

Testicular Torsion Alters the Presence of Specific Proteins in the Mouse Testis as Well as the Phosphorylation Status of Specific Proteins

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ABSTRACT: Testicular torsion followed by torsion repair induces an ischemia-reperfusion injury to the testis that can render the testis aspermatogenic. Previous results have demonstrated this loss of spermatogenesis to be the result of germ cell apoptosis induced by oxidative stress. The present work reports protein changes occurring in the mouse testis 24 hours after repair of a testicular torsion known to induce germ cell apoptosis and severe seminiferous impairment. Total proteins were extracted from sham-operated testes and testes having had 2-hour 720° torsion 24 hours previously. Testicular proteins were separated by 2-dimensional electrophoresis and the resulting gel images were analyzed with image analysis software. Of the over 1100 proteins detected on the average gel, over 700 were consistently appearing in multiple gels, and those protein spot intensities were averaged within sham and torsion groups and compared between the 2 groups. Twenty-three proteins were consistently increased after torsion repair and 48 were decreased. Six proteins, 3

of which increased and 3 of which decreased after torsion repair, were identified by mass spectrometry. The 3 proteins that increased after torsion repair, β 2-tubulin and 2 isoforms of serum albumin, as well as the 3 proteins that decreased after torsion repair, vimentin, phosphoglycerate kinase, and *t*-complex protein 1 β , were for the most part associated with various aspects of cell stress responses. The number of proteins phosphorylated on tyrosine residues exceeded the number of proteins phosphorylated on serine/threonine residues, but among 6 stress-related proteins specifically examined for phosphorylation in sham testes and those examined after torsion repair, increases in threonine phosphorylation of *c*-Jun NH₂ terminal kinase and activating transcription factor 2 were the most prominent. Knowing these proteins and the pathways to which they point will aid in the search for new therapies of oxidative stress in the testis.

Key words: Oxidative stress, testicular proteins.

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Ischemia-reperfusion (IR) injury of the testis occurs with the repair of testicular torsion, a clinical condition most commonly occurring in adolescent boys but also occurring in both younger and older males (Williamson, 1985; Smith-Harrison and Koontz, 1990). The IR injury results in a disruption of spermatogenesis in both humans and laboratory animals that can be permanent (Thomas et al, 1984; Turner, 1984; Smith-Harrison and Koontz, 1990) and in a loss of testicular testosterone secretion, which may be temporary (Anderson and Williamson, 1986; Turner et al, 2005). The torsion-induced disruption of spermatogenesis in the mouse has been shown to be correlated with a cytokine-induced up-regulation of E-selectin in the testicular vascular endothelium, which increases recruitment of neutrophils into the testis (Lysiak

et al, 2001). Neutrophil invasion is concomitant with an increase in intratesticular reactive oxygen species (ROS) and oxidative stress (Turner et al, 1997), which stimulates germ cell-specific apoptosis largely via the intrinsic or mitochondrial pathway (Lysiak et al, 2000).

Interestingly, significant apoptosis has not been reported in Sertoli cells or Leydig cells after repair of testicular torsion, although ROS do disrupt Leydig cell steroidogenesis through perturbation of the mitochondrial membrane (Allen et al, 2004). This may be the reason for the temporary decline in steroidogenesis seen after torsion repair in the rat (Turner et al, 2005). Sertoli cells in the rat testis have also been reported to continue to secrete a relatively normal panel of proteins after torsion repair (Turner and Miller, 1997), although this is not to say that every protein's synthesis and secretion remain unaltered. It simply means that the Sertoli cell's synthetic and secretory apparatus remains intact.

Oxidative stress has been reported to be a factor in a growing number of circumstances associated with male infertility (Samanta and Chainy, 1998; Lucesoli and Fraga, 1999; Pasqualotto et al, 2000; Wellejus et al, 2004); therefore, the present study examined the alterations in

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the mouse testicular proteome after testicular torsion of a degree and duration sufficient to induce oxidative stress and germ cell apoptosis (Turner et al, 1997; Lysiak et al, 2001). In these studies we use a proteomic approach to assess global changes in testicular proteins 24 hours after IR and to identify by mass spectrometry selected, specific proteins that were consistently changed in relative concentration by that treatment. Further, phosphorylation status of testicular proteins was assessed globally with and without IR, as well as phosphorylation of specific, stress-related proteins. The overall hypothesis was that testicular torsion induces changes in specific, stress-related proteins in the testis that would help us identify key pathways involved in IR injury of that organ.

