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Transient ischemia induces apoptosis in the ventral prostate of the rat

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Abstract The mechanisms involved in the castration-induced involution of the ventral prostate (VP) are not fully understood. It was recently reported that castration decreases blood flow in the VP in rats and that this occurs before the apoptotic involution of the organ. However, it is unknown whether a decrease in blood flow may trigger apoptosis in the VP, and this was therefore examined in this study. The right iliac artery was clamped for 1 h in adult male rats. After 24 h of reperfusion, the VPs were frozen or fixed. In situ end-labeling (ISEL) was used to identify apoptotic cells, and testosterone repressed prostatic message-2 (TRPM-2) was measured. Proliferating cell nuclear antigen (PCNA) immunohistochemistry was used to identify proliferating cells. Clamping the right iliac artery reduced blood flow in the right VP to 0.17 of that in the contralateral lobe. This relative ischemia resulted in a threefold increase in the volume density of apoptotic epithelial cells on the treated side, but left cell proliferation unaffected. Testosterone substitution did not change this pattern. This study suggests that a transient period of relative ischemia may induce apoptosis in the rat ventral prostate. This may be of importance for the understanding of castration-induced prostatic involution.

Key words Apoptosis · Ischemia-reperfusion · Blood flow · Ischemia · Prostate · Rat

Introduction

The prostate depends on androgens for its structural and functional integrity. After castration, approximately 80% of the ventral prostate (VP) volume is lost within 7 days [7]. This involution is caused by a decrease in cell proliferation and an increase in apoptotic cell death among the epithelial cells [7, 32], but whether this response is directly caused by androgen deficiency in the epithelial cells, or whether it is secondary to effects in other cells in the prostate remains unknown.

Castration reduces the total volumes of blood vessels and endothelial cells in the VP, and they are normalized by testosterone treatment [9]. This indicates that prostate angiogenesis and blood flow are under androgenic control. Castration decreases blood flow at 1–3 days after castration, and this reduction in blood flow precedes the peak in epithelial cell apoptosis [17]. Thus, it is tempting to speculate that the castration-induced reduction in prostate blood flow may induce a hypoxic situation in the prostate that could trigger the induction of the apoptotic process. Such a relationship between ischemia and apoptosis has been demonstrated in other organs, such as the testis [35], brain [15, 19], kidney [28], and heart [23, 8], after brief periods of ischemia with subsequent reperfusion. Whether the normal prostate responds to ischemia with apoptosis is unknown, but antiangiogenic therapy in experimental prostate cancer induces tumor cell apoptosis [13, 30]. The aim of this study was, therefore, to examine the effects of transient ischemia on prostate epithelial cell apoptosis and proliferation.

Materials and methods

Animals and anesthesia

Adult male Sprague-Dawley rats were purchased from Möllegaard, Denmark. The animals were housed under controlled conditions and given water and pellets ad libitum for at least one week prior to and during the experiments. The experiments were done according to Swedish legislation on animal care and were approved by the local ethics committee on animal protection. At the time of the experiments, the animals weighed 350–500 g.

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The rats were anaesthetized with a 1 : 1 : 2 mixture of Hypnorm (fluanisonum 10 mg/ml and fentanylum 0.2 mg/ml, Janssen Pharmaceutica, Belgium), Dormicum (midazolam 5 mg/ml, Roche, Switzerland) and sterile water (3.0 ml/kg), administered as a single intraperitoneal injection. During the experiment, the animals were kept supine on a thermocontrolled heated pad. Throughout the operations, rats were monitored for pain reaction, and the anesthesia was iterated with 0.05 ml intramuscular Hypnorm, after 45 min or when deemed necessary. A subcutaneous dose of 1 ml saline was given to compensate for fluid losses during the experiment, together with Buprenorphine (Temgesic Meda, Sweden) in a dose of 0.003 mg to control postoperative pain.

Experiment 1

A methodological experiment was carried out to verify the effect of clamping on VP blood flow. The rats were incised with a 3-cm midline incision, which was kept open with self-holding retractors. The aortic bifurcation was identified, and the common iliac artery was dissected free and clamped. Blood flow was measured by using the microsphere method as earlier described [5, 17]. The spheres were injected 5 min after the iliac artery had been clamped. The blood flow to the ischemic VP lobe was decreased to 17% of the value of the flow to the lobe in the contralateral, unischemic side (Fig. 1).

