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Involvement of Nitric Oxide-Mediated Intrinsic Pathway Signaling in Age-Related Increase in Germ Cell Apoptosis in Male Brown-Norway Rats

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We examined, using young and old Brown-Norway rats, the involvement of the nitric oxide (NO)-mediated intrinsic pathway signaling in age-related activation of male germ-cell apoptosis. Increased apoptosis of germ cells was readily observed in the normal-looking testes of old rats. Testicular NO synthase (NOS) activity, assessed by measuring the synthesis of ^{3}H-L-citrulline from ^{3}H-L-arginine, and cytokine-inducible NO synthase (iNOS) levels, assessed by western blot assay, were increased significantly by 90% and 70%, respectively, in the old rats compared to that of young animals. Immunohistochemical analysis of age-related changes in the expression of iNOS in testes confirmed our findings based on western blot assay. Increased NO and germ-cell apoptosis during aging is further associated with cytosolic translocation of mitochondrial cytochrome *c* and poly (ADP) ribose polymerase (PARP) cleavage, thus, suggesting the involvement of NO-mediated intrinsic pathway signaling in age-related increase in germ-cell apoptosis in male Brown-Norway rats.

THE signaling events leading to caspase activation and apoptosis in mammalian cells can be divided into two principal pathways involving either mitochondria (intrinsic) or death receptors (extrinsic) (1–3). Recently, we have demonstrated the involvement of the mitochondria-dependent apoptotic pathway, characterized by Bax translocation, cytochrome *c* release, activation of the initiator caspase 9 and the executioner caspases 3, 6, and 7, and poly (ADP) ribose polymerase (PARP) cleavage in heat-induced male germ-cell apoptosis (4,5). In additional studies using *gld* and *lpr*^{cg} mice, which harbor loss-of-function mutations in Fas L and Fas, respectively (6), we show that the Fas signaling system had little, if any, role in male germ-cell death triggered by mild testicular hyperthermia (7).

Apoptosis has been implicated in a mechanism of germ-cell loss associated with aging in humans as well as in animal models, including Brown-Norway (BN) rats (8–10). However, the mechanisms of increased programmed germ-cell death associated with aging remain uncertain. Nitric oxide (NO) is a rapid messenger molecule that functions at low concentrations as a signal in many diverse physiological processes but at high concentrations (through its toxic derivatives) can cause DNA damage and cell death in a variety of cell types [reviewed in (11)]. NO synthase (NOS), expressed as cytokine-inducible (iNOS), endothelial (eNOS), and neuronal (nNOS) isozymes, oxidize L-arginine to NO and citrulline. It is generally believed that constitutive eNOS and nNOS generate NO that is imperative for blood circulation and signal transmission in the nervous system, whereas uncontrolled high output of NO produced by iNOS can mediate tissue injury and cell death (11). Increased NO production after iNOS induction has been implicated in the impaired gonadotropin secretion associated with aging (12) and in NO-mediated neurotoxicity and cell death presumably through the generation of highly reactive peroxynitrite (13,14). Previously, we have provided evidence of a striking increase in the iNOS expression and NOS activity in the hypothalamus and other regions of the brain of old BN rats (15). In a separate study, we have also noted: a) that the age-related increase in iNOS expression occurs in those hypothalamic regions (such as medial preoptic area and arcuate nucleus) known to control the synthesis and release of GnRH, and b) that the increased iNOS expression is further accompanied by an increase in nitrotyrosine immunoreactivity, a marker of peroxynitrite formation, and neuronal apoptosis (16). Taken together, these results lead to the hypothesis that the increased NO synthesis, through up-regulation of iNOS, may be a key factor in inducing germ-cell apoptosis in aging. To test this hypothesis, in the present study, we analyzed germ-cell apoptosis, NOS activity, in vivo expression of iNOS and nNOS, cytosolic translocation of mitochondrial cytochrome *c*, and PARP cleavage in testes between young and old BN rats.

Methods

Animals

Six young (6 months old) and six old (24 months old) male BN rats were obtained from the National Institute on Aging (Bethesda, MD). Rats were housed in the vivarium

under controlled temperature (22°C) and photoperiod (12-hour light/dark period) with free access to food and rat chow. Animal handling and experimentation were in accordance with the recommendation of the American Veterinary Medical Association and were approved by the Harbor-UCLA Medical Center and Los Angeles Biomedical Research Institute Animal Care and Use Review Committee.