Materials and Methods

Animals and Tissues

Adult male mice (C57BL/6) were anesthetized with an intraperitoneal injection of sodium pentobarbital (0.01 mg/g body weight) and subjected to unilateral testicular torsion on one side and to sham torsion on the opposite side. Testicular torsion was achieved via low, ventral midline incision. Each testis was exposed through the incision, and the gubernaculum and the avascular epididymo-testicular membrane were incised. The testis was rotated 720°. The sham-operated testes were put through this same maneuver but were immediately counter-rotated to original position, and the gubernaculum was reattached with 6-0 monofilament suture. The testis subjected to true torsion was left with the 720° rotation for 2 hours, after which the abdominal incision was reopened and the testis exposed and counter-rotated to its original position. The testis was reattached to the gubernaculum as above and replaced in the scrotum. This degree and duration of torsion has previously been documented to induce germ cell-specific apoptosis in the mouse (Lysiak et al, 2001; Turner et al, 2004). Twenty-four hours after torsion repair all testes were collected for protein extraction.

Protein Extraction and Electrophoresis

Extirpated testes were snap-frozen in liquid nitrogen and stored at -80°C until use. At the time of protein extraction, whole testes were ground with a mortar and pestle chilled in liquid nitrogen and the resultant powder was resuspended in 1 mL of protein extraction/rehydration buffer (6 M urea, 1 M thiourea, 0.5% 2-mercaptoethanol, 1% Triton X-100, 3% 3-[(3-cholmidopropyl)dimethylammonio]-1-propanesulfonate, 0.2% bio-lytes, 20 µg/mL aprotinin, 10 µM E-64, 100 µM leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The samples were placed on ice for 15 minutes and then centrifuged at 14 000 × *g* for 15 minutes at 4°C. The resultant supernatant was collected and an RC-DC protein assay (Bio-Rad, Hercules, Calif) was performed according to the manufacturer instructions to determine protein concentrations.

ReadyGel IPG strips (pH 4.0–7.0; Bio-Rad) were passively rehydrated with 350 µg of protein in 185 µL loading buffer. IPG strips were incubated for 45 minutes at room temperature, then

covered with 2 mL of mineral oil to prevent evaporation and incubated overnight at room temperature. The IPG strips were drained of the mineral oil and transferred to the Protean isoelectric focusing (IEF) tray (Bio-Rad) according to manufacturer protocol. The IEF was performed with conditions of 25 000 volt hours for approximately 6 hours.

Following the IEF, the IPG strips were drained of mineral oil and placed in an equilibration buffer consisting of 6 M urea; 0.375 M Tris-HCl, pH 8.8; 20% glycerol; 2% sodium dodecyl sulfate (SDS); and 2% dithiothreitol for 15 minutes at room temperature on an orbital shaker. The first equilibration buffer was then discarded and the strips were incubated in a second equilibration buffer consisting of 6 M urea; 0.375 M Tris-HCl, pH 8.8; 20% glycerol; 2% SDS; and 2.5% iodoacetamide for 15 minutes at room temperature on an orbital shaker. The equilibrated IPG strips were rinsed in gel-running buffer (25 mM Tris; 192 mM glycine; 0.1% SDS, pH 8.3) and placed on precast, 10.5%–14% SDS polyacrylamide gels. Molecular weight standards were included and electrophoresis was carried out at 125 volts for 2 hours.

Gels (*n* = 5/group) were incubated with a fluorescent stain (Sypro-Ruby; Molecular Probes, Eugene, Oreg) and viewed under ultraviolet light. Digital images were captured and analyzed using PDQuest software (Bio-Rad).

Image Analysis

Gel images were analyzed with PDQuest 7 (Bio-Rad). Image analysis of multiple gels is most effective when the gels are excellent replicates with regard to overall mobility dimensions, intensity of overall staining, clarity of individual protein spots, and absence of artifacts. Thus, image analysis was done on the 3 gel pairs (the paired sham and torsion testes from 3 different animals) that provided the highest replication of those general characteristics.

One of the gels in the sham group was assigned as the reference gel, and an identification number (IDN) was assigned to each protein spot. The spots in all other gels were compared to spots on the reference gel and, where matched by molecular weight and pI location, were assigned the same IDN. The PDQuest software determined the number of protein spots in each gel and the number and IDN of spots common to other gels within the sham group and then the torsion group. This spot identification system was augmented by 1) manual addition of occasional faint spots, which were ignored by the software in some gels but were visually detected in all gels of a group, 2) removal of occasional spots in some gels that were confounded with streaking or detergent micelles, and 3) manual matching when in occasional, complex situations the software could not make a match because of protein smearing or streaking. Individual spot intensities in all 6 gels representing proteins from 3 sham testes and 3 testes 24 hours after torsion repair were determined by the PDQuest software.

Spot quantification data were exported into Excel (Microsoft, Richmond, Wash) and analyzed to verify the average intensity of each protein spot in the gels of the sham and torsion groups. Proteins with average spot intensities that increased or decreased by a twofold measure or more after repair of torsion were noted as being increased or decreased, respectively. Any protein se-

lected for further study appeared in all gels of the sham-operated group.

All gels originally stained with Sypro-Ruby were restained with comassie blue to allow direct visualization. Six proteins (3 that had increased after IR and 3 that had decreased after IR) were selected for identification by mass spectrometry based on the spot's intensity in the comassie-stained gels, the expectation that the spot could be cored from the gel without contamination from adjacent spots, and the further requirement that a protein selected for analysis should be present in every comassie-stained gel examined.

