Involvement of mitogen-activated protein kinases (MAPKs) during testicular ischemia–reperfusion injury in nuclear factor-κB knock-out mice

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Abstract

Nuclear factor kappa-B (NF-κB), extracellular regulated kinase (ERK 1/2) and c-jun-N terminal kinase (JNK) play an important role in testicular ischemia. We investigated the patterns of ERK1/2, JNK and p38 activation in NF-κB knockout (KO) mice subjected to testicular torsion. KO and normal littermate wild-type (WT) animals underwent at 1 h testicular ischemia followed by 24 h reperfusion (TI/R). Sham testicular ischemia–reperfusion mice served as controls. ERK 1/2, JNK and p38 expression by western blot analysis, tumor necrosis factor-α (TNF-α) expression (RT-PCR and western blot analysis) and a complete histological examination were carried out. TI/R caused a greater increase in phosphorylated form of ERK 1/2 in KO mice than in WT animals in either the ischemic testis and the contralateral one. By contrary, active form of JNK and p38 were completely abrogated in both testes of KO mice, while WT animals showed a significant activation of those kinases in both testes. TNF-α expression was markedly reduced in KO mice when compared to WT mice either at the mRNA and the protein level. Finally TI/R-induced histological damage was markedly reduced in KO mice. Our data indicate that NF-κB plays a pivotal role in the development of testicular ischemia–reperfusion injury and suggest that, in the absence of the transcriptional factor, the up-stream signal JNK and p38 may be abrogated while ERK 1/2 activity is enhanced. © 2007 Elsevier Inc. All rights reserved.

Keywords: MAPKs; NF-κB; Testis torsion; TNF-α

Introduction

Testicular torsion is a urological emergency and may cause in humans permanent testicular damage.

In animal models, the severity of testicular damage is related to the time and degree of torsion (Heindel et al., 1990). Although there are various reports for and against this argument, it is generally accepted that unilateral testicular torsion may cause contralateral and ipsilateral testicular damage and results in diminished fertility (Kosar et al., 1997). However, the mechanism underlying testicular damage after torsion has not yet been fully clarified.

Recently, studies from our laboratory demonstrated that the mitogen-activated protein kinase (MAPK) family, in particular extracellular regulated kinase (ERK) 1/2 and c-jun-N terminal kinase (JNK), has a role in the pathogenesis of testicular ischemia–reperfusion injury (Minutoli et al., 2005).

Specifically, active MAPKs are responsible for the phosphorylation of variety of effector proteins, including several transcription factors (Widmann et al., 1999), such as nuclear factor-kappaB (NF-κB), that subsequently bind to the upstream regulatory elements of inducible genes (Kyriakis and Avruch, 2001).

NF-κB 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 ischemia–reperfusion (Ghosh et al., 1998).

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NF-kB exists as a complex of homo- or heterodimers composed of members of Rel proteins (Ghosh et al., 1998). In most resting cells, NF-kB is sequestered within the cytoplasm in an inactive form. After activation, NF-kB complexes translocate into the nucleus and activate transcription of target genes for inflammatory proteins such as tumor necrosis factor-α (TNF-α). This cytokine is produced in testis under normal physiological conditions and plays an important role in maintaining testicular function. Recent studies have demonstrated that the TNF-α is increased after the ischemia and reperfusion of the rodent testis (Lysiak et al., 2003).

Thus, the activation of NF-kB leads to a coordinated increase in the expression of many genes whose products mediate inflammatory and immune responses (Lysiak et al., 2005).