Tissue Preparation

Both young and old rats received an i.p. injection of heparin (130 IU/100 g BW) 15 minutes before a lethal i.p. injection of sodium pentobarbital (100 mg/kg BW) to facilitate testicular perfusion using a whole-body perfusion technique (17). After perfusion with saline, one testis was removed, decapsulated, weighed, snap-frozen in liquid N_2, and stored at −70°C for subsequent determination of NOS activity and iNOS and nNOS levels by western blot assays. The contralateral testes were then fixed by vascular perfusion with either 5% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) or Bouin's solution (Sigma Diagnostics, St. Louis, MO). The testes were removed and processed for routine paraffin embedding. Five-micrometer-thick sections were cut for histological and immunohistochemical observations or in situ detection of apoptosis.

Assessment of Apoptosis

In situ detection of cells with DNA strand breaks was performed in glutaraldehyde-fixed, paraffin-embedded testicular sections by the TUNEL technique (4,5,7,10) using an Apop Tag-peroxidase kit (Chemicon International, San Francisco, CA). In brief, after deparaffinization and rehydration, tissue sections were incubated with proteinase K (20 μg/ml) for 15 minutes, washed in distilled water, and then treated with 2% H_2O_2 for 5 minutes to quench endogenous peroxidase activity. Sections were then incubated with a mixture containing digoxigenin-conjugated nucleotides and TdT in a humidified chamber at 37°C for 1 hour and subsequently treated with antidigoxigenin-peroxidase for 30 minutes at room temperature. To detect immunoreactive cells, the sections were incubated with a mixture of 0.5% DAB and 0.01% H_2O_2 for 6 minutes. Sections were counterstained with 0.5% methyl green, dehydrated in 100% butanol, cleared in xylene, and mounted with Permount (Fisher Scientific, Fairlawn, NJ).

Subcellular Fractionation and Western Blotting

Cytosolic and mitochondrial fractions were prepared as a modification of the procedure described earlier (18). Briefly, saline-perfused testes were homogenized using a Dounce homogenizer in 3 ml of buffer A (0.25 M sucrose, 50 mM HEPES, 10 mM NaCl, 10 mM EDTA, 2 mM dithiothreitol) supplemented with protease inhibitors (Complete Protease Inhibitors; Roche). The crude homogenates were centrifuged at $1000 \times g$ for 10 minutes at 4°C, and the resulting supernatant was centrifuged at $10{,}000 \times g$ for 15 minutes at 4°C to sediment the low-speed fraction containing mainly mitochondria. The mitochondria were washed two times in buffer A and pelleted. The cytosolic and high-speed fractions were isolated following centrifugation of the $10{,}000 \times g$ supernatant fraction at $100{,}000 \times g$ for 60 minutes at 4°C. The resulting supernatant was the cytosolic fraction. Protein concentration was determined using the Bradford method (DC Bio-Rad Assay; Bio-Rad, Hercules, CA).

Western blotting was performed using testicular lysates and subcellular fractions as described previously (4,7). In brief, 50–80 μg of proteins per sample were subjected to electrophoresis on a 4%–12% sodium dodecyl sulfate (SDS)-polyacrylamide gel with MES or MOPS buffer purchased from Invitrogen (Carlsbad, CA) at 200V. Gel was transferred on Immuno-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA) overnight at 4°C. Membranes were blocked in blocking solution (0.05% Tween 20 in Tris-buffered saline and 10% nonfat dry milk) for 1 hour at room temperature then probed using a rabbit polyclonal cytochrome *c* (1:2000; Santa Cruz Biotechnology, Santa Cruz, CA); a rat-specific PARP (1:500; Cell Signaling Technology, Beverly, MA), which recognizes only the cleaved (89 kDa) PARP; a goat polyclonal actin (1:2000; Santa Cruz Biotechnology); and a mouse monoclonal iNOS (1:1000) and nNOS (1:1000; BD Transduction Laboratories, San Diego, CA) antibodies for 1 hour at room temperature or overnight at 4°C with constant shaking. Following three 10-minute washes in TBS-T buffer, membranes were then incubated in antirabbit (Amersham Biosciences, Piscataway, NJ), antigoat, or antimouse immunoglobulin G–horseradish peroxidase (IgG–HRP; Santa Cruz Biotechnology) secondary antibodies at a 1:2000 dilution. All antibodies were diluted in blocking buffer. For immunodetection, membranes were washed three times in TBS-T wash buffer, incubated with enhanced chemiluminescence (ECL) solutions per the manufacturer's specifications (Amersham Biosciences), and exposed to Fuji X-ray film (Fuji Medical Systems, Stamford, CT). To confirm the specificity of the iNOS or nNOS antibody, testicular lysates from old rats were processed in an identical manner, except that the primary antibody was substituted by the mouse control IgG (negative control). The hypothalamus of a 24-month-old BN rat (15) and rat penile smooth muscle cells, which had been induced with bacterial lipopolysaccharide and interferon γ, were used for positive controls for iNOS (19). The cerebellum was used as a positive control for nNOS (19). Band intensities were determined using Quantitity One software from Bio-Rad.

