Insulin-resistance reduces botulinum neurotoxin-type A induced prostatic atrophy and apoptosis in rats

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Abstract

Botulinum neurotoxin-type A (BoNTA) is an emerging therapeutic option for the treatment of benign prostatic hyperplasia. Recent reports indicate increased incidence of benign prostatic hyperplasia in the insulin-resistant individuals. Insulin-resistance is associated with the compensatory rise in the plasma insulin, which is known to have growth-promoting effects. The present study investigated the effect of insulin-resistance on the effectiveness of BoNTA in inducing prostatic atrophy in rats. Sprague-Dawley rats (200–220 g), maintained on normal-pellet or high-fat diet, were injected in the ventral prostate with 5U BoNTA at the end of 9 weeks and were sacrificed 3 weeks later. Ventral prostate was carefully isolated, weighed, fixed and stained to examine the cellular morphology, cell death and proliferation. High-fat diet produced insulin-resistance, hyperinsulinemia and prostatic enlargement in rats. BoNTA caused prostatic atrophy and apoptosis in both insulin-resistant and insulin-sensitive rats. However, the effect of BoNTA was more prominent in insulin-sensitive rats (apoptosis-2 fold, prostatic atrophy-3 fold) as compared to the insulin-resistant rats. Significant increase in the phosphorylation of ERK-1/2 and expression of the proliferating cell nuclear antigen was observed in the prostate of insulin-resistant rats. In the present investigation we report that diet-induced insulin-resistance activates mitogenic signaling of insulin, increases cellular proliferation and reduces BoNTA-induced prostatic atrophy and apoptosis in rats. Results of the present study indicate that the insulin-resistance can affect the therapeutic outcome of BoNTA.

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1. Introduction

Benign prostatic hyperplasia is a highly prevalent condition of the prostate in aging men, characterized by augmented cell proliferation and/or contractility of the gland (Berry et al., 1984). Despite increased prevalence, the pathogenesis of prostatic hyperplasia is not well understood. At present, the combination of α-adrenergic blockers and 5-α-reductase inhibitors (or a combination of both) are major options in the pharmacotherapy of benign prostatic hyperplasia. However, in the clinical management of benign prostatic hyperplasia, surgery remains to be the most effective treatment option (Issa and Regan, 2007). Metabolic syndrome and benign prostatic hyperplasia are common problems in the aging men (Kasturi et al., 2006; Parsons et al., 2006; Zamboni et al., 2003). Experimental and clinical reports provide convincing evidence of the association between insulin-resistance and benign prostatic hyperplasia (Hammarsten et al., 2009; Hammarsten and Hogstedt, 2001; Hammarsten et al., 1998; Mongiu and McVary, 2009; Nandeesha, 2008; Nandeesha et al., 2006; Parsons et al., 2006; Vikram et al., 2010a,b; Vikram et al., 2010c). We have reported increased cell proliferation, contractility and enlargement of prostate gland in the diet-induced insulin-resistant rats (Vikram et al., 2010c). High-fat diet-fed rats provide a rodent model for the prostatic enlargement under insulin-resistant condition. Recently BoNTA has emerged as a therapeutic alternative for the management of lower urinary tract symptoms often associated with benign prostatic hyperplasia, without affecting testosterone-dihydrotestosterone axis (Chuang et al., 2005; Chuang et al., 2006; Oeconomou and Madersbacher, 2010). However, we are not aware of any published data on the efficacy of BoNTA in inducing prostatic atrophy under insulin-resistant conditions. Considering the increased incidences of benign prostatic hyperplasia in insulin-resistant individuals, the present study was aimed at investigating the effect of insulin-resistance on the BoNTA-induced prostatic atrophy and apoptosis in rats. Results of the present investigation clearly demonstrate that insulin-resistance reduces BoNTA-induced prostatic atrophy and apoptosis in rats.

