Changes in Cytosolic Ca\(^{2+}\) Levels Regulate Bcl-xS and Bcl-xL Expression in Spermatogenic Cells during Apoptotic Death*

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Bcl-x exists in two isoforms, the anti-apoptotic form Bcl-xL and the proapoptotic form Bcl-xS. The critical balance between the two forms appears to be important for cell survival; however, it is still not clear exactly how the vital balance is maintained. Using an in vitro spermatogenic cell apoptosis model, this study provides a new insight into the possible role of Ca\(^{2+}\) in regulating the Bcl-xS and Bcl-xL expression. 2,5-Hexanedione, a metabolite of the common industrial solvent n-hexane, caused a significant increase in reactive oxygen species followed by an enhancement of intracellular Ca\(^{2+}\) through the T-type Ca\(^{2+}\) channels. Consequently to the above changes, expression of Bcl-xS increased with a concomitant drop in Bcl-xL expression, thus altering the ratio of the two proteins. Impediment of Ca\(^{2+}\) influx by using a T-type Ca\(^{2+}\) channel blocker pimozide resulted in a decrease in Bcl-xS and an increase in Bcl-xL expression. This caused prevention of mitochondrial potential loss, reduction of caspase-3 activity, inhibition of DNA fragmentation, and increase in cell survival. Alternatively, Ca\(^{2+}\) ionophores caused an increase of Bcl-xS encoding isoform over the Bcl-xL-encoding isoform. Therefore, this study proposes a role for Ca\(^{2+}\) in regulation of Bcl-xS and Bcl-xL expression and ultimately cell fate.

The testis produces mature gametes through the complex process of spermatogenesis that involves stem spermatogonial commitment to proliferation and differentiation, modulated by a network of endocrine and paracrine regulatory signals (1). To maintain testicular homeostasis, very active prosurvival and proapoptotic systems work together to regulate the extent of spermatogenic cell apoptosis in the quest for producing a constant supply of high quality male gametes. The study of mechanisms of spermatogenic cell apoptosis has attracted considerable attention in recent years because of the awareness that environmental agents interfere with spermatogenesis (2, 3). Although Fas/FasL, Bcl-2, and Bcl-xL proteins have been implicated in spermatogenesis, the biological pathways involved are not fully understood. In particular, relatively little is known about the molecular mechanisms regulating the Bcl-xL and Bcl-xS and the outcome of alterations of these proteins in spermatogenic cells. A few reports about testicular Bcl-xL show that down-regulation of the gene after octylphenol exposure leads to cell death (18), severe defects in male germ cells during development in Bcl-x knock-out heterozygous mice (19), and reduction of Bcl-xL expression by anti-androgens, resulting in increased cell death (20). An obstacle in studying the involvement of these proteins in spermatogenic cells in vivo is the inability to create a "spermatogenic cell" only model, and therefore it is impossible to delineate any possible independent action of a death-inducing agent on these cells because of their association with other testicular somatic members.

The presence of active proapoptotic and prosurvival systems in spermatogenic cells provides an opportunity to look at spermatogenic cell death in various death induction models. Previous studies from this laboratory on spermatogenic cell apoptosis using in vitro and in vivo models showed the involvement of the Fas/FasL pathway in estrogen-induced cell death (21, 22). In this study, we have employed a well-proven in vitro spermatogenic cell apoptosis model, this study provides a new understanding of the role of Ca\(^{2+}\) in spermatogenesis.
known testicular toxin 2,5-hexanediol (2,5-HD), 2 which is a metabolite of the common industrial solvents n-hexane and methyl n-butyl ketone. Chronic exposure to 2,5-HD induces peripheral polyneuropathy and testicular injury (23). Using this toxin to induce spermatogenic cell apoptosis, we provide a new insight of a close relationship between Bcl-xS and Bcl-xL with intracellular Ca$^{2+}$, where we demonstrate that increased intracellular Ca$^{2+}$ caused by exposure to 2,5-HD results in a shift toward a relative increase of Bcl-xS encoding isoform over the Bcl-xL isoform. Because the level of Bcl-2 and Bax remained unchanged after 2,5-HD exposure, our results suggest that the effect of 2,5-HD is mediated by reversal of the ratio between apoptosis-inducing and -preventing isoforms of Bcl-x. The apparent regulation of both Bcl-xS and Bcl-xL expression by increased Ca$^{2+}$ induced by either 2,5-HD exposure or Ca$^{2+}$ ionophores sheds a new light on the possible regulation of these molecules in the face of ionic changes.

**EXPERIMENTAL PROCEDURES**

**Materials**

2,5-HD was purchased from Aldrich. The apoptosis detection system was procured from Promega (Madison, WI). Primary and secondary antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Jackson Immunoresearch (West Grove, PA). For color development, Vector immunoperoxidase substrate kit for Western blots was procured from Vector Laboratories Inc. (Burlingame, CA). JC-1, Fluo-3 AM, CM-H2DCFDA, Vybrant apoptosis assay kit, ATP assay kit, and EnzCheck® caspase-3 assay kit were obtained from Molecular Probes (Eugene, OR). Caspase-3 inhibitor (Z-Asp-Gln-Met-Asp-FMK), caspase-8 inhibitor (Z-Leu-Glu-Thr-Asp-FMK) and caspase-9 inhibitor (Z-Leu-Glu-His-Asp-FMK) were purchased from MP biomedicals (Irvine, CA). Taq DNA polymerase was purchased from New England Biolabs (Beverly, MA). All of the reagents used for Western blotting for ECL development were procured from Amersham Biosciences, cyclosporin A, pimozone, verapamil, benzamil, reduced glutathione, o-phthalaldehyde, and any other chemicals unless otherwise mentioned were obtained from Sigma.

