Lipid Peroxidation Activates Mitogen-Activated Protein Kinases in Testicular Ischemia-Reperfusion Injury

Pietro Antonuccio, Letteria Minutoli, Carmelo Romeo,* Piero Antonio Nicòtina, Alessandra Bitto, Salvatore Arena, Domenica Altavilla, Biagio Zuccarello, Francesca Polito and Francesco Squadrito

From the Departments of Medical and Surgical Pediatric Sciences (PA, CR, SA, BZ), Experimental and Clinical Medicine and Pharmacology (LM, AB, DA, FP, FS) and Human Pathology (PAN), University of Messina, Messina, Italy

Purpose: Testicular damage after torsion has been attributed to many mechanisms, of which one is lipid peroxidation of the plasma membrane, which could cause the activation of the mitogen-activated protein kinase family. These proteins are of vital importance for signal transduction pathways and 2 of them, extracellular signal-regulated kinase and c-jun N-terminal kinase, participate in the pathogenesis of testicular ischemia. We investigated whether lipid peroxidation may trigger mitogen-activated protein kinase activation in testicular ischemia-reperfusion.

Materials and Methods: Adult male Sprague-Dawley rats were subjected to 1-hour testicular ischemia, followed by 24 hours of reperfusion. Sham testicular ischemia-reperfusion rats served as controls. Animals were randomized to receive raxofelast, an inhibitor of lipid peroxidation (20 mg/kg intraperitoneally administered 15 minutes before detorsion and 15 minutes after detorsion) or vehicle (1 ml/kg 10% dimethyl sulfoxide/NaCl solution). A group of animals was sacrificed 0, 10, 15, 20, 25 and 30 minutes, and 1, 2 and 3 hours, respectively, after detorsion to evaluate testicular c-jun N-terminal kinase, extracellular signal-regulated kinase and tumor necrosis factor-α activation by Western blot analysis, and mRNA expression and conjugated dienes using a spectrophotometer technique. Another group was sacrificed 24 hours after detorsion to evaluate histological alterations.

Results: Testicular ischemia-reperfusion injury caused a significant increase in the conjugated diene levels, extracellular signal-regulated kinase c-jun N-terminal kinase activity and tumor necrosis factor-α expression in both testes. Furthermore, histological examination revealed marked damage. Raxofelast inhibited these parameters and decreased histological damage.

Conclusions: These data suggest that lipid peroxidation triggers extracellular signal-regulated kinase and c-jun N-terminal kinase activation. Furthermore, mitogen-activated protein kinase blockade might represent a potential therapeutic approach to treatment in patients with unilateral testicular torsion.

Key Words: testis; spermatic cord torsion; JNK mitogen-activated protein kinases; reperfusion injury; rats, Sprague-Dawley

Testicular torsion is a medical emergency that requires immediate diagnosis and treatment to avoid subsequent testicular injury and infertility.1,2 Testicular damage after torsion may arise from oxygen free radicals produced during reperfusion.3 Oxygen free radicals oxidize membrane lipids, proteins and DNA, leading to cellular dysfunction and sometimes cell death.4 They can produce mutations in the genetic code, inducing damage to DNA.

Recent studies from our laboratory using a rat model of testicular torsion also demonstrated that oxidative stress during post-ischemic reperfusion has a pivotal role in activating an interconnected inflammatory cascade that leads to organ damage.5 For these reasons different antioxidant therapeutic strategies have been investigated with the aim of decreasing short-term and long-term testicular reperfusion damage.6,7 The antioxidant vitamin E has an important role in the endogenous defense mechanism since its deficiency is responsible for increased tissue injury caused by oxidative stress.8 In agreement with this hypothesis our recent study also showed that the new antioxidant raxofelast, a vitamin E analogue, strongly attenuates acute testicular damage after torsion.5

Oxidative stress-lipid peroxidation triggers activation of the MAPK family in different tissues.9 To our knowledge its role in MAPK activation after testicular detorsion has not been investigated.

