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Mitogen-Activated Protein Kinase Signaling in Male Germ Cell Apoptosis in the Rat[1]

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ABSTRACT

Programmed germ cell death is critical for functional spermatogenesis. Increased germ cell apoptosis can be triggered by various regulatory stimuli, including testicular hyperthermia or deprivation of gonadotropins and intratesticular testosterone. We have previously shown the involvement of the mitogen-activated protein kinase (MAPK) 14 in apoptotic signaling of male germ cells across species after hormone deprivation. This study investigates the role of MAPK14 in germ cell apoptosis in rats triggered by testicular hyperthermia. The contributions of the MAPK1/3 and the MAPK8 to male germ cell death were also examined after this intervention. We show that 1) testicular hyperthermia results in induction of both MAPK1/3 and MAPK14 but not MAPK8; 2) inhibition of MAPK1/3 has no effect on the incidence of heat-induced germ cell apoptosis, suggesting that MAPK1/3 signaling may be dispensable for heat-induced male germ cell apoptosis; and 3) activation of MAPK14 and BCL2 phosphorylation are critical for heat-induced male germ cell apoptosis in rats. Thus, unlike the hormone deprivation model, heat stress through activation of the MAPK14 signaling promotes germ cell apoptosis by provoking BCL2 phosphorylation, leading to its inactivation and the subsequent activation of the mitochondria-dependent death pathway. These novel findings point to a critical role of MAPK14 in stage- and cell-specific activation of male germ cell apoptosis triggered by hormone deprivation or heat stress.

apoptosis, germ cell apoptosis, MAPK14, MAPK1/3, rat, spermatogenesis, testis

INTRODUCTION

Germ cell apoptosis plays a pivotal role in spermatogenesis [1–3]. This mode of cell death occurs spontaneously during spermatogenesis or can be induced by a variety of apoptotic stimuli, including deprivation of gonadotropins and intratesticular testosterone by gonadotropin-releasing hormone (GnRH) antagonist [1, 2, 4], testicular hyperthermia [5, 6], ischemia-reperfusion [7], exposure to Sertoli cell toxicant [8], estrogen [9], and chemotherapeutic agents [10]. In earlier studies, we took advantage of two different but complementary models (hormone deprivation and heat stress) to define the key signaling pathways for male germ cell death. We have reported that selective deprivation of gonadotropins and testicular T is followed by stage-specific apoptosis of germ cells involving preleptotene and pachytene spermatocytes and round and elongated spermatids at middle (VII and VIII) stages [1, 2, 4]. In subsequent studies, we have demonstrated that transient heat exposure also induces stage-specific activation of apoptosis but at different stages of the spermatogenic cycle [5, 6]. In striking contrast to the hormone deprivation model, a transient exposure of the testes to heat (43°C for 15 min) induces germ cell apoptosis predominantly at early (I–IV) and late (XII–XIV) stages. Pachytene spermatocytes and early spermatids (steps 1–4) at stages I–IV and pachytene, diplotene, and dividing spermatocytes at stages XII–XIV are most susceptible to heat. Thus, the vulnerability of germ cells to apoptosis in these two paradigms is different. Disruption of this orderly process of germ cell death is associated with abnormal spermatogenesis and male infertility [1, 11–14].

Previously, using murine models of testicular hyperthermia or hormone deprivation, we have demonstrated that the mitochondria-dependent intrinsic pathway signaling constitutes a critical component of apoptotic signaling in male germ cells [15–17]. Currently, we know little about the precise upstream signaling pathways by which these stimuli activate the intrinsic pathway signaling and promote apoptosis involving germ cells at different phases of development. Understanding the specific molecular components of the apoptotic pathway in germ cells is an essential step toward the development of novel therapeutic regimens to control accelerated apoptosis during abnormal spermatogenesis as well as more targeted approaches to male contraception.

MAPKs comprise a family of serine/threonine kinases that function as critical mediators of a variety of extracellular signals [18–20]. Members of the MAPK super family include MAPK1/3, MAPK8, and MAPK14. MAPK1 and MAPK3 are activated in response to growth stimuli and promote cell growth, whereas both MAPK8 and MAPK14 are activated in response to a variety of environmental stresses and inflammatory signals and promote apoptosis and growth inhibition [18–20]. The role of these MAPKs in testicular germ cell apoptosis is not well known, and the conclusions of several studies indicate that the regulation of apoptosis by MAPKs varies depending on tissues, the nature of the apoptotic stimulus, and the duration of their activation [18–23]. In a recent study [17], we provided the first in vivo evidence for a signal-transduction pathway involving MAPK14 and NOS2 that, through activation of the intrinsic pathway signaling, promotes male germ

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cell apoptosis in rats after hormone deprivation. However, we do not know whether the MAPK14-mediated intrinsic pathway is also the key pathway for induction of apoptosis in male germ cells after testicular hyperthermia.

The primary objective of this study was to examine whether the MAPK14-mediated intrinsic pathway, as noted after hormone deprivation, is also the key pathway for induction of apoptosis in germ cells after testicular hyperthermia. Given the opposing effects of MAPK1/3 on MAPK8 and/or MAPK14 signaling in some cell lines [reviewed in 20], an additional objective was to examine the possible contributions of MAPK1/3 and MAPK8 to testicular germ cell death triggered by mildly increased scrotal temperature. Our results indicate that the MAPK14-mediated signaling is also the key signaling pathway for heat-induced testicular germ cell apoptosis. However, unlike the hormone deprivation model, this signaling pathway promotes germ cell apoptosis by provoking BCL2 phosphorylation, leading to its inactivation. This results in the perturbation of the BAX/BCL2 rheostat and the subsequent activation of the mitochondria-dependent death pathway. Induction of germ cell apoptosis after heat stress is independent of MAPK1/3 and MAPK8 signaling.

MATERIALS AND METHODS

Animals and Experimental Protocol

Adult (60 days old), male Sprague-Dawley (SD) rats were used. The animals were purchased from Charles River Laboratories (Wilmington, MA) and housed in a standard animal facility under controlled temperature (22°C) and a photoperiod of 12L:12D, with free access to food and water. Testicular heating was performed as described previously [5]. Briefly, after rats were anesthetized with an i.p. injection of pentobarbital (40 mg/kg body weight), their scrota were immersed in a thermostatically controlled water bath either at 23°C (control) or at 43°C (treated) for 15 min, and animals were killed at 0.5, 2, and 6 h after heat treatment.

