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Calpain Inhibitors Prevent p38 MAPK Activation and Germ Cell Apoptosis After Heat Stress in Pubertal Rat Testes

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Testicular injuries like torsion or cryptorchidism can cause massive germ cell death, which could have great impact on male reproductive health. In addition, it has been proposed that modern life style, in the form of underwear or sedentary work position, could increase the testicular temperature, induce germ cell apoptosis, reduce spermatozoa quality and promote male infertility. In this work we showed that a heat stress stimulus induced massive germ cells apoptosis, which was associated with p38 mitogen-activated protein kinase (MAPK) phosphorylation along with an increase in the levels of mRNA encoding calpain 2. Synthetic calpain inhibitors prevented heat stress-induced germ cell apoptosis through inhibition of p38 MAPK phosphorylation. Thus, our results indicate that the blockage of calpains suppresses p38 MAPK phosphorylation, and identifies calpain activation (most likely calpain 2) as an early event in heat stress-induced male germ cell apoptosis.

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Mammalian spermatogenesis involves proliferation of germ cells (spermatogonia), meiosis and the consequent differentiation of haploid spermatids (Matsumoto, 1996; Lagos-Cabre and Moreno, 2008). This process is therefore suitable to address many questions about cell proliferation, differentiation and meiosis in vivo. In addition, it allows the testing of a specific protein function or process in different cell types and under different physiological conditions.

Apoptosis is a process of cell elimination, and in the testis it is believed to function in order to eliminate those germ cells that have not been able to develop a tight interaction with Sertoli cells. Other factors such as breakdown of sex chromosome silencing, absence of testosterone or follicle-stimulating hormone (FSH) can also promote germ cell apoptosis (Henriksen et al., 1996; Vera et al., 2006; Mahadevaiah et al., 2008). It has been shown that apoptosis is characterized by the activation of a family of cysteine proteases named caspases and the fragmentation of DNA driven by calcium activated DNases (CAD) (Taylor et al., 2008).

Apoptosis, among many other processes, is important in controlling the output of spermatozoa and the number of germ cells in a particular differentiation step (Billig et al., 1995; Pentikainen et al., 2003). Spermatogonia type A_{2-4} and spermatocytes in stages I, XII, and XIV are the main cells undergoing apoptosis in adult rat testes (Blanco-Rodriguez and Martinez-Garcia, 1996a,b; Pentikainen et al., 2003). A similar event occurs during puberty of the male rat, when a massive wave of apoptosis peaks at day 25 after birth, in which mainly meiotic cells (spermatocytes) at the pachytene step are involved (Rodriguez et al., 1997; Jahnukainen et al., 2004; Moreno et al., 2006). Recent studies have shown that apoptotic spermatocytes show an up-regulation of the Fas receptor and its transcription factor p53 (Lizama et al., 2007). In addition, pharmacological inhibition of caspase activity suggests that the extrinsic pathway plays an important role in germ cell demise in spermatocytes undergoing apoptosis (Codelia et al., 2008). It was intriguing that in those experiments only 45% of the apoptotic (TUNEL-positive) cells showed Fas receptor

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expression, and that caspase inhibition could prevent a similar percentage of cell death leaving the possibility for other mechanisms to be involved in physiological apoptosis in germ cells (Lizama et al., 2007).

MAPKs comprise a family of serine/threonine kinases that function as critical mediators of a variety of extracellular signals (Ashwell, 2006). Members of the MAPK superfamily include the ERKs, the c-Jun NH2-terminal kinases (JNKs), also known as stress-activated protein kinases, and the p38 MAPKs. Available data from various cell systems other than male germ cells suggest that ERK1 and ERK2 are activated in response to growth stimuli and promote cell growth, whereas both JNKs and p38 MAPKs are activated in response to a variety of environmental stresses and inflammatory signals and promote apoptosis and growth inhibition (Dorion and Landry, 2002; Mittelstadt et al., 2005; Ashwell, 2006). A role for p38 MAPK in male germ cell apoptosis has been suggested after GnRH antagonist treatment. Initiation of germ cell apoptosis is preceded by p38 MAPK activation and induction of inducible nitric oxide synthase (iNOS) (Vera et al., 2006). Despite that pharmacological inhibition of p38 MAPK prevents germ cell apoptosis induced by GnRH antagonist treatment, its downstream effectors are unknown (Vera et al., 2006). Another family of cell stress response proteins are calpains, which constitute a superfamily of intracellular calcium-dependent neutral cysteine proteases whose members are widely expressed in a variety of cells and tissues (Squier et al., 1994; Saez et al., 2006). Some calpains are expressed ubiquitously, while others have restricted tissue expression patterns. Calpains 1, 2, and 11 have been detected in mouse testes and also seem to have some role during fertilization (Rojas et al., 1999; Rojas and Moretti-Rojas, 2000; Ben-Aharon et al., 2005, 2006). In mammals, the large subunit of calpain 1 (CAPN1) and calpain 2 (CAPN2), as well as the small regulatory subunit calpain 4 (CAPN4) are expressed ubiquitously and are the most well-characterized calpains (Blake et al., 2003). Calpains 1 and 2 are expressed in spermatozoa, and ultra-structural studies indicate they are localized between the plasma membrane and the outer acrosomal membrane (Schollmeyer, 1986; Yudin et al., 2000). Calpain 11 expression is restricted to the testis where the protein is detected by day 18 after birth, during mid to late pachytene spermatocyte development. It is present in the acrosomal region of spermatozoa from the cauda epididymis with no expression in the spermatogonia or the post-meiotic spermatids (Ben-Aharon et al., 2006). Despite the presence of these enzymes in both spermatozoa and developing germ cells, their role in the physiology of these cells still remains uncertain.

It is known that many environmental stimuli can induce apoptosis, such as pesticides, hormone deprivation, and heat stress (Shiraishi et al., 2001; Sinha Hikim et al., 2003; Ruwanpura et al., 2008). It has been reported that heat stress induces apoptosis in germ cells of stages I–IV and XII–XIV in adult rats (Lue et al., 1999, 2000, 2002). Heat-induced apoptosis provokes caspase-9 proteolysis and redistribution of the pro-apoptotic protein Bax from a cytosolic to a paranuclear localization (Miura et al., 2002; Vera et al., 2004, 2005). In this model the role of the Fas system is controversial since mice harboring a loss-of-function mutation in FasL or Fas, show a similar degree of apoptosis, while studies in cynomolgus monkeys and mice have shown an up-regulation of Fas at the protein level (Miura et al., 2002; Vera et al., 2004; Jia et al., 2007). Growing evidence supporting caspase-independent cell death in testes after traumatic injury and the suggestive result showing that heat-induced apoptosis of mouse meiotic cells is suppressed by ectopic expression of the testis-specific calpastatin (Shiraishi et al., 2000; Somwaru et al., 2004), prompted us to evaluate the role of these enzymes in both physiological and heat-induced apoptosis during puberty.

Materials and Methods

Animals and chemicals

The potent, cell-permeable, and selective p38 MAP kinase inhibitor PD 169316 was purchased from Merck (Darmstadt, Germany). Caspase-8 (z-IETD-pNA), caspase-9 (z-LEHD-pNA), caspase-2 (Ac-VDVAD-pNA), and caspase-3 (z-DEVD-PNA) substrates, along with the pan-caspase inhibitor (z-VAD-fmk), were purchased from Merck. Purified rabbit calpain and the selective calpain inhibitor MDL28170 were purchased from Sigma (St. Louis, MO). Male Sprague–Dawley rats of 20 days old were acquired from the Animal Facility of our faculty. The rats were housed under a 12L/12D cycle, with water and rat chow being provided ad libitum. They were killed by cervical dislocation. Investigations were conducted in accordance with the rules laid down by the Consortium for Developing a Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching and by the National Research Council. All animal protocols were endorsed by the Chilean National Fund of Science and Technology (FONDECYT).