MAPKs are a group of protein serine/threonine kinases activated by various extracellular stimuli, and they mediate signal transduction from cell surface to nucleus.10 Two of these MAPKs, ERK 1/2 and JNK, were especially investigated for their role. Active MAPKs are responsible for the phosphorylation of various effector proteins, including several transcription factors.11

Recent studies demonstrated that TNF-α activates the stress related kinase pathway after testicular torsion.12 TNF-α is a multifunctional cytokine with effects not only in the proinflammatory response,13 but also in the immunoregulatory response14 and apoptosis. It was shown that this cytokine has a significant role in certain testicular patholo-
gies. We recently reported that MAPK blockade decreases TNF-α expression and improves the testicular damage caused by I/R injury. In the current study we determined whether lipid peroxidation may trigger MAPK activation in T/I/R.

MATERIALS AND METHODS

Animals
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, Maryland. Male Sprague-Dawley rats weighing 250 to 300 gm were used. They were fed a standard diet and had free access to tap water. A 12-hour light-dark cycle was used. Animal temperature was maintained at approximately 37°C using an overhead lamp.

Experimental Protocol
All animals were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium. TI/R was induced by torsion of the left testis with 720-degree twisting of the spermatic cord to produce total testicular occlusion for 1 hour. The same testis was then detorsed. Following 0, 10, 15, 20, 25 and 30 minutes, and 1, 3 and 24 hours of reperfusion, respectively, rats were sacrificed with an overdose of pentobarbital sodium and bilateral orchietomy was performed. Sham operated rats underwent the same surgical procedures as T/I/R rats except for testicular occlusion. Animals were randomized to receive raxoflaxol, an inhibitor of lipid peroxidation (20 mg/kg intraperitoneally administered 15 minutes before detorsion and 15 minutes after detorsion) or its vehicle (1 ml/kg intraperitoneally administered 15 minutes before detorsion and 15 minutes after detorsion) or its vehicle (1 ml/kg intraperitoneally of 10% dimethyl sulfoxide/NaCl solution).

Isolation of Cytoplasmic Protein
Briefly, pulverized testicular sample were homogenized in 1 ml lysis buffer composed of 25 mM tris/HCL, pH 7.4, 1.0 mM egtazic acid, 1.0 mM ethylenediaminetetraacetic acid, 0.5 mM phenyl methylsulfonyl fluoride, 10 μg/ml of each of apro tinin and leupeptin, pepstatin A, and 100 mM Na3VO4 with a Dounce homogenizer. The homogenate was subjected to centrifugation at 15,000 g/ml of each of the testicular section was done in 20 high power fields while

Determination of ERK 1/2, JNK and TNF-α by Western Blot Analysis
Protein samples (50 μg) were denatured in reducing buffer composed of 62 mM tris/HCL, pH 7.4, 1.0 mM egtazic acid, 1.0 mM ethylenediaminetetraacetic acid, 0.5 mM phenyl methylsulfonyl fluoride, 20% sodium dodecyl sulfate, 5% β-mercaptoethanol and 0.003% bromophenol blue, and separated by electrophoresis on 12% sodium dodecyl sulfate polyacrylamide gel. Separated proteins were transferred to a nitrocellulose membrane using transfer buffer composed of 39 mM glycine, 48 mM tris, pH 8.3, and 20% methanol at 200 mA for 1 hour. Membranes were stained with Ponceau stain (0.005% in 1% acetic acid) to confirm equal amounts of protein. They were then blocked with 5% nonfat dry milk in TBS-0.1% Tween for 1 hour at room temperature, washed 3 times for 10 minutes each in TBS-0.1% Tween and incubated with primary phosphorylated antibody for ERK 1/2, JNK (Cell Signaling, Beverly, Massachusetts) and TNF-α (Chemicon International, Temecula, California) in TBS-0.1% Tween overnight at 4°C, diluted 1:1,000. After washing 3 times for 10 minutes each in TBS-0.1% Tween the membranes were incubated with secondary antibody peroxidase conjugated goat anti-rabbit IgG (Pierce, Rockford, Illinois) for 1 hour at room temperature, diluted 1:20,000. After washing the membranes were analyzed by an enhanced chemiluminescence system according to the manufacture protocol (Amersham™). The ERK 1/2, JNK and TNF-α protein signal was quantified by scanning densitometry using a bio-image analysis system (Bio-Profil Celbio, Milan, Italy). Results in each experimental group are expressed as relative integrated intensity compared with that of control normal testis (sham TI/R) measured with the same batch. Equal protein loading was assessed on stripped blots by β-actin immunodetection with rabbit monoclonal antibody (Cell Signaling), diluted 1:500, and peroxidase conjugated goat anti-rabbit IgG (Pierce), diluted 1:15,000. All antibodies were purified by protein A and peptide affinity chromatography.

