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Estrogenic action of commonly used fragrant agent citral induces prostatic hyperplasia

A. A. Geldof, C. Engel, and B. R. Rao

Department of Endocrinology, Academisch Ziekenhuis, Vrije Universiteit, Amsterdam, The Netherlands

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Summary. A rat model for benign prostatic hyperplasia in man (BPH) was investigated. Citral treatment of male Copenhagen rats for 4 months via the transdermal route resulted in a marked hyperplasia of glandular epithelium and interglandular stroma in the ventral prostate. Despite the cellular hyperplasia there was not a significant increase in prostate weight. Investigations of the mechanism of action of citral showed that application of citral directly to the vagina in female, ovariectomized rats resulted in an increased proliferation of vaginal epithelium and a significant increase in the BrdUrd incorporation in vaginal epithelial cells, in short a similar effect to that of estrogen application. In an in vitro assay citral proved to inhibit estrogen binding to estrogen receptors, while no such inhibition was observed with testosterone for androgen receptors. These observations together with the estrogen implication in the BPH and the reported incidence of gynecomastia following exposure to geraniol, a precursor of citral, strongly suggest that the prostatic hyperplasia-inducing capacity of citral may be due to its estrogenic action.

Key words: Citral – Estrogenic – Prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is one of the most common diseases in modern western society, affecting about 85% of men in their eighth decade [4]. Nonetheless, etiological factors and possible preventive measures remain largely obscure. Definitive information might come from animal models. Spontaneous age-related occurrence of BPH is known in dogs [6, 7]. Since castration can inhibit onset of BPH development and androgen-estrogen combination treatment of castrated animals can experimentally induce BPH, endocrine factors have been implicated in its genesis [10, 16, 24].

Furthermore, androgen receptor antagonist and aromatase inhibition have been shown to have an effect on BPH development in these animals [13, 17]. However, BPH in dogs is demonstrably different from the BPH that evolves in man [5, 6, 10, 14, 19]. The use of non-human primates as model for the disease [12, 14] on the other hand, has practical limitations. Spontaneous BPH development in rodents is considered to be an infrequent finding [15]. Experimental induction of rat prostatic hyperplasia by citral, a monoterpene, was reported some years ago [2, 3, 22]. Citral is a volatile monoterpene that occurs in plants and citrus fruits. Due to its strong lemon fragrance it is used commonly as food additive, in cosmetics, detergent and embalming cream.

In the present paper we report on the effects of citral application in male and female rats and on the induction of prostatic hyperplasia. Investigations of the mechanism of citral action suggest that the effects are probably due to its estrogen-like action. The morphological changes observed in rat ventral prostate following citral treatment demonstrate that rodents can be used as an appropriate model for BPH. More importantly, the role of industrial use and consumption of citral-containing products in the etiology of BPH may have to be carefully investigated.

Materials and methods

Animals

Copenhagen rats, originally obtained from the Mammalian Genetics and Animal Production Section, National Cancer Institute, Bethesda, Md, were bred (brother × sister) in our animal facilities. In the experiments young adult male and female animals (ages 4–6 months) were housed in Macrolon cages, two per cage, with commercial chow feed and water ad libitum.

Treatment

Male animals were treated with citral (3,7-dimethyl-2,6-octadienal, Sigma Chemical Company, St. Louis, Mo.) thrice a week for long-term experiments by applying 62 mg (0.7 ml), smeared on the unshaven back skin.

Female, ovariectomized animals were treated with 27 mg (0.3 ml) citral, diluted 1:1 with sesame oil containing 2.8% ethanol, by vaginal application daily for 4 days. Ovariectomized female animals

treated for 4 days by vaginal application of either 5 µg estradiol-17β in 0.03 ml sesame oil containing 2.8% ethanol or of 0.03 ml sesame oil containing 2.8% ethanol formed two groups of control. On the 5th day the female rats were killed by ether overdose and serum was prepared from blood obtained by cardiac puncture. The tissues removed were snap-frozen in liquid nitrogen and stored at −80°C until use.

Blood sampling and steroid measurements

After 28 days of treatment blood samples (0.2 ml) from the animals were withdrawn from the tail vein under mild ether anesthesia and serum was prepared for each individual animal. Serum testosterone and estrogen concentrations were determined using commercially available radioimmune assay kits (TKTT2 and TKE21, respectively; Diagnostic Products Corporation, Los Angeles, Calif.).