2. Material and methods

### 2.1. Animals and experimental design

Animals were approved by Institutional Animal Ethics Committee (IAEC) and were used according to the CPCSEA (Committee for the...
purpose of Control and Supervision of Experimental Animals) guidelines. Experiments were performed on male Sprague-Dawley (SD) rats (200–220 g). Rats were allowed to access the food and water ad libitum. Animals were procured from Institute's Central Animal Facility (CAF) and kept at controlled environmental conditions with room temperature (22 ± 2 °C), humidity (50 ± 10%). The 12 h light (0600–1800 h) and dark cycle was maintained throughout the study. Animals were acclimatized for one week prior to the start of experiment and kept on normal-pellet diet (NPD) or high-fat diet (HFD) for 12 weeks. Glandular injection of BoNTA or saline was given to the subgroup of animals kept on NPD or HFD in the 9th week. The NPD- or HFD-fed rats receiving glandular injection of saline served vehicle control for BoNTA treated NPD- or HFD-fed rats. All the animals were sacrificed after 3 weeks after the glandular injection of BoNTA or saline and ventral prostate was carefully isolated and weighed (Fig. 1A). The left lobe of the ventral prostate was preserved at -80 °C for the molecular studies and right lobe of the ventral prostate was divided into three parts relative to the urethra (distal, intermediate and proximal). Epithelial infoldings in the prostatic acini and morphology of luminal secretory epithelial cells was examined in the histological sections of distal, intermediate and proximal parts of the ventral prostate under hematoxylin and eosin staining. The groups maintained on normal-pellet diet (NPD) or high-fat diet (HFD) were respectively designated as NPD or HFD and interventions (if any) such as glandular injection of saline/BoNTA were indicated by including “Saline” or “BoNTA” to the name of the group. All the animals were killed by cervical dislocation.

2.2. Chemicals

BoNTA was purchased from Allergan Pvt. Ltd. (India). All the primary and secondary antibodies were procured from SantaCruz Biotechnology (USA). TUNEL assay kit and Rat/mouse insulin ELISA kit was procured from Calbiochem (USA) and Linco Research (USA) respectively. Feed ingredients such as NPD (Pranaw Agro Industries, New Delhi, India), lard, cholesterol, DL-methionine (HiMedia, Mumbai, India), vitamin and mineral mix (Sarabhai chemical, Baroda, India) and yeast powder (Pet care, Bangalore, India) were procured from commercial sources. Hematoxylin, eosin, 4′-6-Diamidino-2-phenylindole and DPX mountant were procured from Sigma-Aldrich (USA). Alcohol and xylene were procured from S. D. Fine-chem Ltd. (Mumbai, India).

2.3. Diet for the induction of insulin-resistance in rats

Insulin-resistance was induced in the rats by feeding HFD (5.3 kcal g−1, carbohydrate 17%, protein 25%, fat 58% kcal), while the control rats were fed with NPD (3.8 kcal g−1, carbohydrate 67%, protein 21%, fat 12% kcal). The NPD used to feed the animal was standard rodent chow. The detailed methodology for the HFD preparation has already been described (Srinivasan et al., 2004). In brief, the content of HFD diet includes NPD-powder (36.5%), lard (31%), casein (25%), vitamin-mineral mix powder (6%), cholesterol (1%), DL-methionine (0.3%), Yee-sac powder (0.1%) and sodium chloride (0.1%).

2.4. Glandular injection of BoNTA

Surgery and necropsy of all the animals was done on the necropsy table (Thermo Electron Corporation, USA). Glandular injection of BoNTA was given as previously described (Doggweiler et al., 1998) with some modifications. Briefly, rats were anaesthetized with Thiopentone sodium (50 mg/kg), ventral prostate was carefully exposed and saline/BoNTA (5 U) was injected in the right lobe (200 μl) using Hamilton Syringe (Hamilton Bonaduz AG, Switzerland) (Fig. 1B). Closure of incision was made in layers.