It is demonstrated that the NF-kB involvement may be present in different cell types of the testis (Barnes and Karin, 1997; Mercurio and Manning, 1999; Senfletben and Karin, 2002). More in detail, the p50 and RelA subunits of NF-kB have been identified in the nuclei of spermatocytes just prior to the meiotic division and in spermatids immediately after meiosis. Sertoli cells which support germ cell development were also found to express high levels of nuclear p50 and RelA. Sertoli nuclear NF-kB levels varied depending on the developmental stages of associated germ cells (stage of spermatogenesis) (Delfino and Walker, 1998). In addition, primary cultures of Sertoli cells exhibited elevated levels of nuclear NF-kB-DNA binding activity (Delfino and Walker, 1999). Indeed, NF-kB has been proposed to play a role in testicular ischemia (Lysiak et al., 2005).

In light of these findings, the aim of the present paper was to study the patterns of ERK 1/2, JNK and p38 activation in NF-kB knockout (KO) mice subjected to testicular torsion.

**Materials and methods**

**Animals and treatment**

All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the care and use of Laboratory animals (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, MD, USA). Breeding pairs of NF-kappaB KO mice (p105; b6; 129-Nfkbi161Bal; KO) and normal control littermates WT mice (C57Bl/6; 129; WT), both weighing 22 to 30 g, were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The animals were fed a standard diet ad libitum and had free access to tap water in the laboratory of Section of Pharmacology (Messina, Italy). They were maintained on a 12-hour light/dark cycle at 21 °C. Mice were anesthetized with an intraperitoneal injection of 80 mg/kg of pentobarbital sodium. Testis ischemia–reperfusion injury (TI/R) was induced by torsion of the left testis, with a 720° twisting of the spermatic cord so as to produce a total occlusion of testis for one hour. The same testis was then detorted. Following 0, 10, 30 min and...
1, 3 and 24 h of reperfusion, the animals were sacrificed with an overdose of pentobarbital sodium and bilateral orchidectomies were performed. Sham operated mice underwent the same surgical procedures as TI/R mice except for testicular occlusion. Previous data have suggested that peak expression of MAPKs occurs at time 30 min and thereafter the levels of the kinases decline to the basal levels (Minutoli et al., 2005). In light of these previous observations we investigated the time points 10 and 30 min.

**Isolation of cytoplasmatic protein**

Briefly, pulverized testis samples were homogenized in 1 ml lysis buffer (25 mM Tris/HCl, pH 7.4, 1.0 mM EGTA, 1.0 mM EDTA, 0.5 mM phenyl methylsulfonyl fluoride, aprotinin, leupeptin, pepstatin A (10 μg/ml each) and Na3VO4 100 mM, with a Dounce homogenizer. The homogenate was subjected to centrifugation at 15,000 g for 15 min. The supernatant was collected and used for protein determination using the Bio-Rad protein assay kit (Bio-Rad, Richmond, CA, USA).

**Determination of ERK 1/2, JNK, p38 and TNF-α by western blot analysis**

Protein samples (50 μg) were denatured in reducing buffer (62 mM Tris pH 6.8, 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue) and separated by electrophoresis on an SDS (12%) polyacrylamide gel. The separated proteins were transferred on to a nitrocellulose membrane using the transfer buffer (39 mM glycine, 48 mM Tris, pH 8.3, 20% methanol) at 200 mA for 1 h. The membranes were stained with Ponceau’s (0.005% in 1% acetic acid) to confirm equal amounts of protein and were blocked with 5% non fat dry milk in TBS-0.1% Tween for 1 h at room temperature, washed three times for 10 min each in TBS-0.1% Tween, and incubated with a primary antibody for TNF-α (Chemicon, Temecula, CA, USA) and for the phosphorylated and total form of both ERK 1/2, JNK and p38 (Cell Signaling, Beverly, MA, USA) in TBS-0.1% Tween overnight at 4 °C, diluted 1:1000. After being washed three times for 10 min each in TBS-0.1% Tween, the membranes were incubated with a secondary antibody peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL, USA) diluted 1:20000. After washing, the membranes were analyzed with an enhanced chemiluminescence system according to the manufacturer’s protocol (Amersham, Little Chalfont, UK). The ERK 1/2, JNK, p38 and TNF-α protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). Equal loading of protein was assessed on stripped blots by immunodetection of β-actin with a rabbit monoclonal antibody (Cell Signaling, Beverly, MA, USA) diluted 1:500 and peroxidase-conjugated goat anti-rabbit immunoglobulin G (Pierce, Rockford, IL, USA) diluted 1:15000. All antibodies are purified by protein A and peptide affinity chromatography.
RNA extraction