Immunohistochemical Analyses

Bouin's-fixed, paraffin-embedded testicular sections were deparaffinized, hydrated by successive series of ethanol, rinsed in distilled water, and then incubated in 2% H_2O_2 to quench endogenous peroxidases. Sections were blocked with 5% normal goat serum for 20 minutes to suppress nonspecific binding of IgG, and were subsequently incubated with a dilution of 1:100 iNOS or 1:500 nNOS affinity-purified rabbit polyclonal (BD Transduction Laboratories) antibody (4,5,16,20). Immunoreactivity was detected using biotinylated goat antirabbit IgG secondary antibody followed by avidin-biotinylated horseradish peroxidase complex visualized with diaminobenzidine as per the manufacturer's instructions (rabbit Unitect Immunohistochemistry Detection System; Oncogene, Boston, MA). Slides were counterstained with hematoxylin. In addition, to complement the data on age-related expression of iNOS,

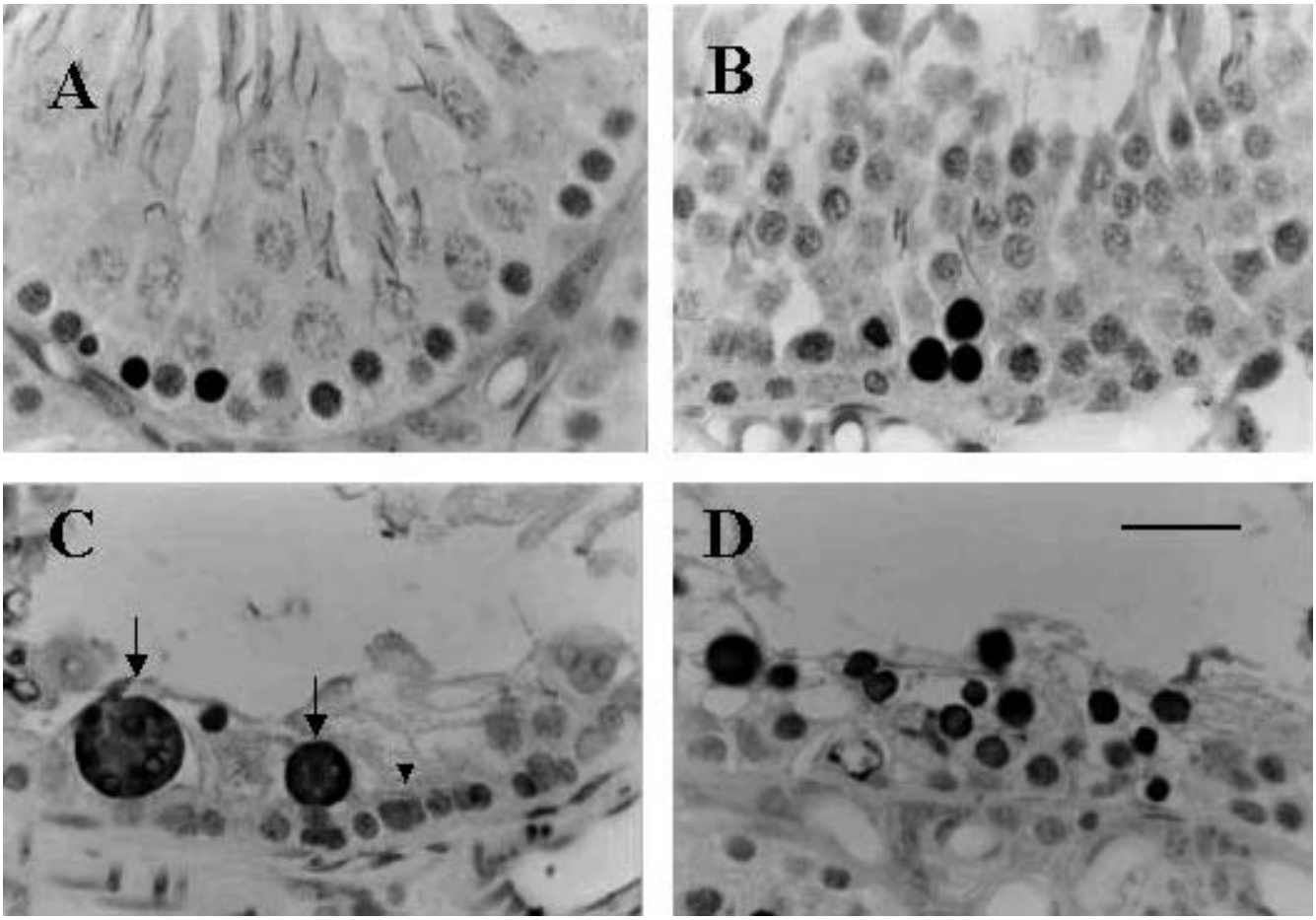

Figure 1. In situ 3′ end-labeling of DNA strand breaks in apoptotic germ cells (detected by TUNEL assay) in young (**A**) and old Brown-Norway rats (**B**–**D**). Methyl green was used as a counterstain. Increased apoptosis of germ cells is clearly evident in old rats, with marked variability in the response of individual tubules. **C**, Apoptotic multinucleated giant cells (arrow) and a Sertoli cell nucleus (arrowhead) are shown in this highly involuted tubule from a 24-month-old Brown-Norway rat. Scale bar, 15 μm.

adjacent testicular sections were also immunostained using an avidin-biotin alkaline phosphatase detection system (Vector Laboratories, Burlingame, CA) with the same mouse monoclonal iNOS antibody used in the Western blot assay according to the manufacturer's instruction. Slides were counterstained with Nuclear Fast Red.

Measurements of NOS Activity

NOS activity in testis homogenates was assessed by measuring the conversion of ^{3}H-arginine to ^{3}H-citrulline as described previously (15). Briefly, the postmitochondrial fraction was passed through Dowex AG50WX-8 (Na^{+}) resin to remove endogenous arginine, and 50-μl aliquots were incubated with 2 μCi/ml resin-purified [^{3}H] L-arginine, 2 mM NADPH, 100 μM L-arginine, and 0.45 mM Ca^{2+} for 30 minutes at 37°C. After eliminating the residual [^{3}H] L-arginine through the resin, [^{3}H] citrulline was counted in the trichloroacetic acid ether-extracted supernatant. Determinations were conducted in triplicate. All values were corrected by the radioactivity eluted in time-zero incubations and were expressed per milligram of soluble protein.