**Animals**

Adult male Wistar rats (Rattus rattus) were obtained from the Small Animal Facility of the National Institute of Immunology (New Delhi). All of the experimental procedures were performed following standards specified by the Institutional Animal Ethics Committee of the National Institute of Immunology. Euthanasia of rats was carried out by carbon dioxide or Ca$^{2+}$ venting isoforms of Bcl-x. The apparent regulation of both Bcl-xS and Bcl-xL isoform. Because the level of Bcl-2 and Bax remained unchanged after 2,5-HD exposure, our results suggest that the effect of 2,5-HD is mediated by reversal of the ratio between apoptosis-inducing and -preventing isoforms of Bcl-x. The apparent regulation of both Bcl-xS and Bcl-xL expression by increased Ca$^{2+}$ induced by either 2,5-HD exposure or Ca$^{2+}$ ionophores sheds a new light on the possible regulation of these molecules in the face of ionic changes.

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**Preparation of Cells**

Spermatogenic cells were prepared as described previously (21, 22, 24). Briefly, decapsulated rat testes were finely chopped in spermatogenic cell culture medium and Ham’s F-12 Dulbecco’s modified Eagle’s medium, and the cells were subjected to serial filtration through Nitex mesh (1000 and 20 μm), Miracloth, and glass wool. The resultant suspension was centrifuged and resuspended in Ham’s F-12 Dulbecco’s modified Eagle’s medium, and flow cytometry was used to check the composition of the cell preparation as described earlier (21, 22, 24).

**Detection of DNA Fragmentation, Phosphatidylserine Exposure, and Cell Viability**

Terminal Deoxynucleotidyl Transferase Enzyme-mediated dUTP Nick End Labeling (TUNEL)—Detection of DNA fragmentation was carried out using a TUNEL assay kit as described previously (21). Formaldehyde (4%) fixed cells were permeabilized with 0.2% (v/v) Triton X-100 for 10 min at room temperature followed by incubation with buffer containing nucleotide mix (50 μM fluorescein-12-dUTP, 100 μM dATP, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 7.6) for 1 h at 37 °C. Propidium iodide staining at 1 μg ml$^{-1}$ was used for measurement of cell viability. Terminal deoxynucleotidyltransferase-labeled and propidium iodide-labeled cells were analyzed by flow cytometry.

Annexin Labeling—For visualization of annexin-V labeling, the procedure was carried out as per the manufacturer’s instructions using a Vybrant apoptosis assay kit. Briefly, the cells after the respective treatments were washed with ice-cold phosphate buffer and suspended in annexin-V binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl$_2$, pH 7.4) and incubated for 15 min with annexin-V conjugated to Alexa fluoro. Subsequently, the cells were washed and resuspended in the binding buffer and analyzed by flow cytometry.

**Flow Cytometry and Microscopy**—Cells (10$^6$ cells ml$^{-1}$) from different experimental groups were run on a BD-LSR flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 20-mV, 488 nm air-cooled argon ion laser and analyzed as described previously (21). All of the signals were detected using the following settings: for fluorescence 1 (green), a 530/28 band pass filter was used, and fluorescence was measured in the log mode; and for fluorescence 3 (red), a 575/26 band pass filter was used, and fluorescence was measured in the log mode. The analyses were performed on 100,000 gated events, and the numeric data were processed using WinMDI shareware. All of the plots are representative of three or four experiments.

After different treatments, the cells were visualized under a Nikon inverted TE-2000 microscope, and the images were captured with a CCD cooled digital camera (Media Cybernetics, Silver Spring, MD) using a Image Pro Plus® version 5.0 software (Media Cybernetics). Filter blocks used for fluorescence were comprised of a blue filter with excitation at 450–490 nm.
Measurement of Mitochondrial Membrane Potential, ROS, and Intracellular Free Ca\textsuperscript{2+}

Mitochondrial membrane potential (\(\Delta \Psi_m\)) was measured using JC-1 probe as described previously (21, 22). Briefly, the cells were labeled with JC-1 (10 \(\mu\)M), a cationic mitochondrial vital dye at 37 °C for 10 min, and the changes in fluorescence were monitored at two different wavelengths as mentioned above. The ratio of the reading at 590 nm to the reading at 530 nm (590:530 ratio) was considered as the relative \(\Delta \Psi_m\) value.

To monitor the level of ROS, the cell-permeant probe CM-H2DCFDA was used as described previously (21). 10\(^6\) cells were incubated with 2 \(\mu\)g ml\(^{-1}\) CM-H2DCFDA for 15 min in the dark; subsequently, treatments were carried out, and fluorescence was monitored at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For each experiment, fluorometric measurements were performed in triplicate and expressed as fluorescence intensity units. All of the measurements were carried out in a Fluostar Optima Spectrofluorometer (BMG Technologies, Offenburg, Germany).