Testes were processed for total RNA isolation using Trizol reagent according to the manufacturer’s protocol. RNA from each sample was dissolved in appropriate volume of TE buffer (10 mM Tris–HCl pH 8.0 and 1 mM EDTA). The RNA solution was quantified at 260 nm by spectrophotometer reading (BioPhotometer, Eppendorf, Germany) and its purity was assessed by the ratio at 260 and 280 nm readings.

Real-time PCR

Total mRNA (5 μg/ml) from each sample was reverse transcribed using High Capacity cDNA Archive Kit according to the manufacturer’s manual (Applied Biosystem, Foster City, CA, USA). Ten nanograms of cDNA was amplified by real-time PCR with 2X TaqMan universal PCR Mastermix (Applied Biosystem), 20× target primer and probe. β-actin was used as the housekeeping gene. Each sample was analysed in duplicates using SDS 7300 (Applied Biosystem). PCR amplification was related to a standard curve. The results were expressed as an n-fold difference relative to normal controls (relative expression).

Histology

Excised mouse testes were longitudinally sectioned and formalin fixed and paraffin embedded. Serial sections (5 μm) were stained with hematoxylin and eosin. Light microscopy of the testicular section was done in twenty high power fields (HPFs). Histological lesions were evaluated in both the tubular and extratubular compartments, using a semiquantitative method, as previously performed (Minutoli et al., 2005), to recognize lobular changes and/or interstitial extravasations. Focal or diffuse tubular changes were objectified, including germ cell detachment or loss in the tubular compartment, and coagulative necrosis. Venular and lymphatic ectasia was quantified using the ocular micrometer of the Zeiss Axioplan microscope. The greatest diameter was recorded on twenty microvessel transverse profiles for each slide as the mean±standard deviation (SD). Histological damage was scored according to the following grades: 0, absent; 1, mild; 2, moderate and 3, severe (Minutoli et al., 2005). To measure testicular edema, a piece of the testicular body was rapidly removed, weighed and blotted dry on filter paper.

Immunohistochemical staining for TNF-α

Paraffin-embedded tissues were sectioned (5 μm), and antigen retrieval was performed using microwave treatment. Tissues were treated with primary antibody against TNF-α (Santa Cruz Biotechnology, Santa Cruz, CA, USA), diluted 1:500 and incubated overnight. Secondary antibody was provided by Dako, and the location of the reaction was visualized with 3,3’-diaminobenzidine tetra-hydrochloride (Dako Cytomation Carpinteria, CA, USA) as chromogen substrate. Sections of human breast carcinoma were processed as positive controls. Slides were then mounted with coverslips.
Statistical analysis

All data are expressed as the mean±SEM. Data were analyzed by ANOVA followed by post hoc evaluation. In all cases, a probability error of less than 0.05 was selected as criterion for statistical significance. For the histological results, statistical analysis was performed using a repeated measures ANOVA test with Dunn multiple comparison post test.

Results

Expression patterns in KO and WT mice before reperfusion

No significant changes were found in the expression of ERK 1/2, JNK, p38, TNF-α mRNA or TNF-α protein either testis of sham-operated KO or WT mice (data not shown). During the ischemic period in TI/R KO and TI/R WT mice, no significant changes were found in the expression of ERK 1/2, JNK or p38 in either the torsioned or the contralateral testis of either mouse strain. During the same time period, TNF-α mRNA and TNF-α protein could not be detected in either testis of either mouse strain.