Protein Identification by Mass Spectrometry

Proteins to be identified were cored from gels with the use of a microblade while under magnification. The cored samples were stored in distilled water at 4°C until analysis. The selected gel spots were subjected to in-gel proteolysis as described previously (Shevchenko et al, 1966). All digest samples were analyzed by nanoflow high-performance liquid chromatography/micro-electrospray ionization on a Thermo LCQ ion trap (San Jose, Calif) operating in the positive ion mode. Peptides were loaded onto home-built, 8-cm-long microcapillary columns (360- μ m outer diameter [o.d.], 75- μ m inside diameter fused silica) packed with YMC brand 5- μ m C18 particles (Waters, Milford, Mass). Peptides were eluted into the mass spectrometer using an acetonitrile/100-mM acetic acid linear gradient (1%–70% acetonitrile over 17 minutes) through an integrated electrospray emitter tip at a flow rate of approximately 60 nL/min. (Martin et al, 2000). The mass spectrometer was operated in the data-dependent mode, which allowed for the acquisition of 1 full-scan mass spectrum followed by 5 sequential MS/MS scans (3-Da window; precursor $m/z \pm 1.5$ Da, collision energy set to 35%, dynamic exclusion time of 1 minute) every 10–15 seconds of the most abundant ions present in the earlier full-scan mass spectrum. All acquired MS/MS spectra were searched against a mouse/rat protein database using the Sequest computer algorithm (Eng et al, 1994), and peptide sequence assignments were accepted if ΔCn score was at least 0.08; X_{corr} for fully tryptic peptides was equal to or greater than 1.8, 2.4, or 3.5 for charge states +1, +2, or +3, respectively; and X_{corr} for partially tryptic peptides was equal to or greater than 3.0 (+2 state) or 4.0 (+3 state).

Detection of Phosphorylated Proteins

Alterations in protein phosphorylation patterns were first investigated with Western blot analysis of proteins separated in 2 dimensions as above and probed with anti-phosphotyrosine (Transduction Labs, Lexington, Ky) and anti-phosphoserine/threonine antibodies (Cell Signaling Technology, Beverly, Mass). Briefly, electrophoresed proteins were electrotransferred to nitrocellulose membranes (Bio-Rad) using standard protocols and blocked with 5% nonfat dried milk and 0.1% Tween 20 in phosphate-buffered saline. The membranes were then incubated overnight at 4°C with either the phosphoserine/threonine or phosphotyrosine antibody ($n = 4$ –7, each, for sham-operated and torsion testes). The samples were subsequently washed and incubated for 1 hour with appropriate secondary antibody, and immunocomplexes were recognized with enhanced chemiluminescence (Super-Signal West Pico, Pierce Chemical, Rockford, Ill).

Also, phosphorylation of specific signal transduction proteins was analyzed using the Bio-Plex[™] bead suspension system (Bio-Rad). This is a multiplexing system allowing the assay of multiple proteins in a single sample. The system utilizes a liquid suspension array of multiple sets of beads internally dyed with different ratios of 2 spectrally distinct fluorophores. This assigns each bead a unique spectral address, and each bead is conjugated to a capture molecule (an antibody directed against a specific protein). The conjugated beads are mixed and incubated with protein samples in a 96-well microplate. Biotin-labeled reporter antibodies directed toward a different epitope on each of the captured proteins are added and samples are drawn into the Bio-Plex array reader. Precise fluid dynamics align the beads in single file through a flow cell in which 2 lasers excite the beads and allow the beads to be identified by their spectral address. The biotin-labeled reporter antibodies bind to the proteins bound to the beads and are detected by high-speed digital signal processors. The fluorescent signals from the rapidly passing beads are translated into quantifiable data by onboard software (Bio-Rad).

In the present experiments, testes were removed 24 hours after the repair of torsion or sham operation and were immediately processed for the Bio-Plex analysis. Briefly, testes were homogenized in 500 μ L of lysis solution (Bio-Rad), vortexed, and set on ice. Forty microliters of 500-mM phenylmethanesulfonyl fluoride was added, and the tissue homogenate was transferred to a clean microcentrifuge tube and frozen at -70°C . The samples were then thawed, centrifuged at $10\,000 \times g$ for 4 minutes and the supernatant collected. Protein concentration was determined using the RC/DC method (Bio-Rad) according to manufacturer instructions. The lysates were adjusted to 500 $\mu\text{g}/\text{mL}$ for use in an assay for 6 different phosphorylated proteins. The phospho-6-plex kit (Bio-Rad) contained antibodies directed against phosphorylated c-Jun NH₂-terminal kinase (JNK), phosphorylated activating transcription factor 2 (ATF2), phosphorylated p38 mitogen-activated kinase (p38 MAPK), phosphorylated signal transducer and activator of transcription 3 (STAT3), phosphorylated extracellular signal-regulated kinases 1 and 2 (ERK1&2), and phosphorylated inhibitor of nuclear factor $\kappa\text{B}\alpha$ ($\kappa\text{B}\alpha$).