Calpain inhibitors synthesis

The calpain inhibitors (Fig. 1) were generated using the following general synthesis route. L-leucine or L-proline was dissolved in NaOH 1 M or $CaCO_3$ 10%. Then, benzenesulphonyl chloride was added with stirring. After 2 h, the mixture was extracted with ethyl ether; and the aqueous layer was acidified with diluted HCl (1:1) to pH 2. The solid was filtered and washed with ethyl ether (3×10 ml) in order to obtain the sulphonamide derivate. At the same time, L-phenylalanine was dissolved with HCl(g) in absolute ethanol, in order to obtain the ester derivate. Then, a mixture of the respective sulphonamide and *N*-hydroxybenzotriazole (HOBT) in dry CH_2Cl_2 at 0°C, under N_2, was treated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). After 1 h at 0°C, a solution of the ether derivate, in dry CH_2Cl_2 was added. The reaction was stirred at 0°C for 1 h and then, at room temperature for 24 h. The solvent was evaporated; the residue was redissolved in ethyl acetate or CH_2Cl_2 and washed with water (3×20 ml). The organic phase was dried over Na_2SO_4 and evaporated under reduced pressure. The obtained residue was washed with ethyl ether in order to obtain the product. The ethyl

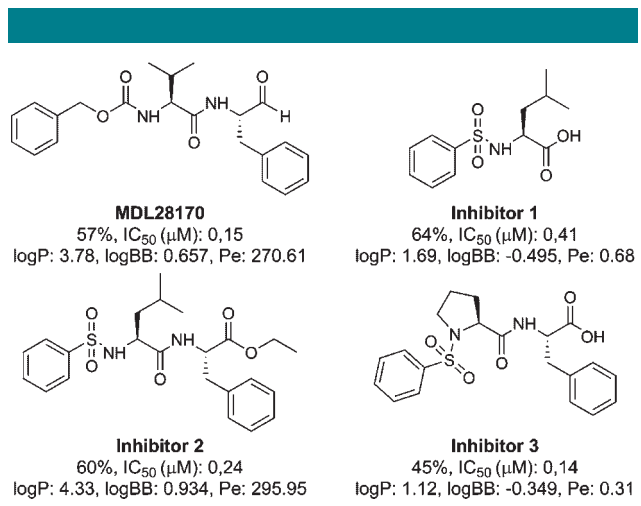

Fig. 1. Calpain inhibitor structures and biological activity of calpain 2 after inhibition. In vitro inhibition (1 μM) and IC_{50} values were obtained using recombinant porcine kidney calpain 2. Log *P*, log BB (blood–brain distribution) and Pe (Permeability in Caco-2 scale (pH 7.4, 500 rpm) $\times 10^{-6}$ cm/sec), were calculated with Pharma Algorithms ADME Boxes v3.06.

ether derivate was treated with HCl, in order to obtain the free carboxylic acid derivate.

Every synthesized molecule was isolated, purified (TLC, extraction with solvents and crystallization) and analyzed (IR, ^{1}H-NMR, ^{13}C-NMR, determination of physical constants and elementary analysis) before its biological evaluation (Benz, 1991; Gutte, 1995). See also online supporting information.

Calpain 2 in vitro inhibition assay

An assay solution containing 0.5 mg/ml casein, 20 mM DTT, 50 mM Tris–HCl (pH 7.4) and 0.03 U (about 0.24 μg) of recombinant porcine kidney calpain II (Merck, Whitehouse Station, NJ) was used. In each well 200 μl of assay solution and 2.5 μl of DMSO was placed, containing inhibitors in different concentrations. The reaction was started by the addition of 50 μl of 20 mM $CaCl_2$ in a test well and 50 μl of 1 mM EDTA in a blank well. After incubations for 60 min at 30°C, an aliquot of the reaction mixture was transferred to another plate containing 50 μl water and 100 μl of Bio-Rad dye reagent concentrate diluted with water (total volume 250 μl). After 15 min, the plate was read in a microplate reader (ELISA) at an absorbance of 595 nm. Non-inhibitor samples were also run as control (1 mM DMSO was used instead), and no-$CaCl_2$ samples as blank (1 mM EDTA was added instead) and the percentage of inhibition was calculated and plotted as the percentage of inhibition of calpain versus log inhibitor concentration. Reported percentages of enzyme inhibition for each inhibitor were obtained at a concentration of 1 μM and the IC_{50} estimation was obtained from the graph (Fig. 1) (Inoue et al., 2003).

ADME parameters predictions

Absorption, distribution, metabolism, and excretion (ADME) parameters were estimated for each inhibitor, using the SMILES entry format (Weininger, 1988). Fragmental or atom-based approaches based on Abraham's solvation rules (Abraham, 1993) were used to predict the octanol/water distribution coefficient (log *P*), intestinal permeability (Pe), and blood–brain distribution coefficient (log BB) parameters with ADME Boxes v3.06 software (Pharma Algorithms, Inc., Vienna, Austria).

Heat shock

Twenty-one-day-old rats were anesthetized with ketamine/xilacine (1 and 75 mg/kg) i.m., and the lower half of the torso of each animal was submerged in a thermostatically controlled water bath at 43°C (treated) for 15 min, after which time the animals were dried and returned to their cages. Rats were killed at different hours after treatment by cervical dislocation (Rockett et al., 2001; Vera et al., 2004).

Intratesticular injections

Twenty-one-day-old rats were anesthetized with ketamine/xilacine (1 and 75 mg/kg) i.m. The testes were exteriorized through a low midline incision. Ten microliters of a solution containing calpain, or p38 MAPK, inhibitors were dissolved in PBS were infused via a 30G needle, inserted through the tunica albuginia with the tip resting in the testicular interstitium. Following drug delivery, the testes were returned to the peritoneal cavity, and the incision was closed. Then, the rats were exposed to heat stress while they were under anesthesia. After different hours of heat stress the animals were sacrificed and the testes were either used for histology or biochemical studies. As a control, PBS was injected into the testes. Three different rats were used for each experimental time.

Immunohistochemistry

Active (phosphorylated) p38 MAPK (p-p38) was detected in paraffin embedded cross-sections of rat testes fixed in Bouin's solution and treated with sodium citrate 0.01 M and pH 6, to expose the antigens. The samples were first treated with 3% H_2O_2 for 10 min, then, to prevent unspecific binding, a standard protein block system (Ultra V block, LabVision, Freemont, VA) was applied for 10 min and the samples were treated with PBS/BSA 1% for 1 h to block. Primary antibody, which detects p38 only when two residues (threonine-180 and tyrosine-182) are phosphorylated (Cell Signaling, Danvers, MA) was applied at a concentration of 2 μg/ml and incubated overnight at 4°C in a humidified chamber after being washed three times for 5 min in a Tris–HCl buffer, pH 7.6 with 0.3 M NaCl and 0.1% Tween-20. Biotinylated secondary antibody, streptavidin–biotinylated–peroxidase complex, amplification reagent (biotinyl tyramide) and peroxidase-conjugated streptavidin were applied step-by-step for 30 min each. Afterwards, incubation slides were washed three times for 5 min in a Tris–HCl buffer, pH 7.6 with 0.3 M NaCl and 0.1% Tween-20. Finally, substrate–chromogen solution consisting of concentrated Tris–HCl and 0.8% H_2O_2 (substrate) and 3,3-diaminobenzidine tetrahydrochloride (DAB) solutions (chromogen) were applied for 1 min and washed in distilled water. Samples were stained with hematoxylin and observed under a phase contrast microscope (Optiphot-2, Nikon, Melville, NY) and photographed with a digital camera (CoolPix 4500, Nikon).

TUNEL analysis

Apoptotic fragmentation of DNA was evaluated by TUNEL analysis in deparaffinized sections of rat testes. The Roche Molecular Biochemical's In Situ Cell Death Detection Kit was used according to the manufacturer's instructions (Roche Applied Sciences, Indianapolis, IN). Samples were observed under phase contrast (Optiphot-2, Nikon) and micrographs were taken with a digital camera (CoolPix 4500, Nikon). TUNEL-positive germ cells, visualized as brown cells due to the peroxidase reaction, were quantified in each tissue section by counting the number of TUNEL-positive cells per seminiferous tubule cross-section in random areas. The apoptotic index was calculated as the average number of TUNEL-positive cells per seminiferous tubule cross-section. Three testicular histological sections were taken per rat (three rats total), with a minimum of 100 randomly selected tubules in each tissue section (a total of 900 tubules per treatment). The data represent the mean (±SD) of rats for each specified age.

Immunofluorescence

Rat testis was fixed in 4% PFA and embedded in paraffin. Sections 5 μm were re-hydrated and then unspecific-binding sites were blocked by incubating sections for 1 h in a PBS plus 2% bovine serum albumin (PBS–BSA) solution. Tissue sections were then incubated overnight with an antibody against p-p38 (2 μg/ml) at room temperature in a humidified chamber. After washing in PBS, tissue sections were incubated with Alexa 488-goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 1 h at room temperature, washed. Next, the sample was processed for TUNEL analysis (see above) and then mounted using a fluorescence protector medium (VectaShield, Burlingame, CA). Sections were observed under phase contrast and fluorescence microscopy (Optiphot-2, Nikon) and photographed with a digital camera (CoolPix 4500, Nikon).