ERK 1/2 expression in TI/R: comparison between KO and WT mice

Both the torsioned or the contralateral testis showed a low constitutive presence of the ERK 1/2 protein in both KO and WT mice after the beginning of the reperfusion (Fig. 1).

Testicular ischemia–reperfusion injury caused a greater increase of phosphorylated ERK 1/2 (p-ERK 1/2) in KO mice than in WT animals (Fig. 1) after 30 min of reperfusion. This higher expression was observed in the
ipsilateral testis and contralateral one of both strains of animals (Fig. 1). No changes were observed in the total form of this kinase in either testis of either KO or WT mice (Fig. 1).

**JNK expression in TI/R: comparison between KO and WT mice**

Very low levels of JNK were found in both the torsioned and the contralateral testes of KO and WT mice immediately after the beginning of reperfusion (Fig. 2).

Phosphorylated JNK (p-JNK) expression increased in both testes of WT mice after 30 min of reperfusion (Fig. 2). By contrast, this protein was completely absent in the torsioned and contralateral testes of KO mice (Fig. 2). No changes were observed in the total form of this kinase in the ipsilateral testis and contralateral one of either strain of animal (Fig. 2).

**p38 expression in TI/R: comparison between KO and WT mice**

Very low levels of p38 were found in both the torsioned and the contralateral testes of KO and WT mice immediately after the beginning of reperfusion (Fig. 3).

Phosphorylated p38 (p-p38) expression increased in both testes of WT mice after 30 min of the reperfusion (Fig. 3).

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**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>WT TI/R</th>
<th>KO TI/R</th>
<th>WT TI/R contralateral</th>
<th>KO TI/R contralateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extratubular edema</td>
<td>2.7±0.49* 0.3</td>
<td>0.4±0.53</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Hemorrhagic extravasation</td>
<td>2.1±0.90* ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Endotubular fluid changes</td>
<td>2.7±0.49* ND</td>
<td>0.4±0.53</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Germ cell changes</td>
<td>2.9±0.38* ND</td>
<td>1.1±0.69*</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Each group was composed of 7 animals. ND = not detectable changes. Histological damage was scored according to the following grades: 0, absent; 1, mild; 2, moderate and 3, severe *p<0.001 vs the corresponding testis in KO I/R.

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*Fig. 6. A. Light microscopy of the ipsilateral testis collected from WT mouse subjected to testicular ischemia-reperfusion injury. Severe hemorrhagic extravasation (arrows) and interstitial edema, microvessel ectasia are obvious. Diffuse germ cell sloughing and hypoxic lesions can also be seen (arrowheads) (H&E original magnification ×200). B. Light microscopy of the contralateral testis harvested from WT mouse subjected to testicular ischemia-reperfusion injury. Co-existence of mild microvessel ectasia (arrows) and regressive germ cells (arrowheads) (H&E original magnification ×200). C. Light microscopy of the ipsilateral testis collected from KO mouse subjected to testicular ischemia–reperfusion injury. Absence of histological lesion (H&E original magnification ×200). D. Light microscopy of the contralateral testis harvested KO mouse subjected to testicular ischemia–reperfusion injury. Absence of histological lesion (H&E original magnification ×200).*
By contrast, this protein was completely absent in the torsioned and contralateral testes of KO mice (Fig. 3). No changes were observed in the total form of this kinase in the ipsilateral testis and contralateral one of either strain of animal (Fig. 3).

TNF-α mRNA and protein expression in TI/R: comparison between KO and WT mice

This inflammatory cytokine was found in both torsioned and contralateral testis of KO and WT mice after the beginning of the reperfusion, but at low expression levels of both mRNA (Fig. 4) and protein (Fig. 5).

After 60 and 180 min of reperfusion, WT mice had a markedly increased TNF-α message (Fig. 4) and protein level (Fig. 5) in both torsioned and contralateral testes following 60 and 180 min of reperfusion. By contrast the mRNA for the inflammatory cytokine was markedly attenuated in KO mice in the ipsilateral and contralateral testes after the reperfusion (Fig. 4), and the same attenuation was observed for the activity of this cytokine (Fig. 5).

