

## Activation of the Nuclear Factor Kappa B Pathway Following Ischemia-Reperfusion of the Murine Testis

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**ABSTRACT:** Ischemia-reperfusion (IR) of the testis results in testicular oxidative stress and germ cell-specific apoptosis. Nuclear factor kappa B (NF- $\kappa$ B) is a nuclear transcription factor involved in the control of a number of cellular processes, and its activation is part of the cellular stress response to a variety of factors including cytokine stimulation, irradiation, and IR. The present study investigates NF- $\kappa$ B activation after IR of the murine testis and potential downstream target genes of that activation. Mice were subjected to a period of testicular ischemia followed by 0–4 hours of reperfusion. Activation of NF- $\kappa$ B was assessed by 1) Western blot analysis of the NF- $\kappa$ B inhibitory protein, I $\kappa$ B $\alpha$ ; 2) immunohistochemistry for I $\kappa$ B $\alpha$ ; and 3) TranSignal NF- $\kappa$ B target gene array (107 genes) analysis. Results demonstrate that I $\kappa$ B $\alpha$  is phosphorylated on serine 32 reaching a peak by 2 hours after IR of the testis. A decrease in total I $\kappa$ B $\alpha$  was also noted at 2 hours after IR, consistent with the rapid degradation of the phosphorylated protein. Phosphorylation and deg-

radation of I $\kappa$ B $\alpha$  is indicative of NF- $\kappa$ B activation. Immunolocalization revealed I $\kappa$ B $\alpha$  specifically in Sertoli cells of the murine testis. Results of the TranSignal target gene array revealed that the expression of 9 genes was consistently changed 2 hours after IR of the testis, 3 of which increased in expression and 6 of which were down-regulated. Most notably, high-mobility group nucleosomal binding domain 1 increased in expression while platelet-derived growth factor B and Wilms tumor homolog decreased. These results suggest that testicular IR releases the suppression of NF- $\kappa$ B by I $\kappa$ B $\alpha$  in Sertoli cells. Activation of the NF- $\kappa$ B pathway in the testis resulted in an alteration of expression of potential NF- $\kappa$ B target genes, some increased while others decreased. The specific roles of these genes in the testicular response to IR remains to be determined.

Key words: Testicular oxidative stress, apoptosis, NF- $\kappa$ B activation.

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Testicular torsion is a multifaceted pathology displaying many of the characteristics of ischemia-reperfusion (IR) injury described in other organs. Testicular torsion or, specifically, torsion of the spermatic cord results in the obstruction of blood flow to the affected testis. In humans surgical intervention is often required to repair the torsion and allow for reperfusion; however, testicular atrophy is a common outcome. In rodent models of IR of the testis permanent loss of spermatogenesis is observed despite the return of blood flow (Turner and Brown, 1993; Lysiak et al, 2000a). Interestingly, this loss of spermatogenesis has been shown to be due to germ cell-specific apoptosis (Turner et al, 1997; Lysiak et al, 2000b) while Sertoli cells appear to function normally (Turner and Miller, 1997). Coinciding with the IR-induced germ cell apoptosis is an increase in neutrophils (Turner et al, 1997) and reactive oxygen species (Lysiak et al, 2002) in the testis. In fact, the recruitment of neutrophils to the testis

after IR is essential for the observed germ cell apoptosis (Lysiak et al, 2001).

Studies investigating the mechanisms of neutrophil recruitment to the testis after IR have demonstrated that E-selectin is a critical endothelial cell adhesion molecule responsible for neutrophil recruitment (Lysiak et al, 2001). E-selectin expressed on endothelial cells mediates the tethering and slow rolling of neutrophils to endothelial cells (Kunkel and Ley, 1996). Recent studies examining the cell signaling pathway regulating E-selectin in the testis after IR have found that there is an increase in the proinflammatory cytokines, interleukin-1 beta (IL-1 $\beta$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ), as early as 0.5 hour after IR (Lysiak et al, 2003). This increase in the proinflammatory cytokines is followed by the activation of the c-jun N-terminal kinase (JNK) stress-related pathway, which in turn is correlated with the expression of E-selectin mRNA and protein and the retention of neutrophils in testicular venules. Direct injection of proinflammatory cytokines into the testis demonstrated that IL-1 $\beta$  but not TNF- $\alpha$  caused activation of JNK and the recruitment of neutrophils to the testis. This indicates that IL-1 $\beta$  is the mediating factor of neutrophil recruitment to the testis after IR (Lysiak et al, 2003).

