Original Article

Role of sex hormone on morphological and histological changes in benign prostatic hypertrophy rats

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Abstract: Background: The prostate, the key secondary male reproductive organ, serves an important function of alkalizing seminal fluid and protecting genetic information in the acidity of the vaginal tract. As males age, the most common urologic condition manifests as an enlargement of the prostate known as benign prostatic hypertrophy (BPH). The purpose of this study is to examine the relationship between hormonal regulation and the morphological changes in BPH. Furthermore, we examine whether the ion-transport pump, H-K-ATPase (HKA), mediates such hormonal regulation. The experiments were designed to test the effects of the primary male androgen, testosterone propionate (TP), as well as the female hormone, estradiol (E2). Methods: The rats were divided into three groups; control group, TP group, and TP+E2 group. Both the TP and the E2 were diluted in vegetable oil and covered to eliminate light exposure. A subcutaneous injection of TP at 3 mg/mL was administered to induce BPH in each rat. After 6 weeks of TP-induced BPH, we divided these rats into two groups. In one group of BPH rats, we injected 60 µg of E2, and in another group of BPH rats, we injected 120 µg of E2 subcutaneously. The rats were sacrificed under anesthesia, and the prostate specimens were dissected. The rat’s body weight and the prostate tissue weight were measured as the organ quotient. Results: The data indicate significant hypertrophy of the luminal cells in rats with 3 mg TP compared to the control (524.542 ± 4.637 vs. 350.583 ± 1.996, P-value < 0.005). Whereas, the group with 60 µg E2 on TP-induced BPH showed significant inhibitory effects compared to TP-induced BPH (385.571 ± 7.265 vs. 524.542 ± 4.637, P-value < 0.005). The experimental group with 120 µg E2 on TP-induced BPH also showed significant inhibitory effects compared to TP-induced BPH (465.857 ± 8.259 vs. 524.542 ± 4.637, P-value < 0.005). The inhibitory effects of the 60 µg E2 group were more significant than the inhibitory effects of the 120 µg E2 group (385.571 ± 7.265 vs. 465.857 ± 8.259, P-value < 0.005), suggesting the importance of maintaining a proper E2:TP ratio. Western blot analysis shows up-regulation of specific bands for HKA alpha subunit at ~97 kDa for TP-induced BPH and down-regulation of HKA in the TP+E2 treatment groups. Conclusions: The results show that TP induces benign prostate hypertrophy. Whereas, E2 is shown to inhibit BPH; the effect of E2 inhibition on BPH requires the optimal ratio between E2 and TP. If such a ratio is not reached, then BPH inhibition will not occur or will be less effective by E2. Both the induction and inhibition of hypertrophic cells suggest that the prostate is under hormonal regulation. The proper E2:TP ratio plays a crucial role in the pathogenesis of BPH. The ratio of E2:TP may lead to new approaches to preventing and treating BPH disease in the future.

Keywords: Benign prostatic hypertrophy (BPH), H-K-ATPase, prostate, ATPase, hormonal regulation

Introduction

Encircling the first part of the urethra, just inferior to the bladder, is a walnut-sized organ of the male reproductive system: the prostate. The main function of the prostate is to secrete a milky fluid that is slightly alkaline and contains an androgen-regulated protein known as prostate-specific antigen (PSA). Being one of the most abundant prostate-derived proteins in seminal fluid, PSA is in charge of enhancing motility and protecting genetic information [1]. As males age, the prostate undergoes an enlargement known as benign prostatic hypertrophy (BPH) [2]. BPH is the most common urologic disease in elderly males, affecting one-third of men in their 60 s, and about half of men by the age of 80 [3]. Common complications include urinary retention, urinary tract infections, and kidney stones [2]. There is a controversial relationship amongst urologists as to whether BPH is a precursor to prostate cancer (PCa). BPH typically arises in the dorsal and lateral lobes of the transition zone, while PCa is an adenocarci-
noma that typically manifests from epithelial cells of the peripheral zone; however, BPH and PCa can co-exist in the transition zone [4, 5]. Although there is no clear evidence regarding a molecular and genetic relationship between BPH and PCa, research suggests that the common denominators of increased incidence with increased age, hormone dependence, and incidence with prostatic inflammation, make for the need of further research [6].

