Insulin Affects Tissue Organization and the Kinetics of Epithelial Cell Death in the Rat Ventral Prostate After Castration

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ABSTRACT: Prostate growth and physiology are regulated by steroid hormones and modulated by multiple endocrine factors. We investigated the action of insulin on the tissue organization and kinetics of epithelial cells in the rat ventral prostate (VP) in response to castration up to 120 h after surgery by using an acute protocol of alloxan-induced diabetes. Diabetes caused a reduction in volume density ($V_v$) and volume of the epithelium. The effects of castration on the epithelium were accelerated in the diabetic animals, as determined by changes in $V_v$ and volume. The smooth muscle cells became atrophic and apparently relaxed in response to castration, in contrast to the spinous aspect observed in nondiabetic castrated rats.

Counting of apoptotic nuclei in the epithelium showed the classical apoptosis peak at 72 hours in nondiabetic rats and an advance of the apoptosis peak to 48 hours after castration in diabetic rats. Insulin restored the time of the peak to 72 hours, suggesting a survival and antiapoptotic effect on VP epithelial cells in both the presence and absence of androgen stimulation. These results were confirmed after immunostaining for cleaved caspase-3. This idea is supported by the observation that insulin also reduced the overall rate of apoptosis at all experimental points analyzed before and after castration.

Key words: Apoptosis, tissue remodeling.

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modulating epithelial cell proliferation (Yono et al, 2005; Vikran et al, 2009), we decided to study the effect of insulin on tissue changes and on the kinetics of epithelial cell apoptosis in the rat ventral prostate after castration. Using a model of alloxan-induced diabetes to follow tissue parameters and apoptosis rate on a daily basis (up to 120 hours after castration), we obtained a series of results demonstrating that insulin represents an important survival factor for the prostate epithelium in an androgen-deprived environment, affecting the rate of epithelial cell apoptosis in the rat VP in response to castration.

**Material and Methods**

**Animals, Treatments, and Tissue Processing**

Seventy-two Wistar male rats aged 75 to 105 days were used. The animals were kept under normal light conditions and received filtered tap water and Purina rodent chow ad libitum. Forty-eight animals received 1–3 intraperitoneal injections of 75 mg/kg alloxan (5,6-dioxuryruracil monohydrate; Sigma Chemical Co, St Louis, Missouri) in 0.1 M citrate buffer, pH 4.4, at 7-day intervals. Before the injections, the animals were fasted for 24 hours and tested for glucosuria and glycermia. Once the animals were determined to have glucosuria (>28 mmol/L) and high glycermia (>350 mg/dL), the animals received an extra injection of alloxan (stabilizing dose) 7 days later. Insulin concentration was below the radioimmunoassay detection levels. Twenty-four animals received exogenous NPH insulin (20 U/kg body weight; Biobras, Montes Claros, MG, Brazil) twice a day (at 0600 and 1800 hours) beginning 24 hours after the stabilizing alloxan injection. This treatment reversed the glucosuria and hyperglycermia. Twenty animals from each group (nondiabetic, diabetic, and diabetic plus insulin) were castrated through scrotal incision under ketamine (80 mg/g body weight) and xylazine (10 mg/g body weight) anesthesia 48 hours after the stabilizing alloxan injection. They were then killed at 24-hour intervals up to 120 hours, resulting in 4 animals per time point per group.

After they were killed by an overdose of anesthetics, the animals were weighed, blood was sampled for the determination of testosterone plasma levels, and the ventral prostates were removed, weighed, and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 24 hours. Fixed prostates were then routinely processed for paraffin or historesin embedding.

Procedures were approved by the University Committee for Ethics in Animal Experimentation (Protocol 1489-1).

**Determination of Testosterone Plasma Levels**

Plasma was separated by centrifugation and stored at –20°C until used. Testosterone and E2 concentrations in the plasma were determined by radioimmunoassay with the use of the Coat-A-Count kit (Diagnostic Products, Los Angeles, California). The sensitivity of the tests was 1.08 ng/dL, and the coefficient of variation was 2.33%.

**Histology and Stereology**

Historesin sections (2 μm) were stained with hematoxylin and eosin for general histology and stereology according to Huttunen et al (1981), as previously described (Antonioli et al, 2004; Garcia-Florez et al, 2005). Volume density (Vv,s) of the epithelium, lumen, stroma (stromal compartment less the smooth muscle cells), and smooth muscle cells was determined from 6 microscope fields per animal (n = 24). As part of an exploratory analysis, the volume of each compartment was determined from the mean weight of the ventral prostate, assuming a tissue specific gravity of 1 g/mL (DeKlerk and Coffey, 1978).