It is well established that IL-1 $\beta$  and TNF- $\alpha$  can mediate their effects through activation of nuclear factor kappa B

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(NF- $\kappa$ B). NF- $\kappa$ B is a family of 5 proteins that are structurally and functionally related and regulate the transcription of a variety of genes. Active NF- $\kappa$ B consists of 2 family members arranged to form either homodimers or heterodimers that then bind to DNA with different specificities (Baeuerle and Baltimore, 1996; Ghosh and Karin, 2002).

Under nonstimulating conditions NF- $\kappa$ B is bound to a member of a structurally related family of proteins termed inhibitors of NF- $\kappa$ B (I $\kappa$ B). This family includes, I $\kappa$ B $\alpha$ , I $\kappa$ B $\beta$ , I $\kappa$ B $\epsilon$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\delta$ , and Bcl-3 (Mercurio and Manning, 1999; Senftleben and Karin, 2002). I $\kappa$ B's binding to NF- $\kappa$ B physically masks the nuclear localization sequences of NF- $\kappa$ B and causes its retention in the cytoplasm. Activation of NF- $\kappa$ B occurs when I $\kappa$ B is phosphorylated and degraded, thus releasing NF- $\kappa$ B for transport to the nucleus where it binds to DNA and directs transcription of its target genes. This activation has been shown to occur after stimulation by TNF- $\alpha$  or IL-1 $\beta$  (Senftleben and Karin, 2002).

The existence of the multiple NF- $\kappa$ B and I $\kappa$ B family members is thought to provide specificity of gene regulation to the many signals that can activate NF- $\kappa$ B. For example, different roles for the activation I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  have been described (Baeuerle and Baltimore, 1996), and both I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  have been localized in the murine testis (Budde et al, 2002) and may play different roles there.

The purpose of the present study was to determine whether IR of the testis with its increase in proinflammatory cytokines alters the phosphorylation and degradation of I $\kappa$ B $\alpha$ , thus indicating the activation of the NF- $\kappa$ B pathway. Further, we sought to determine whether testicular IR would alter the expression of genes known to be NF- $\kappa$ B target genes in other tissues. These studies were conducted with the aim of detecting yet unknown players in the pathway(s) to germ cell apoptosis.

## Materials and Methods

### Reagents and Antibodies

The protease inhibitors leupeptin, phenylmethylsulfonyl fluoride (PMSF), E-64, and aprotinin and the phosphatase inhibitor sodium orthovanadate were all obtained from Sigma (St Louis, Mo). Antibodies against total I $\kappa$ B $\alpha$  (9242) were purchased from Cell Signaling Technology (Beverly, Mass) and against phosphorylated I $\kappa$ B $\alpha$  (serine 32; sc-8404) were obtained from Santa Cruz Biotechnology Inc (Santa Cruz, Calif). Anti- $\alpha$ -tubulin was purchased from Sigma.

### Experimental Testicular Torsion

This work was conducted in accordance with the Society for the Study of Reproduction's *Guiding Principles of the Care and Use of Research Animals*. Adult male C57BL/6 mice were anesthe-

tized with an intraperitoneal injection of 0.01 mg/g body weight of sodium pentobarbital, and the testis was rotated as described by Lysiak et al (2001). Briefly, the testis was exteriorized through a low midline laparotomy, the gubernaculum was divided, and the testis was freed from the epididymo-testicular membrane. The testis was rotated 720° for 2 hours, during which time it remained in the abdomen with a closed incision. Following the 2 hours torsion the incision was reopened, the testis was counterrotated to the natural position, the gubernaculum was re-joined, and the testis was reinserted into the scrotum via the inguinal canal. Testes were examined at the time of repair for the apparent degree of ischemia and reperfusion. Sham operated animals were treated identically except that upon completion of the torsion maneuver the testis was immediately counterrotated.