To further explore the role of these MAPKs in apoptotic signaling of male germ cells, we sought to determine whether a specific inhibitor of each of these molecules [24–29] confers resistance to heat-induced germ cell apoptosis. Specifically, inhibitors used were U0126 for MEK1 and MEK2 [24, 25], SB203580 for MAPK14 [26, 27], and SP60125 for MAPK8 [28, 29] (all from Calbiochem, San Diego, CA). The rationale for using only the hyperthermia model was based on the results of our preliminary study, in which we found no activation of either MAPK1/3 or MAPK8 in hormone-deprivation-induced germ cell apoptosis. Also, we knew from our earlier studies that MAPK14 indeed plays an important role in apoptotic signaling of murine and primate male germ cell apoptosis after hormone deprivation [17, 30, 31]. To evaluate the requirement of MAPKs signaling in regulating germ cell apoptosis, groups of three to five adult SD rats pretreated with an intratesticular injection of 50 μl of vehicle (dimethyl sulfoxide) or 50 μg of a given inhibitor were exposed once to local testicular heating (43°C for 15 min) and killed 6 h later. The inhibitor or vehicle was administered 1 h prior to local testicular heating. Additional groups of three rats received an i.t. injection of vehicle or 50 μg of inhibitor and served as controls. The U0126 dose is extrapolated from an earlier study [25], which showed that i.t. injection of 50 μg of U0126 was effective in blocking AF-2364 [1-(2, 4-dichlorobenzyl)-indazole-3-carbo-hydrazide]-induced ERK activation in the testis. We used the same dose for the MAPK8 or the MAPK14 inhibitor.

Animal handling and experimentation were in accordance with the recommendations 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 control and experimental animals were injected (i.p.) with heparin (130 IU/100 g body weight) 15 min before a lethal i.p. injection of sodium pentobarbital (100 mg/kg body weight) to facilitate testicular perfusion using a whole-body perfusion technique [32]. After perfusion with saline, one testis was removed, decapsulated, and weighed. Portions of testicular parenchyma were snap-frozen in liquid nitrogen and stored at −70°C for subcellular fractionation and Western blotting. The contralateral testes were then fixed by vascular perfusion with Bouin solution (Sigma-Aldrich, St. Louis, MO). The testes were removed and processed for routine paraffin embedding for either in situ detection of apoptosis or immunohistochemistry.

Assessment of Apoptosis

In situ detection of cells with DNA strand breaks was performed in Bouin-fixed, paraffin-embedded testicular sections (5 μm) by the terminal deoxynucleotidyl transferase (TdT)-mediated deoxy-UTP nick end labeling [4, 5, 15–17] using an ApopTag-peroxidase kit (Chemicon International, San Francisco, CA). Enumeration of the nonapoptotic Sertoli cell nuclei with distinct nucleoli and apoptotic germ cell population was carried out at stages XII–XIV of the seminiferous epithelial cycle using an Olympus BH-2 microscope (Olympus, New Hyde Park, NY) with a 100× oil immersion objective. For each rat at least 10 tubules were used. Stages were identified according to the criteria proposed by Russell et al. [33] for paraffin sections. The rate of germ cell apoptosis (apoptotic index) was expressed as the number of apoptotic germ cells per 100 Sertoli cells [17, 30].

Immunohistochemical and Immunofluorescence Analyses

Bouin-fixed, paraffin-embedded testicular sections were immunostained as described previously [15–17, 31]. Primary antibodies included rabbit polyclonal phospho-p44/42 MAPK (1:50), which detects endogenous levels of MAPK1 and MAPK3 only when phosphorylated at threonine 202 and tyrosine 204 (Cell Signaling Technology, Inc., Beverly, MA), phospho-BCL2 (1:50), which specifically detects endogenous levels of BCL2 only when phosphorylated at serine 70 (Cell Signaling), or a rabbit monoclonal phospho-MAPK14 (1:50), which detects MAPK14 only when dually phosphorylated at threonine 180 and tyrosine 182 antibody (Cell Signaling). Immunoreactivity was detected using biotinylated goat anti-rabbit IgG secondary antibody followed by avidin-biotinylated horseradish peroxidase (HRP) complex visualized with diaminobenzidine tetrahydrochloride as per the manufacturer's instructions (rabbit Unitect Immunohistochemistry Detection System; Oncogene Science, San Diego, CA). Slides were counterstained with hematoxylin. Negative and positive controls were run for every assay. The negative controls were processed in an identical manner, except the primary antibody was substituted by the rabbit IgG or pre-incubated with blocking peptides. Testicular sections from monkeys exposed to testicular hyperthermia and/or hormone deprivation by exogenous administration of T were used as positive controls for phospho-MAPK1 and MAPK3 and phospho-BCL2 [31]. Testicular sections form GnRH-antagonist-treated rats were used as a positive control for phospho-MAPK14 [17].

Enumeration of the nonapoptotic Sertoli cell nuclei with distinct nucleoli and phospho-BCL2-positive germ cells was carried out at stages XII–XIV of the seminiferous epithelial cycle 6 h after heating in rats pretreated with or without U0126. Cell count was obtained using an Olympus BH-2 microscope with a 100× oil immersion objective and expressed as the number of phospho-BCL2-positive germ cells per 100 Sertoli cells. For each rat at least 10 tubules were used.

Activation of MAPK1/3 and MAPK14 and phosphorylation of BCL2 in germ cells undergoing apoptosis was detected by the confocal microscopy using double immunostaining as previously described [15–17]. In situ detection of cells with DNA strand breaks was performed in Bouin-fixed, paraffin-embedded testicular sections using an ApopTag-fluorescein kit (Chemicon International). In brief, after deparaffinization and rehydration, tissue sections were incubated with proteinase K for 15 min at room temperature, washed in distilled water, and then incubated with a mixture containing digoxigenin-conjugated nucleotide and TdT in a humidified chamber at 37°C for 1 h and subsequently treated with antidigoxigenin-fluorescein for 30 min in the dark. After fluorescein staining, slides were washed in PBS and incubated with blocking serum for 20 min to reduce nonspecific antibody binding. Slides were then incubated in a humidified chamber overnight at 4°C with a rabbit polyclonal phospho-p44/42 MAPK (1:50), phospho-BCL2 (1:50), or a rabbit monoclonal phospho-MAPK14 (1:50) antibody followed by goat anti-rabbit Texas Red-labeled secondary antibody (1:100; Vector Laboratories, Burlingame, CA) for 45 min at room temperature, washed, and mounted in ProLong Antifade (Molecular Probes, Eugene, OR). For negative controls, sections were either treated only with secondary antibody or with primary antibody pre-incubated with blocking peptide, and no signals were detected. Confocal imaging was performed using a Leica (Deerfield, IL) TCS-SP-MP confocal microscope equipped with a 488-nm argon laser for excitation of green fluorophores such as fluorescein isothiocyanate and a 543-nm helium-neon laser for excitation of red fluorophores such as Texas Red.