**Determination of the Apoptotic Index**

Apoptotic cells were counted by identification of apoptotic nuclei after the Feulgen reaction and after the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) reaction. Historesin sections were hydrolyzed for 1 hour and 32 minutes in 4 N HCl and then incubated with Schiff’s reagent for 45 minutes. After extensive washing, the sections were dehydrated and mounted in Entellan. Six microscope fields per animal (n = 24) were taken at random, and the apoptosis index was determined by dividing the number of apoptotic nuclei by the number of total epithelial cell nuclei in each microscope field, using a ×100 objective. The TUNEL reaction was performed using an in situ cell death detection kit (Roche Diagnostics, Indianapolis, Indiana) to detect DNA fragmentation, following the manufacturer’s instructions. Counting was done as above. Cleaved caspase-3 was located by immunohistochemistry with the use of an anti–cleaved caspase-3 antibody (Cell Signaling Technology, Danvers, Massachusetts) and a routine protocol for immunohistochemistry employed in the laboratory (Augusto et al, 2008). Cleaved caspase-3–positive cells were counted as above.

**Statistical Analysis**

Statistical analyses were performed by analysis of variance followed by the multicomparison post hoc Tukey’s test, at P < .05, with a free trial version of Minitab software.

**Results**

**Morphometrics—Variation of Body and Prostate Weights**

Treatments did not affect Wistar rat body weight within the timeline of the experiment. Castration of the diabetic animals showed a transient nonsignificant decrease in body weight (Figure 1A), and this variation was not seen with simultaneous administration of insulin.

The mean prostate weight in Wistar rats was 0.206 g, corresponding to 0.1% of body weight (Figure 1B and C). The mean VP weight did not change in response to either diabetes or insulin administration to diabetics (Figure 1B and C). Castration caused a 57% reduction in VP weight 120 hours after surgery of nondiabetic rats. In diabetics, castration resulted in progressive reduction
of VP weight, to a level of 51% of that of the controls. Insulin administration accelerated weight loss, attaining the maximum reduction as early as 48 hours after castration (about 58% reduction compared with the respective control), with no further reduction up to 120 hours (Figure 1B and C).

Effects on Plasma Testosterone

The concentration of plasma testosterone was 334.6 ± 204.2 ng/dL. Diabetes induction caused a reduction of about 50% in this number (160.0 ± 52.3 ng/dL), even though in the present experimental conditions and sample size it did not achieve statistical significance. The testosterone concentration partly recovered (217.7 ± 110.0 ng/dL) after insulin administration. Castration caused a more than 99% reduction in testosterone levels in animals of all 3 experimental groups as early as 24 hours after castration.

Variations in Tissue Organization

The VP is a tubulo-acinar structure surrounded by a cylindrical, single-layered epithelium. The lumen is usually large and filled with secretory material. The stromal compartment has sparse cellular and fibrous material. A single layer of smooth muscle cells surrounds the epithelium (Figure 2A). During the timeline of the experiment, diabetes caused few changes in the VP. There was a small reduction in epithelial cell height and a decrease in the secretory content in the acini (Figure 2C). Insulin administration to the diabetic rats affected the growth of the epithelium, which showed an increase in the epithelial infolds, similar to that found in the distalmost ductal regions (Lee et al, 1990). No evident effect of diabetes or insulin administration on diabetic rats was observed in the stroma of noncastrated rats within the timeline of the experiment. Castration caused marked modification of the VP histology. The epithelial cells became short, the lumen was reduced, and the stroma became denser, with many cell types, including mast cells and more evident collagen fibers, 120 hours after castration (Figure 2B). The modifications of the smooth muscle cells consisted of the

Values correspond to the $\bar{x} \pm$ standard deviation (n = 4). Weight reduction was accelerated in the diabetic animals and in the diabetic animals that received insulin. (C) Variation in VP relative weight (expressed as a percentage of body weight) in response to castration in the 3 experimental groups (nondiabetic, diabetic, and insulin-treated diabetic animals). Each group showed a characteristic weight loss rate, but the maximum weight reduction achieved within a 120-hour period was about the same. Values correspond to the $\bar{x} \pm$ standard deviation (n = 4). Asterisks indicated statistically significant variation with respect to the noncastrated animals in each experimental group at $P < .05$. 