The etiology of BPH is poorly understood, making treatment and prevention complex. It is believed that aging and endocrine dysfunction of the hypothalamic-pituitary-gonadal axis contribute to the development of BPH. The primary androgen in males is testosterone. The prostate converts this testosterone into another androgen, dihydrotestosterone (DHT), by the enzyme 5α-reductase, which is shown to have a 2-3 times greater androgen receptor affinity than testosterone itself [7] and facilitates cellular differentiation and proliferation of the prostate [8]. Furthermore, we see that luminal epithelial cells are the primary cells in which the androgen receptor is expressed [9]. Abnormal prostate hypertrophy occurs when there is a disruption of the androgen profile of the prostate, specifically acting on DHT and encouraging proliferation of the tissue [5, 10].

However, proper estrogen levels may play more of a role in prostate growth and development than previously thought. Estrogens, specifically estradiol, play an important role in males, including regulation of gonadotropin feedback, bone maturation, and lipid metabolism. Testosterone is one of the main factors influencing estrogen levels in men. Such changes in estrogen-androgen equilibrium, which occur in midlife, have been subject to study recently and are referred to as endropause [11]. One study revealed a decrease in serum testosterone with a relative rise in serum estradiol levels with advancing age in patients with BPH [12]. With the prostate being an androgen-dependent gland, there is further evidence suggesting that estrogen is necessary in growth and functional maintenance of the prostate [13-15]. The role of a proper androgen-estrogen ratio needs to be established to further understand the physiology and pathology of the human prostate.

The prostate consists of potassium-dependent ion-transporting ATPase pumps that are located in the plasma membrane and function as cation pumps that transfer K⁺ into the cell in exchange for Na⁺ or H⁺. Nongastric H-K-ATPase (HKA) is composed of a catalytic α-subunit (αK⁺H⁻) that performs ATP hydrolysis and ion translocation [16, 17]. Furthermore, ATP12A (former ATP1A1) genes encode αK⁺H⁻ and interact strongly with Na-K-ATPase (NKA) β-isoform (β₁) [7], which is crucial for structural and functional maturation. It is also important for modulation of enzymes’ affinities for cations thus making HKA an αK⁺H⁻-β₁ complex [16, 18, 19], meaning that HKA can mediate Na⁺/K⁺ transport and K⁺ homeostasis in vivo under K⁺ or Na⁺ deprived conditions [18]. Of note, the function of ATP12A protein in the rodent prostate is that of acidification of the semen [7], which is important for the survival of semen in the highly acidic vaginal fluid. Furthermore, ATP12A null mice display loss of acidification and K⁺ absorption in the prostate and the kidney [20-22]. Specifically, non-gastric HKA is required for acidification of luminal prostate fluids and is confined to the lumen-exposed surface of the epithelium [21, 23]. Furthermore, we see that such luminal epithelial cells are the primary cells in which the androgen receptor is expressed [9]. Abnormal prostate hypertrophy occurs when there is a disruption of the androgen profile of the prostate, specifically acting on DHT and encouraging proliferation of the tissue [5, 10].

Methods

Our study involved administration of androgens and estrogens on prostatic cells extracted from male Sprague Dawley outbred rats in specific ratios to observe histological and morphological changes in the prostate cells over specified periods of time. To enhance accuracy of the study, other influences such as environmental and biological influences, like nutritional disparity, were eliminated. It was important to ensure that the biological conditions for all specimens used were similar, meaning that rats were subjected to similar environmental and nutritional conditions.
Animals

Male and female Sprague-Dawley outbred rats were obtained from Harlan® Laboratory, Indianapolis, Indiana. Animals were kept two rats per cage (one male, one female) with free access to food and water. The rats then mated at will and the males were separated from the females postpartum. After 3 weeks, the offspring were separated from the mother and placed two per cage with free access to food and water. The rats were divided into three groups, including one control, one experimental with TP only, and one experimental with TP and E2.

Hormones

Preparation of testosterone propionate (TP): A 500 mL Erlenmeyer flask was filled with 1,500 mg TP (oil soluble: solid: C22H32O3). It was then covered with aluminum foil to avoid light exposure and filled with 500 mL of pure vegetable oil to obtain a 3 mg/mL concentrated TP solution. The solution was then stirred for 48 hours.