Statistical Analysis

The Student t test was used to determine statistical significance for various parameters. Differences were considered significant if $p < .05$.

Results

Testis Size and Germ-Cell Apoptosis

As reported earlier, by 24 months of age, there was a difference in size between the two testes in the same animal (10,21). One testis was often markedly smaller (designated as regressed testis) than the other testis (designated as normal-looking testis because it was of normal weight). The mean testis weight of the normal-looking testis (1.77 ± 0.21) of the 24-month-old rats was significantly ($p < .05$) higher than that of the regressed testis (0.81 ± 0.06) testis, but was similar to that of the young rats (1.72 ± 0.04). Age-related increase in the incidence of germ-cell apoptosis is depicted in Figure 1. Testes from young animals exhibited a low incidence of

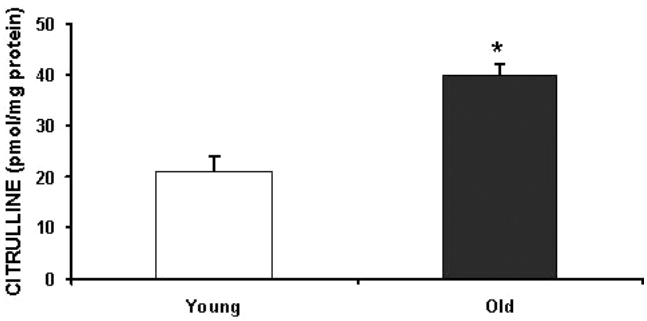

Figure 2. Measurement of testicular nitric oxide synthase activity in young and old Brown-Norway rats. Nitric oxide synthase activity was measured in testis homogenates by the arginine-citrulline conversion assay. Determinations were done in triplicate, and all values were corrected by the amount of radioactivity eluted at time-zero incubation and expressed per milligram of soluble protein. Values are the mean ± *SEM* of five rats per group.

spontaneous germ-cell apoptosis involving primarily A-spermatogonia (Figure 1A) and a few dividing spermatocytes late in meiosis. As shown in Figure 1, increased apoptosis of all three classes of germ cells (spermatogonia, spermatocytes, and spermatids) were readily observed in normal-looking testes of the old BN rats, with marked variability in the response of individual tubules (Figure 1, B–D). A few apoptotic multinucleated giant cells involving round spermatids and Sertoli cell nuclei could also be seen in some tubules (Figure 1, C and D). The regressed testes of these animals displayed only occasional remaining apoptotic germ cells (data not shown); presumably most of the dead cells were eliminated through phagocytosis by the Sertoli cells.