Fig. 1. HFD-feeding induces glucose intolerance in rats. A: Experimental design indicating the time of dietary manipulation (NPD; normal-pellet diet, HFD; high-fat diet), glandular injection of BoNTA and termination of the study. B: Figure showing the site for the injection of BoNTA in the prostate gland. Urinary bladder (UB), ventral prostate (VP) and site of injection for BoNTA (1). C and D: HFD induces glucose intolerance in as determined by IPGTT and area under curve (AUC) of the IPGTT (n = 4). All the values are shown as mean±S.E.M. *P<0.05.
2.5. Biochemical parameters

The blood samples (≈0.8 ml) were collected from the orbital plexus of the rats under light ether anesthesia in heparinized microcentrifuge tubes. The plasma was separated by centrifugation (2500 g×5 min) and analyzed for glucose, triglycerides, total cholesterol (CHOD-POD) and HDL–cholesterol using commercially available spectrophotometric kits (Accurex Biomedical Pvt. Ltd., India). Plasma insulin was estimated by rat/mouse insulin ELISA kit as per the manufacturer’s instruction.

2.6. TUNEL assay

Cell death was detected by TUNEL assay kit according to the manufacturer’s instruction. Briefly, paraffin-embedded sections of the intermediate part of the ventral prostate (right lobe) were deparaffinized, rehydrated and permeabilized with proteinase K. The DNA strand breaks were end labeled with fluorescein tagged nucleotide with terminal deoxynucleotidyl transferase. Cells were counterstained with 4′-6-Diamidino-2-phenylindole. Images were captured by charged coupled device camera attached with the microscope (AXIO Imager, M1 fluorescence microscope. Carl Zeiss, Germany) using ‘Isis’ image analysis software. In total, approximately 4000 cells were examined from each slide and TUNEL positive cells were expressed as percentage of total cells.

2.7. Intraperitoneal glucose tolerance tests

Animals were kept on 6 h fasting for glucose tolerance test and a basal sample was taken, followed by intraperitoneal injection of g-glucose (1000 mg/kg). Blood sample were collected at 5, 15, 30, 60 and 120 min and plasma glucose concentration was determined to assess impairment in the glucose tolerance with time.

2.8. Histological examination

Rats were anesthetized by diethyl ether and killed by cervical dislocation. Ventral prostate was carefully isolated, right lobe was cut and separated in to distal, intermediate and proximal parts relative to the urethra and fixed in 10% formal saline, and paraffin blocks were prepared after completing the routine processing. Sections (5 μm) were prepared from the paraffin blocks and stained with hematoxylin and eosin to examine the cellular morphology. Histological images were captured by charged coupled device (CCD) camera attached with the Olympus microscope (Model BX 51) connected to digital photomicrograph software (OLYMPUS BioReport, CellB). The epithelial infoldings in the prostatic acini and morphology of luminal secretory epithelial cells was examined in the distal, intermediate and proximal parts of the ventral prostate. Cellular height was measured using the photomicrograph software. A minimum of 100 cells from each section was measured, and at least two histological sections of each part of the ventral prostate were examined.

2.9. Immunoblotting and immunohistochemistry

Ventral prostate was homogenized in the radioimmunoprecipitation buffer, sonicated and supernatant containing soluble proteins was isolated by centrifugation (9000 g×10 min). Samples were prepared for the immunoblotting using the supernatant and proteins were resolved on 10–12% SDS-PAGE, transferred to PVDF membrane and analyzed with antibodies against PCNA, MEK, ERK-1/2, P-ERK-1/2 and β-actin. The antigen–primary antibody complexes were incubated with HRP conjugated secondary antibodies and visualized by western blot luminol reagent (SantaCruz Biotechnology, Inc. USA). Image was captured by ImageQuant-350 (Ver. 1.0.2). Quantification was done with ImageQuant TL (GE Healthcare, UK) software and intensity values were normalized with β-actin.

Prostatic sections were deparaffinised with xylene, followed by antigen retrieval by heating in the citrate buffer (10 mM). The prostatic sections were incubated with PCNA polyclonal primary antibody for 24 h. Polyvalent biotinylated goat anti-rabbit secondary antibody and streptavidin peroxidase (STV-HRP) system was used to amplify the signals, followed by detection with diaminobenzidine (DAB) as a chromogen. Slides were counterstained with hematoxylin, dehydrated with graded alcohols and cleared by xylene and mounted in DPX.

2.10. Statistical analysis

Statistical analysis was performed using Jandel SigmaStat statistical software. Significance of difference between two groups was evaluated using Student’s t-test. For multiple comparisons, ANOVA was used and post-hoc analysis was performed with Tukey’s test. Results were considered significant if P values were ≤0.05 (*P≤0.05, **P<0.01, ***P<0.001).