The bead/antibody complexes were vortexed and added to the wells of a 96-well filter plate. Fifty microliters of the tissue lysates as well as positive control samples provided in the analysis kit were added to the wells in duplicate and the plate was incubated for 15 hours with constant agitation. The plate was then vacuum filtered and washed 3 times. Twenty-five microliters of detection antibodies were then added to the wells and the plate was incubated for 30 minutes at room temperature. The plate was again vacuum filtered and washed. Streptavidin-PE was added to each well for 10 minutes, after which time the plate was vacuum filtered, rinsed 3 times, and 125 μL resuspension buffer was added. The 96-well plate was then placed in the BioPlex reader and the data were collected and analyzed as described previously.

Results

Testicular Proteins in Sham-Operated Testes and in Testes 24 Hours After IR

Fluorescent images of Sypro-Ruby-stained gels showed an average of 1182 proteins in extracts from sham testes

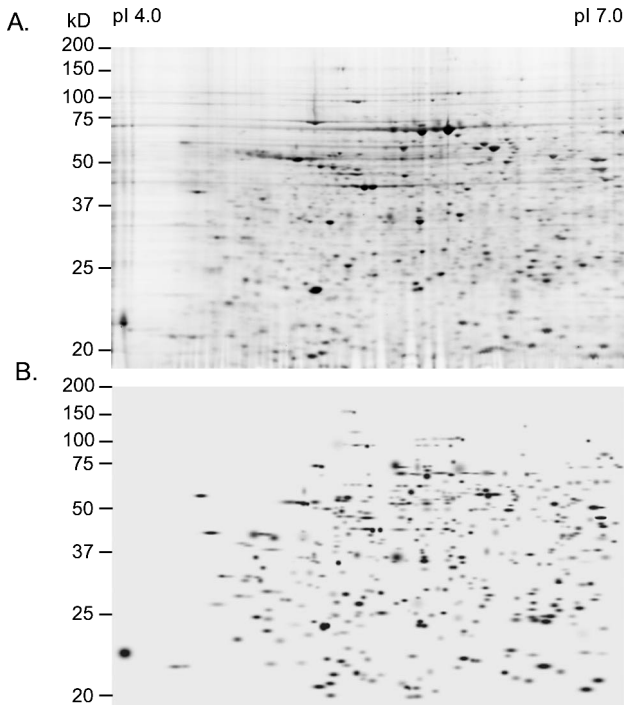


Figure 1. Proteins of the mouse testis with and without ischemia/reperfusion injury induced by testicular torsion. **(A)** typical 2-dimensional electrophoretogram of proteins from the sham-treated mouse testis after staining with Sypro-Ruby. **(B)** Digital composite image showing a summation of the entire group of sham-operated testes. The average density and size of each protein spot in the entire group are illustrated in this composite image. Many of the lighter densities in the A panel were inseparable from background and were eliminated from the composite image, as were those spots that did not appear in replicate gels.

(Figure 1A) and 1076 proteins in testes 24 hours after repair of testicular torsion (not shown). Digital composite images showed all the spots consistently detected by the image analysis system in a group of gels and illustrated average spot densities for the entire group; thus, Figure 1B is a composite image of the entire group of sham-group gels to which the single gel in Figure 1A contributed. The numerical averages for each of these spot densities were generated and fold-changes in average spot density for each protein after IR were calculated.

The image analysis software also produced schematic maps of the composite images to better identify overlapping, but separately detected, proteins and to allow clear identification of consistently increased (Figure 2A) and decreased (Figure 2B) proteins. Seven hundred seventeen different proteins consistently appeared within or between groups and were subject to this analysis. Among these proteins, 23 were detected as having been consistently increased 24 hours after torsion repair (Figure 2A).

In preparation for coring proteins from gels for protein identification by mass spectrometry, the gels had to be stained with comassie blue so proteins could be seen in the visible light range. Relative spot intensities seen with

comassie blue staining correlated well with those seen with Sypro-Ruby fluorescent staining (Figure 1A), although the former staining method was less sensitive and did not detect many of the lighter protein spots. Based on a spot's intensity among the comassie-stained proteins from the testes after IR and on the additional requirement that the spot appear in all 5 gels of the group, 3 of these up-regulated proteins (Figure 2A, spots 2601, 5808, and 6807) were selected for identification by mass spectrometry.

Based on the intensity of Sypro-Ruby staining, 48 proteins decreased by more than or equal to 50% 24 hours after torsion repair (Figure 2B), with 10 of them being completely extinguished. The proteins that disappeared in the IR group had typically been very lightly stained, though consistently appearing, even in the sham group. Based on spot intensity in the comassie blue-stained gels from the sham-operated testis and the additional requirement that the cored spot appear in all 5 gels of the sham-control group, 3 of the proteins (Figure 2B; spots 3701, 8604, and 9505) were selected for identification by mass spectrometry.