NOS Activity and iNOS and nNOS Protein Expression

To test the hypothesis that increased germ-cell apoptosis in aging may be mediated by increased NO production (through induction of iNOS), we compared the effects of age (young and old rats) on NOS activity and on in vivo expression of iNOS and nNOS. NOS activity, measured by the L-arginine/citrulline conversion assay, was increased by 90% in the old animals compared to that of young animals (Figure 2).

To determine the relative contribution of iNOS and nNOS to the observed increase in NOS activity associated with aging, we then assessed the changes in the levels of both the NOS isofoms in testicular lysates between young and old BN rats by Western blotting. The expression of iNOS protein was detectable at 6 months of age, and increased significantly ($p < .05$) by 70% (over the values measured in young animals) in the normal-looking testes (Figure 3). No discernible changes in the iNOS levels were, however, noted between young and old regressed testes. There were also no obvious alterations in nNOS levels between young and old rats (data not shown).

To substantiate our western blot data, we further examined the age-related changes in the in vivo pattern of both iNOS and nNOS by immunocytochemistry. In young rats, using a rabbit polyclonal antibody, we detected an intense iNOS expression in the Leydig cells with little or no immunoreactivity in the Sertoli and germ cells (Figure 4A). In contrast, a marked increase in iNOS immunoreactivity was noted in the Sertoli cell cytoplasm in normal-looking testes from the old animals (Figure 4B). A modest increase in iNOS immunostaining was also noted in germ cells. We further compared iNOS immunostaining in adjacent testicular sections of young and old BN rats using the same mouse monoclonal antibody used in the Western blot assay. We found a noticeable increase in iNOS immunoreactivity in both Sertoli and germ cells of old rats (Figure 4C). Regressed testes of these old animals also exhibited a marked increase in iNOS immunoreactivity in the Sertoli cells (Figure 4D). In contrast, we found no appreciable changes in iNOS staining between Leydig cells from young and old rats. nNOS was detectable in Leydig cells and Sertoli cells in both young and old animals (data not shown), but, unlike iNOS, no age-related changes were apparent.

Increased NOS Activity Through Up-Regulation of iNOS in Aging Is Accompanied by Cytosolic Translocation of Cytochrome c *and PARP Cleavage*

Cytosolic translocation of cytochrome *c* has been proposed to be an essential component in the mitochondria-dependent pathway for apoptosis in various cell systems, including male germ cells (1–4,7). Accordingly, as a first step, we examined the cytochrome *c* release during age-related increase in male germ-cell apoptosis. Testicular lysates were fractionated into cytosolic and mitochondrial fractions, and were analyzed by Western blotting. As shown in Figure 5, little or no cytochrome *c* was detected in cytosol from testes of young rats. In contrast, cytosolic accumulation of cytochrome *c* was clearly evident in testicular lysates from normal-looking testes of old animals. Because the release of cytochrome *c* from mitochondria into the cytosol triggers caspase activation (1–4,7), we then examined the PARP cleavage, a downstream substrate of the executioner caspases, by western blotting. As shown in Figure 6, little or no PARP cleavage product was detected in cytosol from

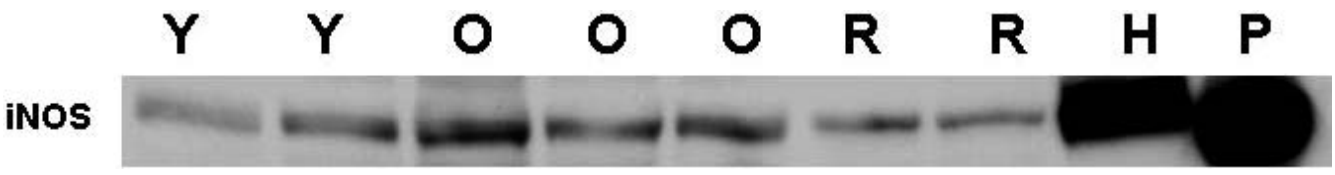

Figure 3. Western blot analysis of cytokine-inducible NO synthase levels in testicular lysates from young and old Brown-Norway rats. Testicular extracts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with antimouse cytokine-inducible NO synthase antibody. The hypothalamus of a 24-month-old rat (15) and rat penile smooth muscle cells, which had been induced with bacterial lipopolysaccharide and interferon γ (19) were used as a positive control. Y, Young testes; O, old normal-looking testes; R, regressed testes; H, hypothalamus; P, penile smooth muscle cells.