Subcellular Fractionation and Western Blotting

Cytosolic and mitochondrial fractions were prepared as described earlier [15–17, 31, 34]. Briefly, testes were homogenized using a dounce homogenizer

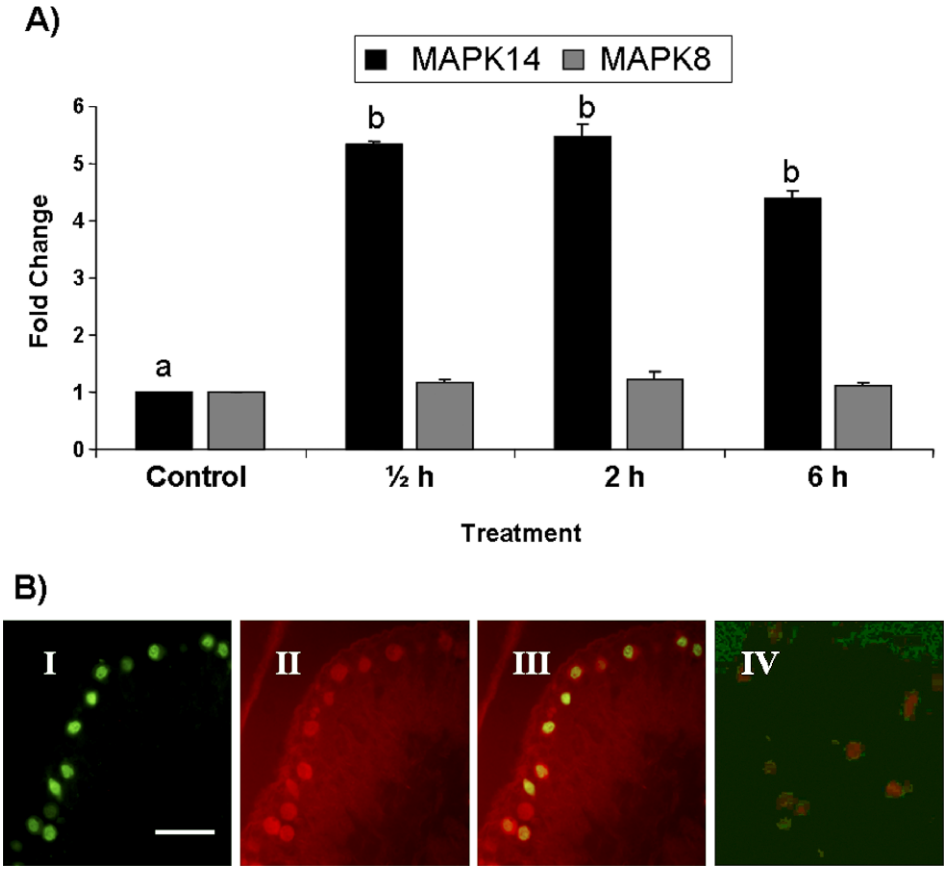

FIG. 1. Testicular hyperthermia results in activation of MAPK14 but not MAPK8. **A**) Time course of activation of MAPK14 and MAPK8 after heat stress, as measured by the TiterZyme Enzyme EIA Kit. Activation of MAPK14, as evidenced by a significant ($P < 0.05$) increase in phospho-MAPK14, was detected at 0.5, 2, and 6 h after heating. Testicular hyperthermia, on the contrary, had no effect on activation of MAPK8. Values are means ± SEM. Means with unlike letters are significantly ($P < 0.05$) different. **B**) Co-staining for TUNEL and phospho-MAPK14 show activation of MAPK14 only in those germ cells undergoing apoptosis at stage XII 6 h after heat treatment (I–III). Green, TUNEL; red, phospho-MAPK14; yellow, colocalization between TUNEL and phospho-MAPK14. Portion of a stage XII tubule from a heat-treated rat pre-incubated with phospho-MAPK14 blocking peptide shows abolition of phospho-MAPK14 immunoreactivity (IV). Bar = 25 μm.

in 3 ml buffer A (0.25 M sucrose, 50 mM Hepes, 10 mM NaCl, 10 mM ethylenediamine tetra-acetic acid, 2 mM dithiothreitol) supplemented with protease inhibitors (Complete Protease Inhibitors, Roche, Indianapolis, IN). The crude homogenates were centrifuged at 1000 × *g* for 10 min at 4°C, and the resultant supernatant was centrifuged at 10 000 × *g* for 15 min 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 × *g* supernatant fraction at 100 000 × *g* for 60 min at 4°C. The resulting supernatant was the cytosolic fraction. The purity of the cytosolic and mitochondrial fractions was assessed by Western blotting using antibodies to actin (1:2000; Sigma-Aldrich) and cytochrome c oxidase subunit IV (COX IV; 1:500; Molecular Probes), respectively.

Western blotting was performed using rat testicular lysates and subcellular fractions as described previously [15–17, 31, 34]. In brief, proteins (50–80 μg) were separated on a 4%–12% SDS-polyacrylamide gel with 2–4-morpholino-ethane-sulfonic acid or 4-morpholine-propanesulfonic acid buffer purchased from Invitrogen (Carlsbad, CA) at 200 V. Gel was transferred on an Immuno-blot PVDF Membrane (Bio-Rad, Hercules, CA) overnight at 4°C. Membranes were blocked in blocking solution (0.3% Tween 20 in Tris-buffered saline and 10% nonfat dry milk) for 1 h at room temperature and then probed using a mouse monoclonal NOS2 (1:500; BD Transduction Laboratories, San Diego, CA) and rabbit polyclonal phospho-p44/42 MAPK (1:500), cytochrome c (1:2000), and phospho-BCL2 (1:2000; Santa Cruz Biotechnology) antibodies for 1 h at room temperature or overnight at 4°C with constant shaking. After three 10-min washes in 0.3% Tween 20 in Tris-buffered saline, membranes were then incubated with a anti-mouse or anti-rabbit (Amersham Biosciences, Piscataway, NJ) IgG-HRP (Santa Cruz Biotechnology) secondary antibody at a 1:2000 dilution. All antibodies were diluted in blocking buffer. For immunodetection, membranes were washed three times in 0.3% Tween 20 in Tris-buffered saline wash buffer, incubated with enhanced chemiluminescence solutions per the manufacturer's specifications (Amersham Biosciences), and exposed to Hyperfilm ECL. Band intensities were determined using Quantity One software (version 4.6.7) from Bio-Rad.

Measurements of Kinase Activation

The TiterZyme Enzyme Immunometric Assay (EIA) kits (Assay Designs, Inc., Ann Arbor, MI) were used for quantitative determination of the phospho-MAPK 1/3, MAPK8, MAPK14 levels in testis lysates as per the manufacturer's instructions. The EIA assay for p38 MAPK activation in testis has recently been validated by us [34]. For example, to detect phospho-MAPK1/3 levels, the kit uses a monoclonal antibody to MAPK1/3 immobilized on a microtiter plate to bind the phospho-MAPK1/3 in the samples or standards. After a short incubation, the excess sample or standard is washed out and a rabbit polyclonal antibody to phospho-MAPK1/3 is added. This antibody binds to the active MAPK1/3 captured on the plate. After a short incubation, the excess antibody is washed out and goat anti-rabbit IgG conjugated to HRP is added, which binds to the polyclonal phospho-MAPK1/3 antibody and generates a signal for chromogenic substrate. The color generated is read at 450 nm. The measured optical density is directly proportional to the concentration of phospho-MAPK1/3 in the samples.