Preparation of estradiol (E2): A 250 mL Erlenmeyer flask was filled with 6 mg E2 (oil soluble: solid: C18H24O2). It was then covered with aluminum foil to avoid light exposure and filled with 100 mL of pure vegetable oil to obtain a 60 µg/mL concentrated E2 solution. The solution was stirred for 48 hours.

Injections and time sequencing: Both the TP and E2 solutions were given subcutaneously (SQ) at a 45-degree angle in the adipose tissue of the rear distal flank. The solution was slightly warmed, and a restraint was used to ensure adequate administration. Needles (22 gauge, 1” length) and syringes (5 cm³) were replaced after each injection. The rats were then marked on their tail to distinguish groupings.

Exactly 7 weeks after initial TP administration, the TP-induced group was sacrificed, and the prostates were dissected and defined as the TP+E2a (60 µg) and the TP+E2b (120 µg) groups.

Euthanasia and prostatectomy

Rats were placed in a large container with a covered cotton ball soaked with isoflurane (inhalational anesthesia). After one minute of exposure to isoflurane, the rat was removed from the container and placed supine on a clean glass plate. The left thoracic cavity was palpated for location of the heart. An injection of 1 mL sodium pentobarbital (euthanasia) was given via cardiac puncture at a 90-degree angle with aspiration to assess for heart puncture. Pressure was maintained over the injection site until expiry was confirmed. Separate needles (20 gauge, 1” length) and syringes (5 cm³) were used for each rat. After euthanasia, the rat’s total body mass was measured. The prostate was then removed, and the urethra and seminal vesicles were detached. The weight of the prostate was measured and divided by the total weight of the rat to determine the organ quotient.

Histopathology

Histopathology methods were used to obtain prostate cell sections, which were placed on microscopic slides for further analysis. The cell images were taken at 100 × and 400 × magnification using a Nikon TE 300 and used to show hormonal effects on the prostate at the cellular level. A calibrated ocular micrometer eyepiece, combined with a stage micrometer, was used to take measurements of prostatic cell lengths in the lateral lobes of the prostate.

Fixation and embedding

Formalin, a dilution of commercial formaldehyde (40% solution of formaldehyde gas in water) in aqueous phosphate buffer, was used as the general fixative. The tissue was then dehydrated in a grading of alcohol-water solutions (15, 30, 50, 70, 85, 90, 100% alcohol, respectively). The tissue was then embedded with paraffin in the following manner. The alcohol was replaced by xylene and then replaced by melted paraffin in the clearing process. The embedding takes place when the paraffin-infiltrated tissue is placed in fresh paraffin and then allowed to cool. A steel knife was mounted in a microtome and used to cut 10 µm tissue.
sections, which were then mounted on a glass microscope slide. After mounting, the tissue sections were stained using hematoxylin (base) and eosin (acid) (H&E). The final step was to permanently mount the sections under a cover-slip using Premount mounting resin. The tissue was then labeled and ready for microscopic examination.

**SDS-PAGE & western blot**

In order to separate the proteins, the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method was used. 100 µL sample with 100 µL of Laemmli’s sample buffer were mixed and incubated at room temperature for 1 hr (50 µL 2-mercaptoethanol + 950 µL Laemmli’s sample buffer). The gel cassette was prepared by removing the tape at the bottom of the gel and assembling the mini protein tetra cell electrophoresis module. The assembly was filled with SDS running buffer up to the edge of the outer plate. The first well was loaded with 20 µL standard protein sample, and small volumes of sample protein (20 µL) dissolved in gel loading buffer were added to each individual well. The gel ran at 200 V for 40 min and was removed gently when finished.