Experiment 2

To explore the effect of different ischemic time periods, the above experiment was repeated, but this time without the blood flow measurement. After the artery had been clamped, the retractors were removed, and the wound was covered with a Kleenex tissue soaked in physiological saline to minimize evaporation. Periods of 10, 30, and 60 min of ischemia were compared. At 90 min of ischemia, the animals showed signs of discomfort after waking up. As this was deemed unacceptable, no ischemic periods longer than 60 min were studied. After the appropriate period of time, the clamp was removed, and the wound was closed with single stitches in the abdominal wall and skin. The animals were then returned to the local animal facility for 12 or 24 h before they were anesthetized in the same way as earlier and killed by thoracotomy. The VPs were then dissected free and immersion fixed in 4% formalin in PBS.

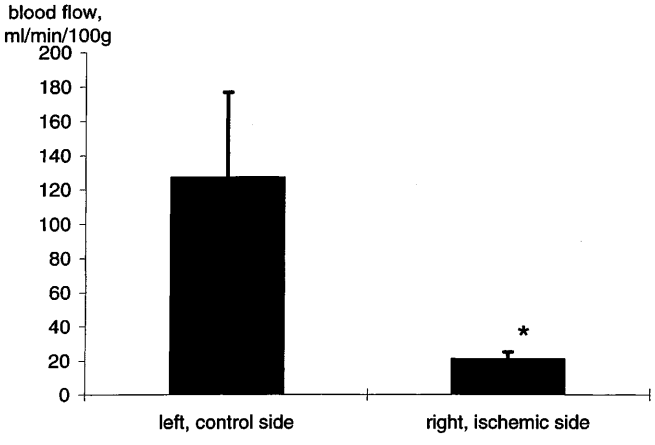

Fig. 1 The blood flow (ml/min per 100 g) to the ventral prostate after clamping the right iliac artery, measured with radioactive microspheres. The blood flow to the clamped side is significantly smaller than to the intact side. Values are means of six animals, error bars are ± SEM, $*P = 0.002$ (Mann-Whitney *U*-test)

Experiment 3

Twenty-four rats were divided into three groups. Group 1 was subjected to a sham operation. In this group, the experiment was done as described above, but the iliac artery was not clamped. Group 2 was subjected to arterial clamping of the right iliac artery for 60 min as described above. In the third group, exactly the same experimental procedure used for group 2 was used, but in addition, 12.5 mg testosterone (Testovirone-Depot, Schering, Germany; 250 mg/ml, diluted in sesame oil) was given subcutaneously 12 h before surgery. This was done to counteract any decrease in testosterone that might arise from operative stress [25, 21].

Detection of apoptosis

In situ end-labeling (ISEL)

The detection of apoptotic cells was done as earlier described [2, 33]. The ventral prostate lobes were fixed in 4% formalin in PBS for 24 h, dehydrated, and embedded in paraffin. Sections 4 μm thick were mounted on poly-L-lysine-coated slides (Sigma, St' Louis, Mo.). The sections were deparaffinized, rehydrated, and digested with pepsin in 0.5% HCl for 5 min with gentle shaking in a 37°C water bath. The digestion was stopped by thorough rinsing with water and buffer A for 5 min. After drying, the slides were incubated for 60 min with buffer A containing 0.01 mM dATP, dCTP, dGTP, biotin-16-dUTP (Boehringer Mannheim Scandinavia). The slides were then rinsed in 0.01% H_2O_2 in PBS for 5 min to block endogenous peroxidase, followed by two 5-min washes in PBS. The sections were incubated with avidin dissolved 1/100 in PBS with 0.1% BSA and 0.5% Tween-20 for 30 min at 20°C before being developed with diaminobenzidine. After immunodetection, the sections were lightly counterstained with Meyer's hematoxylin solution to enhance the morphology. To get negative controls to check for nonspecific peroxidase staining, DNA polymerase I was excluded from the nucleotide mix. Sham-operated rats were used as negative controls.