### Western Blot Analysis

Western blot analysis for total I $\kappa$ B $\alpha$ , phospho-I $\kappa$ B $\alpha$ , and  $\alpha$ -tubulin were performed by isolating testicular proteins at 0.0, 0.5, 2.0, and 4.0 hours after reperfusion of the testis or after sham operations. Briefly, the testes were removed, snap frozen in liquid nitrogen, and stored at -80°C until use. Whole testes were ground with a mortar and pestle chilled in liquid nitrogen. The resultant powder was resuspended in RIPA buffer (0.1% sodium dodecylsulfide [SDS], 1 mM EDTA, 100 mM Tris, 0.15 NaCl, 1.0% deoxycholate, 1.0% triton X-100, pH 7.4) with the addition of protease inhibitors (100  $\mu$ M leupeptin, 1 mM PMSF, 10  $\mu$ M E-64, 20  $\mu$ g aprotinin), and phosphatase inhibitor, (1 mM sodium orthovanadate). The protein suspension was vortexed, incubated on ice for 15 minutes, and centrifuged at 14 000  $\times$  *g* for 15 minutes at 4°C, and the supernatant was collected. The protein concentration in the supernatant was determined using the bicinchoninic assay kit (Pierce, Rockford, Ill), and 50  $\mu$ g of protein per lane was subjected to SDS polyacrylamide gel electrophoresis (PAGE). The gel contents were electrotransferred to nitrocellulose membranes (Bio-Rad, Hercules, Calif); blocked with 5% nonfat dried milk, 0.1% Tween 20 in phosphate-buffered saline (PBS); and incubated overnight at 4°C with an antibody that recognized the form of the protein specifically phosphorylated on serine 32 (1:100 dilution) or an antibody that recognized the total protein (1:500 dilution). The next day membranes were washed and incubated for 1 hour at room temperature with the appropriate peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, Pa). Immunocomplexes were detected with enhanced chemiluminescence (SuperSignal West Pico; Pierce). Densitometry and ImageQuant analysis were subsequently performed.

### Immunohistochemistry

To obtain testes to be sectioned for immunohistochemistry for I $\kappa$ B $\alpha$ , mice were sacrificed at 2 hours after reperfusion of the testis. The testes were removed and placed in Bouin fixative for 6 hours and subsequently paraffin embedded. Tissue sections were deparaffinized, rehydrated, and washed in PBS. Slides were then placed in unmasking solution (Vector Laboratories Inc; Burlingame, Calif), microwaved for 20 minutes, and allowed to cool for 1 hour at room temperature. Subsequently, endogenous peroxidase activity was inhibited by incubating the sections in 3% H<sub>2</sub>O<sub>2</sub> methanol solution for 15 minutes. Slides were then

washed, blocked, and incubated with anti-I $\kappa$ B $\alpha$  antibody overnight at 4°C. The next day slides were washed and incubated with the appropriate biotinylated secondary antibody (Vector Laboratories Inc) for 1 hour at room temperature. Immunocomplexes were visualized with avidin-biotin-peroxidase complex (Vector Laboratories Inc) with diaminobenzidine (Sigma) as the chromagen. All slides were lightly counterstained with hematoxylin.

### Gene Array Analysis

NF- $\kappa$ B gene array analysis was performed using the TranSignal NF- $\kappa$ B target gene array (Panomics, Redwood City, Calif). Briefly, RNA was isolated from liver and from testes 2 hours after IR or sham operation using Trizol reagent (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Twenty micrograms of total testicular RNA was incubated with 5  $\mu$ l of the TranSignal NF- $\kappa$ B primer mix for 2 minutes at 70°C in a thermal cycler (Whatman Biometra, Göttingen, Germany). The temperature was then reduced to 42°C. The cDNA synthesis mixture consisting of labeling mix buffer, biotin-dUTP, reverse transcriptase, and water was then added to the RNA and primer mix and incubated at 42°C for 2 hours. After the incubation, 3  $\mu$ l of denaturing solution was added to each sample and incubated at 68°C for 20 minutes. Samples were then incubated at 72°C for 10 minutes in the presence of neutralization buffer. The resultant biotin-label cDNA probes were then either used immediately or stored at -20°C for future use.

TranSignal NF- $\kappa$ B target gene arrays contain duplicate representations of 107 different genes previously identified as NF- $\kappa$ B target genes in various tissues. Two array membranes representing sham and torsion testes, respectively, were used in each experiment, and the experiment was performed 3 times. Membranes were placed in sterile 50-ml tubes and hydrated with water. Prewarmed hybridization buffer was then added to each tube and incubated at 42°C for 2 hours in a hybridization oven (Hybaid, Franklin, Mass). The labeled cDNA probes were then added to each bottle and hybridized overnight at 42°C. The next day the hybridization mixture was decanted from each bottle and the membranes were washed twice with hybridization wash I buffer for 20 minutes at 42°C. This was followed by 2 washes with prewarmed hybridization wash II buffer for 20 minutes at 42°C. Each membrane was then removed from its hybridization bottle and transferred to a new container containing 20 ml of blocking buffer for 15 minutes at room temperature. Twenty microliters of streptavidin-horseradish peroxidase conjugate was then added directly to the blocking solution, and the membranes were incubated for 15 minutes at room temperature. The membranes were subsequently washed 3 times with wash buffer, each 8 minutes. Detection buffer was then added, and the membranes were incubated for 5 minutes at room temperature. Hybridization signals were detected with a solution of luminol enhancer and peroxide for 5 minutes at room temperature.