Histology

Tissue samples from ventral prostate, submandibular salivary glands and vagina were fixed in phosphate-buffered formalin (4%), and embedded in paraffin. Sections (6 µm) were stained with hematoxylin/eosin.

Immunocytochemistry

To determine DNA-synthesis rate in tissue, animals received intraperitoneal injection (0.2 ml) of bromodeoxyuridin (BrdUrd), 10 mg/ml Hank's balanced salt solution (HBSS), 4 h prior to killing. Tissues were snap frozen in liquid nitrogen and stored at −80°C until sectioning. Cryostat sections (8 µm) were put on gelatin-coated glass slides, dried and fixed in cold acetone (−20°C) for 10 min. After the sections had been washed three times with Tris-buffered saline (TBS); (0.05 M Tris; 0.15 M NaCl; pH 7.6), they were treated with 95% formamide in 0.15 M trissodium citrate at 70°C for 45 min. Sections were rinsed again three times inTBS, pH 7.6 and treated with 0.1 M sodium tetraborate, pH 8.6 for 15 min. After washing three times in TBS (5 min each), the sections were incubated with mouse anti-BrdUrd antibody (DAKOpatts, Denmark), 1:20 in 5% heat-inactivated rabbit serum in PBS (phosphate-buffered saline, pH 7.4) for 1 h in a moist chamber. Additionally, the sections were washed three times in TBS and incubated with rabbit anti-mouse peroxidase-conjugated antibody (RAMPO, DAKOpatts, Denmark, P260), 1:80 diluted in PBS for 1 h in a moist chamber. After three washes with TBS the bound antibody-enzyme complex was visualized by adding diaminobenzidine (0.5 mg/ml Tris buffer, 0.05 M, pH 7.4) +0.3% H_2O_2 (3 drops/3 ml solution) and incubating for 15 min. After three washings with TBS the sections were counterstained using Mayer's hematoxylin for 1 min.

Negative controls for the staining consisted of adjacent sections which were treated using the same procedure as described above, but without first anti-BrdUrd antibody incubation.

Estrogen and androgen receptor analyses

Immature rat uterus tissue was cut into small pieces, pulverized deep-frozen using a microdismemebrator (Braun, Melsungen, FRG) and homogenized in ice-cold buffer (1:5 w/v, Tris 10 mM, EDTA 1 mM, monothioglycerol 12 mM, glycerol 10% v/v, pH 7.4). The homogenate was centrifuged for 10 min at 1000 *g*. The supernatant was centrifuged at 100000 *g* for 60 min to obtain the cytosol fraction as supernatant. The concentration of estrogen receptor (ER) was determined by saturation analysis. Aliquots of cytosol fraction were incubated in duplicate for 16 h at 0–4°C with five dilutions (0.1–1.0 nM) of [2, 4, 6, 7]-^{3}H estradiol-17β (110 Ci/mmol) (Amersham, UK). Non-specific binding was determined at each concentration by adding a 200-fold excess of unlabeled diethylstilbestrol (Sigma, St. Louis, Mo., USA). Citral was added (10, 25 or 50 µl) to the reaction mixture in competition assay. Receptor-bound and free steroid were separated by adding ice-cold buffer, containing charcoal (Tris 10 mM, EDTA 1 mM, dextran 0.01%, charcoal 0.1%, pH 7.4). Following 10 min of incubation, the tubes were centrifuged at 1000 *g* for 10 min. The supernatant was mixed with scintillation fluid (10 ml) and the bound radioactivity counted. A similar type of analysis was also performed using rat prostate cytosol and [2, 4, 6, 7]-^{3}H testosterone (105 Ci/mmol) to determine the possible interaction of citral with androgen receptors.

Results

General effects and organ weight after citral treatment

No toxic reactions were observed, even after prolonged (4 months) citral treatment periods. Locally at the site of application there was keratinization of the skin on the back of some animals accompanied by loss of hair. Weights of the ventral prostate, dorsolateral prostate, and seminal vesicles were not affected significantly by citral treatment (data not shown). To check for citral-induced effects in another kind of glandular tissue [20] submandibular salivary glands were processed for histological examination as control. No changes in the histology of salivary glands could be observed, and the body weight was also not altered by citral treatment.

In ovariectomized female animals treated with citral vaginally there were no macroscopically observable changes except for a slight yellow coloration of the skin surrounding the vagina.