Protein extraction and Western blot assay

Protein extraction was performed by homogenizing isolated seminiferous tubuli in a buffer containing 1 M NaCl, 1 mM EDTA, 10 mg/ml PMSF, 1% Triton X-100, 20 mM Tris–HCl pH 7.4, and centrifuging for 10 min at 13,200 rpm. The samples were run on a 10% polyacrylamide gel (SDS–PAGE) under reducing and denaturing conditions, and then transferred to nitrocellulose at 400 mA for 2 h. Nitrocellulose was blocked with 5% (w/v) non-fat milk, 0.1% Tween in PBS, pH 7.4, and then incubated overnight at 4°C with the following antibodies: anti-p38 MAPK

(pThr180/Tyr182) phosphospecific (0.125 μg/ml, BD Transduction Laboratories, San Diego, CA), anti-total p38 MAPK (0.2 μg/ml, Santa Cruz Biotechnologies, Delaware, DA) or anti-β-actin (0.3 μg/ml, Sigma), as a loading control. Membranes were then incubated with a secondary antibody conjugated with horseradish peroxidase-secondary antibodies (KPL, Gaithersburg, MD) diluted 1:3,000 in blocking solution for 1 h at room temperature, and complexes detected by ECL (Pierce Biotechnology, Rockford, IL).

Caspase activity measurement

Caspase activity assays were performed as previously described (Ding et al., 2000; Moreno et al., 2006; Lizama et al., 2007). Briefly, isolated seminiferous tubuli were homogenized in a buffer containing 1 M NaCl, 1 mM EDTA, 10 mg/ml PMSF, 1% Triton X-100, 20 mM Tris–HCl pH 7.4. Caspase substrates labeled with chromophore p-nitro aniline (pNA) were used and upon cleavage a yellow color was produced, measured by a spectrophotometer at 405 nm. The amount of product generated was calculated by extrapolation of a standard curve of free pNA. One international unit (IU) was defined as the amount of caspase hydrolyzing 1 μM of pNA/min at 25°C. Results are expressed in units of enzyme per milligram of tissue (U/mg tissue). The results are presented as the mean of six different rats.

Cell cycle analysis by flow cytometry

Seminiferous tubuli were separated by continuous pipetting in 1.5 ml KHB (Krebs-Henseleit buffer plus 1% BSA) medium (2 g/L D-glucose 0.141 g/L magnesium sulfate [anhydrous] 0.16 NaH_2PO_4 0.35 g/L KCl and 6.9 g/L NaCl) with 15 μl of a collagenase solution (0.5 mg/ml) added. Tubuli were decanted while maintaining Leydig and blood cells suspended in the medium, which was consequently discarded. Collagenase causes the tubule walls to release germ and Sertoli cells. Using a syringe with a 21G needle the tubules were further destroyed and the individual cells liberated. Finally the solution of individual cells was filtered through a 50 μm filter. To analyze cell cycles, the individual cells in KHB solution were pelletted and then fixed in 70% ethanol overnight. As described by Riccardi and Nicoletti (2006) on the day of analysis the cells were pelletted and washed once with phosphate buffered saline (PBS). The pellet was then dissolved in a cell cycle buffer containing 0.1% sodium citrate, 0.3% Triton X-100 (both Sigma-Aldrich Co., St. Louis, MO), 50 μg/ml propidium iodide and 50 μg/ml RNase A (both Invitrogen Corporation, Carlsbad, CA) dissolved in distillate water. The samples were then analyzed within 10 min of buffer addition in a Coulter Epics XL cytometer; 10,000 gated events were acquired.

RT-PCR

Total RNA was isolated using TRIzol-Reagent (Invitrogen Corporation). First, complementary DNA was made using 5 μg total RNA in the presence of Superscript III reverse transcriptase (Invitrogen Corporation) and random primers. After the RT reaction, 51 μl of the incubation mixture was used as a template for the subsequent PCR reaction. Several primer sets were used to obtain the PCR products of calpain 1 forward 5′-AGGAACTACCCAGCTACCTT-3′: Reverse 5′-GGATCTGGTCATCTAGTTCC-3′: calpain 2 forward 5′-AGAGAAGAAGGCTGACTACC-3′: reverse 5′-CTGAAAAACTCAGCCACGAG-3′, calpain 11 forward 5′-CGTATGGTCAACCTCATGGA-3′ and GAPDH 5′-TCCACCACCCTGTTGCTGTA-3′ and reverse 5′-ACCACAGTCCATGCCATCAC-3′ The reaction was initiated at 94°C for 1 min, followed by 94°C for 30 sec, 60°C for 45 sec, and 72°C for 1 min for 30 cycles, and a final extension at 72°C for 5 min. Aliquots of the PCR products were run in a 1% agarose gel and stained with 0.1 g/ml ethidium bromide. Digital pictures were take using a photocamera NIKON (CoolPix 4500, Nikon) and the intensity of each band (number of pixels) was calculated using the software program Image J v 1.41, a public domain, Java-based image processing program developed at the National Institutes of Health (http://rsb.info.nih.gov/ij/). The values are given in arbitrary units (AU) which represents the ratio of the number of pixels between the band of interest and that of GAPDH, as a loading control.

Statistical analysis

For mean comparisons, we used analysis of variance (ANOVA). When the ANOVA 18 test showed statistical differences, the Tukey post-test was used to discriminate 19 between groups. Statistical significance was defined as $P < 0.05$ (Sokal, 1995). Statistical analyses were performed using GraphPad Prism version 5.0 for 21 Windows (GraphPad Software, San Diego, CA, www.graphpad.com).

Results

The ability of the different molecules to inhibit calpain activity was assayed in vitro using purified porcine calpain 2 and casein as substrate. Results showed that among all the synthesized molecules only three showed acceptable values of inhibition. In vitro, the most potent inhibitor was inhibitor 3 with an IC_{50} of 0.14 μM, followed by inhibitor 2 (IC_{50} of 0.24 μM) and then inhibitor 1 (IC_{50} of 0.41 μM). As a reference we used MDL28170, a widely used calpain inhibitor, which in our hands showed an IC_{50} of 0.15 μM (Fig. 1). Thus, our inhibitors showed to properly inhibit calpain activity in vitro.

Calpains and caspases are both cystein proteases, and they share many cellular substrates. In addition, caspase inhibitors like z-VAD-fmk are also able to inhibit calpain activity. We showed that inhibitors 1 and 2 did not effect caspase-3, -2, -8, or -9 activities (Fig. 2). However, we found that even though inhibitor 3 did not affect caspase-3 and caspase-2 activity, it was able to significantly increase caspase-8 and caspase-9 activity (Fig. 2, $^{*}P < 0.05$; $^{***}P < 0.001$).

Using these three synthesized calpain inhibitors, we wanted to determine whether they could also prevent processes driven by calpain activation in vivo. To this end, we chose germ cell apoptosis induced by heat stress, a process which has been suggested to induce calpain activation (Somwaru et al., 2004).

Heat stress induces apoptosis through calpain activation

First, we wanted to establish which of the previously described calpain isoforms expressed in mouse testes, is present and activated by heat stress in rat testes. We observed that heat stress (43°C for 15 min) induced a significant amount of apoptotic cells in 20-day-old rat testes 24 h after treatment (Fig. 3C). Histological examination of the samples showed many germ cells undergoing apoptosis (Fig. 3C). RT-PCR of heat-treated testes showed there was no change in calpain 1 mRNA (Fig. 3A,B). However, we detected a significant increase in calpain 2 mRNA, and concomitantly a decrease in calpain 11 mRNA (Fig. 3A,B, $^{*}P < 0.05$). Next, we asked if the increase in the levels of calpain 2 mRNA indicated a role for calpains in germ cell apoptosis. To answer this question we injected calpain inhibitors into the testes of 20-day-old rats, submitted them to heat stress and assayed apoptosis by TUNEL. Histological examination showed that there was an important reduction in the number of TUNEL-positive cells in the heated testes pre-treated with inhibitor 1 or inhibitor 2 as compared to those heated without inhibitor pre-treatment (Fig. 4A). A quantitative study indicated that a significant decrease in apoptosis was observed after inhibitor 1 treatment and to a lesser but still significant extent after inhibitor 2 treatment (Fig. 4B, $^{**}P < 0.01$; $^{***}P < 0.001$). Inhibitor 3 did not significantly reduce the number of apoptotic cells after heat stress. As a control we used