Changes in intracellular Ca\textsuperscript{2+} concentration were monitored with the fluorescent probe fluo-3AM as described previously (21). Briefly, the cells were loaded for 30 min at 25 °C with 5 \(\mu\)M fluo-3AM-containing 1 \(\mu\)M pluronic acid F-127 for proper dispersal and 0.25 mM sulfipyrazone, an organic anion transport inhibitor to reduce leakage of the fluo-3 dye. Just before use, the cells were washed with medium to remove nonhydrolyzed fluo-3AM. Fluorescence measurements were performed at an excitation wavelength of 488 nm and an emission wavelength of 530 nm. For each experiment, fluorometric measurements were performed in triplicate and expressed as fluorescence intensity units. All of the measurements were carried out in a Fluostar Optima Spectrofluorometer (BMG Technologies, Offenburg, Germany).

Intracellular Free Ca\textsuperscript{2+} Measurement of Mitochondrial Membrane Potential, ROS, and Intracellular Free Ca\textsuperscript{2+}

Intracellular Free Ca\textsuperscript{2+} Measurements

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DNA Analysis

Cells (10\(^6\) cells ml\(^{-1}\)) from the control and experimental groups were mixed with 55 \(\mu\)l of Triton X-100 lysis buffer containing 0.5 mM EDTA, 1 mM Tris-HCl, pH 8.0, 0.5% Triton X-100 and incubated on ice for 20 min and subsequently centrifuged at 12,000 \(\times\) g for 30 min. The supernatant was extracted with 1:1 mixture of phenol/chloroform and precipitated in cold ethanol and sodium acetate. After RNase treatment, to each sample 5 \(\mu\)l of loading buffer was added and incubated for 30 min at 37 °C. The samples were analyzed on agarose (1.2%) gel.

Caspase-3 Assay

Caspase-3 activity in the cells was determined as per the manufacturer’s instructions using an EnzChek caspase-3 assay kit (Molecular Probes). Briefly, the cells (10\(^6\) cells ml\(^{-1}\)) after the respective treatments were washed with ice-cold phosphate buffer (pH 7.2), and the cell pellets were lysed in cell lysis buffer (10 mM Tris, 200 mM NaCl, 1 mM EDTA, 0.001% Triton X-100). Subsequently, after centrifugation, the supernatants from each sample were mixed with Z-Asp-Glu-Val-Asp-AMC substrate solution. A standard curve of AMC ranging from 0 to 100 \(\mu\)M was run with each set of samples. Control without enzyme was used in each assay to determine the background fluorescence of the substrate. As an additional control to some selected samples, 1 \(\mu\)l of the 1 mM Ac-Asp-Glu-Val-Asp-CHO (aldehyde) the caspase-3 inhibitor stock solution, was added. Fluorescence was measured at an excitation wavelength of 342 nm and an emission wavelength of 441 nm in a Fluostar Optima spectrofluorometer (BMG Technologies).

Reverse Transcription-PCR

Total RNA was isolated from untreated and untreated cells using TRIzol reagent (Invitrogen). About 2 \(\mu\)g of total RNA was used to synthesize cDNA, and 5\% of these were used in PCR. Oligo(dT)\(_{18}\) primers (0.5 \(\mu\)g/\(\mu\)l), dNTP, and SuperScript II reverse transcription (Invitrogen) were used for cDNA synthesis reaction. The specific primers are as follows: Bcl-xL (sense), 5’-GTTAATCTGGGGTGCCATTTG-3’; Bcl-xL (antisense), 5’-TGGATCCAAGGCTCTAGGTG-3’; Bcl-xS (antisense), 5’-CAGGGCATATCGACACGC-3’; Bcl-xS (sense), 5’-CAGGGGACAGCATATCAGAGC-3’.

A cycle profile consisted of 2 min at 94 °C, followed by 30 s at 94 °C for denaturation, 30 s at 60 °C for annealing, and 30 s at 72 °C for extension for Bcl-xL and Bcl-xS. For Bcl-xS, the annealing temperature was 58 °C. All of the PCR amplification reactions were carried out for 30 cycles each. For visualization, the resulting PCR products were resolved on 1.2% agarose gel and stained with ethidium bromide.
Immunochemistry

Cells (10^7) from the control and experimental groups were fixed in 2% paraformaldehyde for 15 min. Subsequently, the cells were washed two times in phosphate-buffered saline and blocked in 3% normal goat serum containing 0.1% saponin at room temperature for 30 min. The cells were then incubated with the primary antibody at the concentration of 1:100 for 1 h at 37°C followed by secondary antibody conjugated to Alexa fluor at a concentration of 1:100 for 30 min at 7°C. Subsequently the cells were washed and resuspended in phosphate-buffered saline for flow cytometry.

Statistical Analysis

Paired comparisons were conducted using a paired t test, and all of the data are presented as the means ± S.E. The differences were considered significant at a 0.05 level of confidence.