RNA Extraction
Testes were processed for total RNA isolation using TRIzol® reagent according to the manufacturer protocol. RNA from each sample was dissolved in 50 μl sterile ribonuclease-free water, quantitated spectrophotometrically and stored at −80°C or used immediately for PCR.

Real-Time PCR
Per sample 5 ng mRNA were reverse transcribed using a High Capacity cDNA Archive Kit according to the manufacturer manual (Applied Biosystems, Foster City, California). cDNA (2 μl) was amplified by real-time PCR with 2 × TaqMan® universal PCR mastermix, 20 × target primer and probe with β-actin serving as the housekeeping gene. Each sample was analyzed in duplicate using an ABI™ Prism® 7300. PCR amplification was related to a standard curve. Results are expressed as the n-fold difference relative to normal controls (relative expression).

CD Evaluation
Estimation of the tissue content of CD was done to evaluate the extent of lipid peroxidation in damaged tissue. Samples of testicular were frozen at −80°C until assay. Samples were collected in polyethylene tubes and then washed with 1 ml butylated hydroxytoluene (1 mg/ml in phosphate buffer).

After drying in absorbent paper the samples were frozen at 4°C until analysis. Biochemical CD assay required previous lipid extraction from tissue samples by chloroform/methanol (2:1). The lipid layer was dried under nitrogen atmosphere and dissolved in cyclohexane. The CD testicular content was measured at 232 nm using a spectrophotometric technique. The amount of tissue CD is expressed in as Δ ABS/mg.

Histology
Excised rat testes were longitudinally sectioned, formalin fixed and paraffin embedded. Serial sections (5 μm) were stained with hematoxylin and eosin. Light microscopy of the testicular section was done in 20 high power fields while
blinded to previous treatment. Histological lesions were evaluated in the tubular and extratubular compartments using a semiquantitative method, accounting for interstitial extravasations, and tubular and extratubular features. Focal or diffuse tubular changes were also evaluated, including germ cell sloughing or loss and coagulative necrosis. Venular and lymphatic ectasia was objectified using the ocular micrometer of an Axioplan® microscope. The greatest diameter was recorded on 20 microvessels transverse profiles per slide as the mean ± SD. Histological damage was scored in accordance with certain grades, including 0—absent, 1—mild, 2—moderate and 3—severe.

To measure testicular edema a piece of the testicular body was rapidly removed, weighed and plotted dry on filter paper. The extent of testicular edema was calculated by measuring water content. Testicular tissue was weighed before and after desiccation at 95°C for 24 hours. The difference between wet and dry tissue weight was calculated. The degree of edema is expressed as a percent the tissue wet weight.

**Statistical Analysis**

All data are expressed as the mean ± SD. Data were analyzed by ANOVA for multiple comparisons of results. Duncan’s multiple range test was used to compare group means. In all cases a probability error of less than 0.05 was considered statistically significant. For histological results statistical analysis was performed using a repeated measures ANOVA test with the Dunn multiple comparison post test.

**Drugs**

Raxofelast is a lipid peroxidation inhibitor. Compounds were administered intraperitoneally in dimethyl sulfoxide/NaCl 0.9% (1:10 volume per volume) solution. All substances were prepared fresh daily and administered in a volume of 1 ml/kg.

**RESULTS**

**Testicular Tissue CD Levels**

Determination of testicular CD was performed to estimate free radical damage in the tissue. Low CD levels were measured in testes from sham TI/R animals treated with vehicle or raxofelast at any reperfusion time. There were no differences among the groups or between the ipsilateral and contralateral sides in the same group (fig. 1). CD levels in each testis of TI/R animals treated with vehicle increased at 10 minutes and remained relatively constant after 25 minutes of reperfusion (fig. 1). Raxofelast administration significantly decreased the CD concentration in treated rats in the I/R and the contralateral testis vs sham TI/R and TI/R animals (p <0.005).