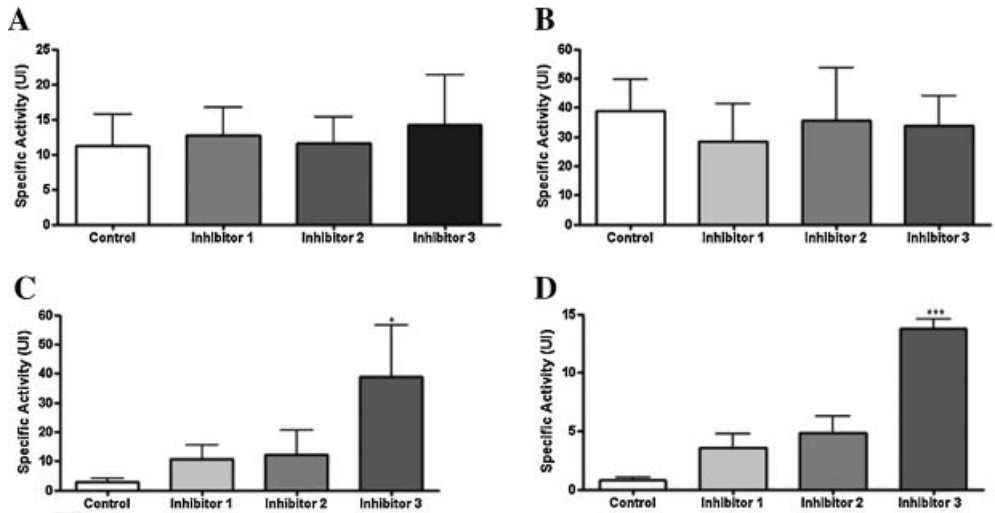

Fig. 2. Calpain inhibitors do not inhibit caspase activity. In vitro evaluation of caspase-3 (A), caspase-2 (B), caspase-8 (C), and caspase-9 (D) activity in absence (white bars) or presence of 10 μM inhibitors 1, 2, or 3. None of the inhibitors reduce the activity of caspase-3 or caspase-2, but inhibitor 3 increased the activity of caspase-8 and caspase-9. Each bar represents the mean (±SD) of three independent experiments ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$).

the well-known selective and cell-permeable calpain inhibitor MDL28170 (MDL), which significantly prevented heat-induced apoptosis after 24 h. Intratesticular injection of inhibitors alone did not have any effect in the apoptosis index as compared with controls (data not shown).

An alternative way to examine apoptosis is to quantify the number of cells in sub-G1 by flow cytometry. Results from three independent experiments showed $5.4 \pm 0.42\%$ of the cells were in the sub-G1 fraction (apoptotic cells) in 20-day-old rats without any treatment (Fig. 5A,F). After heat stress the level of apoptosis (sub-G1 cells) increased to $15.71 \pm 0.72\%$, which was significantly higher than controls (Fig. 5F, $^{***}P < 0.001$). It was also evident that there was a reduction in the number of germ cells in G0/G1 in the heated testes, as compared to non-treated animals. Inhibitors 1 and 2 significantly prevented the increase in sub-G0/G1 cells after heat treatment ($6.48 \pm 0.28\%$ and $8.78 \pm 0.85\%$, respectively), a decrease to levels similar to those measured in non-treated animals. Concomitantly, there was an increase in the percentage of cells in G1/G0, as compared to temperature-treated rats, reaching values similar to control animals, in testes treated with inhibitors 1 and 2 (Fig. 5B,C,F, $^{***}P < 0.01$). Interestingly, control testes showed a significantly smaller percentage of cells in S phase compared to temperature-treated, regardless to pre-incubation with calpain inhibitors (Fig. 5F). Inhibitor 3 did not show any effect on the number of cells in sub-G0/G1 or G0/G1 compared to heat-treated animals. Inhibitor 2 and inhibitor 3 also promoted a decrease in the percentage of cells in G2/M phase, as compared to control or temperature-treated rats (Fig. 5F, $^{**}P < 0.01$). Thus, two independent techniques, histology (TUNEL) and flow cytometry, showed similar results: inhibitors 1 and 2, but not inhibitor 3, significantly reduced apoptosis after heat stress in germ cells.

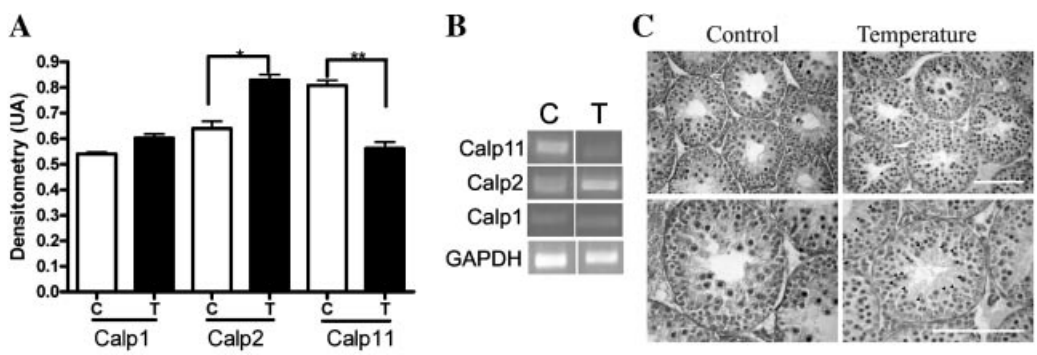

Fig. 3. Calpain 2 increases after heat-induced apoptosis. A: The graph shows the levels of each calpain isoform mRNA of control (white bars) and treated testes (black bars) using as a reference the level of GAPDH. Each bar represents the quantification of three independent experiments (mean ± SD) $^{*}P < 0.05$, $^{**}P < 0.01$. B: The levels of calpain 1 (Calp1), calpain 2 (Calp2), and calpain 11 (Calp11) were evaluated by RT-PCR in control (C) and heat-treated (T) testes 24 h after treatment (43°C, 15 min). C: Histological sections (PAS-hematoxylin) of control and heat-treated seminiferous tubuli. The increase in apoptotic cells is clearly observed in the treated testis (black arrowheads). The lower row is a magnification of the upper row. Bar is 100 μm.

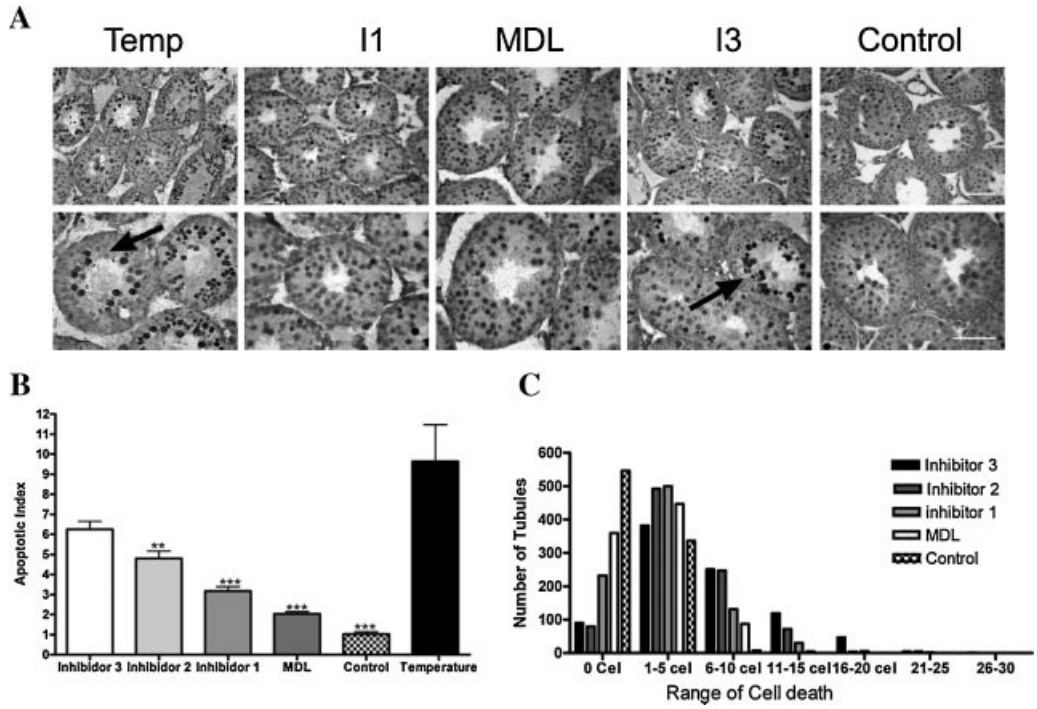

Fig. 4. Calpain inhibitors prevent heat-induced apoptosis. Testes were injected with a solution containing inhibitor 1 (I1), inhibitor 2 (I2), inhibitor 3 (I3) or the general calpain inhibitor MDL28170 (MDL), then subjected to a heat shock and apoptosis evaluated 24 h later. A: Histological examination of TUNEL staining at low (upper row) and high magnification (lower row) showed that both MDL and inhibitor 1 showed a high capacity of prevention in the formation of apoptotic cells. Dark dots show TUNEL-positive (apoptotic) cells (arrows). B: Quantification of the histological sections; inhibitor 1, inhibitor 2 and MDL, but not inhibitor 3, showed a significant reduction in the apoptotic index as compared to controls ($^{**}P < 0.01$; $^{***}P < 0.001$). C: The graph shows the frequency of tubuli with different numbers of apoptotic cells (TUNEL-positive) in control and treated animals. Bar is 100 μm.

Mammalian testes have different cell populations with different amounts of DNA, among which are haploid spermatids. Since these cells only have half the amount of DNA of other cell types, we wondered if they could account for the sub-G0 population observed in control animals. We isolated both pachytene (meiotic cells) and haploid spermatids and analyzed them by flow cytometry. Results showed that purified pachytene cells present a homogeneous population (Fig. 5E). On the contrary, purified haploid cells showed a single sharp peak in a region different from sub-G0 cells (compare Fig. 5D and A). Therefore, the events observed in control and treated cells indeed correspond to sub-G0 cells, probably both pachytene and spermatogonia in apoptosis.