The comassie blue-stained proteins consistently increased 24 hours after torsion repair were β -tubulin (IDN#2601), and two different isoforms of a serum albumin precursor (5808 and 6807; Table 1). The 3 decreased proteins identified from the comassie blue-stained gels were vimentin (3701), chaperonin containing TCP1, subunit 2 β (8604), and phosphoglycerate kinase 2 (PGK2; 9505; Table 1).

Protein Phosphorylation

Phosphorylation of testicular proteins was altered 24 hours after IR, as shown by both Western blot and Bio-Plex analyses. Western blot analysis of proteins from the sham-operated testis typically detected 5 to 6 proteins that were phosphorylated on serine/threonine residues (Figure 3A). A similar number of phosphorylated proteins were detected after IR (Figure 3B), but the phosphorylation associated with some spots had increased, while others had decreased. There was considerable sample-to-sample variation, but the consistent changes induced by IR are illustrated in Figure 3. Proteins 1 to 5 showed little or no phosphorylation signal in sham-treated testes (Figure 3A), but the phosphorylation signal in those spots increased in all 4 samples examined after IR (Figure 3B). Protein 6 appeared in 3 out of 4 blots of sham-treated and IR testes and was not affected by the treatment. Spots 8, 9, and 10 consistently showed reduced phosphorylation signal after IR. Four proteins (7, 11, 12, and 13) are typical of those appearing in only 1 or 2 blots after testicular IR but were never present in sham-operated testes.

The variable pattern of increase and decrease in serine/threonine phosphorylation was seen again in the analysis

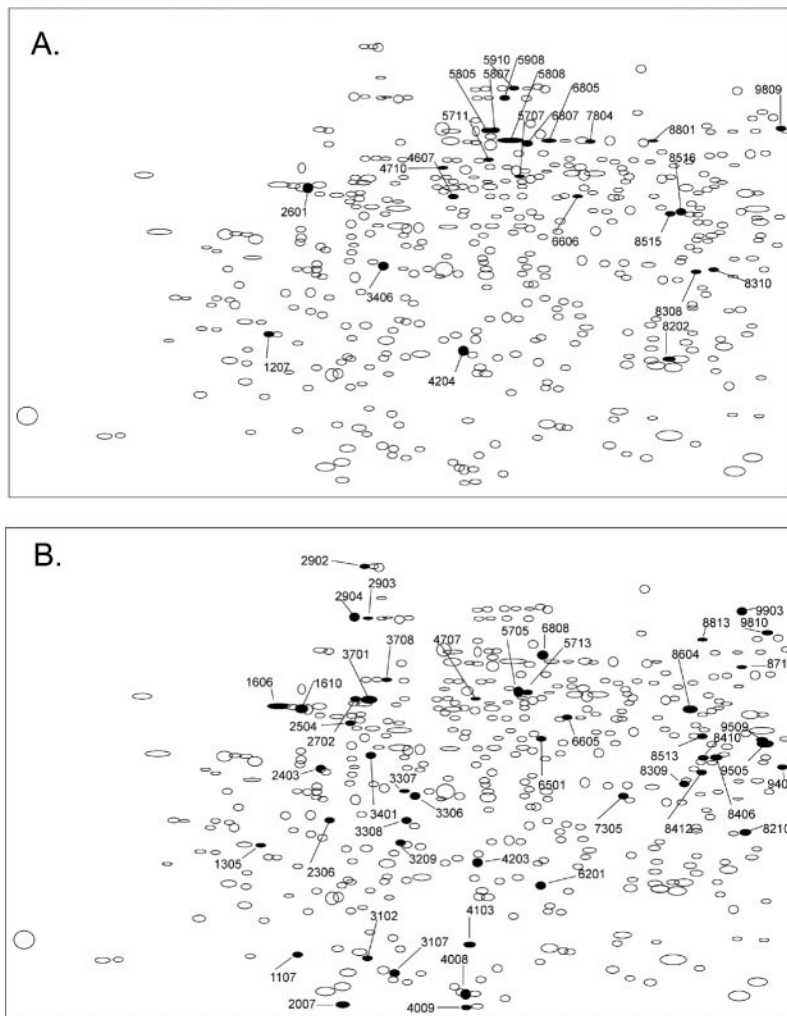


Figure 2. Digital schematic maps of the proteins detected in Figure 1B. This method of illustration more clearly shows where the image analysis system detected overlap among multiple proteins. (A) Identification (IDN) numbered and darkened protein spots are those which increased by at least a twofold measure 24 hours after torsion repair. (B) Numbered and darkened spots are those in which spot intensity declined by at least half within 24 hours of torsion repair.