To obtain semiquantitative numerical data, the resultant films were scanned on a densitometer and imported into ImageQuant. Numerical values corresponding to the optical density were determined for each target gene, and values were corrected for background using the application's local average method. The local average method determines the average density of pixels

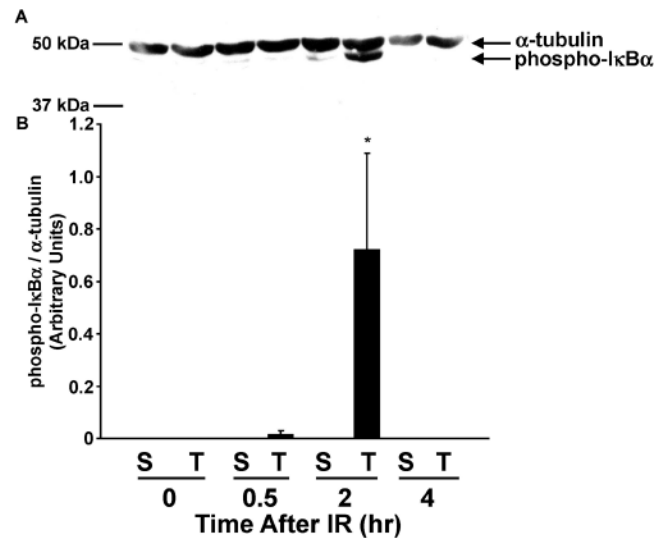


Figure 1. (A) Representative Western blot of phospho-I $\kappa$ B $\alpha$  at various times after ischemia-reperfusion (IR) of the testis (S = sham and T = torsion). Membranes were simultaneously blotted for  $\alpha$ -tubulin for a load control. (B) Relative increase in phosphorylated I $\kappa$ B $\alpha$  after IR of the testis. I $\kappa$ B $\alpha$  is phosphorylated by 2 hours after IR. Histogram bars represent the mean plus or minus standard error of the mean of 5 separate blots. Asterisk indicates  $P < .05$ .

surrounding the target gene hybridization spot and subsequently corrects the volume report for background. The background corrected volume report for each gene on the arrays was then normalized to ubiquitin expression as a load control. Genes were assigned to 1 of 3 groups, those genes that were highly expressed in sham testes (upper third of pixel intensities), those genes that were moderately expressed (middle third of intensities), and those that were less abundantly expressed (lower third of intensities). Within these groups data were examined to identify only those genes that were consistently up- or down-regulated in triplicate experiments.

### Statistical Analysis

All statistical evaluations were either by analysis of variance followed by the Tukey range test or the Student's  $t$  test ( $P < .05$ ) after evaluation of each data set by the Chauvenet criterion for homogeneity.

## Results

### Activation of NF- $\kappa$ B After IR of the Testis

Activation of the NF- $\kappa$ B pathway in the testis after IR was examined indirectly by Western blot analysis for phosphorylation and degradation of the NF- $\kappa$ B inhibitory protein, I $\kappa$ B $\alpha$ . Phosphorylated I $\kappa$ B $\alpha$  was detected as a single band of approximately 47 kD in Western blots of electrophoresed proteins (Figure 1A). A significant increase in phosphorylated I $\kappa$ B $\alpha$  occurred by 2 hours after IR, but this phosphorylated product was not detectable 4 hours after IR (Figure 1A and B).