Statistical Analysis

Statistical analyses were performed using the SigmaStat 2.0 Program (Jandel Cooperation, San Rafael, CA). The Student-Newman-Keuls test after one-way repeated-measures ANOVA was used for statistical significance. Differences were considered significant if $P < 0.05$.

RESULTS

Testicular Hyperthermia Results in Activation of MAPK14 but Not MAPK8

Given that MAPK14 signaling is critical for murine as well as primate male germ cell apoptosis triggered by hormone deprivation [17, 30, 31], we considered the possibility that testicular hyperthermia may also trigger MAPK14 activation. To test this hypothesis, we examined the effect of heat stress on MAPK14 activation. Activation of MAPK14, as evidenced by a significant ($P < 0.05$) increase (by 5.3-fold) in phospho-MAPK14 levels in testis lysates, was detected within 0.5 h of heating and remained active thereafter through out the treatment period (Fig. 1A). Activation of MAPK14 in the testis was also ascertained by immunocytochemistry and

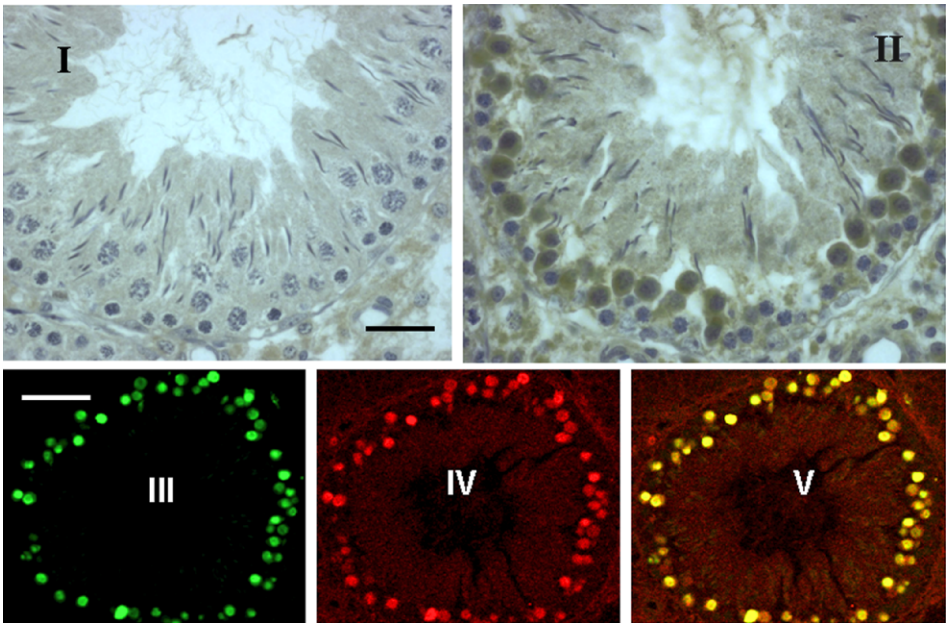

FIG. 2. Testicular hyperthermia results in serine phosphorylation of BCL2 in germ cells. **I** and **II**) Portions of stage XII tubules from control and a rat that had been exposed to short-term local testicular heating shows serine phosphorylation of BCL2 only in heat-susceptible late pachytene spermatocytes 6 h after heating (**II**). Bar = 25 μm. (**III–V**) Confocal images of late pachytene spermatocytes at stage XII from a heat-treated rat show TUNEL (green), phospho-BCL2 (red), and colocalization of TUNEL and phospho-BCL2 (yellow) in apoptotic germ cells 6 h after heat treatment. Bar = 50 μm.

confocal microscopy using a phospho-specific antibody, which detects MAPK14 only when dually phosphorylated at threonine 180 and tyrosine 182. Co-staining for TUNEL and for phospho-MAPK14 confirmed activation of MAPK14 only in those germ cells undergoing apoptosis (Fig. 1B, I–III). No such immunostaining was noted when the primary antibody was pre-incubated with phospho-MAPK14 blocking peptide (Fig. 1B, IV). Interestingly, activation of MAPK14 preceded the induction of apoptosis, which suggests that MAPK14 may play a role during heat-induced germ cell apoptosis.

Like MAPK14, the MAPK8 signaling pathway has been implicated in the apoptotic response of various cell systems exposed to a variety of environmental stresses, inflammatory signals, or injury [35–38]. We, accordingly, examined the effect of heat stress on MAPK8 activation. As shown in Figure 1A, testicular hyperthermia had no effect on MAPK8 activation.

Testicular Hyperthermia Further Results in Serine Phosphorylation of BCL2, Leading to its Inactivation in Germ Cells

Because the phosphorylation status of BCL2 plays an important role in its prosurvival activity [38–41], and this can be induced by MAPK14 [42, 43], we next examined whether the increased germ cell apoptosis after heat stress is associated with BCL2 phosphorylation. Compared with control, where no staining was detected, we found marked increase in the serine-phosphorylated form of inactive BCL2 only in heat-susceptible germ cells (Fig. 2, I and II). Co-staining for TUNEL and phospho-BCL2 further confirmed phosphorylation of BCL2 only in those germ cells undergoing apoptosis (Fig. 2, III–V).

SB203580 Prevents NOS2 Induction and BCL2 Phosphorylation, Suppresses Cytochrome C Release, and Confers Resistance to Heat-Induced Male Germ Cell Apoptosis

To further explore the role of MAPK14 signaling, we examined whether SB203580, a specific inhibitor of MAPK14 [26, 27], could prevent or attenuate heat-induced male germ cell apoptosis. We analyzed the changes in the incidence of germ cell apoptosis (expressed as numbers per 100 Sertoli cells) at stages XII–XIV in rats pretreated with or without SB203580 after heat stress. As shown in Figure 3, apoptotic index was very low in controls (15.4 ± 2.7). No significant changes in the apoptotic index were noted between control and SB203580-treated (22.3 ± 0.23) rats. Compared with both controls, mild testicular hyperthermia within 6 h resulted in a marked activation of germ cell apoptosis (451.1 ± 18.2). SB203580 at this dose level significantly ($P < 0.05$) attenuated such heat-induced germ cell apoptosis by 50.6%.

We next investigated the mechanism by which SB203580 protects germ cell apoptosis induced by heat stress. Given that NO-mediated intrinsic pathway signaling plays an important role in male germ death in rats as well as in men [17] and that can be trigged by MAPK14 activation [17], we examined the effect of SB203580 on NOS2 induction and cytochrome c release from mitochondria. As shown in Figure 4A, induction of NOS2 (~1.5-fold) was clearly evident 6 h after heat treatment, and that could be significantly ($P < 0.05$) suppressed by inhibition of MAPK14. Western blot analysis also revealed a significant ($P < 0.05$) increase (by 2.1-fold) in phospho-BCL2 levels in testicular lysates after heat treatment (Fig. 4A). Pretreatment with SB203580 significantly ($P < 0.05$) suppressed (by 23.0%) such heat-induced phosphorylation of BCL2. Inhibition of NOS2 induction and BCL2 phosphorylation is further accompanied by suppression of cytochrome c release from mitochondria (Fig. 4B).