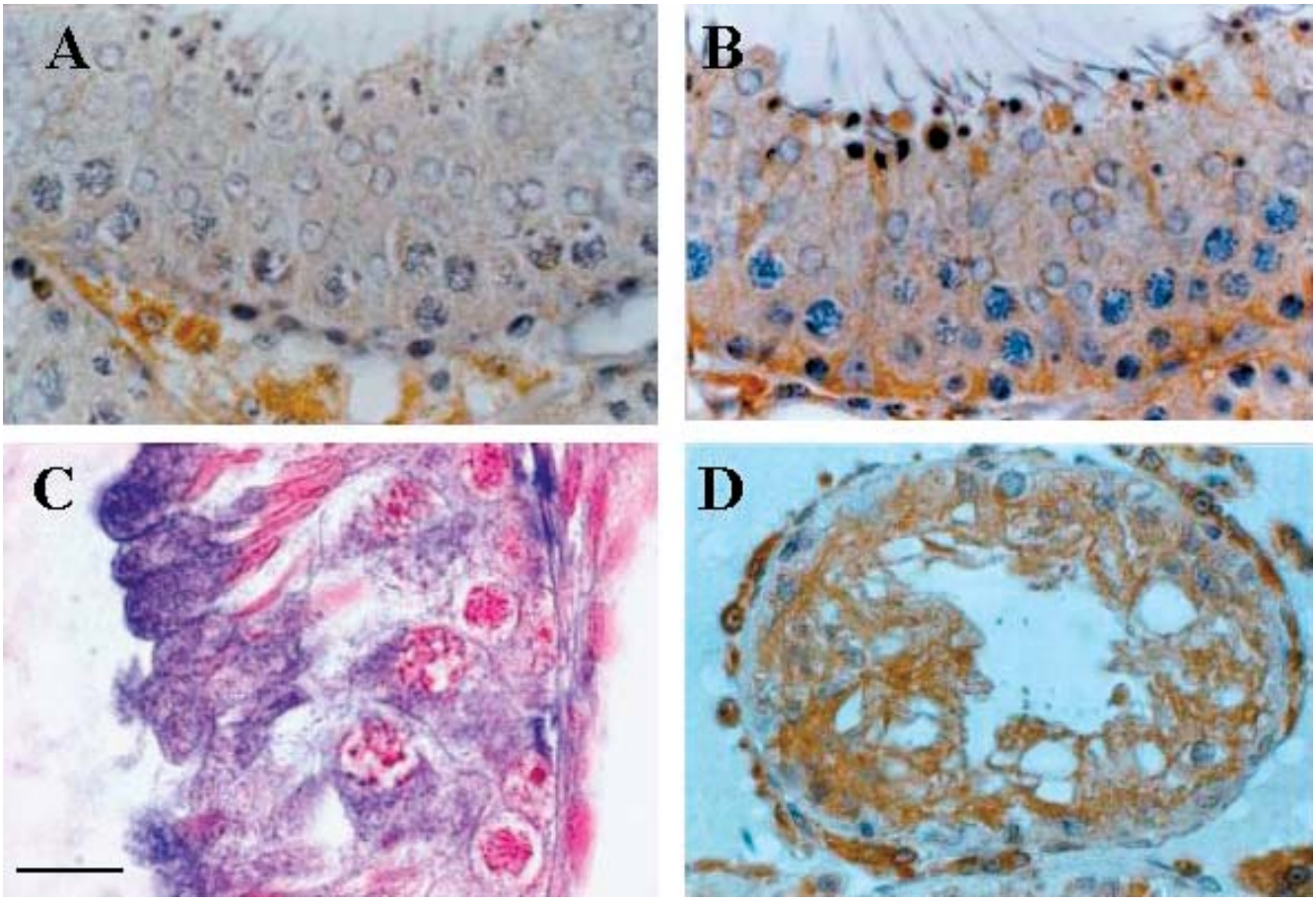

Figure 4. Immunocytochemical analysis of age-related changes in the in vivo expression of cytokine-inducible NO synthase (iNOS) in the Brown-Norway rats. Affinity-purified rabbit polyclonal (**A**, **B**, and **D**) and mouse monoclonal (**C**) iNOS antibodies were used. Testes were fixed by perfusion with Bouin's fluid, embedded in paraffin, sectioned, and immunostained using a diaminobenzidine (**A**, **B**, and **D**) or alkaline phosphatases-based (**C**) detection system. **A**, Portion of a stage VIII tubule from a young rat shows weak iNOS expression in the Sertoli and germ cells. iNOS expression is most striking in the Leydig cells. **B**, Portion of the same stage tubule from a normal-looking testis from an old rat showing a marked increase in iNOS immunoreactivity in the Sertoli-cell cytoplasm. Increased cytosolic immunoreactivity is also noted in the germ cells. **C**, Immunolocalization of iNOS in a normal-looking tubule from a 24-month-old rat using a mouse monoclonal antibody. Note a noticeable increase in iNOS immunoreactivity in both germ cells and the Sertoli cells. **D**, A highly regressed tubule from a 24-month-old rat exhibits a marked increase in iNOS immunostaining in the Sertoli cells. Scale bar, 15 μm.

