC12 or HeLa cells were bound to DNA-cellulose to identify the immunoreactive Ku70 band with DNA-binding activity. Lanes 1, 2, Cytosolic extracts (30 μ g) from rabbits subjected to 15 min of ischemia and 18 hr of reperfusion; lanes 3, 4, cytosolic extracts (30 μ g) from rabbits subjected to 60 min of ischemia and 18 hr of reperfusion; lane 5, bound fraction from 60 μ g of sample shown in lane 1; lane 6, bound fraction from 60 μ g of sample shown in lane 3; lane 7, bound fraction from 60 μ g of PC12 cell lysate; lane 8, bound fraction from 7 μ g of HeLa cell lysate; lane 9, 30 μ g of PC12 cell lysate; and lane 10, 7 μ g of HeLa cell lysate. Samples were separated by SDS-PAGE and were immunoblotted with rabbit anti-Ku70 antibody with detection by enhanced chemiluminescence. The heavily stained band of $M_r \sim 50,000$ was observed in the absence of primary antibody. The identity of the immunoreactive band of $M_r \sim 65,000$ in all samples (open arrowhead) is not known, but it did not bind preferentially to DNA-cellulose.

strand transitions such as hairpin and bubble structures (Blier et al., 1993; Falzon et al., 1993). Both Ku subunits have single-stranded DNA-dependent ATPase activity, and Ku70 also functions as a helicase that may aid in binding to nicked chromatin (Cao et al., 1994; Tuteja et al., 1994; Ochem et al., 1997). Ku alone bound to DNA can promote loop formation (Cary et al., 1997) and stimulate intermolecular ligation of nonhomologous DNA fragments presumably by tethering ends (Ramsden and Gellert, 1998). Until recently it was proposed that Ku antigen was the regulatory component of DNA-PK, recruiting and anchoring the catalytic subunit to breaks in DNA. Recent studies, however, demonstrate that Ku and DNA-PKcs can bind independently to free ends of DNA (Yaneva et al., 1997; Hammarsten and Chu, 1998). DNA-PKcs is activated after binding to DNA ends, but Ku binding to the same DNA further enhances activity. This led to a model in which Ku is proposed to bind to free ends of DNA and subsequently to translocate to an internal position allowing DNA-PKcs to bind to the free ends (Lieber et al., 1997; Yaneva et al., 1997; Hammarsten and Chu, 1998). Ku stabilizes the DNA-DNA-PKcs interaction and activates the enzyme under physiological conditions, but how this achieves DNA repair has not been elucidated.

The present study demonstrated an increase in DNA binding of Ku in the nuclear fraction after 18 hr of reperfusion after occlusion for 15 min. Reperfusion after 60 min of occlusion led to the loss of Ku antigen DNA binding both in the cytosolic and nuclear fractions. Reduction in DNA binding was confined to the region of the spinal cord subjected to reduced blood flow during occlu-

sion. The magnitude of changes in DNA-binding activity did not correlate with the changes in protein levels of Ku70 and Ku80 detected by immunoblotting. The low level of Ku antigen expression in rabbits versus primates, however, makes an accurate quantification of protein levels difficult. It is estimated that expression of Ku and DNA-PKcs is 50-fold lower in rodents and rabbits than in primates (Celis et al., 1987; Anderson and Lees-Miller, 1992; Wang et al., 1993; Finnie et al., 1995). Furthermore, the immunodominant epitopes of Ku recognized by human autoantibodies and some monoclonal antibodies are nonlinear and are not conserved in rodents (Porges et al., 1990; Wen and Yaneva, 1992; Wang et al., 1993). Thus it could not be concluded in the present study whether increased DNA binding in animals subjected to 15 min of ischemia and 18 hr of reperfusion was caused by translocation of the Ku antigen from cytoplasm to nucleus. Reduction in DNA binding in animals occluded for 60 min was in part attributable to loss of the Ku protein, but conformational changes, posttranslational modifications, or alter-