These results suggest that heat stress induces an increase in calpain 2 mRNA levels and that calpain inhibitors could prevent the apoptosis observed after this stimulus in 20-day-old rats.

Involvement of p38 MAPK in heat stress-induced germ cell death

Next we wanted to determine the possible relationship between calpain activation and p38 MAPK, another important response element during cell stress. Activation of p38 MAPK in the testis was assessed by immunohistochemistry and Western blot using a phosphospecific antibody, which detects the protein only when both threonine-180 and tyrosine-182 are phosphorylated (p-p38). P-p38 was detected in controls and heated testes after 24 h (Fig. 6B,C,E). Heat stress triggered significant germ cell apoptosis along with an increase in the levels of p-p38 6 h after treatment (Fig. 6A,B). Assays at earlier times did not show an increase in p-p38 (data not shown). Immunohistochemistry for p-p38 showed that in control testes from 21-day-old rats it was localized mainly in pachytene spermatocytes and some Sertoli cells (Fig. 6C). Heat stress promoted p-p38 re-localization, and 6 h after treatment, the label was clearly shown on some spermatogonia and luminal pachytene spermatocytes (Fig. 6D). Apoptotic (picnotic) cells showed a strong label of p-p38 24 h after treatment (Fig. 6E, arrows). In order to determine the possible role of p-p38 in heat-stress-induced germ cell apoptosis, we evaluated the effect of pharmacological inhibition of this kinase. Results showed that the inhibitor PD169316 (1 μM) significantly prevented heat-induced apoptosis (Fig. 6A, $^{**}P < 0.01$), and the label pattern of p-p38 in seminiferous tubuli was similar to that observed in controls (Fig. 6F, compare with C). In addition, a double labeling study showed that most of the p-p38 positive cells were TUNEL-positive cells, strongly suggesting that the increased in active p38 related to apoptosis rather than other cell process (Fig. 7, arrows). Thus, these results suggest that p-p38 is associated with heat-stress induced apoptosis (43°C for 15 min), and that germ cell death is prevented, at least partly, by the inhibition of p38 MAPK.

Finally, we wondered if there was a functional relationship between p38 MAPK activation and calpain activity. To this end, we induced apoptosis by heat stress in rat testes previously injected with calpain inhibitor 1 or 2 and then we assayed the activation of p38 MAPK. Results showed that the number of apoptotic cells positive for p-p38 increased significantly after heat stress (Fig. 8A,B, $^{*}P < 0.05$). However, an intratesticular injection of 10 μM of either inhibitor 1 or inhibitor 2 significantly prevented this increase in the number of apoptotic cells positive for p-p38 (Fig. 8A,B, $^{*}P < 0.05$). This result correlated with the prevention of the increase in the level

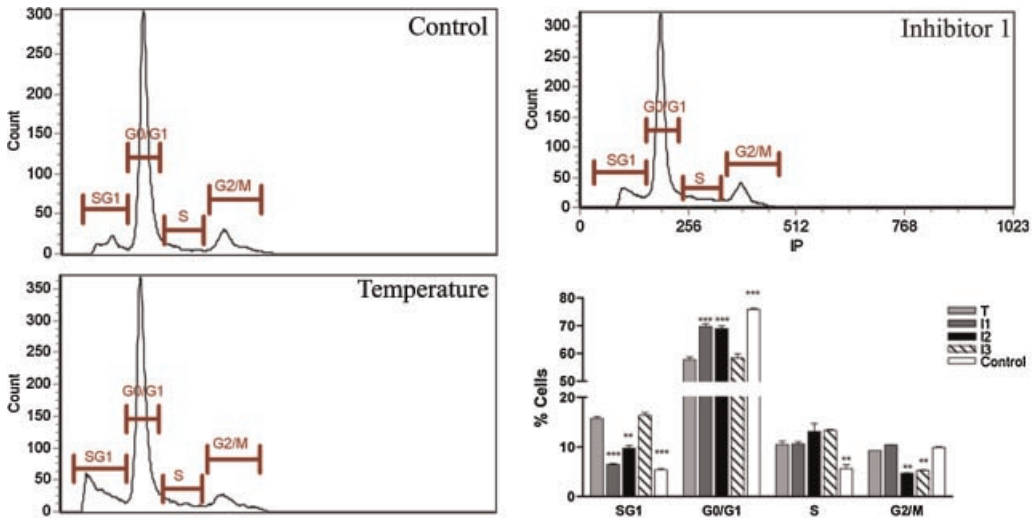

Fig. 5. Heat-induced apoptosis evaluated by flow cytometry. The histograms show testicular cells of controls, heat-treatment (temperature) or heat-treatment in the presence of calpain inhibitor (inhibitor 1), distributed according to cell cycle stage (G1/G0, S, and G2/M). Note that temperature treated-cells show a significant increase in cells in the sub-G1 stage, suggesting they are in apoptosis. D: The percentages of cells in the different stages of the cell cycle and of cells in apoptosis are shown in graph. The inhibitor-treated bars were compared to heat stress bars (n = 3, $^{**}P < 0.05$, $^{***}P < 0.01$).

of p-p38 observed 6 h after heat treatment, which was significantly prevented by inhibitor 1 or inhibitor 2 (Fig. 8 C).

Therefore, these results show that germ cell apoptosis induced by heat stress is mediated by p-p38, a downstream effector of calpain activation.

Discussion

Calpains in heat-induced male germ cell death

Much of the recent attention to the role of calpains in apoptosis has been due to the cross-talk between the caspase and calpain cascade (Gomez-Vicente et al., 2005). Conflicting roles for calpain activity in the stimulation and suppression of apoptosis have been proposed. Calpain activity has been previously shown to play a pro-apoptotic role via activation of caspases-3 and -12 and the cleavage of the pro-apoptotic Bax and Bid proteins (Wood et al., 1998; Wood and Newcomb, 2000; Mandic et al., 2002). On the other hand, calpain mediated cleavage of p53 and caspases-7 and -9 have been found to suppress apoptosis (Atencio et al., 2000; Chua et al., 2000). To complicate matters further, calpains have recently been demonstrated to mediate the activation of p53 and consequent

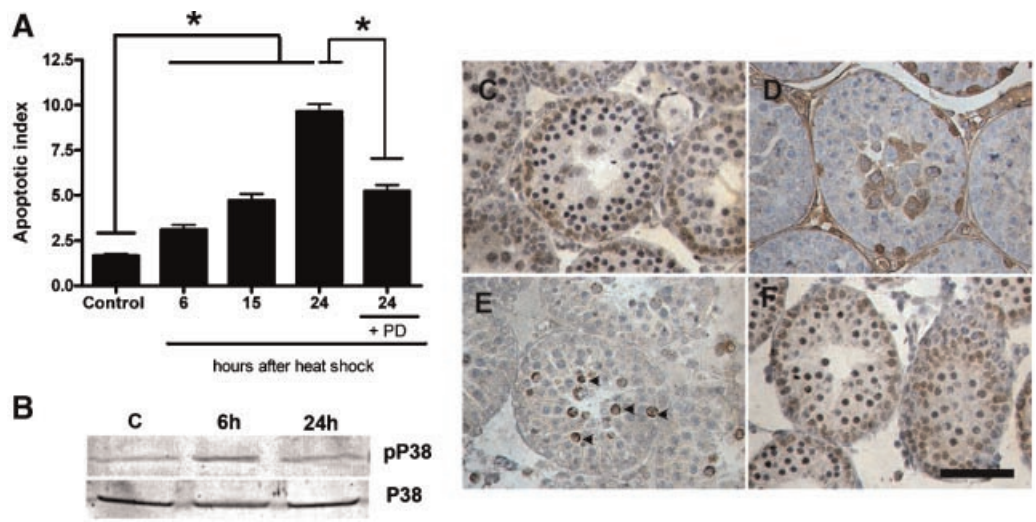

Fig. 6. p38 MAPK participates in heat stress-induced apoptosis. A: Apoptosis, as evaluated by the mean of apoptotic germ cells per seminiferous tubuli (±SD) increases steadily starting 6 h after treatment. Apoptosis is prevented in the presence of an inhibitor of p38 MAPK (1 μM PD169316), $^{*}P < 0.05$. B: Western blot of p-p38 MAPK and p38 MAPK in (C) Immunohistochemistry of p-p38 in control testis o 6 (D) and 24 (E) h after heat shock. F: p-p38 MAPK immunohistochemistry in heated testis injected with the p38 MAPK inhibitor. The arrows show apoptotic cells stained with the antibody against p-p38 MAPK. Bar 100 μm.