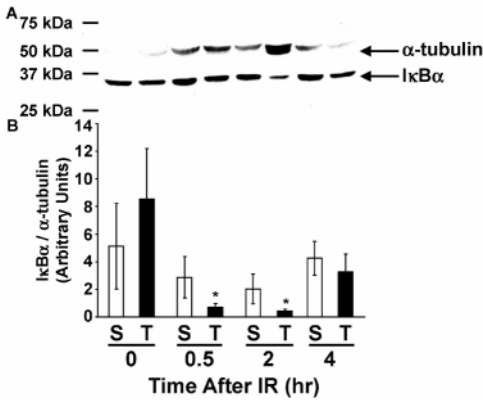


Figure 2. (A) Representative Western blot of total IκBα at various times after ischemia-reperfusion (IR) of the testis (S = sham and T = torsion). Membranes were simultaneously blotted for α-tubulin for a load control. (B) Relative expression of total IκBα after IR of the testis. Nuclear factor kappa B is reduced by 2 hours after IR and phosphorylated IκBα is ubiquitinated and runs at approximately 47 kd (just under the α-tubulin band). Histogram bars represent the mean plus or minus standard error of the mean of 5 separate blots. Asterisk indicates *P* < .05.

Western blot analysis for total IκBα revealed a major band at 33 kd that decreased at both 0.5 and 2 hours after IR of the testis (Figure 2A and B). The decrease in this 33-kd band corresponds in time with the appearance of a 47-kd phosphorylated IκBα band in Figure 1A. This shift in molecular weight most likely represents the phosphorylation and ubiquitination of the 33-kd form.

*Localization of IκBα in the Testis*

Immunohistochemical analysis of total IκBα in the testis revealed a pattern of specific immunoreactivity resembling that of Sertoli cells in the seminiferous tubules (Figure 3). Other cell types in the testis were not immunoreactive.

*Activation of NF-κB Target Genes*

NF-κB target gene arrays were used to determine whether IR caused the alteration of NF-κB target gene expression in concert with the phosphorylation and degradation of IκBα. To ensure specificity of the NF-κB target gene ar-

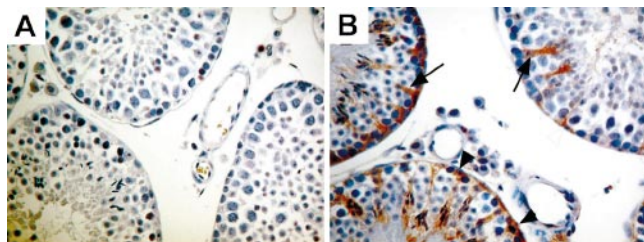


Figure 3. Immunolocalization of IκBα in the mouse testis. (A) Omission of the primary antibody, serving as a negative control. (B) IκBα immunoreactivity is localized to Sertoli cells (arrows). Original magnification, 219×.

**Testis Liver**

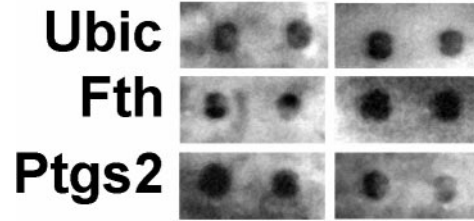


Figure 4. Selected gene expression in liver and testis as detected by the nuclear factor kappa B target gene array. Prostaglandin-endoperoxide synthase-2 (*ptgs2*) is an example of a gene expressed more in the testes than liver. Ferritin heavy chain (*fth*) is an example of a gene expressed more in the liver than in testes, and ubiquitin (*ubic*) is an example of a gene that is expressed similarly in the 2 tissues. This quality control comparison was done to ensure the microarray's sensitivity to anticipated changes in NF-κB activation.

ray, liver RNA was compared with normal testis RNA. Numerous target gene expressions were different between testis and liver. For example, ferritin heavy chain (*Fth*) gene was expressed more in the liver than in the testis and the prostaglandin-endoperoxide synthase-2 (*Ptgs2*) gene was less in the liver than the testis (Figure 4). Other genes like ubiquitin (*ubic*) were expressed equivalently in both tissues (Figure 4). Major consistent gene expressions on the arrays using sham testis RNA included genes like *gapdh*, *pdgfb*, *vegfc*, and *wt-1* (Figure 5), the genes for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), platelet-derived growth factor B (PDGF-B), vascular en-