Figure 8. Immunoblots of Ku subunits and DNA-PK catalytic subunit. Rabbits were subjected to 0, 15, or 60 min of ischemia and 4 or 18 hr of reperfusion as indicated. Each lane is an extract from an individual animal. **A**, Aliquots (30 μ g) of low-lumbar cytosolic extracts were sequentially immunoblotted with rabbit anti-Ku70 (bottom) followed by rabbit anti-Ku80 (top). The lower band of the doublet stained by the Ku70 antibody was identified as Ku70 by DNA-cellulose binding. The right lane is an aliquot of HeLa cell lysate. **B**, Aliquots (50 μ g) of the low-lumbar cytosolic extracts were sequentially immunoblotted with monoclonal anti-DNA-PKcs (top) followed by rabbit anti-Ku80 (bottom). The left lane (lane 1) is an aliquot (18 μ g) of HeLa cell lysate. The positions of the intact DNA-PKcs and the 150 kDa fragment are indicated by filled arrows. The open arrow indicates an immunoreactive band reported previously to be unrelated to DNA-PKcs. The positions of the prestained molecular weight markers are indicated on the right without arrowheads. Detection of antibody on the blots was by enhanced chemiluminescence.

Figure 9. Expression of DNA-PKcs, Ku subunits, PARP, and fodrin after ischemia and reperfusion. Rabbits were subjected to 0, 15, or 60 min of ischemia and 18 hr of reperfusion. Total soluble protein extracts of low-lumbar spinal cord were prepared as described in Materials and Methods. *Top, Panels 1–4*, Equal amounts of extracts (50 μ g) from individual animals were separated by SDS-PAGE, immunoblotted sequentially with monoclonal anti-DNA-PK catalytic subunit, monoclonal anti-PARP, rabbit anti-Ku70, and rabbit anti-Ku80 antibody, and detected using enhanced chemiluminescence. *Bottom, Panel 5*, A new blot of the same extracts (50 μ g) was prepared and immunoblotted with a monoclonal anti- α -fodrin antibody. The *left lane* in all panels is an aliquot (15 μ g) of HeLa cell lysate. The *band* migrating at ~82 kDa in two of the four right lanes in the Ku70 panel was observed when the primary antibody was omitted. Animals subjected to 60 min of ischemia and 18 hr of reperfusion showed reduced expression of DNA-PKcs, Ku70, Ku80, and PARP. The *bottom panel* shows that the 250 kDa protein (*open arrow* in the top panel) and the injury-induced fragments of 150 and 120 kDa recognized by the anti-DNA-PKcs antibody are identical to fodrin and its proteolytic products. The positions of prestained molecular weight markers are indicated on the left.

ations in the association of Ku with other proteins may also contribute.

It is not known whether DNA-binding activity of the Ku antigen is regulated. Various studies indicate that posttranslational modifications and conformational changes do occur. The

ratio of Ku80 charge variants identified in two-dimensional gels (Celis et al., 1987; Stuiver et al., 1991) differs in quiescent versus proliferating cells. Ku subunits are substrates for DNA-PKcs, but phosphorylation has not been demonstrated to affect heterodimerization or DNA binding (Jin and Weaver, 1997), al-

Figure 10. Time course of PARP and fodrin proteolysis during reperfusion of ischemic spinal cord. Total soluble protein extracts were prepared from control rabbits or those subjected to 60 min of ischemia and 4 or 18 hr of reperfusion. *Top*, Equal amounts of extracts (100 μ g) were separated by SDS-PAGE and immunoblotted with monoclonal anti-PARP antibody. *Bottom*, The blot was stripped and then immunoblotted with monoclonal anti- α -fodrin antibody. Detection was by enhanced chemiluminescence. *Lanes 1, 2, 4, 5*, Extracts of the human neuroblastoma cell line SH-SY5Y were analyzed to compare migration of PARP and its 85 kDa proteolytic fragment. Aliquots (40 μ g) of SH-SY5Y that were incubated with 5 mM EGTA for 24 hr to induce PARP cleavage (*lanes 1, 5*) or left untreated (*lane 4*) were analyzed. *Lane 3* contains a spinal cord extract (100 μ g) from a rabbit subjected to ischemia and reperfusion. In *lane 2*, aliquots of the spinal cord extract in *lane 3* and the treated SH-SY5Y extract in *lane 1* were mixed and analyzed to show that the spinal cord extract interfered with detection of the 85 kDa PARP fragment. A decrease in the amount of 110 kDa PARP and proteolysis of fodrin to yield 150 and 120 kDa fragments occurred within 4 hr of reperfusion in animals occluded for 60 min. In SH-SY5Y cells, EGTA treatment for 24 hr induced cleavage of PARP but not fodrin (*lane 5*). The positions of the prestained molecular weight markers are indicated on the *right*.