Morphometry

The slides were examined under a light microscope with an 11×11 square lattice fitted in the ocular as earlier described [31]. The volume density of apoptotic (ISEL-positive) epithelial cells was calculated by counting the number of hits (grid line intersections) falling over apoptotic cells and the total number of hits falling over epithelial cells. At least 100 fields were examined at ×400 magnification in each prostate. In total, the number of epithelial cells examined varied between 5000 and 10 000 per prostate. This was considered to be an adequate number of cells for obtaining statistically representative data [32].

Northern blotting of testosterone repressed prostatic message-2 (TRPM-2)

The VPs were snap frozen in liquid nitrogen and kept at −70°C until the experiments were made. Approximately 20 mg of frozen tissue was homogenized with a knife homogenized in Trizol, and total RNA was prepared according to the manufacturer's protocol (Life Technologies, Sweden). RNA (20 μg) was electrophoresed on a 1% agarose gel in 10 mM sodium phosphate buffer, before being blotted onto a nylon filter (3 M). After blotting, the filters were routinely stained with methylene blue to certify a successful blot and equal loading of RNA in the wells. The filters were crosslinked and prehybridized for 4 h and then hybridized for 16 h with (α-32P) dCTP-labeled randomly primed probes for TRPM-2 and β-actin (Megaprime, Amersham, UK), mainly according to Brändström [2]. The hybridization solution had an activity of 2×10^6 CPM/ml. The signals were visualized and quantified by densitometry, using the Molecular Analyst scanning equipment and accompanying software (Biorad, Sweden). The expression of

β-actin was used to normalize for differences in RNA loaded onto the gel. Untreated VPs and VPs obtained 2 days after castration were used as negative and positive controls, respectively.

Quantification of cell proliferation rate

The volume density of proliferating cells in the VP was quantified with morphometry as described above. Proliferating cells were identified by using immunohistochemistry for proliferating cell nuclear antigen (PCNA) [16]. Sections of paraffin-embedded VPs were treated with methanol to suppress endogenous peroxidase activity, washed with PBS, and incubated overnight with a primary monoclonal anti-PCNA antibody (Dako M879, Dakopatts, Denmark). The sections were then incubated with a secondary biotinylated antibody for 30 min, followed by avidin-biotin complex (ABC) reagents for 45 min and peroxidase substrate for development for 15 min. Between incubations, the sections were washed for 10 min in PBS. After immunodetection, the sections were lightly counterstained with haematoxylin.

Statistics

Values are expressed as mean ± standard error of the mean (SEM). Comparisons between groups were made using the non-parametric Mann-Whitney *U*-test with support from the statistical program SPSS for Windows (version 7.5). *P*-values less than 0.05 were considered significant.

Results

The ischemia model

After clamping the right iliac artery, the blood flow in the right VP lobe, as measured with the microsphere method, was reduced to 0.17 of the value in the contralateral lobe (Fig. 1). The ischemia-reperfusion time that resulted in a maximal apoptotic response was obtained after 60 min of arterial clamping followed by 24 h of reperfusion (Fig. 2).

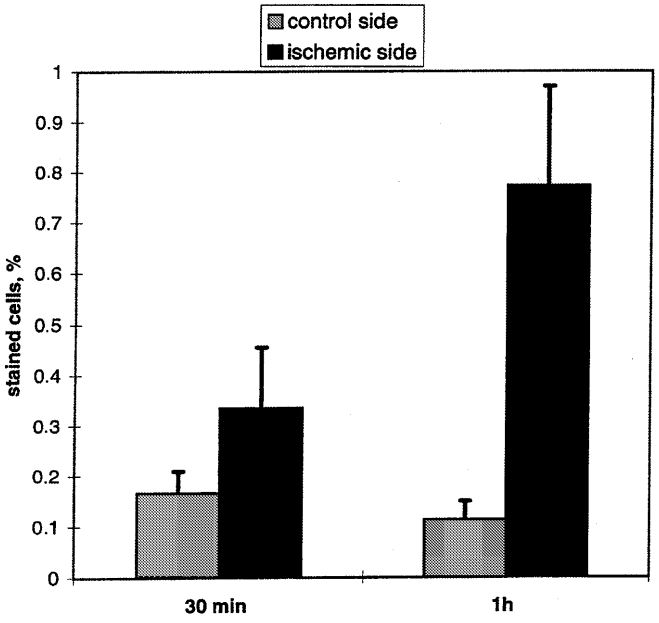

Fig. 2 The volume density of apoptotic [in situ end-labeling (ISEL)-positive] cells in the prostate epithelium at different times of ischemia (30 min and 1 h). The volume of apoptotic cells was higher in the VPs subjected to 1 h of ischemia. Values are means ± SEM, $n = 3$ in each group