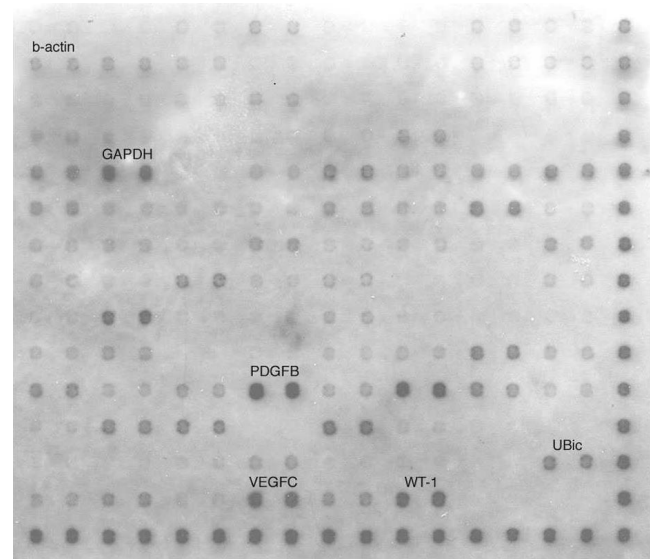


Figure 5. Representative nuclear factor kappa B target gene array on RNA from a sham testis. Four examples of genes that were prominently and consistently expressed are noted in uppercase letters. Two additional genes to be used as load controls are noted in lowercase letters. The bottom row and right-hand column are biotinylated DNA control lanes.

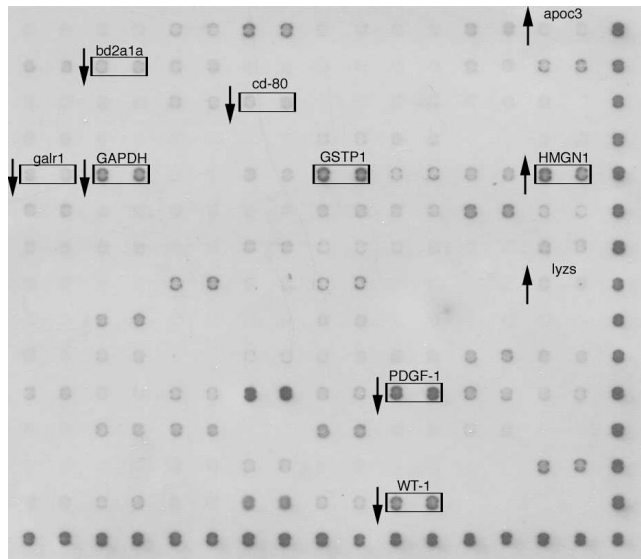


Figure 6. Typical NF- $\kappa$ B target gene array on RNA from a testis 2 hours after ischemia-reperfusion. Highly expressed genes showing consistent changes in triplicate arrays are noted in uppercase letters over boxed hybridization spot pairs. Each gene is represented in duplicate. Intermediately expressed genes showing consistent changes are noted in lowercase letters over boxed spot pairs, and low expression genes showing consistent changes are indicated by lowercase letters over spot pairs with no boxes. Some spots (eg, lyzs) were very faint and reproduce poorly. Arrows indicate the direction of change in 3 out of 3 arrays.

dothelial growth factor (VEGF) isoform c, and Wilms Tumor (WT)-1 protein, respectively. Of the 3 genes on the array to be used as load controls,  $\beta$ -actin ( $\beta$ -actin), *gapdh*, and *ubic* (Figure 5), only *ubic* was relatively unchanged between arrays representing sham and IR testes; therefore, all data were normalized to the average value for *ubic* for each array.

Of the 107 genes on the NF- $\kappa$ B targeted gene array, many exhibited changes, either positive or negative, in individual runs of the experiment, but the standard was maintained that in order to be a gene of interest the gene had to change in a consistent direction in 3 out of 3 experiments. With that standard, the potential NF- $\kappa$ B target genes in the testis were reduced to 9 (Figure 6). Of the genes that were considered highly expressed, 4 changed in expression after IR. The mRNA for high-mobility group nucleosomal binding domain 1 (*hmg1*) consistently increased by approximately 40%, and the mRNAs for *gapdh*, *pdgf-1* (platelet-derived growth factor B), and *wt-1* (the mouse Wilms tumor homolog) were consistently decreased in expression by an average 45%, 25%, and 26%, respectively.

Of the genes that were moderately expressed, the mRNAs for *cd80* (CD80 antigen) and *bcl2-A1a* (Bcl-2 related protein A1a) were consistently down-regulated in testes after IR by an average 38% and 27%, respectively. In the category of genes that showed low expression, the mRNAs for *apoc3* (apolipoprotein C-III) and *lyzs* (lyso-

zyme) were consistently up-regulated in the testis after IR (an average 13-fold and twofold increase over sham values, respectively), and *galr1* (Galinin receptor-1) was consistently down-regulated and reduced by an average 70% between IR and sham testes.

There were numerous genes that changed in the same direction in only 2 of the 3 experiments. An interesting example is *gstp1* (glutathione-S-transferase-1), a prominently expressed gene (Figure 6). This gene increased by an average 62% in 2 experiments, but declined in the third.