Table 1. Testicular proteins identified by mass spectrometry

Gel IDN*	Increase (I) or Decrease (D)†	Protein Name	Accession Number
2601	I	B2 tubulin	NP 666228
5808	I	Serum albumin precursor	PO7724
6807	I	Serum albumin precursor	PO7724
3701	D	Vimentin	CA69019
8604	D	t-Complex protein 1β	P80314
9505	D	Phosphoglycerate kinase 2	P09041

* Identification number (IDN) of spots cored from gels illustrated in Figure 2.

† Mean increase or decrease in spot density by at least twofold measure between sham and torsion testes.

of specific-protein phosphorylations using the Bio-Plex technology (Table 2). JNK, ATF-2, and IκBα are each phosphorylated on serine or threonine, but JNK and ATF-2 were significantly increased ($P < .05$) after IR by an average 53% and 50%, respectively, while IκBα phosphorylation remained virtually unchanged (Table 2).

Tyrosine phosphorylation patterns (not shown) were much more complex than the serine/threonine phosphorylation patterns (eg, 30–40 phosphorylated proteins were detected on each protein blot from sham-operated testes, and there was also more variability between blots). As with the serine/threonine phosphorylations, IR increased detectable tyrosine phosphorylation of some proteins, while it decreased tyrosine phosphorylation in others. A difference was that a high proportion of proteins with tyrosine phosphorylation occurred in only 1 gel pair (sham vs IR within the same animal); thus, these individ-

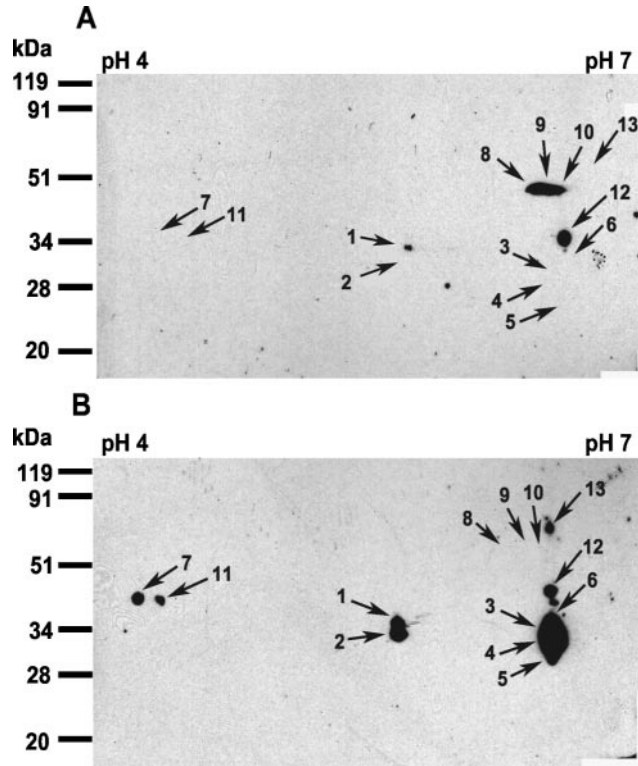


Figure 3. Serine/threonine phosphorylation in sham-treated testis proteins (A) and in proteins from testes 24 hours after repair of torsion (B). Phosphorylation of proteins 1 through 4 consistently increased after torsion repair, and phosphorylation of proteins 8 to 10 consistently decreased. Spots 7 to 13 were never present in sham-testis extracts and appeared in only 1 or 2 posttorsion samples.

ual phosphorylations did not consistently occur within the groups of testes. Overall, tyrosine phosphorylations typically appeared decreased rather than increased after torsion repair (not shown).

The Bio-Plex analysis showed that STAT3, p38 MAPK, and ERK1&2, which are all phosphorylated on tyrosine residues, were variably affected by torsion (Table 2). Average STAT3 and ERK1&2 phosphorylation values were increased by 23% and 20%, respectively, 24 hours

after torsion repair, but only the increase in STAT3 phosphorylation was statistically significant ($P < .05$). p38 MAPK remained essentially unchanged (Table 2).

Discussion

Testicular responses to experimental torsion have been studied by a number of labs, with some investigators focusing on attempts to reduce oxidative stress in the testis after IR (Akgur et al, 1994; Uz et al, 2002; Ozkan et al, 2004), while others have worked at dissecting the underlying cell and molecular mechanisms of testicular injury (Lysiak et al, 2001; Shiraishi et al, 2001; Powell et al, 2002). Both approaches have as their ultimate goal the determination of effective therapies to improve testicular salvage in cases of testicular torsion in the human.

Previous reports from this lab have partially detailed an across-compartment pathway leading to germ cell apoptosis after IR. Early in this pathway, cytokine induction of stress-related kinases leads to E-selectin expression by the testicular vascular endothelium (Lysiak et al, 2003). E-selectin expression is associated with the neutrophil recruitment into the testicular interstitial compartment that occurs after torsion repair (Turner et al, 1997; Lysiak et al, 2001), which is contemporaneous with intratesticular ROS production (Turner et al, 1997), increases in Bax and caspase expression (Lysiak et al, 2000), and germ cell apoptosis inside the seminiferous tubule compartment (Turner et al, 1997; Lysiak et al, 2001).