MAPK8 Signaling Pathway Is Not Critical for Heat-Induced Male Germ Cell Apoptosis

To determine if MAPK8 also plays a role in apoptotic signaling in male germ cells, we examined whether SP600125, a selective inhibitor of MAPK8 [28, 29], could prevent or attenuate heat-induced germ cell apoptosis. We analyzed the changes in the incidence of germ cell apoptosis (expressed as numbers per 100 Sertoli cells) at stages XII–XIV in rats pretreated with or without SP600125 for 6 h after heat treatment. Results are summarized in Figure 5. Compared with controls, mild testicular hyperthermia within 6 h resulted in a marked activation of germ cell apoptosis. Inhibition of MAPK8 had no discernible effects on heat-induced germ cell apoptosis.

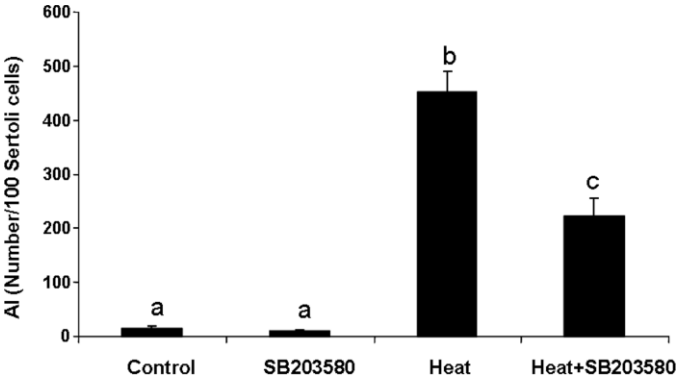

FIG. 3. SB203580 confers resistance to heat-induced germ cell apoptosis. Apoptotic indices (AI, number of apoptotic germ cells per 100 Sertoli cells) at stages XII–XIV in rats treated with vehicle (control), SB203580, heat, or heat + SB203580 show that pretreatment with SB203580 significantly ($P < 0.05$) prevents heat stress-triggered apoptosis in germ cells. Values are means ± SEM. Means with unlike letters are significantly ($P < 0.05$) different.

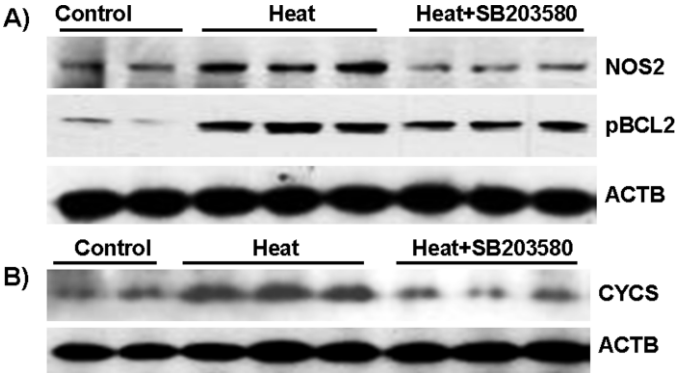

FIG. 4. SB203580 confers resistance to heat-induced germ cell apoptosis by suppressing NOS2 induction, BCL2 phosphorylation, and cytosolic translocation of cytochrome c (CYCS). Representative Western blots of testicular lysates from control, heat, and heat + SB203580-treated rats show effective suppression of NOS2 and BCL2 phosphorylation (pBCL2) (**A**) and cytosolic translocation of CYCS during heat-induced male germ cell death (**B**). Data are representative of three to five animals at each treatment group from one of three separate experiments.

No significant changes in the apoptotic index were noted between control and SP-600125-treated rats. These results, together with the above findings that testicular hyperthermia had no effect on MAPK8 activation, thus indicate that MAPK8 signaling is dispensable for heat-induced germ cell apoptosis in the testis.

Testicular Hyperthermia Results in Stage- and Cell-Specific Activation of MAPK1/3

Given the opposing effects of MAPK1/3 and MAPK14 on apoptosis, we then examined the contribution of MAPK1/3 in heat-induced germ cell death. We found MAPK1/3 activation after heat treatment, as evidenced by immunohistochemistry (Fig. 6, A–D) as well as by immunoblotting (Fig. 6E). Immunohistochemistry further revealed MAPK1/3 activation only in the Sertoli cells involving exclusively heat-susceptible early (I–IV) and late (XII–XIV) stages within 0.5 h of heating (Fig. 6, B–D). Thus, the activation of MAPK1/3 in the Sertoli cells is indeed upstream of activation of germ cell apoptosis, which was first detected 6 h after heating [6, 15]. By 6 h, immunostaining for phospho-MAPK1/3 was, however, evident only in those selective heat-susceptible germ cells (Fig. 6F, I–III). Co-staining for TUNEL and for phospho-MAPK1/3 further confirmed activation of MAPK1/3 only in those germ cells undergoing apoptosis (Fig. 6F, IV–VI). Heat-induced activation of MAPK1/3 was also ascertained by EIA assay. Compared to controls, levels of phospho-MAPK1/3 increased significantly ($P < 0.05$) by 2.0-, 2.5-, and 2.7-fold, respectively, at 0.5, 2, and 6 h after heat treatment. Together, these initial experiments suggest that MAPK1/3 signaling may contribute to the induction of male germ cell death after heat stress.

MAPK1/3 Signaling Pathway Is Not Critical for Heat-Induced Male Germ Cell Apoptosis

The above studies led us to hypothesize that activation of both MAPK1/3 and MAPK14 may be needed for heat-induced germ cell apoptosis. To test this hypothesis, we examined whether U0126, which specifically inhibits MAPK-extracellular signal-regulated kinase 1 and 2 (MEK1/2) activity, an upstream kinase of MAPK1/3 [24], could prevent or attenuate heat-induced germ cell apoptosis. Pretreatment with 50 μg of U0126 significantly ($P < 0.05$) prevented heat-induced MAPK1/3 activation, as evidenced by an EIA assay, by 77.4% (154.5 ± 13.1 vs. 34.9 ± 2.8 pg/100 μg of protein). Of note, inhibition of MAPK1/3 had no effect on activation of MAPK14 after heat treatment (Fig. 7).

We then evaluated the efficacy of U0126 in preventing or attenuating heat-induced germ cell apoptosis. We analyzed the changes in the incidence of germ cell apoptosis (expressed as numbers per 100 Sertoli cells) at stages XII–XIV in rats pretreated with or without U0126 6 h after heat treatment (Fig. 8). Compared with controls (16.0 ± 2.0), mild testicular hyperthermia within 6 h resulted in a marked activation of germ cell apoptosis (515.1 ± 18.2). Inhibition of MAPK1/3 activation had no discernible effects on heat-induced germ cell apoptosis. No significant changes in the apoptotic index were noted between control and U0126-treated (22.3 ± 0.2.3) rats.