**Time Course of ERK 1/2 in TI/R**

During the ischemic period ERK 1/2 activity was not significantly changed (data not shown). In addition, low levels ERK 1/2 activity were found in the I/R and the contralateral testis immediately after the beginning of reperfusion in...
sham occluded and reperfused animals at any reperfusion time (fig. 2). ERK1/2 activity in both testes increased as early as 10 minutes and it achieved peak levels at 30 minutes of reperfusion (fig. 2). ERK 1/2 protein was also investigated 2 hours after reperfusion. However, we did not find any activation of this kinase in the I/R or in the contralateral testis (fig. 2).

The administration of raxofelast, an inhibitor of lipid peroxidation, markedly inhibited MAPK activity in each testis vs sham TI/R and vs TI/R plus vehicle obtained from 7 different animals (p < 0.05 and < 0.01, respectively, fig. 2). The same pharmacological treatment did not affect JNK activity in the sham treated group (fig. 3).

**Time Course of TNF-α in TI/R**

No detectable TNF-α activity was observed during the ischemic period. This inflammatory cytokine was investigated at 60 and 180 minutes of reperfusion in the I/R and the contralateral testis (fig. 4). Low levels of the inflammatory cytokine were observed in the I/R and the contralateral testis immediately after the beginning of reperfusion, and in ischemic and reperfused rats, and sham treated animals (data not shown). A significant increase in TNF-α activity in both testes was noted at 60 minutes, which attained a peak at 180 minutes of reperfusion (fig. 4). The administration of raxofelast, an analogue of vitamin E, markedly attenuated TNF-α in each testis vs sham TI/R and vs TI/R plus vehicle obtained from 7 different animals (p < 0.01). However, it did not modify the inflammatory cytokine in the sham TI/R group (fig. 4).

**Real-Time PCR Evaluation of TNF-α mRNA**

Inflammatory cytokine mRNA was slightly increased in the I/R and the contralateral testis immediately after the beginning of reperfusion (data not shown). A significant increase in TNF-α mRNA in each testis was observed at 60 minutes and it attained a peak at 180 minutes of reperfusion (fig. 5). The administration of raxofelast, an analogue of vitamin E, markedly attenuated the message for TNF-α in both testes vs sham TI/R and vs TI/R plus vehicle obtained from 7 different animals (p < 0.01). However, it did not modify inflammatory cytokine mRNA in the sham group (fig. 5).
FIG. 5. Quantitative mRNA expression of inflammatory cytokine TNF-α in animals treated with 20 mg/kg raxofelast intraperitoneally administered 15 minutes before and 15 after detorsion or its vehicle, 1 ml/kg NaCl 0.9% solution intraperitoneally with quantitative data corrected for actin. a, I/R testes; b, contralateral testes. Bars represent mean + SD of 7 experiments. Asterisk indicates p <0.01 vs sham TIR. Pound sign indicates p <0.01 vs TIR plus vehicle.

Histology
The table lists histological findings. We studied 20 high power fields per section. Testes from sham TI/R rats receiving vehicle or raxofelast showed unmodified tubular and extratubular compartments without inflammatory infiltrates or hemorrhagic lesions. Mean microvessel diameter was 14.25 ± 1.08 μm. In contrast, TI/R rats treated with vehicle had severe edema, venular and lymphatic vessel ectasia (130.4 ± 9.12 μm), and focal germ cell hypoplasia vs sham TI/R animals (p <0.001, see table and fig. 6, A).

In contralateral testes I/R produced a lesser extent of extratubular edema and microvessel ectasia (78.01 ± 7.12 μm) with mild hypospermatogenesis vs IR in the same group (p <0.001, see table and fig. 6, B). Raxofelast treated rats showed mild edema, venular/lymphatic ectasia (45.18 ± 6.06 μm), hypospermatogenesis in the IR testis and a normal-appearing contralateral testis (see table, and fig. 6, C and D).

DISCUSSION
TI/R injury causes oxidative stress and irreversible damage in the ipsilateral and in the contralateral testes. Although reperfusion is essential for the survival of ischemic tissue, it can also generate additional cell injury, which has been attributed to neutrophil infiltration and ROS generation. When ROS generation exceeds the defense mechanisms, oxidative stress is generated, which contributes to reversible or irreversible cell injury. In this regard, recent reports indicate that superoxide dismutase mimic enzymes that metabolize ROS and can partially prevent TI/R injury. Antioxidants would theoretically have a dual effect in TI/R injury since it would limit damage evolution, decreasing free radicals generated by lipid peroxidation, and counteract the ROS mediated activation of inflammatory reaction. In fact, our previous studies have demonstrated that antioxidant raxofelast decreases oxidative stress and the morphological changes that follow TI/R. It was also suggested in another experimental model that the oxidative stress-lipid peroxidation phenomenon triggers MAPK pathways.