though it was shown to upregulate the ATPase activity of Ku (Cao et al., 1994). Activity of the DNA-PK complex is regulated during the cell cycle, but the mechanism is unknown (Lee et al., 1997). Endogenous Ku antigen isolated from cultured cells is found in both cytoplasmic and nuclear fractions by immunoblotting (Lees-Miller et al., 1995; Peterson et al., 1995; Fewell and Kuff, 1996), but human p70 or p80 expressed individually in nonprimate cells is localized immunocytochemically to the nucleus (Wang et al., 1994). There is an increase in immunoreactivity of the Ku antigen in the nuclei of cells grown at high density or after contact with other cells without affecting the cellular distribution of Ku proteins analyzed by immunoblotting (Fewell and Kuff, 1996). Because the immunodominant epitopes recognized by the antibodies used are nonlinear (Porges et al., 1990; Wen and Yaneva, 1992), this suggests that extracellular signals can illicit conformational changes or alter epitope accessibility in the Ku antigen.

In the present study, the 460 kDa catalytic subunit of DNA-PK decreased during reperfusion of animals occluded for 60 min. DNA-PKcs is cleaved in human cells exposed to agents that induce apoptosis, generating fragments of ~230–250, 150–165, and 120 kDa (Ajmani et al., 1995; Casciola-Rosen et al., 1995; Han et al., 1996; Song et al., 1996; Teraoka et al., 1996; McConnell et al., 1997). No loss of Ku antigen was noted in cells undergoing apoptosis induced by UV irradiation, anti-Fas antibody, or various chemical treatments (Casciola-Rosen et al., 1995; Han et al., 1996; Song et al., 1996; Teraoka et al., 1996; McConnell et al., 1997) but was observed in activated human peripheral blood lymphocytes that undergo apoptosis (Ajmani et al., 1995). Proteolysis of DNA-PKcs appears to be mediated by a caspase-3-like activity, because *in vitro* this enzyme generates the same array of fragments observed *in vivo*. The 150 and 120 kDa frag-

ments generated by ischemia and reperfusion in the present study and recognized by a DNA-PKcs antibody appear to be derived from α -fodrin and not DNA-PKcs. Caspase-3-generated DNA-PKcs fragments of 150–165 and 120 kDa are derived from the C-terminal half of the molecule and would not be predicted to react with the monoclonal anti-DNA-PKcs antibody used in this study, which was produced using a peptide antigen from a more N-terminal region.

It is well documented that injury from ischemia and reperfusion is associated with cleavage of α -fodrin to generate a 150 kDa C-terminal fragment (Seubert et al., 1989; Saido et al., 1993; Yokota et al., 1995). Calpain I is one enzyme responsible for cleavage at this site, but numerous proteases cleave fodrin in this hypersensitive region (Harris and Morrow, 1988). More recently it was shown that caspase-3 cleaves fodrin nine residues C-terminal to the major calpain I site and further processes the fragment to 120 kDa (Cryns et al., 1996; Nath et al., 1996; Wang et al., 1998). It was demonstrated in several models that apoptosis is accompanied by cleavage of fodrin, with a 140–150 kDa doublet appearing first followed by the 120 kDa product (Martin et al., 1995; Cryns et al., 1996; Nath et al., 1996; Wang et al., 1998). Furthermore in cells lacking caspase-3, the 120 kDa fragment is not generated in response to apoptotic stimuli (Jänicke et al., 1998; Zheng et al., 1998). Therefore the cleavage pattern of fodrin observed in this study is consistent with activation of caspase-3 activity as well as that of calpain I within 4 hr after onset of reperfusion. This agrees with other studies demonstrating caspase-3 activation in models of reversible cerebral ischemia in rat (Chen et al., 1998; Namura et al., 1998) and spinal cord ischemia in rabbits (Hayashi et al., 1998). The appearance of the 150 kDa breakdown product in samples from control animals and those occluded for 15 min is consistent with previous reports of

low levels of the 150 kDa fragment in untreated cells or tissue (Cryns et al., 1996; Nath et al., 1996).