testes of young rats. In contrast, PARP cleavage product was clearly detected in testicular lysates from normal-looking testes of old BN rats.

Discussion

Male reproductive aging in the BN rat, similar to men, is accompanied by a gradual decline in testosterone production and loss of germ cells and Sertoli cells through increased apoptosis (10,21,22). However, the underlying mechanisms for germ-cell loss are not well understood. In the present study, using young (6-month-old) and old (24-month-old) BN rats, we examined the involvement of the NO-mediated intrinsic pathway signaling in age-related activation of male germ-cell apoptosis. In the 24-month-old BN rats, as expected (10,21,23), one testis frequently regressed, whereas the contralateral testis remained relatively normal. Such age-related variation in testes size in the same animal was unique to the BN rats, as other strains such as Sprague-Dawley rats did not experience similar changes in testis weight associated with aging (24,25). Increased apoptosis of spermatogonia, spermatocytes, and spermatids was readily observed in the normal-looking testes of old rats, with marked variability of response in apoptotic rate in individual tubules. The regressed testes of these old animals displayed occasional remaining apoptotic germ cells. We further note that aging in BN rats, as in hypothalamus and other brain regions (15,16), is associated with increased NO production in the testis. Increased NOS activity in the testes concurrent with aging is independent of nNOS. The endogenous factors that induce iNOS expression in the aging testis are unknown, but by analogy with what is known on experimental iNOS induction, it can be postulated that this may occur through an increase in either the circulating levels or at the tissue levels of cytokines. Indeed, a growing body of evidence demonstrates exaggerated production of proinflammatory cytokines in blood as well as in various murine tissues in aging (26–28).

In earlier studies, we as well as others have reported that reproductive aging in the BN rat is accompanied by decreased serum testosterone levels associated with functional deficits

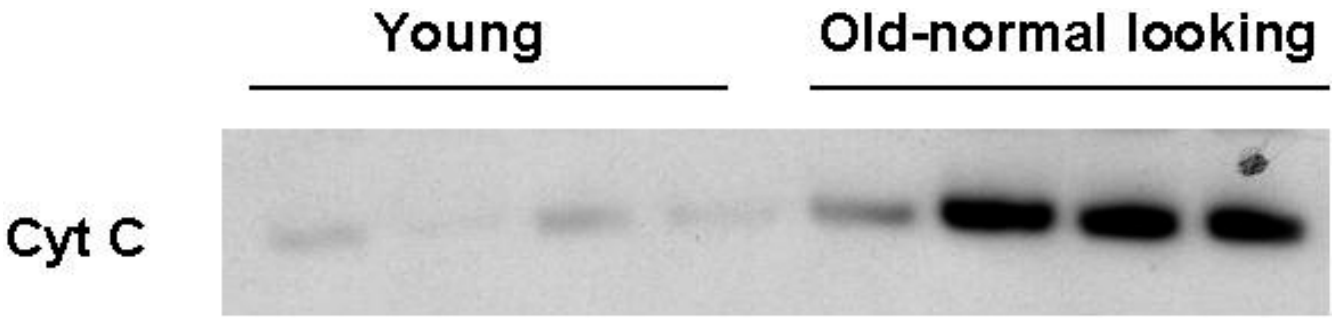

Figure 5. Western blots of cytosolic fractions of testicular lysates from normal-looking testes of old Brown-Norway rats show accumulation of mitochondrial cytochrome *c* in the cytosol. The same blot was reprobed with actin antibody and used as a control for equal loading.