Histology

The histological findings are summarized in Table 1. Twenty high power fields for each section were analyzed. No histological changes were observed in either testis of sham-operated KO or WT mice (results not shown). Mean microvessel diameter was 19.12±2.13 μm.

WT mice subjected to testicular ischemia–reperfusion had severe lobular hemorrhagic extravasations, venular and lymphatic vessel ectasia (102.31±12.09 μm), significantly greater than microvessel diameter of KO ischemic testis (p<0.001), together with edema, germ cell hypobiosis and/or diffuse coagulative necrosis associated with high amounts of endotubular fluids (Table 1 and Fig. 6A) after 24 h of reperfusion.

As a consequence of ischemia/reperfusion in WT animals, contralateral testis showed mild microvessel ectasia (41.03±5.11 μm), significantly greater than microvessel diameter of KO contralateral testis (p<0.001), without an excess endotubular fluid and without hypoxic cell changes (Table 1 and Fig. 6B) after 24 h of reperfusion.

By contrast, the ipsilateral testis and contralateral one of KO mice subjected to testicular ischemia–reperfusion, neither showed significant histological changes, nor microvessel ectasia (21.18±3.02 μm and 19.31±11.09 μm) (Table 1 and Fig. 6C and D) after 24 h of reperfusion.

Immunostaining for TNF-α

Immunohistochemical analysis revealed absence of immunopositivity for TNF-α in sham-operated WT mice (Fig. 7A). By contrast, TNF-α was strongly produced in the Leydig cells in
Discussion

Testicular torsion is a multifaceted pathology displaying many of the characteristics of ischemia–reperfusion injury described in other organs. Surgical intervention is often necessary to re-establish blood flow; however, testicular atrophy may still ensue, depending on the degree of rotation and duration of the torsion.

Pathophysiological mechanisms that regulate such alterations have been ascribed to direct damage caused by ischemia during torsion and to secondary effects due to reperfusion during the untwisting of the spermatic cord (Greenstein et al., 2001). Ischemia and reperfusion of testis is an important phenomenon that has not been completely defined from the perspective of the molecular signalling pathways. In this regard, previous reports from our laboratory have demonstrated the important role of the MAP kinases (Minutoli et al., 2005) in the pathogenesis of the torsion of the testis. Our data concur with others’ experimental evidence suggesting that ischemia and reperfusion of the murine testis stimulates JNK (Lysiak et al., 2003).

In the current study, we found an augmented p-ERK 1/2 expression and the complete absence of P-JNK and p38 in both testes of WT mice (Fig. 7B) subjected to testicular ischemia–reperfusion. Additionally, KO mice subjected to testicular ischemia–reperfusion showed a weak staining for the cytokine in the Leydig cells (Fig. 8).

A first tentative hypothesis could be that p-ERK 1/2 is over-expressed to overcome the lack of p-JNK, p-p38 and NF-κB. However, this “compensatory mechanism” is not able to efficiently activate the inflammatory cascade: as a matter of fact KO mice have reduced TNF-α levels and reduced tissue damage.

Alternatively, the higher expression of p-ERK 1/2 in KO mice might unmask a protective role for this kinase. In fact, it has been shown that in myocardial ischemia–reperfusion or in the “phenomenon” of preconditioning, early activation of MAPKs may contribute to cardioprotection in the so-called reperfusion injury kinase (RISK) pathways (Hausenloy and Yellon, 2004). However, this hypothesis deserves further examination in future experiments.

Although the precise mechanism by which NF-κB mediates the effects of ischemia and reperfusion of testis remains undetermined, it is of interest that activation of both p-JNK and p-p38 is abrogated in KO mice.