While running the gel, a piece of polyvinylidene difluoride (PVDF) membrane was cut to the same size as the filter paper. Two plastic dishes were prepared—one filled with methanol and the other filled with Western blot transfer buffer. The membrane was incubated in the methanol for 15 sec and then equilibrated for 5 min in the transfer buffer. The transfer stack was assembled, and the whole tank was filled with Western blot transfer buffer. The transfer stack was assembled, and the whole tank was filled with Western blot transfer buffer. Ice and a stirrer were then placed in the tank, as well. The gel ran at 100 V and < 350 mA for 1 hr. To reduce nonspecific binding, the membranes were soaked in 10% skim milk (15 mL PBS + 1.5 g dry milk, pH 7.2) for 1 hr at room temperature and again overnight at 4°C. The membrane was incubated with primary antibody-anti-proton pump/HKAα (anti-HKAα, code no.-D031-3, clone-1H9, subclass-Mouse IgG1, quantity-100 µg, concentration-1 mg/mL; Medical and Biological Laboratory Co., Ltd., Japan)-diluted with PBS (pH 7.2) containing 1% skim milk for 1 hr at room temperature. Aliquot 100 µL of anti-HKAα into 20 tubes, each 5 µL of antibody. The application was to put 5 µL anti-HKAα into 5 mL PBS + 1% skim milk. The membrane was then washed with PBST (0.05% Tween-20 in PBS) 3 times for 5 min each time. Then the membrane was incubated with 1:10,000 horseradish peroxidase (HRP)-conjugated anti-mouse IgG diluted with 1% skim milk (in PBS, pH 7.2) for 1 hr at room temperature (20 mL PBS + 2 g milk + 2 µL secondary antibody). The membrane was washed with PBST 6 times for 5 min each time and incubated with chemiluminescence reagent for 1 min. The extra reagent was removed from the membrane by dabbing with a paper towel and sealed in a plastic wrap to avoid sensitive light. The membrane was exposed to an X-ray film in a dark room for 5 min and developed as usual (developer solution + water-fixer solution). Resultant band densities were analyzed using ImageJ® computer software and converted into graphs comparing area densities using Microsoft Excel®.

**Statistics**

The data is expressed as mean ± standard error where appropriate. For the statistical analysis of luminal cell size, the micrometer readings were measured, and the statistical analysis was performed. The Western blot analysis was performed using ImageJ® software. Analysis of variance and t-test were used where appropriate to determine statistical significance. P-values < 0.05 were considered statistically significant.

**Results**

The experimental groups of rats we selected were TP-induced and TP+E2-induced. All rats were under normal dietary conditions. The rats were then separated into four groups depending on hormonal regulation: control, TP-induced, TP+E2α-induced, and TP+E2β-induced. Follow the histopathology procedure (mentioned in the methods section). Cell size was measured by ocular and stage micrometer. An average and standard deviation of the total measurements per slide were calculated to determine hypertrophy in each tissue section. These results were then compared to the control group under no external hormonal administration.

**Organ quotient**

An analysis of the organ quotient (OQ) was determined by dividing the weight of the dis-
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Table 1. Effects of TP and E2 on organ and cell size

<table>
<thead>
<tr>
<th>Hormonal Regulation</th>
<th>Organ Quotient (g)</th>
<th>Cell Size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.102 ± 0.013 (n = 6)</td>
<td>350.583 ± 1.996 (n = 72)</td>
</tr>
<tr>
<td>TP</td>
<td>0.180 ± 0.010 (n = 5)</td>
<td>524.542 ± 4.637 (n = 83)</td>
</tr>
<tr>
<td>TP+E2a</td>
<td>--</td>
<td>385.571 ± 7.265 (n = 14)</td>
</tr>
<tr>
<td>TP+E2b</td>
<td>--</td>
<td>465.857 ± 8.259 (n = 14)</td>
</tr>
</tbody>
</table>

Abbreviations are: TP, testosterone propionate; E2, estradiol. *TP = 3 mg, E2 = 60 μg; **TP = 3 mg, E2 = 120 μg.

Figure 1. Organ quotient (OQ) of the weight of the prostate compared to the total body weight of the rat (before dissection). The 3 mg/mL TP-induced BPH group yielded a 0.18% OQ compared to the control group at 0.10% (P-value = 0.0006).