Figure 1. Body and ventral prostate (VP) weight variation. (A) Body weight variation in response to diabetes and castration. Values correspond to $\bar{x} \pm$ standard deviation (n = 4). Little significant variation was observed in body weight during the experiment. (B) Variation in VP weight in response to castration in the 3 experimental groups (nondiabetic, diabetic, and insulin-treated diabetic animals). (C) Variation in VP relative weight (expressed as a percentage of body weight) in response to castration in the 3 experimental groups (nondiabetic, diabetic, and insulin-treated diabetic animals). Each group showed a characteristic weight loss rate, but the maximum weight reduction achieved within a 120-hour period was about the same. Values correspond to the $\bar{x} \pm$ standard deviation (n = 4). Asterisks indicated statistically significant variation with respect to the noncastrated animals in each experimental group at $P < .05$. 

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acquisition of a spinous aspect (irregular outline) and the adoption of a multilayer organization around the regressing epithelium, similar to previous reports (Antonioli et al, 2004, 2007).

Most of these aspects were observed in the castrated diabetic animals (Figure 2D), except for those related to the smooth muscle cells, which lacked the aspects commonly seen in castrates and are relaxed. Insulin administration had little effect on the morphological aspects of the VP in castrated diabetic rats, except for fewer cells observed in the stroma (Figure 2F).

Stereology demonstrated that the epithelium is the predominant compartment of the VP, corresponding to a volume density (Vv) of 47% and a mean volume of 153 μL in noncastrated nondiabetic animals (Figure 3A and E). Induction of diabetes caused a reduction in the Vv and volume of the epithelium. Insulin administration did not reverse this reduction within the timeline of the experiments (Figure 3A and E). Counterbalancing this reduction in the epithelium was an increase in the Vv of the lumen, which was even larger after administration of insulin (Figure 3B). These variations were insufficient to cause a significant change in the volume of the lumen (Figure 3F). Stromal Vv and volume were not affected within the timeline of the experiment (Figure 3C and G), considering the short period of exposure to the diabetic condition and insulin administration. Similar to the epithelium, the Vv of the epithelium and the volume occupied by smooth muscle cells was smaller in diabetic animals, and even smaller after treatment with insulin (Figure 3D and H).

Epithelial Vv and volume showed a significant reduction only 120 hours after castration of nondiabetic rats (Figure 3A and E). In diabetic rats, the reduction in epithelial Vv is more rapid, achieving significance as early as 24 h after castration (Figure 3A). The already reduced epithelial Vv in diabetic animals treated with insulin was little affected by castration but, like nondiabetic rats, was significantly reduced only by 120 hours after castration (Figure 3A), although with different kinetics, a maximum reduction in epithelial volume of 65% was noted for the 3 experimental groups 120 hours after castration (Figure 3E).

Castration had little effect on the luminal and stromal Vv and volume up to 120 hours after surgery in the 3 experimental groups (Figure 3B and C, 3F and G). Androgen deprivation caused no reduction in the Vv and volume of the smooth muscle cells in nondiabetic animals but promoted significant reduction in the Vv and volume observed in diabetic animals. Insulin reverted this effect, and no significant reduction was observed in insulin-treated diabetics (Figure 3D and H), confirming the histological observations described above.

**Kinetics of Apoptosis After Castration**

Feulgen-stained prostate sections allowed for the identification of apoptotic nuclei, which were characterized by pyknosis or fragmentation (Figure 4A). The percentage of apoptotic nuclei identified after the Feulgen reaction in the VP in intact rats was 0.7%. Diabetes induction by alloxan did not affect this percentage. However, insulin administration to diabetic rats caused a 92% reduction in the number of apoptotic nuclei (Figure 4A). Orchiectomy caused a 550% increase in the apoptotic index 72 hours after surgery. Ninety-six hours after castration, this index dropped 32%, returning to basal levels by about 120 hours (Figure 4A). In diabetic rats, there was an advance of the 72-hour peak to 48 hours (Figure 4A). Insulin administration caused significant reduction in the percentage of apoptotic nuclei in noncastrated rats and at different time points after castration, in addition to restoring the peak to 72 hours (Figure 4A).