of individual Leydig cells, and loss of germ cells through increased apoptosis [reviewed in (22)]. We have also shown that low circulating levels of testosterone in this rat model is further associated with a decreased LH pulse amplitude and a reduced GnRH and gonadotropin responsiveness to excitatory amino acid agonist (29,30). In contrast, we found no significant alterations in intratesticular testosterone concentrations between young and normal-looking testes from old BN rats (10,21). On the basis of the observations that the activation of apoptosis occurred despite the presence of normal levels of intratesticular testosterone (10,21,22), we suggest that the causal factor for increased germ-cell apoptosis in the old rats may not be endocrine in nature, but may be related to oxidative stress factors [reviewed in (11)]. Several lines of evidence indicate that increased NO synthesis through up-regulation of iNOS plays a major role in the induction of apoptosis in various cell systems (11,14,31,32). Thus, an attractive possibility is that increased NO production, after induction of iNOS, may contribute to spermatogenic damage through increased germ-cell apoptosis in BN rats associated with aging. This concept is further supported by another line of evidence showing that up-regulation of testicular iNOS after treatment with bacterial lipopolysaccharide causes significant germ-cell loss (33,34). Decisive evidence that iNOS plays an important role in testicular germ-cell apoptosis derives from our recent studies of knockout animals (20). iNOS knockout mice have enlarged testis and increased sperm number, and exhibit stage-specific suppression of spontaneous germ cell apoptosis. These mice also confer partial resistance to heat-induced male germ-cell apoptosis. However, the possibility that the observed age-related increase in NO production and germ-cell apoptosis could also be contributed by eNOS can not be excluded on the basis of the data presently available.

One mechanism by which NO can induce apoptosis is through activation of p38 mitogen-activated protein kinase (p38 MAPK), which, in turn, stimulates Bax translocation to mitochondria, resulting in cytosolic translocation of cytochrome *c* and subsequent activation caspases (35–37). Indeed in the present study, we found cytosolic translocation of cytochrome *c* and PARP cleavage in the activation of germ-cell apoptosis associated with aging. This is consistent with our prior work indicating the involvement of the

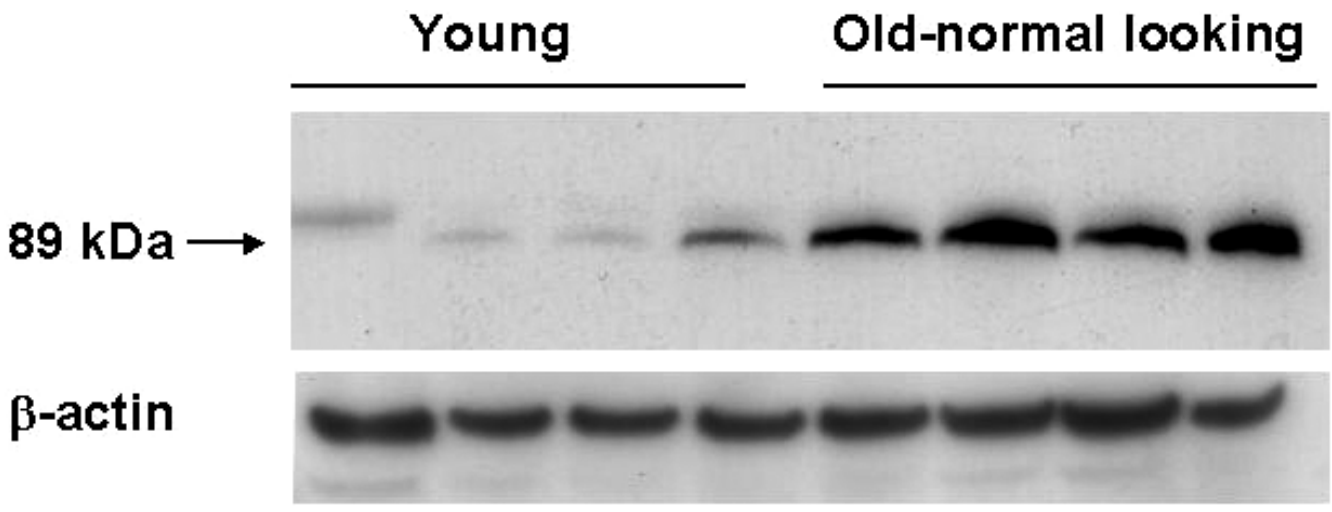

Figure 6. Testicular lysates from young and old Brown-Norway rats were analyzed using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with a rat-specific rabbit polyclonal antibody, which specifically recognizes the cleaved poly (ADP) ribose polymerase. The same blot was reprobed with actin antibody and used as a control for equal loading. Note accumulation of cleaved poly (ADP) ribose polymerase in testicular lysates from normal-looking testes from old Brown-Norway rats.

mitochondria-dependent (intrinsic) pathway signaling in testicular germ-cell death triggered by heat stress (4,5,7). Collectively, these data clearly establish the importance of NO-mediated intrinsic pathway signaling in age-related increase in germ cell apoptosis in male BN rats.

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