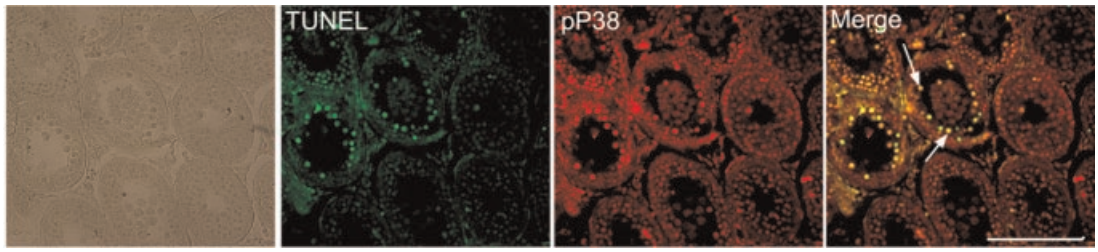

Fig. 7. Co-localization of TUNEL and p-p38 expression after heat-stress. A: Light microscopy, TUNEL, and p-p38 were detected in the same paraffin-embedded cross tissue section of 21-day-old rats 6 h after heat stress. Merge image showing that most of the TUNEL germ cells have a p-p38 signal at the plasma membrane (arrows). Bar 100 μm.

apoptotic cell death in mouse neuronal cells following DNA damage (Sedarous et al., 2003). In this work we have shown that heat stress induces the expression of calpain 2 mRNA, suggesting an important role of this isoform in this cell death model. A logical explanation for these results is that calpain 2 might have a role in the heat-stress response. This explanation is further supported by the finding that heat-stress induced germ cell death is prevented by calpain inhibitors. We showed that inhibitors 1 and 2 prevented the apoptosis induced by heat stress in 21-day-old rats. We have showed this effect by using two different experimental approaches: TUNEL in histological sections and quantifying the percentage of sub-G0/G1 population. In mitotic cells, cell cycle analysis is a useful tool to test whether cells undergo cell death by measuring sub-G0/G1 group. However, again, testicular germ cells are not single-type cells. The peak before G1 could just represent spermatids with hyper-condensed DNA in late stages. However, previous work using flow cytometry and histology have shown that haploid round spermatids are first detected in 24–25 days old rats. Since we used 21-day-old rats in our experiments, it is very unlikely that the sub-G0/G1 peak was due to haploid cells (Simorangkir et al., 1997; Malkov et al., 1998).

In this work we showed that following heat stress there is an increase in the expression of calpain 2 and a decrease of calpain 1 mRNA, while the mRNA of calpain 1 remains unchanged. In this context we hypothesized that probably calpain 1 or

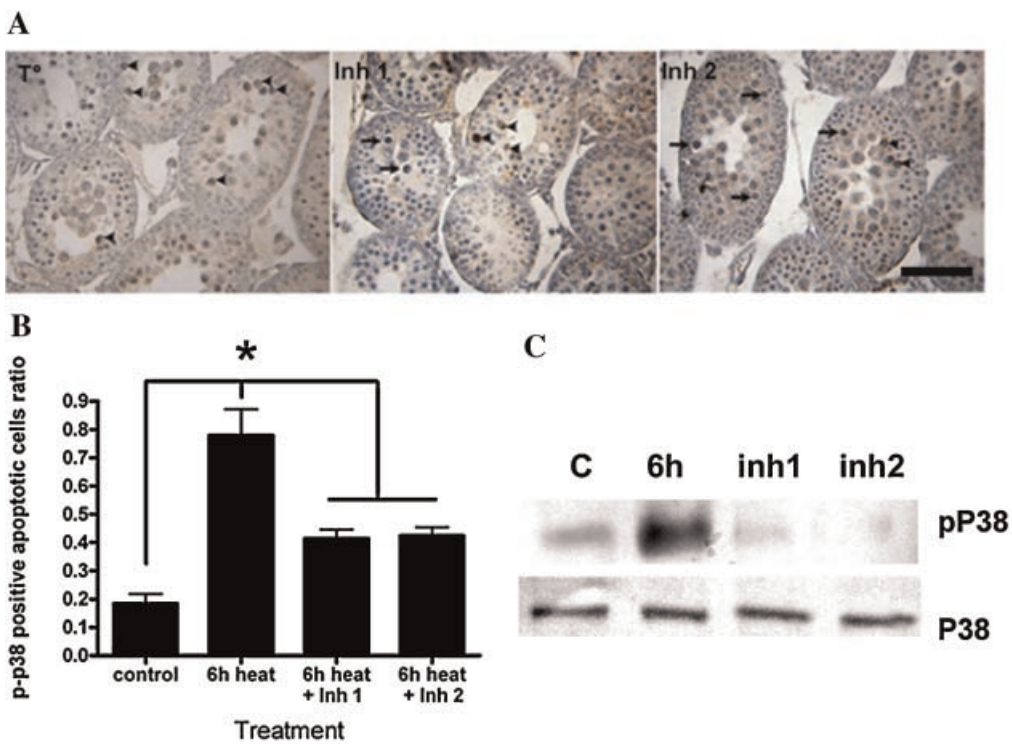

Fig. 8. Calpain inhibitors 1 and 2 prevent p38 MAPK activation after heat stress. A: Immunohistochemistry of p-p38 in testis 6 h after a heat shock (T). The arrows show positive cells stained with an antibody against p-p38. Pre-incubation with inhibitor 1 or inhibitor 2 shows that the number of labeled cells is similar to control (0 h). B: The number of apoptotic cells showing p-p38 labeling increases significantly after heat stress, which is prevented in rats pretreated with calpain inhibitor 1 or inhibitor 2, $^{*}P < 0.05$. The data in each bar was obtained by counting a total of 150 apoptotic cells from three different rats. C: The increase in phosphorylation of p38 (p-p38) induced 6 h after heat shock is prevented by inhibitor 1 or inhibitor 2 treatment. Bar 100 μm.

calpain 11 might have roles different from apoptosis in testis biology. For example, according to the expression pattern of calpain 11 during spermatogenesis and its localization in spermatozoa, it has been suggested that it functions in regulating calcium-dependent signal transduction events during meiosis and sperm functional processes (Ben-Aharon et al., 2005, 2006). A possible explanation for calpain 11 mRNA decrease upon heat stress is that the cell types expressing this mRNA (meiotic spermatocytes) are those undergoing apoptosis, and that this observation is an indirect effect of germ cell demise. Another explanation is that heat stress promotes degradation of the calpain 11 mRNA, without a significant increase in its translation rate (Ben-Aharon et al., 2005, 2006). On the other hand, calpain 1 has been observed to participate in sperm capacitation, and has also been found to have high expression levels in spermatocytes entering meiosis (Aoyama et al., 2001). Thus, it is possible that calpain 1 has functions related to signaling events in differentiation/fertilization, rather than apoptosis (Aoyama et al., 2001; Ben-Aharon et al., 2005).

We used new synthetic calpain inhibitors (Fig. 1) that do not inhibit caspases activity in vitro. Interestingly, the inhibitor with the highest in vitro activity (inhibitor 3) was not the most potent under in vivo conditions. This inhibitor was less efficacious than inhibitors 1, 2 and MDL in vitro, and is predicted to have lower cell permeability. Inhibitor 3 is larger than inhibitor 1, but blood–brain estimated distribution is lower, suggesting that the volume occupied by this molecule probably renders it more difficult to pass the more size restrictive hemato-testicular barriers. Similar results were reported for a series of calpain inhibitors structurally related to those used in this study, which have been shown to induce apoptosis in cancer cells by enhancing p53-dependent apoptosis or by caspase-3 activation (Guan et al., 2006). The calpain inhibitors used in this study are not thought to inhibit caspases, because these proteins are characterized by almost absolute specificity for aspartic acid in the P_1 position (Cohen, 1997; Denault and Salvesen, 2002). In addition, we have shown that inhibitors 1 and 2 do not inhibit caspases-3, -2, -8, and -9 activity, suggesting effects were indeed due to the inhibition of calpain activity. It was intriguing that caspase-8 and -9 activities increased in the presence of inhibitor 3, consistent with its lack of apoptosis inhibition after heat stress, and the fact that caspases 8 and 9 are direct substrates of calpain 2 (Chua et al., 2000). Although IC_{50} values evaluate intrinsic inhibitory potency, the effect in whole cell systems is determined by many other factors such as drug stability, cellular penetration, or in vivo metabolism. It is also possible that this inhibitor may have some non-specific activities or that it acts as both an inhibitor of calpain and an activator of caspases. In fact, it has been shown that caspases behave as allosteric enzymes and it is possible that this inhibitor interacts with this site in caspases (Hardy et al., 2004).

In summary, our results show that heat stress induces germ cell apoptosis driven by both caspase (previous reports) and calpain activity.