JNK is especially called stress-activated protein kinase, since it is additionally activated by cellular stresses such as reactive oxygen species and proinflammatory cytokines, including IL-1β and TNF-α (Sugden and Clerk, 1998). Although these
cellular stresses activate NF-κB as well as JNK, we have demonstrated that the lack of NF-κB causes the absence of JNK activation. This observation is in agreement with previous findings in other models of organ damage produced in mice lacking the gene for NF-κB (Kawano et al., 2005).

MAPKs are an evolutionary conserved family of serine/threonine kinases, involved in the regulation of gene expression, cell proliferation, cell survival and death (Kyriakis and Avruch, 2001). The main task of these activated proteins is the targeted phosphorylation of transcription factors such as NF-κB (Barnes, 1997). Therefore it could be speculated that JNK is turned off in mice lacking the gene for NF-κB during testicular ischemia–reperfusion injury because of the absence of its specific target.

NF-κB has been shown to have a pivotal role in several physiological and pathological conditions of testis (Lysiak et al., 2005; Lilienbaum et al., 2000). Indeed, the transcription factor activates inflammatory cascade which involves cytokines which include TNF-α. The importance of this cytokine in the pathogenesis of testicular torsion is well recognized (Minutoli et al., 2005; Lysiak et al., 2003; Lysiak, 2004).

Consequently, in this study, we analyzed the message of the inflammatory cytokine as well as the mature protein. Our results indicate that genetically modified mice showed markedly reduced testis TNF-α expression when compared to WT mice in both torsioned and contralateral testes.

A possible explanation for this finding could be that in the absence of the transcriptional factor, the latter step of the inflammatory cascade is kept from being fully activated.

Histological features correlated very well with the biochemical “picture” of a blunted inflammatory phenotype. As a matter of fact mice lacking the gene for the transcription factor showed neither significant histological changes, nor microvessels ectasia in both ischemic and contralateral testis, thus confirming that ischemia and reperfusion of testis is mainly a NF-κB-dependent pathological condition. Additionally, immunohistochemical analysis showed a greater immunopositivity for TNF-α in the Leydig cells in testes of WT mice subjected to testicular ischemia–reperfusion compared to KO mice suggesting that inflammatory process is particularly increased in interstitial tissue of twisted testis.

On the basis of our observations we tried to define a possible pathophysiological mechanism in the ischemia–reperfusion injury as summarized in Fig. 9.

Overall, our experiments have also shown a contralateral testicular damage with an inflammatory pattern, i.e. MAPKs, similar to that observed in twisted testes. The presence of contralateral involvement has been previously recognized by investigating other pathogenesis mechanism(s): acute testicular torsion causes oxidative stress and irreversible damage both in the twisted ipsilateral testis and in the untwisted contralateral testis (Ozkan et al., 2004; Saba et al., 1997; Kosar et al., 1997).

However, the mechanism of contralateral damage is to date unclear. One theory postulates that there is a decrease in contralateral testicular blood flow as a reflex to an afferent stimulus (Andiran et al., 2000; Prillaman and Turner, 1997). Overall, whatever the triggering mechanism and in light of our experimental findings, the cascade of pathological events occurring in both testes appear very similar.

Conclusions

Taken together, the present results confirm that NF-κB plays a pivotal role in the development of testicular ischemia–reperfusion injury and suggest that, in the absence of the transcriptional factor, the up-stream signals JNK and p38 may be abrogated while ERK 1/2 expression is over-activated. However, it is difficult to skip from gene knockout studies, in which the activity of a given kinase is completely abolished, to drug therapy, where a partial inhibition may be sufficient to achieve the desired therapeutic effect. Furthermore, since NF-κB and related kinases are involved in the expression of genes that mediate transformation, proliferation, invasion, angiogenesis, and metastasis on one hand and apoptosis, immunity, and hematopoiesis on the other, all the alternative molecules involved in the inflammatory pathways should be tested with particular caution.

References