Prostatic histology after citral treatment

Young adult male rats treated as controls showed a columnar epithelial lining of glandular acini and sparse stromal interstitial tissue in their ventral prostate (Fig. 1). The glandular lumen was filled with eosinophilic material. In distal ducts in the periphery of the ventral prostate the glandular epithelium appeared to be proliferative. In all the six male rats treated for 5 weeks with topical citral application on the unshaven skin of the back, slight changes were noted in the histology of the ventral prostate. In these rats a limited degree of epithelial hyperplasia could be observed (Fig. 2). After 2 and 4 months of treatment these changes became progressively more pronounced, and a marked proliferation of stromal tissue was clearly observed (Fig. 3, 4). No treatment-induced changes were found in either gross morphology or histology in the seminal vesicles or in the salivary glands of citral-treated animals.

Cellular proliferation in prostate during citral treatment

Attempts were made to monitor cellular proliferation in the ventral prostate during 5- and 10-day citral treatment cycles of male rats by immunocytochemical demon-

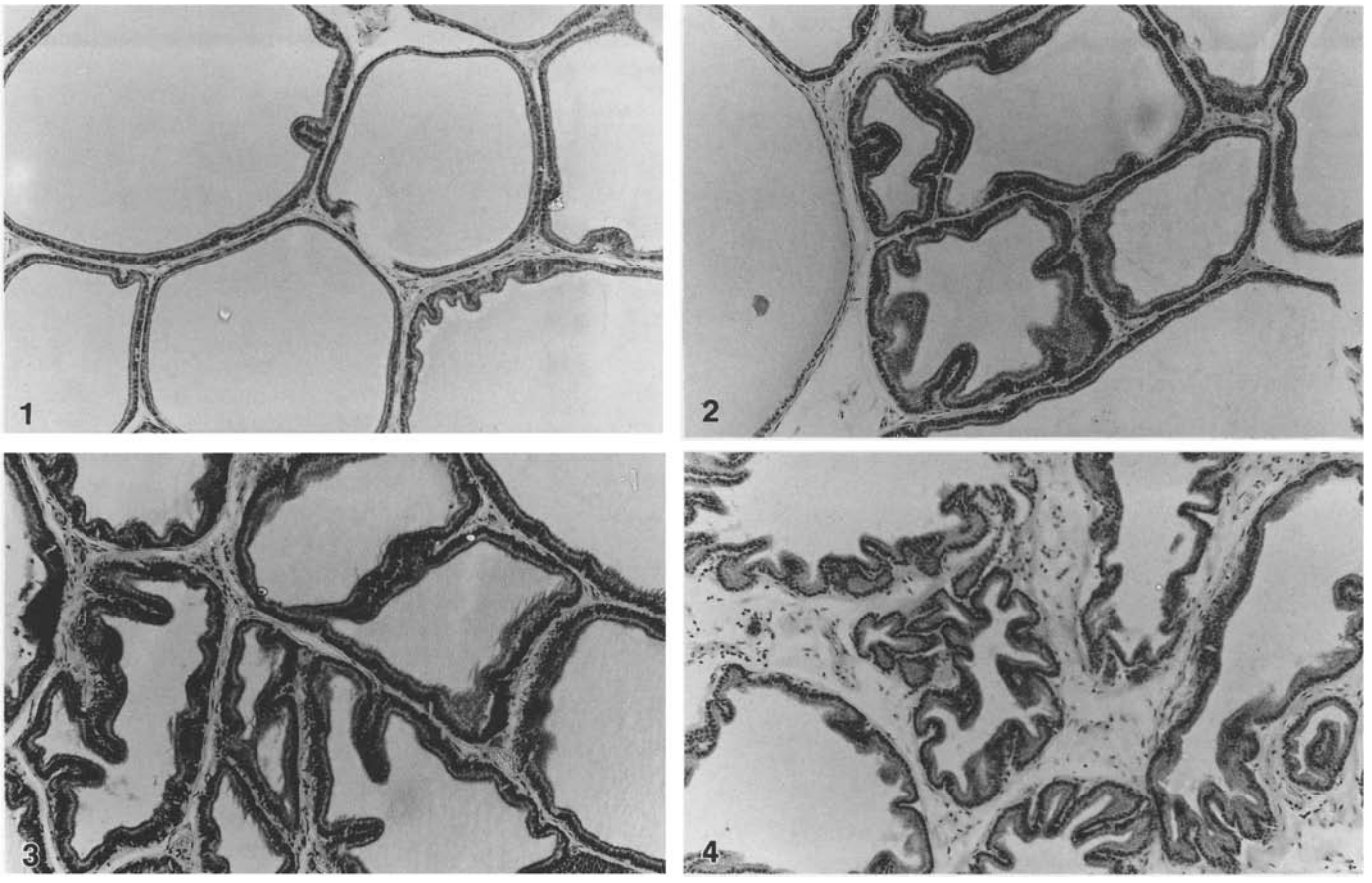

Fig. 1. Photomicrograph of ventral prostate from untreated, control male Copenhagen rat. H&E; ×105