Involvement of p38 MAPK in heat-stress-induced apoptosis in male germ cells

Heat is a stress injury that induces the activation of several genes in different cell types. Heat induces a significant increase in male germ cell apoptosis both in adult and pubertal rats and mice. It has been previously shown in adult rats that stages I–V and XII–XIV of spermatogenesis are the most susceptible to this injury (Lue et al., 1999, 2000). We have shown here that p38 MAPK is an early response element, since its activated form was significantly increased 6 h after the stimulus. We did not detect any obvious increase in p38 activation at earlier time points (data not shown), as has been shown in adult rats (Jia et al., 2009; Johnson et al., 2008). It is possible that this discrepancy is related to the response of other cell types present in the adult organ, such as round and elongating spermatids. We observed a striking increase in the label of p-p38 in both spermatogonia and pachytene spermatocytes, suggesting that these two cell types are susceptible to heat injury. This is different to that reported in adult testes, where only pachytene spermatocytes seem to be susceptible to activation of the apoptosis program (Jia et al., 2009; Johnson et al., 2008). Thus, these data suggest a difference in response to heat stress in the pubertal and adult testis. Activation of p38 MAPK has also been reported to be present in germ cell apoptosis induced by antagonist of GnRH treatment and testosterone deprivation of human seminiferous tubules (Vera et al., 2006); two stimuli clearly related to stress signaling. We have shown here that only a small percentage of germ cell undergoing apoptosis have detectable levels of p-p38 in control testis (Fig. 7B). In contrast, after heat stress most of the apoptotic germ cells have high levels p-p38. Thus, after, heat stress almost all apoptotic germ cells express p-p38, which clearly contrast with the situation in control and calpain-treated testis (Fig. 7B). This interesting finding could be explain in term of different mechanisms of apoptosis elicited between physiological and heat stress condition.

In this regard, the re-location of p-p38 from a cytoplasmatic to a nuclear localization seems to be and important step in heat stress induced apoptosis. This hypothesis is supported by the observation that calpains inhibitors prevent germ cell apoptosis and p-p38 re-location. Our Western blot data clearly show an increase in p-p38 after heat stress, but according to the immunohistochemical pictures, there are more p-p38 than in heat-treated organ. This apparent contradiction may be explained because control and heated testes slides were not performed at the same time, and it took a longer time to develop the color in control than in heated testes in order to have both groups with similar staining levels. Thus, difference in the time of developing color mirror the differences in p-p38 levels between controls and heat-treated testis, as evaluated by Western blot. In this way, the immunohistochemical images give only qualitative information about the localization of p-p38.

We have shown here that calpains are one of these stress response elements in dying germ cells and that they have the ability to induce p38 MAPK activation. Calpains are proteases activated by an increase in cytosolic calcium. Our results show that two synthetic inhibitors of these enzymes are able to prevent p38 MAPK activation, which suggests that calpain activation is an early event, and closely related to p38 MAPK activation, during male germ cell apoptosis induced by a heat stress stimulus. In accordance with our results, it has been recently shown that hyperthermia-induced caspase-2 activation leads to mitogen-activated protein kinase 14 (MAPK14 or p38) activation (Jia et al., 2009). Therefore, it seems that the pathway induced by hyperthermia-caused germ cell apoptosis involves protease (probably calpain 2 and caspase-2) and then p38 MAPK activation. In this work we have shown that our calpain inhibitors did not inhibit the activity of caspases-3, -2, -8, or -9. Since the inhibition of caspase-2 prevents heat-induced apoptosis (Johnson et al., 2008), it is possible that calpain activation is an event upstream of caspase action. Alternatively, it is possible that hyperthermia triggers more than one death pathway, which may converge in p38 MAPK activation and inducible NO. This hypothesis is supported by data showing that calpains induce caspase activation and BAX processing/translocation to the mitochondria, events that have already been shown under hyperthermia conditions (Dorion and Landry, 2002; Vera et al., 2004, 2005; Jia et al., 2009; Johnson et al., 2008). However, it remains to be shown whether heat stress induces calpain-mediated BAX and caspase activation.

Thus, our results strongly suggest the participation of calpains, likely calpain 2, as a key element in heat stress-induced apoptosis in mammalian germ cell apoptosis.

Acknowledgments

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Literature Cited

Abraham MH. 1993. Scales of solute hydrogen-bonding: Their construction and application to physicochemical and biochemical processes. Chem Soc Rev 22:73–83.

Aoyama T, Ozaki Y, Aoki K, Kunimatsu M, Tada T, Sasaki M, Suzumori K. 2001. Involvement of mu-calpain in human sperm capacitation for fertilization. Am J Reprod Immunol 45:12–20.

Ashwell JD. 2006. The many paths to p38 mitogen-activated protein kinase activation in the immune system. Nat Rev Immunol 6:532–540.

Atencio IA, Ramachandra M, Shabram P, Demers GW. 2000. Calpain inhibitor I activates p53-dependent apoptosis in tumor cell lines. Cell Growth Differ 11:247–253.

Ben-Aharon I, Brown PR, Etkovitz N, Eddy EM, Shalgi R. 2005. The expression of calpain 1 and calpain 2 in spermatogenic cells and spermatozoa of the mouse. Reproduction 129:435–442.

Ben-Aharon I, Brown PR, Shalgi R, Eddy EM. 2006. Calpain 11 is unique to mouse spermatogenic cells. Mol Reprod Dev 73:767–773.

Benz G. 1991. In: Trost BM, Flemming I, editor. Synthesis of amide and related compounds comprehensive organic synthesis. Oxford, UK: Pergamon Press. pp 381–417.

Billig H, Furuta I, Rivier C, Tapanainen J, Parvinen M, Hsueh AJ. 1995. Apoptosis in testis germ cells: Developmental changes in gonadotropin dependence and localization to selective tubule stages. Endocrinology 136:5–12.

Blake JA, Richardson JE, Bult CJ, Kadin JA, Eppig JT. 2003. MGD: The mouse genome database. Nucleic Acids Res 31:193–195.

Blanco-Rodriguez J, Martinez-Garcia C. 1996a. Induction of apoptotic cell death in the seminiferous tubule of the adult rat testis: Assessment of the germ cell types that exhibit the ability to enter apoptosis after hormone suppression by oestradiol treatment. Int J Androl 19:237–247.

Blanco-Rodriguez J, Martinez-Garcia C. 1996b. Spontaneous germ cell death in the testis of the adult rat takes the form of apoptosis: Re-evaluation of cell types that exhibit the ability to die during spermatogenesis. Cell Prolif 29:13–31.

Chua BT, Guo K, Li P. 2000. Direct cleavage by the calcium-activated protease calpain can lead to inactivation of caspases. J Biol Chem 275:5131–5135.

Codelia VA, Cisternas P, Moreno RD. 2008. Relevance of caspase activity during apoptosis in pubertal rat spermatogenesis. Mol Reprod Dev 75:881–889.

Cohen GM. 1997. Caspases: The executioners of apoptosis. Biochem J 326:1–16.

Denault JB, Salvesen GS. 2002. Caspases: Keys in the ignition of cell death. Chem Rev 102:4489–4500.

Ding HF, Lin YL, McGill G, Juo P, Zhu H, Blenis J, Yuan J, Fisher DE. 2000. Essential role for caspase-8 in transcription-independent apoptosis triggered by p53. J Biol Chem 275:38905–38911.

Dorion S, Landry J. 2002. Activation of the mitogen-activated protein kinase pathways by heat shock. Cell Stress Chaperones 7:200–206.

Gomez-Vicente V, Donovan M, Cotter TG. 2005. Multiple death pathways in retina-derived 661W cells following growth factor deprivation: Crosstalk between caspases and calpains. Cell Death Differ 12:796–804.

Guan N, Korukonda R, Hurh E, Schmittgen TD, Donkor IO, Dalton JT. 2006. Apoptosis induced by novel aldehyde calpain inhibitors in human tumor cell lines. Int J Oncol 29:655–663.

Gutte B. 1995. Peptide: Synthesis, structures and applications. London, UK: Academic Press. pp 39–91.

Hardy JA, Lam J, Nguyen JT, O'Brien T, Wells JA. 2004. Discovery of an allosteric site in the caspases. Proc Natl Acad Sci USA 101:12461–12466.

Henriksen K, Kangasniemi M, Parvinen M, Kaipia A, Hakovirta H. 1996. In vitro, follicle-stimulating hormone prevents apoptosis and stimulates deoxyribonucleic acid synthesis in the rat seminiferous epithelium in a stage-specific fashion. Endocrinology 137:2141–2149.

Inoue J, Nakamura M, Cui YS, Sakai Y, Sakai O, Hill JR, Wang KK, Yuen PW. 2003. Structure-activity relationship study and drug profile of N-(4-fluorophenylsulfonyl)-L-valyl-L-leucinal (SJA6017) as a potent calpain inhibitor. J Med Chem 46:868–871.