Oxidants activate cytosolic and membrane bound phospholipase A_2 with the subsequent production of arachidonic acid metabolites, which subsequently stimulate ERK and JNK activity, leading to the ultimate stimulation of the transcription of several mitogen responsive genes. Membrane involvement in the downstream activation of MAPK suggests that the free radical induced, chain breaking reaction of lipid peroxidation might represent a reasonable signal for MAPK activation TI/R injury.

Moreover, we have also previously observed strong JNK and ERK 1/2 activation in the ipsilateral and contralateral testes after testicular torsion. The mechanism of this contralateral damage is unclear. One theory postulates a decrease in contralateral testicular blood flow as a reflex to an affenter stimulus. However, whatever the triggering mechanism may be, the cascade of pathological events occurring in both testes is similar. It has been shown that in myocardial I/R or the phenomenon of preconditioning early MAPK activation may contribute to cardioprotection in so-called reperfusion injury salvage kinase pathways. Therefore, for similarity we hypothesized that JNK and ERK 1/2 activation in the contralateral testis might represent a protective mechanism. In the current study each MAPK was not significantly expressed during the ischemic period, but rather began to increase at 5 minutes, peaked at 30 to 40 minutes and decreased by 1 hour after testis detorsion. Furthermore, no detectable ERK 1/2 and JNK activity was observed 2 hours after reperfusion. ERK is activated primarily in response to growth factors or mitogen, while JNK activation depends on ultraviolet irradiation, protein synthesis inhibitors, osmotic stress and inflammatory cytokines.

To confirm the key role of oxidative stress in the pathogenesis of TI/R injury we performed the current experiment...
with the vitamin E analogue raxofelast, which has a hydrophilic character and powerful antioxidant properties. This group of vitamin E analogues shows no systemic toxicity even following a high dose of up to 1 gm/kg.20 Raxofelast administration resulted in a marked decrease in ERK 1/2 and JNK activation.

Recent data demonstrated an increase in TNF-α expression after testicular reperfusion.12 In agreement with this finding we have also previously found marked TNF-α expression in the ischemic, reperfused testis.15 As observed for MAPK, the contralateral testis also showed a robust increase in proinflammatory cytokine content, thus, confirming that TI/R injury also causes marked impairment bilaterally. In addition, the level of CD, a marker of lipid peroxidation, was significantly increased in the 2 testes after testicular torsion, confirming the effect of I/R on the injured side and on the contralateral side. The administration of vitamin E analogue decreased CD levels in each testis.

Histological features correlated well with the biochemical assay. Indeed, by inhibiting lipid peroxidation raxofelast blunted the inflammatory cascade, decreasing histological modifications and also resulting in less damage to the contralateral testis, observed 24 hours after reperfusion in extratubular and endotubular compartments.

CONCLUSIONS

Taken together these findings led us to hypothesize that exaggerated lipid peroxidation might represent at least in part one of the most important signals responsible for the activation of these MAPKs in TI/R injury. These data indicate that antioxidants may represent a potential therapeutic approach to the management of testicular torsion. Furthermore, treatments aimed at blunting oxidative stress are extremely important for decreasing problems related to infertility. This is the case in testicular torsion in humans, in whom pharmacological protection is possible only after the ischemic event has occurred but before complete reperfusion is induced by surgical intervention. In fact, our results clearly show that the compound provided dramatic protection in each gonad, almost completely reversing histopathological changes and biochemical parameters.

ACKNOWLEDGMENTS

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### Abbreviations and Acronyms

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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CD</td>
<td>conjugated dienes</td>
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<tr>
<td>ERK 1/2</td>
<td>extracellular signal-regulated kinase</td>
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<td>I/R</td>
<td>ischemia reperfusion</td>
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<td>JNK</td>
<td>c-jun N-terminal kinase</td>
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<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>TBS</td>
<td>tris buffered saline</td>
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<td>TI/R</td>
<td>testicular I/R</td>
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REFERENCES