Counting the number of nuclei exhibiting DNA fragmentation (Figure 4B), as revealed by the TUNEL reaction, confirmed the major aspects of the apoptosis kinetics shown by counting apoptotic nuclei. The mean percentage of nuclei undergoing DNA fragmentation in the epithelial cells of the nondiabetic rat VP was 1.23%. Diabetes induction by alloxan caused a decrease in the apoptotic index of about 81% compared with the noncastrated, nondiabetic rats. Castration caused a progressive increase in the percentage of TUNEL-positive nuclei up to 72 hours. In diabetic animals, castration resulted in an advance of the peak to 48 hours. Mirroring the counts of the apoptotic nuclei recognized by characteristic morphology, insulin treatment of diabetic animals caused a decrease in the percentage of TUNEL-positive nuclei in both nondiabetic or diabetic rats before castration and also resulted in a greater number of them at 72 hours.

Identification and counting of cleaved caspase-3 showed a similar kinetics of apoptosis under the 3 experimental conditions, especially by demonstrating an advance of the peak found at 72 to 48 hours in diabetic rats and an overall reduction in the number of apoptotic cells after insulin administration to diabetic rats (Figure 4C). The numbers obtained after counting cells immunostained for cleaved caspase-3 were 2-fold higher than those obtained for apoptotic nuclei.

**Discussion**

In the present study, we demonstrated that insulin regulates the regressive changes of the VP in response to castration by affecting the histological organization of
Figure 2. Histological aspects of the rat ventral prostate under different experimental conditions. (A, B) Nondiabetic animals. (A) The normal epithelium (Ep) consists of a single layer of cylindrical polarized cells with basal nuclei. The epithelial cells delimit a lumen (L) filled with secretory material. The stroma is sparse, with few cells and fibrillar components. (B) Castration results in short epithelial cells and smaller luminal spaces. The stroma exhibits a higher density of smooth muscle cells (SMCs), other cell types, and extracellular matrix components. The SMCs appeared in multiple layers and showed an irregular outline (spinous aspect). 

(C, D) Diabetic animals. (C) Diabetes caused a reduction in the epithelial cell height and less evident changes in the stroma, with a slightly higher concentration of the extracellular matrix fibrils. (D) Castration of diabetic rats accelerated the reduction in epithelial cell height but seemed to delay the stromal modifications; in particular, the smooth muscle cells appeared relaxed and did not show the irregular outline observed in the nondiabetic castrated animals. 

(E, F) Insulin-treated diabetic animals. (E) Insulin administration increased epithelial cell height, and the epithelium showed several infolds. (F) Insulin administration to diabetic castrated rats appeared not to contribute to restoration of the histological aspects of the nondiabetic castrated rats. The details show aspects of the epithelial and smooth muscle cells in each experimental condition. A–F, scale bars = 100 μm. Insets, scale bars = 50 μm.
Figure 3. Stereology of tissue compartments of the rat ventral prostate (VP) corresponding to the relative volume (Vv%) (A–D) and the volume (mL) (E–H) of the epithelium, lumen, stroma, and smooth muscle cells. The reduction in epithelial Vv% (A) and volume (E) is accelerated in diabetic rats and diabetic rats receiving insulin, but the maximum reduction after 120 hours is about the same. Little variation was observed in the Vv% (B) and volume (F) of the lumen in the timeline of the experiments, except that these parameters were smaller for the nondiabetic animals. A significant reduction of the volume (G), but not the Vv% of the stroma, was observed for the nondiabetic castrated rats, whereas
Experimental group at diabetic animals and diabetic animals receiving insulin than for the nondiabetic animals in both noncastrated and castrated conditions. Values affected after the acute model of alloxan-induced smooth muscle cell compartments were the most epithelial cell apoptosis. Of the tissue compartments, as well as the kinetics of we examined the changes in histology and organization of these changes were less evident for the diabetic rats. The Vv% of the VP relative weight. To understand this effect, the first 2 approaches have been used before and were shown to be comparable (Bruni-Cardoso et al, 2009). Notably, the number of cells stained for cleaved caspase-3 was 2-fold higher than the number of apoptotic nuclei, probably because the former stains the cytoplasm and has a better chance of being sampled in a tissue section with the same thickness. We also

Curiously, the maximum reduction observed in the epithelium in nondiabetic animals did not differ from that observed in diabetic animals. Taken together, these results reinforce the idea that progressive regression depends on stromal remodeling, which was previously suggested by García-Flórez et al (2005) and confirmed recently in a detailed study of MMP-2, -7, and -9 expression in the VP after castration (Bruni-Cardoso et al, 2009).