Effects of 60 min of ischemia followed by 24 h of reperfusion on prostate cell apoptosis and proliferation

The prostate lobes subjected to ischemia showed an increased amount of apoptosis (Figs. 3 and 4). The volume density of apoptotic (ISEL-positive) cells in treated lobes was increased three-fold over the volume density in the control lobes (Fig. 3). A similar relationship between control and treatment sides, but on a lower level, was

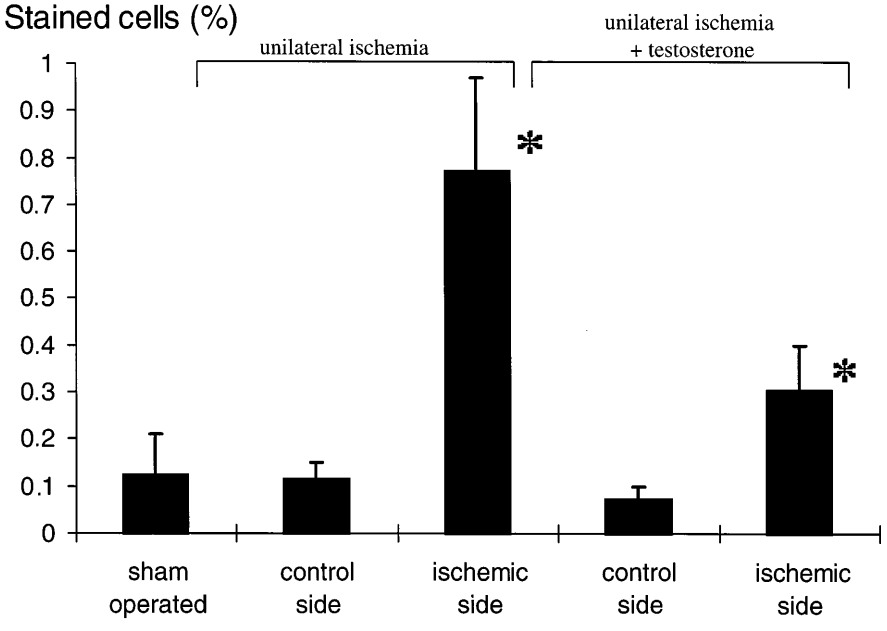

Fig. 3 The volume density of apoptotic (ISEL-positive) epithelial cells after 1 h of unilateral ischemia and 24 h of reperfusion. The volume of apoptotic cells was significantly higher in the VPs subjected to ischemia and reperfusion than in the control lobes and in the sham-operated animals. Administration of testosterone prior to surgery diminished the induction of apoptosis in both the ischemic and control sides. Values are means ± SEM, $n = 5$ animals in each group. *$P < 0.05$ (Mann-Whitney *U*-test)