Fig. 2. Photomicrograph of ventral prostate from male Copenhagen rat treated for 5 weeks (three times weekly) with topical citral application. H&E; ×105

Fig. 3. Photomicrograph of ventral prostate from male Copenhagen rat treated for 2 months (three times weekly) with topical citral application. H &E; ×105

Fig. 4. Photomicrograph of ventral prostate from male Copenhagen rat treated for 4 months (three times weekly) with topical citral application. H&E; ×105

Table 1. Proliferation rate (BrdUrd incorporation) in gladular epithelium of ventral prostate acini of Copenhagen rats, following daily citral treatment (Means ± SD; $n = 6$)

	$BrdUrd^+$ cells (%)
Untreated control	0.35 ± 0.53
Citral for 1 week	0.31 ± 0.47
Citral for 2 weeks	0.06 ± 0.05

stration of BrdUrd incorporation. No significant changes in BrdUrd-positive cells either in glandular epithelium or in stroma within the ventral prostate could be detected (Table 1).

Steroid hormone levels in male rats after citral treatment

After 1 and 2 weeks of daily treatment with citral on the skin of the back blood was drawn from six male animals and sera prepared. Sera from 6 untreated control rats were also prepared. Serum levels of testosterone and estradiol-17β were determined by radioimmunoassay (Table 2). There appeared to be no treatment-induced alterations in steroid levels.

Estradiol levels in female rats after citral treatment

Five female, ovariectomized animals received a daily citral application in the vagina for 4 days. Two control groups consisted of five female, ovariectomized animals each; one receiving daily vaginal application of 5 μg estradiol-17β and the other receiving vaginal application with vehicle (sesame oil) only. Levels of estradiol-17β measured in sera after citral treatment did not significantly differ from those measured in controls, while there was a significant increase in estrogen-treated rats (Table 3). Testosterone was not detectable.

Table 2. Serum steroid levels in male Copenhagen rats after daily citral treatment (means ± SD; $n = 6$)

	Testosterone (nmol/l)	Estradiol-17β (nmol/l)
Untreated control	3.18 ± 1.10	0.033 ± 0.016
Citral for 1 week	2.93 ± 1.99	0.032 ± 0.012
Citral for 2 weeks	4.01 ± 2.05	0.030 ± 0.008

Table 3. Serum estradiol-17β levels in ovariectomized female Copenhagen rats after different vaginal treatments, daily for 4 days

	Serum estradiol level (nmol/l)
Untreated controls	0.030 ± 0.006
Citral-treated	0.014 ± 0.010
Estrogen-treated	0.070 ± 0.017

Table 4. Proliferation rate (BrdUrd incorporation) in vaginal epithelial cells of female, ovariectomized rats following 4 days of treatment (means ± SD; $n = 6$)

	BrdUrd-positive epithelial cells (%)
Control-treated	1.90 ± 2.60
Citral-treated	7.05 ± 3.36
Estrogen-treated	12.50 ± 3.40

Table 5. Citral interaction with radiolabeled (^{3}H-estradiol-17β) binding to estrogen receptor in uterine cytosol

Incubation	Net bound (dpm)[a]
Uterine cytosol alone	14116
Uterine cytosol + 10 µl citral	8965
Uterine cytosol + 25 µl citral	7439
Uterine cytosol + 50 µl citral	4205

[a] Separation of bound from unbound steroid with dextran-coated charcoal

Cellular proliferation in vagina during citral treatment

BrdUrd incorporation was measured by immunocytochemical means in vaginal tissue from five ovariectomized female animals following daily application of citral for 4 days. Control groups consisted of five female, ovariectomized animals each; one group received daily vaginal application of 5 µg estradiol-17β and the other received vaginal applications of vehicle (sesame oil) only. After 4 days of treatment animals from all three groups were killed on the 5th day, after incorporation of BrdUrd. Positively stained epithelial cells were counted and expressed as percentage of epithelial cells in the section (Table 4).

The number of proliferating epithelial cells in the vagina appeared to be increased significantly after citral treatment ($P < 0.05$), while the proliferation was highest in estrogen-treated group.