Because MAPK1/3 activation is also known to induce BCL2 phosphorylation [44], we next examined the effect of U0126 on BCL2 phosphorylation in germ cells. We counted the number of phospho-BCL2-positive germ cells at stages XII–XIV in rats pretreated with or without U0126 after heat treatment. Cell counts were expressed numbers per 100 Sertoli

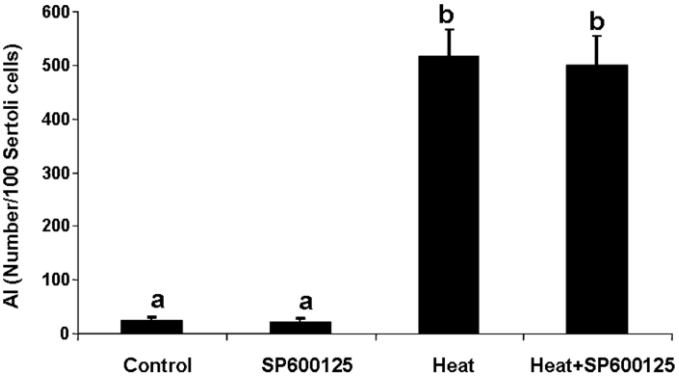

FIG. 5. SP600125 does not protect heat-induced germ cell apoptosis. Apoptotic indices (number of apoptotic germ cells per 100 Sertoli cells) at stages XII–XIV in rats treated with vehicle (control), SP600125, heat, or heat + SP600125 show that SP600125 has no discernible effects on heat-induced germ cell apoptosis. Values are means ± SEM. Means with unlike letters are significantly ($P < 0.05$) different.

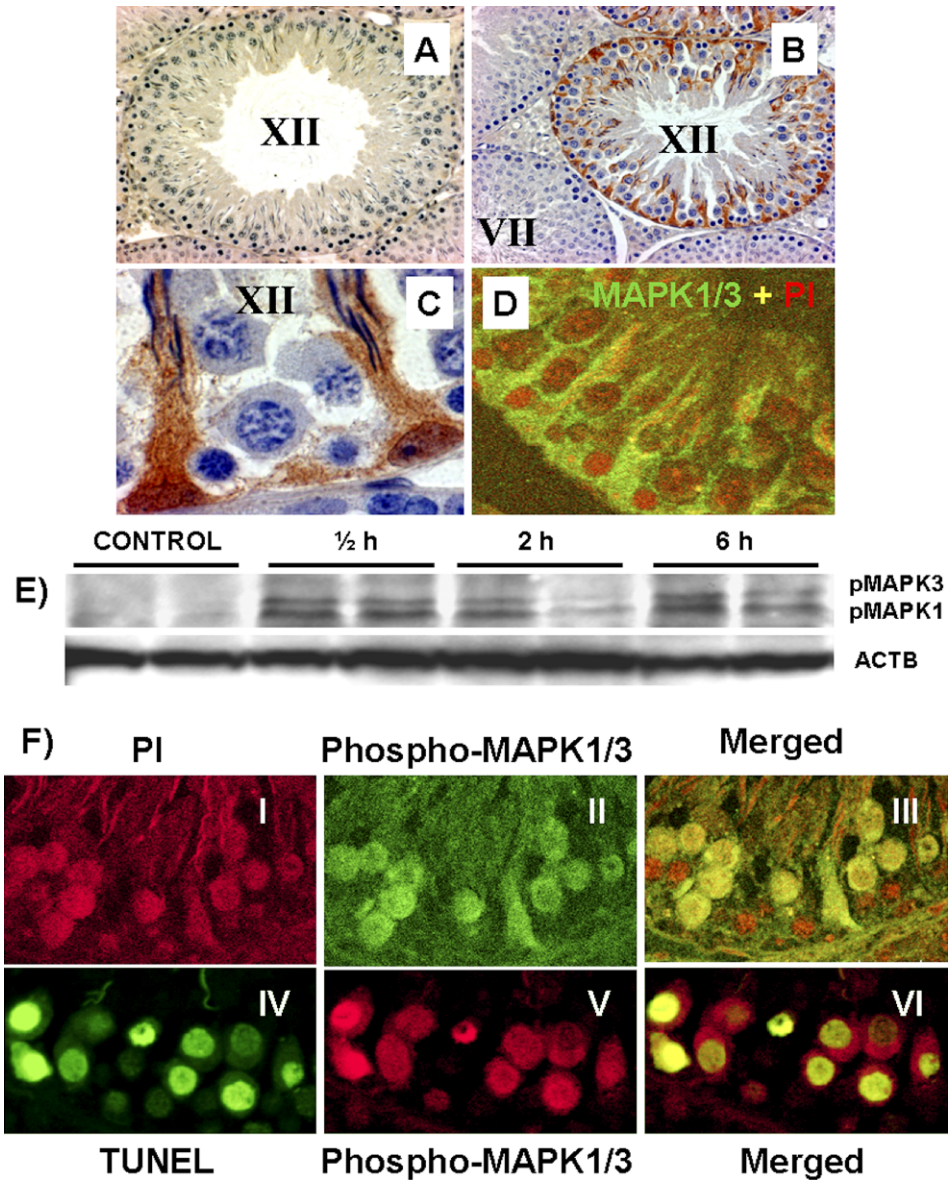

FIG. 6. Heat stress does activate ERK in the testis. **A–D**) Immunocytochemical analysis of MAPK1/3 activation after heat stress. Testicular sections from a control rat (**A**) and rats that had been exposed once to short-term testicular heating (**B–D**) show activation of MAPK1/3 in Sertoli cells at a heat-sensitive stage (XII) but not at the middle stage (VII) after 0.5 h of heating. Hematoxylin or propidium iodide (PI) was used as a counter stain. **E**) Western blot analysis of MAPK1/3 activation after testicular hyperthermia. Data are representative of three to five animals at each time point from one of three separate experiments. Molecular weights are 44 kDa (pMAPK3) and 42 kDa (pMAPK1). **F**) Activation of MAPK1/3 in heat-susceptible pachytene spermatocytes 6 h after heating. Confocal images of a stage XII tubule from a heat-treated rat exhibit phospho-MAPK1/3 immunoreactivity only in heat-susceptible late pachytene spermatocytes 6 h after heat treatment (I–III). No such immunoreactivity is detected in early spermatocytes and elongated spermatids. Chromatin was stained with PI. Confocal images of late pachytene spermatocytes at stage XII tubules show TUNEL (green), phospho-MAPK1/3 (red), and colocalization of phospho-MAPK1/3 and TUNEL (yellow) in apoptotic germ cells 6 h after testicular hyperthermia (IV–VI). Original magnification ×200 (**A**, **B**); ×600 (**C**, **D**, and **F**).

cells. Compared with controls (16.0 ± 5.7) or inhibitor only (9.7 ± 7.7), testicular heating within 6 h resulted in a marked increase in the number of phospho-BCL2-positive germ cells (448.3 ± 35.2). No significant changes in the number of phospho-BCL2-positive germ cells were noted between heat and heat+U0126-treated (457.6 ± 48.3) rats. Together, these results indicate that MAPK1/3 signaling may be dispensable for heat-induced germ cell apoptosis in the testis.