An analysis of the micrometer measurements of cell length in the lateral lobe of the prostate examines the association of TP and E2 with BPH. In the rats, the epithelial cell sizes of the control group vs. the TP-induced (3 mg) BPH group were 350.583 ± 1.996 μm (n = 72) vs. 524.542 ± 4.637 μm (n = 83) (Figure 2). The results suggest that TP at a concentration of 3 mg/mL induces significant hypertrophy in cell size. The cell sizes of the rats treated with TP+E2a (3 mg TP, 60 μg E2) vs. TP+E2b (3 mg TP, 120 μg E2) were found to decrease to 385.571 ± 7.265 (n = 14) vs. 465.857 ± 8.259 μm (n = 14) compared to the TP-induced BPH; 524.542 ± 4.637 μm (n = 83) (Figure 2). A statistical comparison of the cell size under TP-induced BPH vs. inhibitory conditions by E2a or E2b (Figure 3) shows the inhibitory effects of the 60 μg E2 group were more significant than the inhibitory effects of the 120 μg E2 group (385.571 ± 7.265 vs. 465.857 ± 8.259, P-value < 0.005), suggesting the importance of maintaining a proper E2:TP ratio.

Western blot

By using anti-HKAα primary antibody, we were able to detect bands at ~97 kDa in epithelial cells of the lateral lobe in the rodent prostate and the stomach (Figure 4). HKα levels were increased (P < 0.005) by 2-fold at 5 min in the TP-induced treatment group. Furthermore, we observed HKα levels decreased (P-value < 0.005) in the TP+E2-induced group as compared to the TP-induced group (Figures 5, 6).

Discussion

The development of BPH is related to particular histological changes that take place in the prostate cells as a result of changes in the estrogen/androgen ratio. With increasing age, the serum level of estrogen/androgen in elderly men is normally 1/120-1/80 and, in extreme cases, the prostate can reach serum levels of 1/8 [25]. Therefore, a change in the E2:TP ratio could induce BPH. The results of this study collaborate with the previous research findings. Relative prostate masses show that BPH of the lateral lobe was induced by 3 mg injections of TP after just 3 weeks. Significant hypertrophy was observed in the luminal cells of the lateral lobe of the 3 mg TP groups.

The action of estrogens as regulators of the function and development of the prostate is significant. With the prostate being the model organ for androgen-dependent growth and development, estrogens are also responsible for regulating growth, development, and function through interactions with and maintenance of...
particular ratios of androgens [26]. Biologically, estrogens repress the hypothalamic-pituitary-gonadal axis, a process that has direct effects on the testes [26]. As a result, androgen production by the testes is inhibited. Estrogen regulation is achieved through multiple routes, two of which include action on prolactin and action on prostatic cells. In the latter, E2 is produced by the local aromatization of TP and acts on the prostate cells to inhibit cell growth. Aromatase reactions convert TP into E2, with aromatase being most prevalent in adipose cells [27]. Thus, as males age, TP decreases, metabolism slows and abdominal fat increases, in turn, increasing E2 levels relative to TP levels. Furthermore, disruption of serum TP and altered levels of DHT in the prostate encourage proliferation of the prostate tissue (consistent with DHT theory; [28]). While there are many different theories speculating the specifics regarding hormonal deregulation (DHT hypothesis, embryonic reawakening theory, stem cell theory), further research is needed to

**Figure 2.** Images of the cell size in the lateral lobe of the rodent prostate. Significant hypertrophy of the luminal cells is seen in the TP-induced (3 mg/mL) group (524.542 ± 4.637), while significant inhibition of luminal hypertrophy is seen in the both TP+E2-induced (E2a: 60 µg/mL vs. E2b: 120 µg/mL) groups (385.571 ± 7.265 vs. 465.857 ± 8.259, respectively). *A = 100 × zoom, B = 400 × zoom.

**Figure 3.** An analysis of the micrometer measurements of the cell length in the lateral lobe of each group shows that 3 mg/mL TP significantly induces cellular hypertrophy (P-value < 0.005), while the TP+E2 groups show significant inhibition on TP-induced hypertrophy. Then, the ratio of the TP+E2a (3 mg TP, 60 µg E2) group shows better inhibitory effects on hypertrophy compared to the ratio of the TP+E2b (3 mg TP, 120 µg E2) group. This demonstrates the importance of maintaining a proper E2:TP ratio in control over prostate enlargement.
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In this study, significant findings were observed between the experimental groups and the control group. Specifically, the rat prostate-body weight percentage, or the organ quotient (OQ), of the control was 0.10% vs. 0.18% in the TP-induced BPH group. Furthermore, the epithelial cell lengths show that TP-induced BPH (3 mg) increases the hypertrophy of the lateral prostate cells, confirming that androgens, in particular TP, and estrogens, in particular E2, are vital in the development of BPH.