Diabetic animals showed smaller smooth muscle cell volumes, which was consistent with their atrophic histological appearance. Within the timeline of the experiment, very little effect was observed on the volume of the smooth muscle cells in nondiabetic rats, suggesting that at least 1 week of androgen deprivation is necessary to achieve significant alterations in the smooth muscle volumes in the VP (Antonioli et al, 2005). However, these cells became even more atrophic in the diabetic rats, and this effect was not reversed by insulin, causing both groups to show significantly smaller smooth muscle cell volumes 120 hours after castration, compared with the nondiabetic rats. Accordingly, insulin is an important factor for the maintenance of smooth muscle cells in culture (Gerdes et al, 1996).

The reduction of epithelial cells in response to castration is partially attributed to their deletion by apoptosis. Kinetics of epithelial cell death shows a classical peak at 72 hours after castration (Kerr and Searle, 1973; Kyprianou and Isaacs, 1988; Isaacs et al, 1994). Here, we show that the peak of apoptosis is advanced to 48 hours in diabetic animals and restored to 72 hours on insulin administration. The results are based on cell counts made after Feulgen staining, TUNEL labeling, and cleaved caspase-3 immunostaining. The first 2 approaches have been used before and were shown to be comparable (Bruni-Cardoso et al, 2009). Notably, the number of cells stained for cleaved caspase-3 was 2-fold higher than the number of apoptotic nuclei, probably because the former stains the cytoplasm and has a better chance of being sampled in a tissue section with the same thickness. We also

these changes were less evident for the diabetic rats. The Vv% (D) and volume (H) of the smooth muscle cells were consistently smaller for the diabetic animals and diabetic animals receiving insulin than for the nondiabetic animals in both noncastrated and castrated conditions. Values correspond to the ± standard deviation. Asterisks indicated statistically significant variation with respect to the noncastrated animals in each experimental group at P < .05.
showed that insulin administration reduced the overall rate of apoptosis in both noncastrated and castrated animals. Insulin levels are positively correlated with VP size (Vikran et al, 2009), and blocking insulin production during sexual maturation compromises prostate growth (Soudamani et al, 2005). This occurs because insulin regulates the production of testosterone by affecting the hypothalamic-hypophyseal-testes axis (Kirchick et al, 1979) and producing local effects through insulin receptors (Stattin et al, 2001). In addition to these growth-promoting effects, the present results suggest that insulin also functions as a survival and antiapoptotic factor for the VP epithelium, in either the presence or absence of androgen stimulation (ie, before and after castration).

Insulin-like growth factor 1 (IGF-1) was identified as a major regulator of epithelial cell survival in the rat VP, which is lost by androgen deprivation (Ohlson et al, 2006). Androgen deprivation led not only to the reduction in local production of IGF-1, but also increased IGF binding proteins (IGFBPs; especially IGFBP-3, which is involved in mediating epithelial cell apoptosis) (Firth and Baxter, 2002) and reduced the ability of the surviving cells to respond to exogenous IGF-1 by reducing the amount of insulin receptor substrate 1 (IRS-1) and increasing its level of inhibitory phosphorylation at serine 307 (Ohlson et al, 2006).
the other hand, type I IGF receptor (IGF1-R) conditional abrogation led to uncontrolled epithelial cell proliferation and prostate hyperplasia (Sutherland et al. 2008). These changes in IRS-1 are, at least in part, responsible for the peripheral resistance to insulin in castrated animals and its reversal in response to testosterone (Holmang and Bjorntorp, 1992) and seem to be responsible for the failure of insulin administration to rescue the histological effects described here within the timeline of the experiments.

We suggest that the survival effect (manifested as an increased time for apoptosis) is mediated by insulin signaling and cross-talk with the IGF-I pathway, and that the antiapoptotic pathway (seen as a reduction in the rate of apoptosis) is manifested by modulating the turnover of the androgen receptor and the rate of nuclear depletion after castration. Our laboratory is currently engaged in dissecting these signaling pathways.

Testosterone deficiency compromises insulin production and insulin receptor expression in muscle, liver, and adipose tissue (Muthusamy et al, 2007). Together, the data reported in this study demonstrate the existence of cross-talk between insulin and androgens, confirming, at least in part, the working hypothesis that endocrine hormones modulate the effects of androgens on the prostate gland. Working out this hypothesis seems important because the results might correlate aging, obesity, and diabetes with prostate physiology. The findings presented here are relevant because they reinforce the idea that hyperinsulinemia, diabetes, or both are risk factors in prostate diseases (ie, prostate hyperplasia and cancer) and prognostic factors for the resurgence of androgen-independent prostate cancer after orchectomy.

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