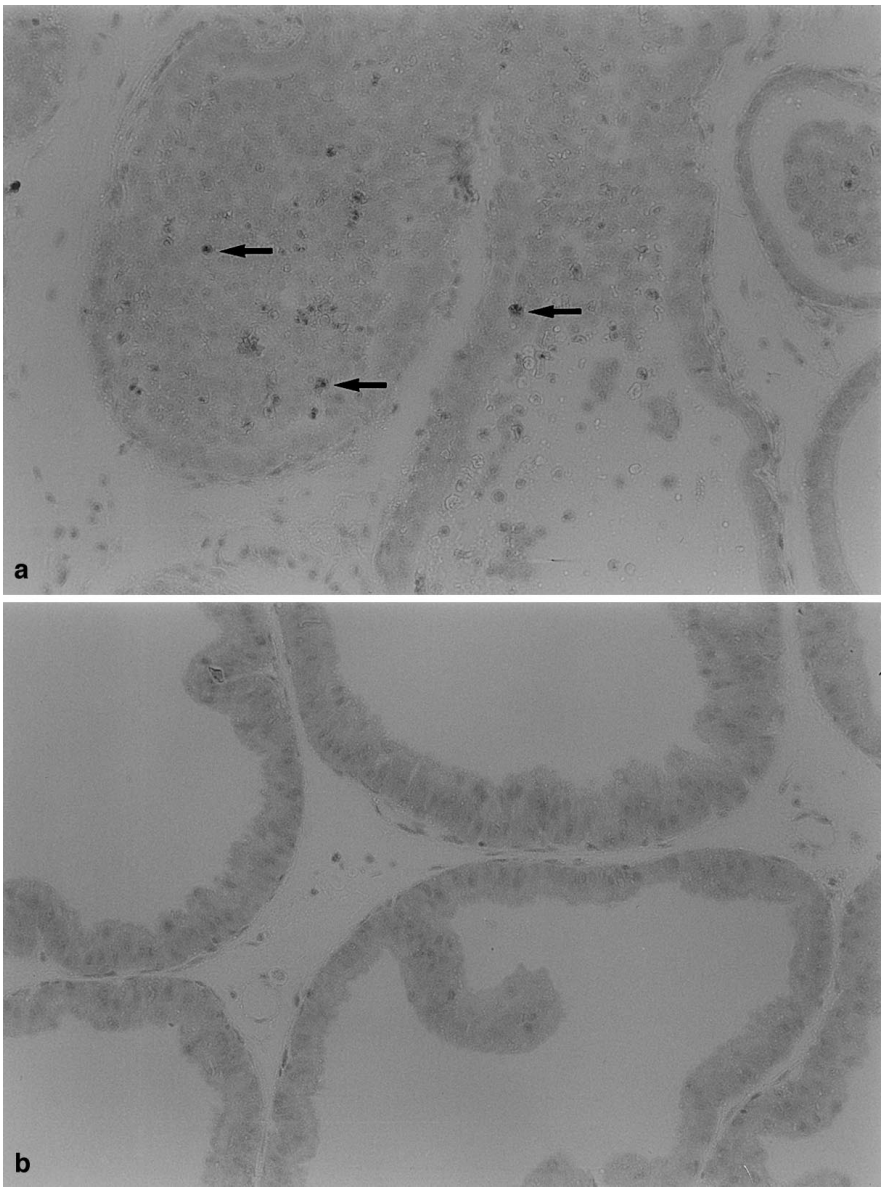

Fig. 4 ISEL section (×400 magnification) from the ventral prostate lobes in an animal subjected to 1 h of ischemia and 24 h of reperfusion **a** and in a sham-operated animal **b**. Several apoptotic cells are observed in the epithelium (*arrows*) in **a**

found in the rats treated with unilateral VP lobe ischemia and testosterone. Unilateral ischemia did not induce any apparent effects on prostate epithelial cell proliferation (Fig. 5).

Northern blotting

The analysis of TRPM-2 expression with Northern blotting showed a single band with a size of 2 kb (the same size as the TRPM-2 probe) in the VPs obtained two days after castration, which were used as a positive control. In the VPs of intact rats and in the VPs subjected to ischemia, weaker signals were seen (Table 1). β-actin was used as an internal standard to normalize for any differences in loading, and its expression was found to be relatively constant. In Table 1, the amount of TRPM-2 is expressed as the relative amount of signals for TRPM-2 densitometrically normalized to the amount of signals for β-actin.

In sham-operated, ischemic, and control lobes of the VPs, similar levels of TRPM-2 were detected. This is probably because the stress of the operation causes a decrease in testosterone levels, which overshadows the effect of ischemia on the expression of TRPM-2. In the animals given testosterone, however, the levels of TRPM-2 were substantially higher in the ischemic side of the VP than in both the control side of the VP and in sham-operated rats.