DISCUSSION

In the present study, using a rat model of testicular hyperthermia [5, 6], we investigated the contributions of MAPKs to male germ cell apoptosis. Our data constitute the first demonstration that 1) testicular hyperthermia results in stage- and cell-specific activation of both MAPK1/3 and MAPK14 but not MAPK8, 2) inhibition of MAPK1/3 has no effect on the incidence of heat-induced germ cell apoptosis, and 3) activation of MAPK14 and BCL2 phosphorylation are critical for heat-induced male germ cell apoptosis in rats.

The MAPK1/3 signaling pathway, though essential for controlling cell proliferation and differentiation, could also play a role in cell death [reviewed in 20]. However, its role in apoptosis remains controversial, with several studies suggesting that it may play either a proapoptotic [44–46] or an antiapoptotic [47, 48] role in this process. Available evidence also suggests that heat stress can activate MAPK1/3 [49, 50]. In the later study [50], we demonstrated the activation of MAPK1/3 in monkey Sertoli cells after testicular hyperthermia. Indeed, in the present study, we found activation of MAPK1/3 within 0.5 h of heating in the Sertoli cells at heat-susceptible stages. By 6 h, immunostaining of active MAPK1/3 was evident mostly in germ cells undergoing apoptosis. At present, we do not know the possible significance of our findings. These observations, however, do suggest that not only germ cells but also Sertoli cells may be affected by heat treatment. In a recent study, we demonstrated that heat treatment through activation of MAPK1/3 induces dedifferentiation of adult Sertoli cells into immature states in monkeys [50]. It is also pertinent to note here that MAPK1/3 plays an important role in

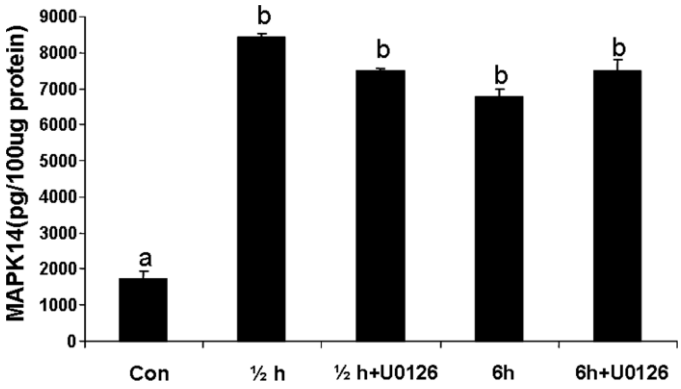

FIG. 7. U0126 has no effect on heat-induced activation of MAPK14. Values are means ± SEM. Means with unlike letters are significantly ($P < 0.05$) different. Con, control; ug, microgram.

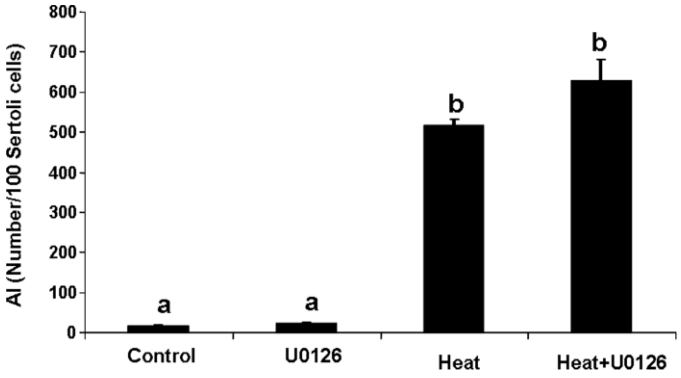

FIG. 8. U0126 does not protect heat-induced germ cell apoptosis. Apoptotic indices (number of apoptotic germ cells per 100 Sertoli cells) at stages XII–XIV in rats treated with vehicle (control), U0126, heat, or heat + U0126 show that inhibition of MAPK1/3 has no discernible effects on heat-induced germ cell apoptosis. Values are means ± SEM. Means with unlike letters are significantly ($P < 0.05$) different.

regulating Sertoli-germ cell adherens junction (AJ) such as ectoplasmic specialization (ES) in the testis [25, 51]. There have been studies indicating that disruption of ES structure and function can induce germ cell apoptosis [25, 52]. Thus, it remains possible that activation of MAPK1/3 in the Sertoli cells after heat stress could perturb Sertoli-germ cell AJ dynamics at the heat-sensitive stages and promote, via yet-to-be-identified mechanisms, germ cell apoptosis [25, 52].

To evaluate the requirement of the MAPK1/3 signaling pathway, we examined whether U0126, a specific inhibitor of MAPK-ERK 1 and 2 (MEK1/2) signaling, could prevent or attenuate heat-induced germ cell apoptosis. We found that inhibition of MAPK1/3 has no effect on the incidence of heat-induced germ cell apoptosis. Relevant to this is the demonstration that MAPK3-/- mice are viable and fertile [53]. Collectively, these results suggest that MAPK1/3 signaling may be dispensable for heat-induced germ cell apoptosis. However, we can not rule out the possibility that activation of MAPK1/3 in the Sertoli cells, which precedes the initiation of apoptosis after heat stress, could sensitize these germ cells to apoptosis through perturbation of Sertoli cell function [50] and/or AJ dynamics [25, 52].

The BCL2 family of proteins governs the mitochondria-dependent pathway for apoptosis [54–56]. Recently, we reported that induction of male germ cell apoptosis after withdrawal of gonadotropins and i.t. T is associated with an increase in BAX and a decrease in BCL2 levels in the mitochondrial fractions of testicular lysates, suggesting a perturbation of the BAX/BCL2 rheostat in the mitochondria [17]. It is conceivable that the signal for activation of cytochrome c-mediated death pathway emanates from such perturbation of the BAX/BCL2 rheostat in the mitochondria. However, unlike our hormone derivation model, we found that heat-induced male germ cell apoptosis is preceded by an increase in BCL2 levels (BAX levels remained unaffected) in the mitochondria [15]. Immunocytochemistry further revealed increased expression of BCL2 only in heat-susceptible germ cells [6]. The obvious question raised by these observations is why these germ cells are dying in spite of the enhanced expression of BCL2 and with no change in the BAX expression in the mitochondria. The enhanced expression of BCL2 in the absence of BAX could imply possible loss of its apoptotic function. A growing body of evidence indicates that serine phosphorylation of BCL2 leads to its inactivation and its ability to form dimers with BAX and, therefore, results in the loss of its antiapoptotic function [38–41]. In the present study, we found increased levels of the serine-phosphorylated form of the inactive BCL2 in heat-susceptible germ cells after heat treatment compared with controls, where no such staining was detected. Co-staining for TUNEL and phospho-BCL2 confirmed phosphorylation of BCL2 only in those germ cells undergoing apoptosis. Consistent with the involvement of MAPK14 signaling in phosphorylation of BCL2 [42, 43], here we show activation of MAPK14 as early as within 0.5 h of heating. The activation of MAPK14 preceded the induction of germ cell apoptosis, which was first detected 6 h after heat treatment [6, 15]. Most importantly, we further show that SB203580 effectively suppressed cytochrome c release and significantly ($P < 0.05$) prevented heat-induced germ cell apoptosis. It is thus conceivable that the signal for activating the mitochondria-dependent pathway during heat-induced male germ cell apoptosis emanates from MAPK14-mediated inactivation of BCL2 through phosphorylation and the subsequent