Concurrent with the degradation of DNA-PKcs and fodrin in the RSCIM, a decrease in the amount of the enzyme PARP was observed. PARP is activated by DNA single- and double-strand breaks and adds poly(ADP-ribose) chains to nuclear proteins including itself. Automodification of PARP promotes its release from damaged DNA and allows access to repair proteins (Satoh and Lindahl, 1992). PARP is degraded during apoptosis to 85 and 24 kDa peptides (Nicholson et al., 1995; Tewari et al., 1995). Proteolysis is attributed mainly to caspase-3 activation, but PARP, as well as DNA-PKcs, is cleaved in cells lacking caspase-3 indicating that unidentified caspases with similar specificity are also activated by apoptosis (Jänicke et al., 1998). Because fodrin cleavage indicates that caspase-3 is activated in the RSCIM, it is likely that caspase-3 also contributes to proteolysis of DNA-PKcs and PARP during reperfusion after a 60 min occlusion.

Numerous proteins are *in vitro* substrates for DNA-PKcs although the critical *in vivo* substrates have not been identified. Substrates include transcription factors, hsp90, the C-terminal domain of RNA polymerase II, and proteins that respond to DNA damage (tumor suppressor p53, replication protein A, and Ku subunits) (Anderson and Lees-Miller, 1992; Anderson and Carter, 1996). In general efficient phosphorylation requires DNA-PKcs and substrate to be bound to the same DNA molecule. Of particular interest is the recent finding that DNA damage induced by ionizing radiation or DNA alkylating agents increases the association of DNA-PKcs and c-Abl (Kharbanda et al., 1997). DNA-PKcs phosphorylates and activates c-Abl. Activated c-Abl can phosphorylate DNA-PKcs, causing its dissociation from DNA and inactivation (Chan and Lees-Miller, 1996). Activation of c-Abl is associated with induction of cell death, but the signaling pathways are not defined. Another target of DNA-PKcs is p53 (Anderson and Lees-Miller, 1992). The DNA-PK complex, in concert with an unknown nuclear factor, phosphorylates and activates p53 in response to DNA damage, including that initiated by hydrogen peroxide (Woo et al., 1998). An increase in p53 expression was reported after cerebral ischemia, but increased activity was not shown directly (Chopp et al., 1992; Li et al., 1997). Activation of p53 is associated with either arrest of the cell cycle to allow repair or induction of apoptosis.

The ability of the DNA-PK complex to respond to DNA damage suggests that it is poised to control activation and assembly of other enzymes and proteins involved in repair and/or pathways leading to cell death after injury because of ischemia and reperfusion. The process of cell death induced by ischemia and reperfusion in animal models of stroke displays a continuum of characteristics of apoptosis and necrosis depending on the model and severity of insult (Héron et al., 1993; Linnik et al., 1993; MacManus et al., 1993; Tominaga et al., 1993; Li et al., 1995; Kato et al., 1997; Mackey et al., 1997; Petito et al., 1997). In other systems, it has been proposed that excessive oxidative stress-induced DNA damage initiates apoptosis. A recent study showed that noncycling neurons rejoined DNA strand breaks induced by x-irradiation at a slower rate than did astrocytes and underwent apoptotic-like cell death (Gobbel et al., 1998). Thus reperfusion after short ischemic insults may lead to limited DNA damage that activates repair by nonhomologous end joining and base excision repair. After longer durations of ischemia, extensive oxidative damage to DNA, protein, and lipid may initiate signaling cascades that abort the repair pathways by degradation of the necessary components.

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