Citral interaction with estrogen and androgen receptors

To determine the effects of citral on estrogen binding to estrogen receptors (ER), increasing amounts of citral were incubated together with uterine cytosol and ^{3}H-labeled estradiol-17β at 4°C for 16 h. After separation of bound from unbound steroid with dextran-coated charcoal, the bound radioactivity was measured. Increasing amounts of citral in the incubation mixture proved to inhibit ^{3}H-estradiol-17β binding to ER (Table 5). A similar dose–response relationship was not observed with radiolabeled testosterone binding to prostate cytosol androgen receptor (data not given).

Discussion

Citral, owing to its pleasant fragrance, is widely used as a food additive, in cosmetics, and in detergents. Experiments using radiolabeled citral have indicated rapid absorbance and metabolism in the skin after dermal exposure [11]. Interestingly, in our present investigations citral application to the unshaven skin was found to induce epithelial and stromal hyperplasia in male Copenhagen rat ventral prostate. These results confirm earlier reports published by others [2, 3, 22]. However, it must be pointed out that we, unlike others, have failed to observe any significant increase in prostate weight following citral treatment in spite of significant cellular proliferation and an increase in the glandular cell numbers. Unlike our present investigations, in which full grown adult male rats received citral three times a week, other investigators used a higher daily dose of citral in young animals, at a time when there was regularly steady growth in prostate weight [2, 3, 22]. Our investigations demonstrate that citral can indeed induce prostatic cellular hyperplasia, without clear prostatic hypertrophy, in rodents.

Estrogens have been used experimentally to induce prostatic hyperplasia in animals [12, 17]. In addition, estrogens have been implicated in the pathogenesis of BPH in man. In particular, a high estrogen concentration could be localized within the BPH stromal nuclei [18]. Prevalence of ER has been demonstrated in nuclei of prostatic urethra, of the periurethral prostatic duct and of the interglandular prostatic stroma of BPH tissue [21]. The above information supports an involvement of estrogen or an estrogenic action in BPH in man.

We have demonstrated for the first time that citral competes with estradiol-17β for ER, while no such competition was observed with testosterone for androgen receptors. Since anti-estrogens also bind to ER, compe-

tition with estrogen for ER alone may also suggest an anti-estrogen action of citral in estrogen-responsive target tissue. On the other hand, the magnitude of the effect of citral on epithelial proliferation in the vagina and the significant increase in BrdUrd incorporation compared with controls clearly suggest an estrogenic action. However, the proliferation induction effect on vaginal epithelial cells in ovariectomized female animals appeared to be smaller than that induced by estradiol-17β application, suggesting that citral is a weak estrogen. The reported case of gynecomastia in a man exposed to geraniol, a precursor of citral is further strong evidence for considering citral to be estrogenic [1]. All the above results strongly suggest that the induction of prostatic hyperplasia by citral is probably due to its estrogenic action mediated through ER.

Another putative action that has to be considered for citral could be derived from the observation that it has an ability to inhibit tumor induction by carcinogens in mice [9]. It has been suggested that this action of citral is due to an antiretinoid effect. A possible role of retinoic acid metabolism in prostatic hyperplasia has yet to be investigated.

Animal models for the study of human BPH have been attempted in such species as dog and primates. Ethically and economically such models are not ideal. A mouse model in which prostatic hyperplasia can be induced by transplanting fetal urogenital sinus tissue into the prostate gland of a syngeneic adult mouse has been developed [8]. Histological hyperplasia of the resulting chimeric organ proved to be dependent on androgens [23]. However, the development and the availability of a simple rodent model for prostatic hyperplasia, which should be both versatile and experimentally accessible to provide statistically meaningful and reproducible results, will overcome some of the problems related to the development of an animal-model for BPH and allow some insight into the pathologic factors in the genesis of this disorder in man. Citral-induced hyperplasia in the ventral prostate of rat could offer such a model.

Presently citral, citral analogues and precursors are used extensively in perfumes and detergents and in food additives. Since citral is readily absorbed through the skin and since it has been demonstrated that it can induce prostate hyperplasia, the use of citral and citral-like compounds in the etiology of BPH in man should be investigated. In any such investigations, the emphasis should be placed not on the dose of the compounds used but on the determination of the effects of the absorbed and cumulated dose in persons exposed to citral and citral-like compounds.

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Dr. B. R. Rao
Department of Endocrinology
Academisch Ziehenhuis
Vrije Universiteit
P.O. Box 7057
1007 MB Amsterdam
The Netherlands