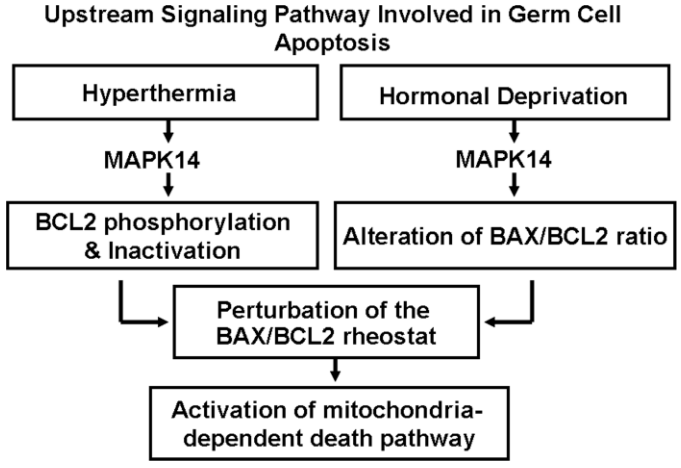

FIG. 9. Upstream signaling pathway involved in germ cell apoptosis triggered by mild testicular hyperthermia or by hormonal deprivation. In both models the induction of apoptosis is triggered by a MAPK14-mediated pathway. Activation of MAPK14, through changes in the ratio of BAX and BCL2 in the mitochondria, triggers intrinsic pathway signaling and promotes germ cell apoptosis in response to a lack of hormonal stimulation [17]. The same signaling pathway is also the key pathway for heat-induced testicular germ cell apoptosis. However, unlike the hormone deprivation model, this signaling pathway promotes germ cell apoptosis by provoking BCL2 phosphorylation, leading to its inactivation, thereby resulting in the perturbation of the BAX/BCL2 rheostat and the subsequent activation of the mitochondria-dependent death pathway.

activation of the mitochondria-dependent death pathway. The present observation is significant because it may help to explain our earlier seemingly paradoxical observation of increased germ cell apoptosis in spite of high levels of BCL2 in rats after heat stress [6, 15] and in monkeys after T and/or heat treatments [31]. Together, these data further suggest an important corollary for apoptotic regulation of male germ cells by BCL2 family members after heat treatment.

The reasons for the limited effectiveness of SB203580 in protecting heat-induced germ cell apoptosis remain unknown. One obvious possibility is that the dose of SB203580 is insufficient. Consistent with this is the demonstration that SB203580 at this dose level significantly ($P < 0.05$) but not completely prevented heat-induced NOS2 induction, BCL2 phosphorylation, and cytochrome c release. In this context it is important to note that we found limited (60.2%) protection against heat-induced germ cell apoptosis in mice by minocycline [57], which effectively inhibits MAPK14 activation, NOS2 induction, cytochrome c release and, as a consequence, caspase activation [58, 59]. The possibility that a caspase-independent mechanism triggered by death effectors other than MAPK14, such as apoptosis-inducing factor or endonuclease G [60], also cannot be excluded on the basis of the data presently available. Nevertheless, the present clearly demonstrates a protective role of SB203580 in heat-induced testicular germ cell apoptosis.

Data reported herein indicate that testicular hyperthermia has no effect on MAPK8 activation. We have further shown that inhibition of MAPK8 has no effect on the incidence of heat-induced germ cell apoptosis. The present findings are in accord with the earlier observations that MAPK8, MAPK9, and MAPK10 knockout mice are viable and fertile [61]. Collectively, these results suggest that MAPK8 signaling is dispensable for testicular germ cell apoptosis triggered by heat stress.

In summary, we have demonstrated the upstream signaling pathway by which both testicular hyperthermia and hormone deprivation activate the intrinsic pathway signaling and promote male germ cell apoptosis (Fig. 9). A MAPK14-mediated pathway, through changes in ratio of BAX and BCL2 in the mitochondria, activates intrinsic pathway signaling and promotes male germ cell apoptosis in response to a lack of hormonal stimulation. The same signaling pathway is also the key pathway for heat-induced testicular germ cell apoptosis. However, unlike the hormone deprivation model, this signaling pathway promotes germ cell apoptosis by provoking BCL2 phosphorylation, leading to its inactivation, thereby resulting in the perturbation of the BAX/BCL2 rheostat and the subsequent activation of the mitochondria-dependent death pathway.

Heat has long been recognized as a risk factor responsible for decreased sperm counts in men [62]. In a recent study, we further demonstrated that indeed germ cell apoptosis plays an important role in the organized regression of spermatogenesis in men after hormone deprivation and/or heat stress [63]. Notably, heat, in combination with hormone deprivation, enhances the suppression of spermatogenesis further than either treatment alone [63]. Rise in testicular temperature in conditions such as cryptorchidism and varicocele has been shown to cause oxidative stress, leading to increased germ cell apoptosis and male infertility [14, 64]. Despite the recognition that increased germ cell apoptosis plays an important role in male infertility, the underlying mechanisms of increased germ cell apoptosis in these conditions are not well known. In concert with our results in rat, Zhang et al. [65] have found increased expression of BCL2 and no change in the expression BAX in experimental cryptorchidism-induced testicular germ cell apoptosis in rhesus monkeys. In contrast, FAS signaling, characterized by lower levels of soluble FAS in the seminal plasma of infertile men with varicocele, has been implicated in the apoptotic response of germ cells in patients with varicocele [66]. Recently, Lee et al. [67] found overexpression of BCL2 in the internal spermatic vein of patients with varicocele. Thus, our understanding of the precise signal transduction pathways culmination in activation of testicular germ cell apoptosis in those conditions is still far from complete. We emphasize that the present study was designed to elucidate the precise upstream signaling pathway by which heat stress activates the intrinsic pathway signaling and promotes stage- and cell-specific activation of germ cell apoptosis. We believe that defining the signal transduction pathway may lead to novel targets that may be used in the future for regulation of male fertility or treatment of male infertility.

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