The two groups of rats injected with both TP and E2 (E2a: 60 μg and E2b: 120 μg) 3 weeks after TP-induced (3 mg) BPH showed a significant inhibition of BPH. It was observed that the lateral lobes of the TP+E2 groups were much smaller than the TP-induced BPH group, similar to that of the control group. Furthermore, E2 at a concentration of 60 μg (TP+E2a) showed significantly more inhibition than the group with an E2 concentration of 120 μg (TP+E2b). Higher E2 concentration achieves less inhibition and may actually induce BPH by potentially stimulating androgen receptors (AR). This suggests that not only is it pivotal in maintaining the balance of androgens and estrogens but, more specifically, the proper E2:TP ratio plays an important role in the pathogenesis of BPH. If an optimal ratio is not maintained, meaning a fluctuation in TP and/or E2, BPH is likely to occur.

Our studies indicate that TP increases hypertrophy of the luminal prostate cells and may demonstrate an alteration in enzymatic activity of non-gastric HKA, consistent with results from Nakamura et al. [29]. According to Streif et al., cellular apoptosis is characterized in part by intracellular acidification, K+ loss and cell shrinkage [7, 30]. HKA activation is important in antagonizing intracellular acidification and may attenuate cell apoptosis by countering K+ loss and apoptotic cell shrinking [7]. HKA plays
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an important role in K+ homeostasis and mediation of Na+/K+ transport as a αβ complex [16, 18]. In this study, we identified up regulation of HKα (~97 kDa) in TP-induced BPH rats (Figure 6), which suggests that HKA mediates hormonal regulation and expression. Further, we identified down regulation of HKα in the TP+E2-induced groups compared to the TP-induced BPH group. Such deranged expression pattern might be from an alteration in gene transcription.

While the study conducted shows valuable evidence of organ growth in TP-induced BPH, it was limited in some manners. Foremost, a larger sample size of rats would increase the confidence and decrease the uncertainty, especially in the TP+E2 groups. Also, fluctuating more dosages (5-10 each) of hormone variation would provide more insight on the specific E2:TP ratio. With this being said, future research needs to be done increasing the sample size and increasing the amount of hormonal variation (both TP and E2) the rat receives. From there, further research should be conducted towards establishing an optimal ratio and a potential link between BPH and prostate cancer at a molecular level.

Conclusion

Both BPH and carcinogenesis of prostate cancer are accepted to be in the transition zone of the prostate. The cell images and measurements thus were taken from the lateral lobes of the transition zone in rodents, and TP-induced BPH was found to hold true through our experimental results. Rats who were injected with 3 mg/mL TP show a significant increase in prostate growth. Furthermore, epithelial cell lengths show that 3 mg/mL TP increases the hypertrophy of the lateral prostate cells. Such a finding suggests that androgens serve a vital function in the development of BPH and potentially other prostate dysplasias, like PCa. Our studies indicate that TP-induced BPH demonstrates up regulation while the TP+E2-induced groupings demonstrate down regulation in enzymatic activity of H-K-ATPase, suggesting that HKA mediates hormonal regulation in the prostate. Furthermore, rats injected with 60 μg/mL E2 and 3 mg/mL TP (TP+E2) show a significant inhibition of BPH. Both induction and inhibition of hypertrophied cells suggest that the prostate is under hormonal regulation. With this being said, the group injected with 120 μg/mL E2 and 3 mg/mL TP (TP+E2) shows significant inhibition of BPH, but significantly less inhibition compared to the TP+E2 group. The proper E2:TP ratio plays an important role in the pathogenesis of BPH. If the optimal ratio is not maintained, it can lead to BPH and possibly other pathological conditions like PCa. Such knowledge of optimizing E2:TP in humans may help to prevent or cure BPH in the future.

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Disclosure of conflict of interest

None.

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