RESULTS

2,5-HD Induces Apoptosis of Spermatogenic Cells in Vitro—Previous studies describe exposure to 2,5-HD as an apoptosis-inducing event in spermatogenic cells (3). To investigate death pathways associated with 2,5-HD exposure, we used an in vitro spermatogenic cell culture system (21) consisting of both spermatocytes and spermatids, the spermatids being the most abundant cells that exist in intimate association with the spermatocytes within the seminiferous epithelium. Fig. 1A shows the cell preparation used for the study as described previously (21, 22). Exposure to 2,5-HD caused cell death over a period of 10 h with about 50% cells dying by 8 h as measured by propidium iodide staining (Fig. 1B). To identify whether this death occurred through the apoptotic pathway, several biochemical tests were carried out. Genomic DNA prepared from spermatogenic cells after 10 h of exposure showed a clear DNA ladder, a hallmark of apoptosis (25), in the 2,5-HD treated group (Fig. 1C, lane b), whereas genomic DNA from control cells did not show such a feature (Fig. 1C, lane a). Initiation of DNA fragmentation was evident earlier around 6 h after toxin treatment that was visible as TUNEL-positive cells. Fragmented ends of DNA were labeled with fluorescein isothiocyanate-tagged dUTP, and flow cytometric measurements showed a distinct shift in about 65% of cells at 6 h (Fig. 1D). Apoptosis was also identifiable by the ability of the apoptotic cells to bind to annexin-V. This is because cells undergoing apoptosis exhibit disorganization of the plasma membrane, followed by the externalization of certain phospholipids such as phosphatidylserine (26), which binds to annexin-V with high affinity, allowing the detection of annexin-V conjugated to fluorescein isothiocyanate by flow cytometry. After 3 h of exposure to the toxin, the cells showing annexin-V-positive staining were around 79% as compared with 8% in the controls (Fig. 1E). Finally, initiation of DNA fragmentation was evident from 3 h onwards (Fig. 1C, lane c). Parallel studies showed that 71% of treated cells exhibited cleavage of poly(ADP-ribose) polymerase (PARP), which is a substrate for caspase-3, and this cleavage is a hallmark for apoptosis in vitro once exposed to 2,5-HD.

Ca^{2+} Changes Occur with 2,5-HD—Given the available literature showing a close relationship between Ca^{2+} and apoptosis (27), we investigated whether Ca^{2+} changes occurred in response to 2,5-HD. Our data demonstrate that intracellular Ca^{2+} concentration increases...
approximately by five times that of controls after 2,5-HD exposure as shown by increase in cellular fluorescence in fluo-3-labeled cells (Fig. 2A). The actual increase in \( \text{Ca}^{2+} \) started at about 2 h and reached a peak around 4 h and formed a plateau thereafter. This increase could occur through either the pooling of intracellular \( \text{Ca}^{2+} \) sources or influx of \( \text{Ca}^{2+} \) from extracellular media. Chelation of extracellular \( \text{Ca}^{2+} \) with EGTA resulted in a total inhibition of \( \text{Ca}^{2+} \) increase (Fig. 2A), providing evidence that extracellular ions were the primary source and no pooling from intracellular stores was involved. This influx of \( \text{Ca}^{2+} \) from extracellular sources occurs through multiple mechanisms like voltage gated
pumps, Na\(^+\)/Ca\(^{2+}\) exchangers in reverse mode, Ca\(^{2+}\) ATPase, and non-selective cation channels. Inhibitors of voltage gated L-type Ca\(^{2+}\) channels, namely verapamil and nifedipine, were not able to block Ca\(^{2+}\) entry, whereas the presence of a T-type Ca\(^{2+}\) channel blocker pimozide during 2,5-HD treatment completely abrogated Ca\(^{2+}\) increase (Fig. 2B). Benzamil and bepridil, two Na\(^+\)/Ca\(^{2+}\) exchanger blockers, were ineffective in preventing 2,5-HD-induced Ca\(^{2+}\) entry (Fig. 2C). The above data suggested that T-type channels were the primary route of Ca\(^{2+}\) entry. The constant influx of Ca\(^{2+}\) was maintained by T-type Ca\(^{2+}\) channels as the addition of pimozide at 60 and 120 min of treatment decreased Ca\(^{2+}\) influx to near basal levels (Fig. 2D). Fig. 2E shows the presence of increased Ca\(^{2+}\) in spermatids and spermatocytes after 2,5-HD exposure (Fig. 2E, panel c) and the lack of fluo-3 labeling in cells pretreated with pimozide (Fig. 2E, panel e) that stained like controls (Fig. 2E, panel a). This part of the data essentially suggested that there was a Ca\(^{2+}\) increase from about 2 h after 2,5-HD exposure, and the primary route of Ca\(^{2+}\) entry was the T-type Ca\(^{2+}\) channel. Looking at the consequences of pimozide-induced inhibition of Ca\(^{2+}\) entry, we found that there was a significant block in 2,5-HD-induced cell death in the presence of pimozide (Fig. 2F). Therefore, the above data clearly established a link between 2,5-HD-induced cell death and increased intracellular Ca\(^{2+}\).