## Discussion

IR of the rodent testis results in an increase in the proinflammatory cytokines, IL-1 $\beta$  and TNF- $\alpha$  (Lysiak et al 2003). A role for IL-1 $\beta$  has been demonstrated in the activation of the stress-related kinase, JNK, ultimately leading to E-selectin expression in testicular endothelial cells and neutrophil recruitment (Lysiak et al, 2003). The recruitment of neutrophils to the testis after IR is essential for the observed germ cell-specific apoptosis (Lysiak et al, 2001). Since these proinflammatory cytokines can also activate the NF- $\kappa$ B pathway, we sought to determine whether NF- $\kappa$ B is activated after testicular IR and, if so, what genes are affected by its activation. Results of the present study demonstrate that I $\kappa$ B $\alpha$  is phosphorylated and shifts in molecular weight, suggesting that it is ubiquitinated 2 hours after IR of the murine testis (Figures 1 and 2). Further, total I $\kappa$ B $\alpha$  was immunolocalized in Sertoli cells, suggesting NF- $\kappa$ B activation in that cell type after IR (Figure 3). I $\kappa$ B $\alpha$  phosphorylation is an indicator of NF- $\kappa$ B activation, and that activation was confirmed by employing an NF- $\kappa$ B gene target array (Figures 4 through 6).

In most cells NF- $\kappa$ B remains sequestered in the cytoplasm until activation by certain stimuli such as proinflammatory cytokines, bacterial and viral products, physical stress, oxidative stress, phorbol esters, protein kinase C, or cell mitogens (Baeuerle and Baltimore, 1996). Upon binding to specific cell surface receptors, TNF- $\alpha$  and IL-1 $\beta$  can induce NF- $\kappa$ B activation by activating members of the mitogen activated protein kinase/ERK kinase kinase (MEKK)-related family including NF- $\kappa$ B-inducing kinase (NIK) and MEKK1. Activation of NIK and MEKK1 phosphorylate and activate 2 major components of the I $\kappa$ B kinase complex, I $\kappa$ B kinase  $\alpha$  and  $\beta$  (IKK $\alpha$  and IKK $\beta$ ), but biochemical as well as targeted gene knockout studies have determined that IKK $\beta$  and not IKK $\alpha$  is the target for cytokine induced NF- $\kappa$ B activation (Senftleben and Karin, 2002). I $\kappa$ B $\alpha$  is recruited to the complex and subsequently phosphorylated on serine residues at positions 32 and 36 (DiDonato et al, 1997). This

phosphorylation keys a rapid ubiquitination and degradation through the proteasome pathway.

Following IR of the murine testis I $\kappa$ B $\alpha$  is phosphorylated on serine residue 32 (Figure 1) and most likely ubiquitinated (Figures 1 and 2). We have previously demonstrated that TNF- $\alpha$  and IL-1 $\beta$  mRNA are up-regulated 0.5 hour after IR of the testis (Lysiak et al, 2003); thus, as hypothesized, phosphorylation of I $\kappa$ B $\alpha$  follows the production of the proinflammatory cytokines.

This is not the first report of I $\kappa$ B modulation in the testis, but it is the first to report localization in the Sertoli cells. Budde et al (2002) described the presence of both I $\kappa$ B $\alpha$  and I $\kappa$ B $\beta$  in the murine testis and reported that I $\kappa$ B $\alpha$  is less expressed than I $\kappa$ B $\beta$ . These investigators went on to show that I $\kappa$ B $\beta$  is localized to haploid spermatids, but they did not localize I $\kappa$ B $\alpha$ . We have examined for I $\kappa$ B $\alpha$  specifically, since it is the form induced by the proinflammatory cytokines of interest in IR injury of the testis. I $\kappa$ B $\alpha$ -specific staining was detected only in Sertoli cells (Figure 3); thus, NF- $\kappa$ B activation in the testis may be regulated in different cell types (spermatids and Sertoli cells) by the specific expression of different I $\kappa$ B family members.

Previous results from our laboratory have demonstrated that IR of the testis results in germ cell-specific apoptosis (Turner et al, 1997; Lysiak et al, 2000b). A significant increase in the number of apoptotic germ cells occurs by 24 hours after IR of the murine testis, but a trend toward increased apoptosis begins by 4 hours (Lysiak et al, 2001).