3. Results

3.1. HFD-feeding induces insulin-resistance in rats

Significant increase in the body weight of HFD-fed rats was observed as compared to the NPD-fed rats. HFD-feeding led to altered lipid profile as evident from increase in the triglyceride, total cholesterol and LDL–cholesterol level as compared to NPD-fed rats. HFD-feeding induced glucose intolerance, mild hyperglycemia and hyperinsulinemia in the experimental animals (Table 1 and Fig. 1C–D).

Table 1

<table>
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<tr>
<th>Parameters/Group</th>
<th>NPD</th>
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<th>NPD + BoNTA</th>
<th>HFD</th>
<th>HFD + Saline</th>
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<td>5</td>
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<td>Initial</td>
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<td>220.7 ± 3.3</td>
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<td>215.0 ± 2.1</td>
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<tr>
<td>Terminal</td>
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<td>360.0 ± 13.5</td>
<td>355.4 ± 8.5</td>
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<td>404.2 ± 12.7</td>
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<td>142.7 ± 13.6</td>
<td>137.4 ± 6.4</td>
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<td>98.6 ± 2.9</td>
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<td>Cholesterol (mg/dl)</td>
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<td>95.0 ± 15.7</td>
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<td>Triglyceride (mg/dl)</td>
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<td>Plasma insulin (mg/ml)</td>
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<td>2.5 ± 0.2</td>
<td>2.1 ± 0.2</td>
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All the values are shown as mean ± S.E.M. *P<0.05, **vs. NPD, ***vs. NPD + Saline and ****vs. NPD + BoNTA.
3.2. **Effect of insulin-resistance and glandular injection of BoNTA on the prostate gland**

HFD-feeding with or without glandular injection of saline led to significant ($P<0.001$) increase in the weight of the ventral prostate as compared to the NPD-fed rats. Glandular injection of BoNTA reduced prostate weight in both NPD as well as in HFD-fed rats as compared to the respective vehicle control group. However, the effect of BoNTA was more prominent in the NPD-fed rats as compared to the HFD-fed rats. Insulin-sensitive rats demonstrated approximately 3 fold higher decrease in the weight of prostate gland as compared to the insulin-resistant rats. Although, BoNTA was injected in the right lobe of the ventral prostate, both lobes (right and left) demonstrated atrophy (Fig. 2A–B). Histological examination of the ventral prostate (distal, intermediate and proximal parts of the right lobe) revealed follicular dilation and flattening of the epithelium without inflammatory infiltrate in BoNTA treated rats. However, glandular injection of saline did not lead to any change in the cellular morphology. Increased infoldings of epithelial layer was observed in the prostatic acini of the HFD-fed rats as compared to the age matched controls (Fig. 3A–H and I).

**Fig. 2.** Effect of insulin-resistance on the BoNTA-induced prostatic atrophy. A: Effect of insulin-resistance on the BoNTA induced prostatic atrophy (n=5–6). B: Representative photomicrographs showing the effect of insulin-resistance and BoNTA treatment on the prostate size. All the values are shown as mean±S.E.M., *$P<0.05$, **$P<0.01$, ***$P<0.001$ and ns $P>0.05$ (ns — not significant).

**Fig. 3.** Effect of insulin-resistance and BoNTA treatment on the cellular morphology. A–H: Representative photomicrographs of the prostate at different magnification indicating the normal cellular morphology in the control rats (A and E), follicular dilation and flattening of the epithelium in the BoNTA treated control rats (B and F), increased follicular infoldings in the insulin-resistant rats (C and G) and follicular dilation and flattening of epithelium in the BoNTA treated insulin-resistant rats (D and H). I: BoNTA treatment led to decrease in the height of prostatic epithelial cells. All the values are shown as mean±S.E.M.
3.3. Effect of insulin-resistance and glandular injection of BoNTA on the cellular proliferation

Significant increase in the PCNA level was observed in the prostate of HFD-fed rats as compared to the NPD-fed rats irrespective of the glandular injection of the saline or BoNTA. The results obtained with the immunoblotting were further confirmed by the immunohistochemical staining of the prostatic sections for PCNA (Fig. 4).