ROS Increases after 2,5-HD Exposure—High intracellular Ca\(^{2+}\) often causes an increase in ROS levels (28); therefore, ROS was measured by labeling the cells with the ROS-sensitive probe CM-H\(_2\)DCFDA that largely detects H\(_2\)O\(_2\) and hydroxyl radicals. A sharp increase was noted at 50 min, thereby suggesting that ROS increase occurred prior to Ca\(^{2+}\) influx (Fig. 3A). This increase gradually declined, reaching control levels by 80 min, showing a transient nature of the increase. When an antioxidant GSH (5 mM) was used, the increase in the ROS level was inhibited (Fig. 3A). If ROS elevation was responsible for Ca\(^{2+}\) increase, then a decline in ROS levels should reduce Ca\(^{2+}\) influx. GSH (5 mM) could successfully reduce Ca\(^{2+}\) increase (Fig. 3B), providing a link between ROS and Ca\(^{2+}\). These data suggested that 2,5-HD-induced Ca\(^{2+}\) increase was due to a prior increase in ROS generated by 2,5-HD.

Several observations described above indicated mitochondrial involvement; therefore, mitochondrial functions were checked. There was a decline in the \(\Delta \Psi _{m}\) as measured by the potentiometric probe JC-1 between 3 and 4 h during 2,5-HD treatment (Fig. 3C). This fall in potential could be rescued by preincubation of the cells with 20 \(\mu\)M pimozide (Fig. 3C). This suggested that increased Ca\(^{2+}\) could be the cause for a decline in \(\Delta \Psi _{m}\), because the fall in \(\Delta \Psi _{m}\) occurred after the Ca\(^{2+}\) increase. The loss of \(\Delta \Psi _{m}\) could occur through two mechanisms: through the formation of permeability transition pore or through the rupture of the outer mitochondrial membrane. We exposed the cells to cyclosporin A (5 \(\mu\)M), an inhibitor of the pore during 2,5-HD exposure. Because cyclosporin A was able to block the fall in potential (Fig. 3C), it was apparent that the mitochondrial megachannel was involved. Loss of \(\Delta \Psi _{m}\) is a reflection of mitochondrial dysfunction, and this was corroborated by a loss of ATP that occurred after 2,5-HD exposure (Fig. 3D). If ROS was involved in Ca\(^{2+}\) increase and Ca\(^{2+}\) to the fall in \(\Delta \Psi _{m}\), as established earlier, the scavenging of ROS should be able to inhibit the fall in \(\Delta \Psi _{m}\). GSH was able to reduce the loss in \(\Delta \Psi _{m}\) (Fig. 3E), thus providing a link between ROS and Ca\(^{2+}\) to \(\Delta \Psi _{m}\) loss. However, this does not rule out the possibility that ROS itself could contribute to \(\Delta \Psi _{m}\) loss. Fig. 3F shows the staining of spermatogenic cell mitochondria with the potentiometric probe JC-1, where the controls are predominantly stained red (Fig. 3F, panel b) showing normal \(\Delta \Psi _{m}\) whereas the toxin-treated cells exhibit predominantly green mitochondria, showing a loss of \(\Delta \Psi _{m}\) (Fig. 3F, panel f). The visualization of the pimozide-treated cells with predominantly red mitochondria (Fig. 3F, panel h) confirmed our data that pimozide was able to prevent the fall in \(\Delta \Psi _{m}\). The data presented in this part suggest the involvement of mitochondria in 2,5-HD-induced apoptosis.

2,5-HD-induced Cell Death Is Caspase-dependent—In caspase-dependent cell death as opposed to a caspase-independent one, the executioner caspase, caspase-3 is activated. There was a significant increase in caspase-3 activity by 4 h after 2,5-HD exposure; however, in the presence of 50 \(\mu\)M caspase-9 inhibitor (Z-Leu-Glu-His-Asp-FMK), the increase in caspase-3 activity was abrogated (Fig. 4A), confirming the involvement of the mitochondrial pathway. It was found that anti-oxidant GSH that reduced ROS levels and pimozide that restricted Ca\(^{2+}\) entry could inhibit caspase-3 activity (Fig. 4B). Caspase-3 inhibitor (Z-Asp-Glu-Met-Asp-FMK) was used as a positive control (Fig. 4B). Arguably, if the above agents were able to reduce caspase-3 activity, cell survival should increase even after 2,5-HD exposure because caspases will be inactive in the presence of the inhibitors. When cell death was checked, caspase-3 and caspase-9 inhibitors were able to reduce cell death as opposed to caspase-8 inhibitor that was unable to rescue the cells from dying (Fig. 4C). The oligonucleosomal fragmentation of DNA that occurs during apoptosis is due to activation of caspase-activated DNase (25). Both caspase-3 and caspase-9 inhibitor and pimozide were able to reduce oligonucleosomal fragmentation of DNA (Fig. 4D) essentially showing that inhibition of caspase-3 activity brought about by the caspase inhibitors and pimozide reduced DNA cleavage through inhibition of caspase-3 activity.