Since phosphorylation of I $\kappa$ B $\alpha$  is an indirect assessment of NF- $\kappa$ B activation, we performed NF- $\kappa$ B targeted gene array analysis on RNA from sham testes and testes 2 hours after IR. Identifying changes in NF- $\kappa$ B target genes at 2 hours after IR of the testis would not only provide further evidence that NF- $\kappa$ B is activated, but screening those downstream genes would suggest targets for further study in their potential role in germ cell-specific apoptosis or Sertoli cell survival. Clearly, gene arrays hybridized with whole-testis RNA will reflect contributions from the entire testis, but I $\kappa$ B $\alpha$  was localized to Sertoli cells and the timing of the RNA extraction relative to IR was chosen to match the time of maximum I $\kappa$ B $\alpha$  activation (Figure 1); thus, activation of NF- $\kappa$ B target genes is very likely in Sertoli cells.

Of the 107 genes in the NF- $\kappa$ B targeted gene array, 9 demonstrated consistent changes 2 hours after IR of the testis. *Hmgn1* was consistently up-regulated. Its protein product is a member of a family of proteins that regulate DNA-related activities like transcription, replication, and repair (Bustin, 1999). Since *Hmgn1* is involved in these DNA-related activities it is not surprising that it would be increased when a transcription factor like NF- $\kappa$ B is activated. Both PDGF-B, a growth factor with roles in

cell proliferation and differentiation (Hwang et al, 2003), and WT1, a transcription factor known primarily for controlling gene expression during embryonic kidney development (Armstrong et al, 1993), were down-regulated after IR of the testis. Interestingly, both PDGF-B (Loveland et al, 1995) and WT1 (Del Rio-Tsonis et al, 1996) have been reported to be produced by Sertoli cells.

Loveland et al (1995) not only found PDGF-B in Sertoli cells, but found PDGF receptor subunits as well, and neither ligand nor receptor was detected in germ cells. This suggests that PDGF-B may not play a direct role in germ cell survival but may have a paracrine mechanism of action. Indeed, a role for PDGF-B in gonocyte proliferation has been previously described (Li et al, 1997). WT1 gene expression in Sertoli cells has been found associated with specific states of germ cell maturation and has been shown to regulate sex-determining gene (SRY; Hossain and Saunders, 2001), suggesting that the WT1 gene product may have a role in spermatogenesis. Further, even though NF- $\kappa$ B is usually thought to increase gene transcription, there is evidence that activation of NF- $\kappa$ B can result in inhibition of specific gene transcription (Todorov et al, 2004).

Of the genes that were moderately expressed in the testis *cd80*, whose product is typically expressed on immune cells, and *bcl2a1a*, whose product has previously been described to have an antiapoptotic role in hematopoietic cells (Zong et al, 1999), were both decreased after IR. In the less abundantly expressed category *ApocIII*, which encodes a protein associated with chylomicrons and low- and high-density lipoproteins (Aalto-Setälä et al, 1996), and *Lyzs*, which encodes a small protein that cleaves carbohydrate chains and is important in antibacterial responses (Mir, 1977), were both up-regulated after IR. *Galr1* in the less abundantly expressed group encodes a rhodospin-like, G protein-coupled receptor that binds the neuropeptide galinin (Iismaa and Shine, 1999). This gene was down-regulated after IR. None of these latter genes have been previously reported in the testis, and their roles in germ cell survival or death remains unknown.

Activation of the NF- $\kappa$ B pathway in Sertoli cells is suggested by the immunolocalization of I $\kappa$ B $\alpha$  in those cells. Sertoli cells remain present and active in the hours and even days after IR injury to the rodent testis (Turner and Miller, 1997); thus, their role is presumably an indirect one in channeling a variety of cell signals that will potentially lead to germ cell apoptosis. Another possibility is that the pathways involved may also play important roles in Sertoli cell survival after IR. The present results direct our interest to gene expressions heretofore unknown in the testis and to their potential activities subsequent to IR injury, whether the ultimate effect is on Sertoli cells or germ cells.

IR of the testis results in germ cell-specific apoptosis. Testicular torsion of the murine testis followed by repair is an excellent model system to study the many facets of this pathology. Dissecting the intracellular signaling pathways activated after testicular IR, including discovering target genes of selected transcription factors, will not only aid in understanding the molecular pathway(s) to germ cell apoptosis after IR but may well provide insight into IR injury in general. Our goal is to exploit these pathways for the development of therapies to protect the at-risk organ after an IR episode.

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