3.4. Effect of insulin-resistance and glandular injection of BoNTA on the phosphorylation of ERK1/2

Significant increase in the phosphorylated-ERK1/2/ERK1/2 was observed in the prostate of HFD-fed rats as compared to the NPD-fed rats. BoNTA treatment did not led to any appreciable change in the phosphorylation of ERK-1/2 either in NPD or HFD-fed rats (Fig. 4).

3.5. Effect of insulin-resistance and glandular injection of BoNTA on the apoptosis

Glandular injection of BoNTA-induced significant increase in the frequency of TUNEL positive cells in the ventral prostate of NPD- and HFD-fed rats as compared to the respective diet matched normal and vehicle controls. However, the frequency of TUNEL positive cells were found to be significantly lower in the BoNTA treated insulin-resistant rats as compared to the insulin-sensitive rats (Fig. 5).

Fig. 4. Effect of insulin-resistance and BoNTA treatment on the cellular proliferation and MEK/ERK signaling. A: Immunohistochemical localization of proliferating cell nuclear antigen (PCNA) in the prostatic epithelial cells. B: Significant increase in the PCNA level was observed in the HFD-fed rats as compared to the normal-pellet diet NPD-fed rats. C: Significant increase in the p-ERK1/2 was observed in the HFD-fed rats as compared to the normal-pellet diet NPD-fed rats. All the values are shown as mean±S.E.M., *P<0.05, **P<0.01, a vs. NPD, b vs. NPD+Saline and c vs. NPD+BoNTA.

4. Discussion

Epidemiological reports indicate increased incidence of benign prostatic hyperplasia in the insulin-resistant individuals (Ozden et al., 2007; Vikram et al., 2010a). Recent studies propose BoNTA as a potential candidate for the management of benign prostatic hyperplasia (Economou and Madersbacher, 2010) considering its ability to induce prostatic atrophy and apoptosis in rodents (Doggweiler et al., 1998; Nishiyama et al., 2009; Silva et al., 2009b) as well as in humans (Silva et al., 2008). Here, we report that insulin-resistance activates mitogenic signaling of insulin, increases cellular proliferation and reduces BoNTA-induced prostatic atrophy and apoptosis in rats.

Prostatic hyperplasia does not develop spontaneously in rats unlike humans and dogs (Kobayashi et al., 2009). However, the rodent prostate responds to different hormones and has been extensively utilized as an experimental model to study the pathogenesis of prostatic hyperplasia (Mahapokai et al., 2000) and to screen potential drug candidates for treating benign prostatic hyperplasia (Nanda et al., 2009; Thiyagarajan et al., 2002; Veeresh Babu et al., 2010). Previous studies indicating remarkable decrease in the weight of prostate gland in the hypoinsulinemic (induced by selective β-cell toxins streptozotocin) rats and prostatic enlargement in the hyperinsulinemic rats underlines the crucial role of insulin in prostatic growth (Cai et al., 2001; Escobar et al., 2009; Rahman et al., 2007; Vikram et al., 2010b; Vikram et al., 2008; Yono et al., 2005). Several epidemiological reports also suggest hyperinsulinemia as an important risk factor for benign prostatic hyperplasia (Hammarsten and Hogstedt, 2001; Nandeesha et al., 2006; Vikram et al., 2010a).
Previously we have reported enlargement of prostate in the insulin-resistant rats (Vikram et al., 2010c) and the same experimental model has been utilized in the present study to investigate the effect of BoNTA on the ventral prostate under insulin-resistant condition.