Expressions of Bcl-xL and Bcl-xS Proteins Change on 2,5-HD Exposure—Because Fas/Fasl have been reported to be intimately associated with spermatogenic cell apoptosis, we checked the levels of these two proteins after treatment. There was no change in Fas/Fasl after 2,5-HD treatment, suggesting that the Fas/Fasl pathway was not involved in the current model (Fig. 5A). Because mitochondria appeared to be involved in 2,5-HD-induced apoptosis, the profile of pro- and anti-apoptotic proteins of the Bcl-2 family were checked because mitochondria is the site of function of these proteins. There was no change in Bcl-2 and its antagonist Bax protein after treatment with 2,5-HD (Fig. 5B). Alternatively, two other Bcl-2 family members, Bcl-xS and Bcl-xL could be involved; therefore, the levels of these two proteins were checked, and after 2 h of 2,5-HD exposure there was a decline in Bcl-xL protein and an increase in Bcl-xS protein, indicating a change in ratio (Fig. 5B). In a number of systems, a change in the Bcl-xS and Bcl-xL ratio has been implicated in cell death (10, 13). If such was the case in spermatogenic cells and if this ratio change is prevented, arguably cells should not undergo apoptosis. EGTA and pimozide that interfere with intracellular Ca\(^{2+}\) increase caused a distinct down-regulation of Bcl-xS when given along with 2,5-HD (Fig. 5C). The relative expression levels of Bcl-xL and Bcl-xS as measured by densitometry is shown in Fig. 5 (D and E). The ratio of Bcl-xS/Bcl-xL is shown in Table 1, where a distinct increase of the ratio of Bcl-xS to Bcl-xL is seen in the 2,5-HD-treated groups as compared with controls or 2,5-HD treatment in the presence of pimozide.

Increase in Cellular Ca\(^{2+}\) Induces Bcl-xS Expression—Because 2,5-HD induced a Ca\(^{2+}\) increase followed by Bcl-xS increase, we tried to investigate whether cellular Ca\(^{2+}\) increase brought about by ionophores would lead to such changes. Expressions of Bcl-xL and Bcl-xS protein were checked by analyzing cells stained for the proteins by flow cytometry after inducing an increase of intracellular Ca\(^{2+}\) with Ca\(^{2+}\) ionophores. We used two Ca\(^{2+}\) ionophores, ionomycin and A23187, to raise cellular Ca\(^{2+}\) levels and followed the Bcl-xS and Bcl-xL changes. The data show a distinct increase in cells staining for Bcl-xS in the 2,5-HD
group that was effectively reduced in the presence of pimozide (20 μM) (Fig. 6A, panel a). In the presence of elevated intracellular Ca\(^{2+}\) brought about by A23187, Bcl-xS expression was increased (Fig. 6A, panel b), but Bcl-xL expression was reduced (Fig. 6A, panel c). Similarly, exposure of the cells to ionomycin (1 μM) increased Bcl-xS staining (Fig. 6A, panel d) but reduced Bcl-xL staining (Fig. 6A, panel e). Reverse transcription-PCR performed on RNA isolated from the cells of the various treatment groups showed that 2,5-HD treatment increased the level of Bcl-x mRNA (Fig. 6B). Fig. 6 (C and D) shows the expressions of alternatively spliced products of Bcl-x, the Bcl-xL and the Bcl-xS, respectively. A reduction of Bcl-xL mRNA expression after 2,5-HD exposure in comparison with controls was observed (Fig. 6C). However, when pimozide was present during treatment, this reduction was not detected (Fig. 6C). The presence of ionomycin (1 μM) and A23187 (100 nM) showed a decline in Bcl-xL expression as compared with the controls (Fig. 6C). Bcl-xS expression increased after 2,5-HD exposure in comparison with the group where pimozide was present (Fig. 6D). The presence of ionomycin (1 μM) and A23187 (100 nM) showed a distinct increase in Bcl-xS expression (Fig. 6D), demonstrating a relationship between elevated Ca\(^{2+}\) and increased Bcl-xS expression.

**DISCUSSION**

The purpose of this study was to understand the molecular mechanisms of toxin-induced apoptosis of spermatogenic cells where 2,5-HD,
a n-hexane metabolite, was used as a model toxin. The new finding emerging from the use of this model of 2,5-HD-induced spermatogenic cell apoptosis is the close relationship between modulation of intracellular Ca\(^{2+}\) and alterations in the ratio of pro- and anti-apoptotic proteins Bcl-xS and Bcl-xL. Synthesis of the data show that (i) 2,5-HD was able to induce apoptotic death in spermatogenic cells in the absence of any other testicular cells; (ii) ROS generation occurred in response to 2,5-HD; (iii) there was an increase in intracellular Ca\(^{2+}\); (iv) an increase in Bcl-xS expression with a corresponding decline in Bcl-xL expression occurred; (v) Ca\(^{2+}\) ionophores induced similar changes as that of 2,5-HD; (vi) 2,5-HD interfered with mitochondrial function; and (vii) the death induced was caspase-dependent.

Through the evolution of multicellular organisms, the Ca\(^{2+}\) ion has emerged as a crucial intracellular signaling molecule. Not only does this ion facilitate signaling, under circumstances of cellular stress it initiates the cell death process by a variety of mechanisms (27). In the present study where we have used 2,5-HD as a model toxin for studying spermatogenic cell apoptosis, Ca\(^{2+}\) seems to be pivotal in steering the cell toward death. This in vitro model was ideal for the study because it contained spermatocytes and spermatids in the ratio at which they would be in close association in the seminiferous epithelium (29, 30).

The model of 2,5-HD-induced death satisfies the criteria of apoptotic phenotype because cells under 2,5-HD treatment showed internucleosomal DNA degradation, PARP cleavage, cytochrome c release in the cytosol, and phosphatidylserine exposure. Biochemical changes including caspase-3 activation and mitochondrial potential loss were also evidence for an operative apoptotic pathway.