Jahnukainen K, Chrysis D, Hou M, Parvinen M, Eksborg S, Soder O. 2004. Increased apoptosis occurring during the first wave of spermatogenesis is stage-specific and primarily affects midpachytene spermatocytes in the rat testis. Biol Reprod 70:290–296.

Jia Y, Hikim AP, Lue YH, Swerdloff RS, Vera Y, Zhang XS, Hu ZY, Li YC, Liu YX, Wang C. 2007. Signaling pathways for germ cell death in adult cynomolgus monkeys (Macaca fascicularis) induced by mild testicular hyperthermia and exogenous testosterone treatment. Biol Reprod 77:83–92.

Jia Y, Castellanos J, Wang C, Sinha-Hikim I, Lue Y, Swerdloff RS, Sinha-Hikim AP. 2009. Mitogen-activated protein kinase signaling in male germ cell apoptosis in the rat. Biol Reprod 80: 771–780.

Johnson C, Jia Y, Wang C, Lue YH, Swerdloff RS, Zhang XS, Hu ZY, Li YC, Liu YX, Hikim AP. 2008. Role of caspase 2 in apoptotic signaling in primate and murine germ cells. Biol Reprod 79:806–814.

Lagos-Cabre R, Moreno RD. 2008. Mitotic, but not meiotic, oriented cell divisions in rat spermatogenesis. Reproduction 135:471–478.

Lizama C, Alfaro I, Reyes JG, Moreno RD. 2007. Up-regulation of CD95 (Apo-1/Fas) is associated with spermatocyte apoptosis during the first round of spermatogenesis in the rat. Apoptosis 12:499–512.

Lue YH, Hikim AP, Swerdloff RS, Im P, Taing KS, Bui T, Leung A, Wang C. 1999. Single exposure to heat induces stage-specific germ cell apoptosis in rats: Role of intratesticular testosterone on stage specificity. Endocrinology 140:1709–1717.

Lue Y, Hikim AP, Wang C, Im M, Leung A, Swerdloff RS. 2000. Testicular heat exposure enhances the suppression of spermatogenesis by testosterone in rats: The "two-hit" approach to male contraceptive development. Endocrinology 141:1414–1424.

Lue YH, Lasley BL, Laughlin LS, Swerdloff RS, Hikim AP, Leung A, Overstreet JW, Wang C. 2002. Mild testicular hyperthermia induces profound transitional spermatogenic suppression through increased germ cell apoptosis in adult cynomolgus monkeys (Macaca fascicularis). J Androl 23:799–805.

Mahadevaiah SK, Bourc'his D, de Rooij DG, Bestor TH, Turner JM, Burgoyne PS. 2008. Extensive meiotic asynapsis in mice antagonises meiotic silencing of unsynapsed chromatin and consequently disrupts meiotic sex chromosome inactivation. J Cell Biol 182:263–276.

Malkov M, Fisher Y, Don J. 1998. Developmental schedule of the postnatal rat testis determined by flow cytometry. Biol Reprod 59:84–92.

Mandic A, Viktorsson K, Strandberg L, Heiden T, Hansson J, Linder S, Shoshan MC. 2002. Calpain-mediated Bid cleavage and calpain-independent Bak modulation: Two separate pathways in cisplatin-induced apoptosis. Mol Cell Biol 22:3003–3013.

Matsumoto AM. 1996. Spermatogenesis. In: Adashi EY, Rock JA, Rosenwaks Z, editors. Reproductive endocrinology, surgery, and technology. New York: Lippincott-Raven. pp 2385.

Mittelstadt PR, Salvador JM, Fornace AJ, Jr., Ashwell JD. 2005. Activating p38 MAPK: New tricks for an old kinase. Cell Cycle 4:1189–1192.

Miura M, Sasagawa I, Suzuki Y, Nakada T, Fujii J. 2002. Apoptosis and expression of apoptosis-related genes in the mouse testis following heat exposure. Fertil Steril 77:787–793.

Moreno RD, Lizama C, Urzua N, Vergara SP, Reyes JG. 2006. Caspase activation throughout the first wave of spermatogenesis in the rat. Cell Tissue Res 325:533–540.

Pentikainen V, Dunkel L, Erkkila K. 2003. Male germ cell apoptosis. Endocr Dev 5:56–80.

Riccardi C, Nicoletti I. 2006. Analysis of apoptosis by propidium iodide staining and flow cytometry. Nat Protoc 1:1458–1461.

Rockett JC, Mapp FL, Garges JB, Luft JC, Mori C, Dix DJ. 2001. Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. Biol Reprod 65:229–239.

Rodriguez I, Ody C, Araki K, Garcia I, Vassalli P. 1997. An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J 16:2262–2270.

Rojas FJ, Moretti-Rojas I. 2000. Involvement of the calcium-specific protease, calpain, in the fertilizing capacity of human spermatozoa. Int J Androl 23:163–168.

Rojas FJ, Brush M, Moretti-Rojas I. 1999. Calpain-calpastatin: A novel, complete calcium-dependent protease system in human spermatozoa. Mol Hum Reprod 5:520–526.

Ruwanpura SM, McLachlan RI, Stanton PG, Loveland KL, Meachem SJ. 2008. Pathways involved in testicular germ cell apoptosis in immature rats after FSH suppression. J Endocrinol 197:35–43.

Saez ME, Ramirez-Lorca R, Moron FJ, Ruiz A. 2006. The therapeutic potential of the calpain family: New aspects. Drug Discov Today 11:917–923.

Schollmeyer JE. 1986. Identification of calpain II in porcine sperm. Biol Reprod 34:721–731.

Sedarous M, Keramaris E, O'Hare M, Melloni E, Slack RS, Elce JS, Greer PA, Park DS. 2003. Calpains mediate p53 activation and neuronal death evoked by DNA damage. J Biol Chem 278:26031–26038.

Shiraishi K, Naito K, Yoshida K. 2000. Inhibition of calpain but not caspase protects the testis against injury after experimental testicular torsion of rat. Biol Reprod 63:1538–1548.

Shiraishi K, Naito K, Yoshida K. 2001. Nitric oxide promotes germ cell necrosis in the delayed phase after experimental testicular torsion of rat. Biol Reprod 65:514–521.

Simorangkir DR, Wreford NG, De Kretser DM. 1997. Impaired germ cell development in the testes of immature rats with neonatal hypothyroidism. J Androl 18:186–193.

Sinha Hikim AP, Lue Y, Diaz-Romero M, Yen PH, Wang C, Swerdloff RS. 2003. Deciphering the pathways of germ cell apoptosis in the testis. J Steroid Biochem Mol Biol 85:175–182.

Sokal RR. 1995. Biometry: The principles and practice of statistic in biological research. New York: W.H. Freeman. 887 p.

Somwaru L, Li S, Doglio L, Goldberg E, Zirkin BR. 2004. Heat-induced apoptosis of mouse meiotic cells is suppressed by ectopic expression of testis-specific calpastatin. J Androl 25:506–513.

Squier MK, Miller AC, Malkinson AM, Cohen JJ. 1994. Calpain activation in apoptosis. J Cell Physiol 159:229–237.

Taylor RC, Cullen SP, Martin SJ. 2008. Apoptosis: Controlled demolition at the cellular level. Nat Rev Mol Cell Biol 9:231–241.

Vera Y, Diaz-Romero M, Rodriguez S, Lue Y, Wang C, Swerdloff RS, Sinha Hikim AP. 2004. Mitochondria-dependent pathway is involved in heat-induced male germ cell death: Lessons from mutant mice. Biol Reprod 70:1534–1540.

Vera Y, Rodriguez S, Castanares M, Lue Y, Atienza V, Wang C, Swerdloff RS, Sinha Hikim AP. 2005. Functional role of caspases in heat-induced testicular germ cell apoptosis. Biol Reprod 72:516–522.

Vera Y, Erkkila K, Wang C, Nunez C, Kyttanen S, Lue Y, Dunkel L, Swerdloff RS, Sinha Hikim AP. 2006. Involvement of p38 mitogen-activated protein kinase and inducible nitric oxide synthase in apoptotic signaling of murine and human male germ cells after hormone deprivation. Mol Endocrinol 20:1597–1609.

Weininger D. 1988. SMILES, a chemical language and information system. 1. Introduction to methodology and encoding rules. J Chem Inf Comput Sci 28:31–36.

Wood DE, Newcomb EW. 2000. Cleavage of Bax enhances its cell death function. Exp Cell Res 256:375–382.

Wood DE, Thomas A, Devi LA, Berman Y, Beavis RC, Reed JC, Newcomb EW. 1998. Bax cleavage is mediated by calpain during drug-induced apoptosis. Oncogene 17:1069–1078.

Yudin AI, Goldberg E, Robertson KR, Overstreet JW. 2000. Calpain and calpastatin are located between the plasma membrane and outer acrosomal membrane of cynomolgus macaque spermatozoa. J Androl 21:721–729.