We have previously used the rat testis to examine for testicular proteins, the synthesis of which is consistently and significantly increased or decreased after IR (Turner and Miller, 1997). We found a limited number of proteins in which synthesis was consistently altered after torsion repair. This was interpreted to mean that Sertoli cell function remains relatively robust after IR. On a related note, Sertoli cells persist in seminiferous tubules after periods of torsion that eliminate germ cells through apoptosis, and

Table 2. Phosphorylated proteins detected with the Bio-Plex technology listing their phosphorylated residues, fluorescence values (mean \pm SEM), and percent increase or decrease from control values 24 hours after repair of testicular torsion

Phosphorylated Protein*	Residues Phosphorylated	Flourescence Value, Control†	Flourescence Value, Torsion†	Increase or Decrease‡
STAT3	Tyrosine	77.0 \pm 3.6	95.0 \pm 4.868§	+23
JNK	Threonine	93.8 \pm 4.2	143.7 \pm 16.1§	+53
p38 MAPK	Tyrosine	63.5 \pm 4.4	61.8 \pm 2.65	-3
ATF2	Threonine	119.8 \pm 6.8	179.7 \pm 15.3§	+50
ERK1&2	Tyrosine	113.7 \pm 9.5	136.2 \pm 6.2	+20
I κ B α	Serine	87.5 \pm 3.8	83.0 \pm 1.9	-5

* STAT3 indicates signal transducer and activator of transcription 3; JNK, c-Jun NH₂-terminal; p38 MAPK, p38 mitogen-activated; ATF2, activating transcription factor 2; ERK1&2, extracellular signal-regulated kinases 1 and 2; and I κ B α , inhibitor of nuclear factor.

† n = 6 for all values.

‡ Percent increase or decrease of mean posttorsion value from mean control value.

§ Significantly different from controls, $P < .05$.

Sertoli cells have not themselves been seen to undergo apoptosis after torsion.

Despite the fact that the synthesis of detected proteins was little altered by degrees and durations of torsion sufficient to induce aspermatogenesis in the rat (Turner and Miller, 1997), it remained possible that activation or inhibition of existing proteins (binding proteins, enzymes, etc) is an important aspect of the testicular injury that occurs after IR (Lysiak et al, 2003, 2005), even though those changes may not be specific to the IR injury. We have continued our studies in the mouse because of the greater molecular biology resources available in that species.

An average of over 1100 proteins were detected in individual gels separating proteins of the sham-operated mouse testis (Figure 1), many of them faintly stained and not consistently appearing in replicate gels. A total of 717 spots were consistently present and used for the comparison between the sham and torsion groups to determine the increased and decreased proteins in this mouse model of testicular torsion (Figure 2). As was the case with the results of Turner and Miller (1997), relatively few proteins were consistently and significantly altered after torsion repair. Of the 23 proteins with increased spot densities 24 hours after IR, 3 were chosen for identification based on the ability to confidently identify and isolate them from commassie blue-stained gels. One of the proteins with increased spot density after IR was identified as a β 2-tubulin, a major microtubule constituent that increased by a 2.4-fold measure (Table 1). A major source of testicular tubulin is likely to be the condensing spermatids and spermatozoa in the seminiferous tubules, but the increase in the protein occurs despite the fact that germ cells are being lost through apoptosis 24 hours after torsion repair (Lysiak et al, 2001). The reason for this discrepancy is obscure, but a developmental "switch" in some β -tubulin genes has been reported (Slaughter et al, 1987), and it is possible that the testicular dystrophy induced by IR has recapitulated such a switch "on," leading to the increased abundance of the β 2-tubulin protein, although the cell source of such a switch is unknown.

Two of the proteins that increased after IR were isoforms of serum albumin precursor, which increased by 2.4-fold and 2.3-fold measures, respectively (Table 1). Albumin is not known to be synthesized in the testis, although in the rat testis a related protein, testibumin, is (Shaha et al, 1988). Interestingly, Melsert et al (1991) reported that albumin stimulates Leydig cell steroid production in a dose-dependent manner in vitro when added to testicular fluid. While serum albumin is known to be distributed in the interstitial space in the testis (Christensen et al, 1985), it has been presumed to be there as a transudate from serum, and we are unaware of any suggestion of local synthesis. Serum albumin is known to be

synthesized in places other than the liver, and in other cell types it has been postulated to be part of the cellular redox sensing apparatus (Holderman et al, 2002). Thus, the presence of serum albumin precursor in testis extracts indicates that testicular cells (type unknown) synthesize albumin, either as a part of their protection mechanism against oxidative stress or as a facilitator of Leydig cell steroidogenesis under stress conditions.