Spermatogenic cell death in response to a variety of agents and during development involve the Fas/FasL pathway (4, 5, 21, 22). In contrast, in this model, there was no change in the level of constitutive Fas or FasL during the toxin treatment, suggesting a Fas-independent death pathway being triggered. This was further confirmed by the inability of caspase-8 inhibitor to prevent cell death because the Fas/FasL-initiated pathway would involve caspase-8. A role of caspase-9 was evident from the ability of caspase-9 inhibitor to reduce caspase-3 activity, DNA fragmentation, and cell death. Caspase-9 involvement is a post-mitochondrial event; therefore, it was apparent that 2,5-HD-induced cell death occurred through the mitochondrial pathway. This was further confirmed by the release of cytochrome c in the cytosol. To further illuminate the mechanistic imperatives of the operative mitochondrial pathway, the profile of the Bcl-2 family of proteins was checked because Bcl-2 is known to prevent apoptosis induced by a wide range of agents.
suggesting that multiple pathways to cell death converge in a step that can be regulated by Bcl-2. A number of studies report the involvement of Bax, Bcl-2, and Bcl-w proteins during spermatogenesis (7). Regulated spatial and temporal expression of Bcl-w is required for normal testicular development and continued spermatogenesis. Bax-deficient mature male mice demonstrate increased cell death and dramatic tes-

**FIGURE 5.** Bcl-xS and Bcl-xL but not Fas/FasL are involved in 2,5-HD-induced apoptosis. A, no change in Fas/FasL staining of Western blots of cell lysates probed with anti-Fas and anti-FasL antibody. B, Western blots of cells treated with 2,5-HD at different time points probed with anti-Bcl-2, anti-Bax, anti-Bcl-xL, and anti-Bcl-xS antibodies shows no change in Bcl-2 and Bax profile but a distinct down-regulation of Bcl-xL and up-regulation of Bcl-xS. The data are representative of at least three independent experiments. C, changes in expression of Bcl-xS and Bcl-xL in the presence of 2,5-HD, 2,5-HD + EGTA, and 2,5-HD + pimozide (10 and 20 μM). The data are representative of at least three independent experiments. Note the decrease in Bcl-xS staining in the presence of pimozide and EGTA. D and E, graphs showing the densitometric measurements of Western blots of Bcl-xS and Bcl-xL expression. The data represent the means ± S.E. (n = 3). *, p < 0.05, 2,5-HD versus control; ¶, §, and **, p < 0.05, 2,5-HD, 2,5-HD + EGTA, 2,5-HD + pimozide (10 and 20 μM) versus 2,5-HD.

**TABLE 1**

| Time  | Control | 2,5-HD | 2,5-HD + EGTA | 2,5-HD + pimozide | EGTA only |
|-------|---------|--------|--------------|------------------|-----------|
| 0 min | 0.30 ± 0.03 | 0.31 ± 0.03 | 0.30 ± 0.02 | 0.31 ± 0.01 | 0.30 ± 0.03 |
| 60 min| 0.41 ± 0.04 | 3.85 ± 0.38 | 0.33 ± 0.03 | 0.33 ± 0.02 | 0.34 ± 0.03 |
| 120 min| 0.43 ± 0.04 | 5.85 ± 0.58* | 0.33 ± 0.03 | 0.33 ± 0.02 | 0.35 ± 0.02 |
| 150 min| 0.44 ± 0.04 | 11.20 ± 1.12* | 0.35 ± 0.02 | 0.35 ± 0.03 | 0.35 ± 0.03 |

* p < 0.05 as compared with respective controls.
Bcl-xS and Bcl-xL in Spermatogenic Cell Apoptosis

**FIGURE 6. Inhibition of Ca\(^{2+}\) entry reduces Bcl-xS expression.**

A, cells stained with anti-Bcl-xL and anti-Bcl-xS antibodies followed by staining with a secondary antibody conjugated to fluorescein isothiocyanate and analyzed by flow cytometry. The data show a distinct increase in cells staining for Bcl-xS (panel a) in the 2,5-HD group that is effectively reduced in the presence of pimozide (20 \(\mu\)M) (panel c), Treatment of the cells with A23187 increased Bcl-xS expression (panel b) but reduced Bcl-xL staining (panel c). Similarly, exposure of the cells to ionomycin (1 \(\mu\)M) increased Bcl-xS staining (panel d) but reduced Bcl-xL staining (panel e). The data are representative of at least three independent experiments. B, detection of Bcl-x mRNAs. B shows increased expression of Bcl-x mRNA in 2,5-HD-treated cells. C, note the reduction of Bcl-xL expression after 2,5-HD exposure in comparison with the groups where pimozide (20 \(\mu\)M) was present or the control group. The presence of ionomycin (1 \(\mu\)M) and A23187 (100 \(\mu\)M) shows a decrease in Bcl-xL expression. D, detection of Bcl-xS mRNA by reverse transcription-PCR. Note the increase of Bcl-xS expression after 2,5-HD exposure in comparison with the groups where pimozide was present or the control group. The presence of ionomycin (1 \(\mu\)M) and A23187 (100 \(\mu\)M) shows an increase in Bcl-xS expression.