Discussion

The present study demonstrates that 1 h of transient relative ischemia followed by reperfusion results in

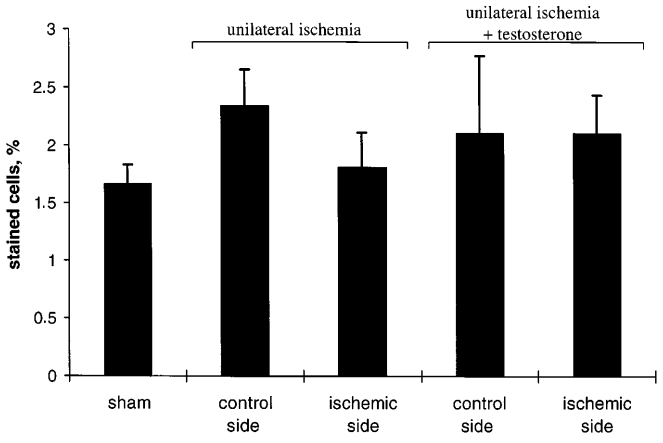

Fig. 5 The volume density of proliferating epithelial cells [proliferating cell nuclear antigen (PCNA)-labeled] after 1 h of unilateral ischemia and 24 h of reperfusion (values are means ± SEM, $n = 5$ animals in each group). The volume density of proliferating cells was not influenced by ischemia

Table 1 The relative amount of TRPM-2 expression in the ventral prostate of rats belonging to different experimental groups as indicated below. The amount of TRPM-2 is expressed as the relative amount of TRPM-2 signals densitometrically normalized to the signals for β-actin. Intact rats are given the arbitrary value 1 to simplify comparison

Treatment groups	TRPM-2 expression
Intact VP	1
2 days castrated VP	100
Sham operated	28.8
Control side	30.0
Hypoxic side	12.7
Testosterone substituted sham operated	2.33
Testosterone substituted control side	1.00
Testosterone substituted hypoxic side	23.0

epithelial cell apoptosis but leaves cell proliferation unaffected in the rat ventral prostate. This finding is in accordance with experiments done in other organs, such as the testis, brain, kidney, and heart, where short-term ischemia induced by arterial clamping also results in apoptosis [8, 15, 19, 23, 28, 35]. Clamping the iliac artery did not result in complete ischemia in the ventral prostate, but blood flow was reduced to 0.17 of the control value. As a comparison, blood flow was decreased to 0.44 and 0.24 of the pretreatment value 1 and 3 days after castration [17]. It is therefore not unlikely that the magnitude and duration of the castration-induced decrease in blood flow in the ventral prostate could be sufficient to induce cell death.

The relative increase in apoptosis seen after ischemia was not abolished by the administration of testosterone, suggesting that an androgen-independent pathway is partly involved. The observation that the volume density of apoptotic cells and the expression of TRPM-2 were reduced in the testosterone-supplemented experimental animals may, however, suggest a possible interaction between testosterone and ischemia. If so, this further supports the hypothesis that a reduction in blood flow may act together with other factors in the castrated rats to induce involution of the ventral prostate. The increased apoptosis in the contralateral side of the ischemia-treated rats could be due either to a decrease in the blood flow resulting from vessels crossing the midline or to the decrease in testosterone that results from the stress of surgery [25, 21].

The mechanisms behind the induction of apoptosis after ischemia have not been established. Ischemia in itself could cause fragmentation of the DNA by triggering the genetic program leading to apoptosis, but the induction of apoptosis might also be related to the reperfusion after the ischemic period [20]. Reperfusion may induce the production of oxygen radicals that damage the DNA sufficiently for the cells to undergo apoptosis [34]. The elimination of reactive oxygen species (ROS) by scavenger substances, such as glutathion, has been shown to inhibit the apoptosis of cultured cells, indicating the need for ROS to induce apoptosis [27, 10]. On the other hand, others have described apoptosis after ischemia without reperfusion [26, 14], and Tsujimoto et al. [29] did not observe any influence from scavengers or from scavenger inhibitors on the development of apoptosis in vitro. The present finding that cell death was induced by ischemia-reperfusion could also be the result of substances released from other cells, such as tissue macrophages or mast cells, which have been shown to be activated and degranulated in response to hypoxia [1]. Macrophages store and release cytokines such as transforming growth factor-β (TGF-β) in their granules [6], and these substances have been observed to cause apoptosis in several cell types [11, 22].

Conclusions

Transient and relative ischemia may induce apoptosis in the glandular epithelium in the ventral prostate of the rat. As castration results in a rapid decrease in prostate blood flow, it is suggested that this decrease may be involved in triggering the subsequent involution of the organ.

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