Glandular injection of BoNTA led to atrophy of the prostate gland in both insulin-sensitive as well as insulin-resistant rats. However, it was found to be more effective in the insulin-sensitive rats as compared to the insulin-resistant rats. Reduced effect of the BoNTA on the prostate of insulin-resistant rats can be attributed to the compensatory rise in the plasma insulin level, which is known to have growth-promoting effects. To further investigate the effects of BoNTA at cellular level, cell proliferation and apoptosis were examined in the prostate gland. Significant increase in the PCNA level, irrespective of the glandular injection of saline or BoNTA, clearly indicates increased cell proliferation in the prostate of insulin-resistant rats. TUNEL assay was performed to assess the cell death in the ventral prostate in response to the BoNTA treatment. Although, BoNTA significantly increased the frequency of TUNEL positive cells in both insulin-sensitive and insulin-resistant rats, the frequency of TUNEL positive cells was found to be significantly higher in the insulin-sensitive rather than insulin-resistant rats (Fig. 5).

Previous studies have reported that glandular injection of BoNTA induces apoptosis, decreases cellular proliferation and α1A-adrenergic receptor expression in the rodent prostate without affecting the androgen receptor expression (Chuang et al., 2006). However, in the present investigation, we did not find any appreciable difference in the PCNA level either in the insulin-sensitive or insulin-resistant rats following BoNTA treatment. Our results were different from previous reports probably on account of (i) use of lower dose and/or (ii) examination of the PCNA level after three weeks of the glandular injection of BoNTA. In consistence with the results of previous studies (Chuang et al., 2006; Nishiyama et al., 2009), BoNTA treatment induced follicular dilation and flattened epithelium in both normal as well as in insulin-resistant rats. Although BoNTA –treated insulin-resistant rats had higher prostate weight than controls (NPD), the dilation of the prostatic follicles and flattened epithelium indicates loss of the secretory activity. This can be attributed to the BoNTA-induced temporary chemical denervation produced by inhibiting release of acetylcholine and norepinephrine (Silva et al., 2009b).

In addition to maintaining plasma glucose level, insulin has growth-promoting actions. The IRS/PI3-Kinase signaling of insulin is mainly concerned with the metabolic effects, whereas MEK/ERK signaling is responsible for its mitogenic actions. It has been reported that in insulin-

![Fig. 5. Effect of insulin-resistance and BoNTA treatment on the apoptosis. A: Representative photomicrographs indicating the increased incidences of TUNEL positive cells in the prostatic sections of BoNTA injected rats. B: Significant increase in the TUNEL positive cells was observed in the prostate of BoNTA treated rats as compared to respective diet matched controls. However, the frequency of TUNEL positive cells was significantly higher in the normal rats as compared to the insulin-resistant rats. All the values are shown as mean ± S.E.M., *P<0.05, **P<0.01, ***P<0.001 and ns P>0.05 (ns - not significant).]
resistance, IRS/PI3-Kinase pathway is impaired and MEK/ERK pathway remains unaffected (Jiang et al., 1999). Previously we have reported enhanced activation of ERK-1/2 during hyperinsulinemia as a possible explanation for the increased cellular proliferation and prostatic enlargement in hyperinsulinemic rats (Vikram et al., 2010c). Significant increase in the phosphorylation of ERK1/2 was observed in the prostate of insulin-resistant rats irrespective of BoNTA treatment. This indicates that insulin-resistance associated compensatory hyperinsulinemia results in the over-activation of the mitogenic signaling of insulin in the prostate gland. The anti-apoptotic effect of insulin has been demonstrated both in vitro as well as in vivo experiments. Insulin is thought to exert its anti-apoptotic effects by changing the expression of Bcl-xl and activation of ERK-1/2 during hyperinsulinemia as a possible explanation for the increased cellular proliferation and prostatic enlargement in hyperinsulinemic rats (Augustin et al., 2003; Frances et al., 2010; Wu et al., 2007). Insulin is thought to exert its anti-apoptotic effects by changing the expression of Bcl-xl and activation of ERK-1/2 during hyperinsulinemia as a possible explanation for the increased cellular proliferation and prostatic enlargement in hyperinsulinemic rats (Augustin et al., 2003; Frances et al., 2010; Wu et al., 2007).


In vitro as well as in vivo experiments. Insulin is thought to exert its anti-apoptotic effects by changing the expression of Bcl-xl and activation of ERK-1/2 during hyperinsulinemia as a possible explanation for the increased cellular proliferation and prostatic enlargement in hyperinsulinemic rats (Augustin et al., 2003; Frances et al., 2010; Wu et al., 2007).


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