In particular, atrophy (31) as a result of disordered seminiferous tubules with an accumulation of atypical premeiotic germ cells but no mature haploid sperm (32). In this study, the absence of alterations in Bcl-2 expression in the spermatogenic cells treated with 2,5-HD made it highly unlikely that Bcl-2 was important in prolonging cell survival. Bax, the 21-kDa protein that can associate with Bcl-2 and alter its functions without any actual alterations in Bcl-2 levels (33), also remained unchanged. In this study, there was no translocation of Bax to the mitochondria (data not shown), showing that in the absence of increased expression no translocation event occurred. It therefore appeared that Bcl-2 and Bax were not the major players in 2,5-HD-induced apoptosis.

An alternative possibility of apoptosis control would be to influence the ratio of the two Bcl-x isoforms. Bcl-x is related to Bcl-2, but in many cases the patterns of Bcl-x expression are strikingly different from those reported previously for Bcl-2, suggesting that these two genes regulate cell survival differently (34). Alternative splicing generates two isoforms of Bcl-x. The large isoform Bcl-xL that contains all of the regions of homology to Bcl-2 protects against several forms of apoptosis (35); the short isoform Bcl-xS, which lacks two of the homology regions, BH1 and BH2, promotes apoptosis by counteracting Bcl-2 and Bcl-xL function (35). Therefore, the ratio between the two isoforms could decide whether a given stimulus will cause apoptosis or not. It is generally believed that the ratio of death antagonists, such as Bcl-2 and Bcl-xL, and death agonists, such as Bax and Bcl-xS, plays a major role in the fate of the cell following an apoptotic stimulus (36). Our model demonstrated an increase in Bcl-xS and a decrease in Bcl-xL isoforms, resulting in an increase in the ratio of Bcl-xS to Bcl-xL. Bcl-xS being proapoptotic, this observation suggested strongly that it was the primary initiator of cell death. Several other studies in a variety of systems have shown that an increase in the ratio of Bcl-xS and Bcl-xL is indicative of an increase in apoptotic death (13, 37, 38). It appears that although never demonstrated before, this system operates in spermatogenic cells as well.

One of the major mechanisms of Bcl-2-mediated cytoprotection is protecting mitochondrial function (36). Mitochondrial involvement was further corroborated by the loss of the mitochondrial potential and decline in ATP levels following 2,5-HD treatment. Interestingly, cyclosporin A, an inhibitor of permeability transition pore (39), could inhibit the fall in mitochondrial potential, indicating that the 2,5-HD-mediated mitochondrial effects occurred through the opening of the permeability transition pore. These data are consistent with the recognition that Bcl-xL plays a role in the maintenance of mitochondrial integrity, and consequent to a significant loss in Bcl-xL and an increase in Bcl-xS, the fall in \(\Delta \Psi_m\) occurs.

If the Bcl-xS to Bcl-xL ratio was involved in spermatogenic cell death, then reversal of the ratio should result in increased cell survival. We were able to modulate these levels by interfering with Ca\(^{2+}\) changes induced by the toxin. The significant increase in Ca\(^{2+}\) induced by the toxin and the effects consequent to lowering of the intracellular Ca\(^{2+}\) levels by T-type channel blocker pimozide clearly implicated Ca\(^{2+}\) in the cytotoxicity of the toxin. T-type channels are the most abundant channels in rodent spermatogenic cells (40–42), and under 2,5-HD exposure, they appear to be primary operators for Ca\(^{2+}\) entry. Ca\(^{2+}\) appeared to be related to the expression of Bcl-xS and Bcl-xL messenger RNA, and protein because inhibition of Ca\(^{2+}\) entry resulted in the lowering of Bcl-xS and increase in Bcl-xL message and protein. Importantly, Ca\(^{2+}\) ionophores were able to mimic 2,5-HD-induced changes, providing credence to the suggestion that high intracellular Ca\(^{2+}\) leads to an increase in Bcl-xS and lowering of Bcl-xL. Ca\(^{2+}\) alterations induced by ionomycin and chelators are known to alter Bcl-2/Bax ratio (43),...
whereas tumor necrosis factor α expression increases Ca^{2+} and suppresses Bcl-2 (15).

It is well established that there is a close relationship between accumulation of mitochondrial Ca^{2+} and opening of the mitochondrial permeability transition pore (44). Frequently, when cytosolic Ca^{2+} increases, mitochondria accumulates some of this Ca^{2+}, leading to the opening of the mitochondrial transition pore. It is possible that consequent to 2,5-DH treatment, the increase in cytosolic Ca^{2+} contributed to an elevation of mitochondrial Ca^{2+}, leading to transition pore opening. It is therefore a possibility that a decrease in Bcl-xL and an increase in Bcl-xS could be closely related to changes in mitochondrial Ca^{2+}.

Therefore, this study provides a new possibility that toxin-induced cytosolic Ca^{2+} increase in spermatogenic cells alters the Bcl-xS to Bcl-xL ratio, leading to mitochondrial changes resulting in apoptotic death. In addition, this study suggests that ion channel alterations in the cell could signal a change in alternative splicing of Bcl-x leading to up-regulation of a given isoform. Further studies are required to probe into the mechanistic details of the dependence of alternative splicing of Bcl-x on the ionic environment of the cell.

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