The number of proteins with decreased spot density after IR (48) was more than double the number with increased density (23). The 3 protein spots chosen for identification were vimentin, *t*-complex protein 1 β , and testis-specific PGK2. Vimentin is an intermediate filament protein that has been reported to have a role in Leydig cell testosterone secretion, and that role has been speculated to be through its function in the transport of cholesterol to the outer mitochondrial membrane (Bilinska et al, 1997). Vimentin was reduced by approximately 54% in the mouse testis 24 hours after IR. That reduction in intermediate filament may be associated with an acute decrease in testicular venous testosterone concentrations in the mouse, such as that previously reported for the rat testis 24 hours after torsion repair (Turner et al, 2005). Further, the afore-mentioned increase in serum albumin precursor may be a part of the general mechanism attempting to compensate for or repair this loss of Leydig cell function.

The *t*-complex protein 1 β is an actin-binding chaperonin that was reduced by 52% after IR of the mouse testis. This protein is one of many *t*-complex proteins in germ cells, and its reduction may be a reflection of the disturbance to spermatogenesis and the loss of germ cells by apoptosis that occurs by 24 hours after torsion repair. Finally, PGK2 was reduced by 55% after IR. PGK2 is an enzyme localized to spermatocytes and spermatids and is involved in the metabolism of glucose and fructose (Naz and Vanek, 1998). The reason for a testis-specific isoform and the specific role of that isoform in spermatogenesis is unknown, but the decline in the protein in testis extracts after IR may well be due to the loss of healthy germ cells, as indicated above.

While the identified proteins from the 2-dimensional (2-D) electrophoresis were other than well-known stress-related pathway molecules, they could be common, endpoint proteins bearing the result of such pathway activations. Enzyme activation is commonly by phosphorylation of the inactive molecule, and these phosphorylations can be on serine, threonine, or serine residues of the native protein. Thus, in addition to the identification of proteins from among those that had increased or decreased after torsion repair, changes in protein phosphorylation were also studied using Western blots with anti-phosphoserine/threonine and anti-phosphotyrosine antibodies as probes. Very few proteins were consistently phosphor-

ylated on serine/threonine residues (Figure 3). While over 8 times more proteins were phosphorylated on tyrosine than on serine/threonine (not shown), those results were also highly variable, making interpretation difficult. Phosphorylations detected on specific residues may vary depending on the antibody used, but with the present antibodies, in no case did the phosphorylated proteins identifiably co-migrate with spots on the Sypro-Ruby gels discussed previously. Alternatively, Bio-Plex technology was used to determine quantitative changes in the phosphorylation of 6 specific stress-related kinases, 3 of them phosphorylated on serine/threonine, 3 of them on tyrosine (Table 2).

The most prominent quantitative changes in protein phosphorylation occurred with JNK and ATF2, both of them threonine phosphorylations. Detection of JNK and ATF2 phosphorylation increased by an average of 53% and 50%, respectively. Both increases were statistically significant ($P < .05$). ATF2 is a member of the cAMP-response element-binding protein family and is a leucine zipper protein involved in cellular stress responses; further, ATF2 is a known target of JNK in the mediation of those cell-stress responses (Hayakawa et al, 2003). The more modest changes in phosphorylated STAT3 and ERK1&2 may indicate a stimulation of cell-repair events via pathways involving those molecules.

In summary, IR injury induced by testicular torsion causes significant changes in the presence of selected testicular proteins, but among an average of over 1100 proteins detectable on 2-D electrophoresis, relatively few were consistently altered in spot density 24 hours after torsion repair. The increases or decreases (depending) of 6 identified proteins from those gels, vimentin, β 2-tubulin, PGK2, *t*-complex protein 1 β , and 2 isoforms of serum albumin precursors, could be rationalized, but they gave little information regarding the predominant pathway(s) within 24 hour of IR injury. Protein phosphorylation studies demonstrated that phosphorylation patterns among the testicular proteins were highly variable, although typically more tyrosine phosphorylation was detected than serine/threonine phosphorylation. None of the phosphorylated proteins on 2-D gels matched spot locations with consistently appearing proteins on Sypro-Ruby-stained gels. On the other hand, when specific protein phosphorylations were studied by Bio-Plex analysis, 2 proteins phosphorylated on threonine residues; JNK and ATF2 were quantitatively the most predominant. JNK and ATF2 have previously been shown to undergo activation within minutes of testicular reperfusion (Lysiak et al, 2003), but the present results demonstrate a prolonged activation still detectable 24 hours later. Whether this is a persistence of the acute process detected earlier by Western blot analysis (Lysiak et al, 2003), but now seen at a longer time point through use of the more sensitive Bio-Plex analysis, or

whether it is a rebound activation particularly influencing delayed events is yet to be determined. By whatever means, it is likely an extension of the pathophysiology of the testis after IR injury, and identification of proteins altered either by increases or decreases in expression or by phosphorylation status will provide insight into specific pathways affected in the testis after torsion. These proteins and pathways may offer targets for potential therapies for the rescue of the testis after torsion or other oxidative stress injuries to the testis.

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