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Inhibition of Testosterone Secretion by Digitoxin in Rat Testicular Interstitial Cells

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Abstract Both in vivo and in vitro experiments were conducted to determined the effects of digitoxin on the secretion of testosterone, and its underlying mechanisms including testicular adenosine 3′:5′-cyclic monophosphate (cAMP), and the activities of steroidogenic enzymes. Male rats were injected with digitoxin, human chorionic gonadotropin (hCG), or hCG plus digitoxin *via* a jugular catheter. Blood samples were collected immediately before and at 30 and 60 min after the challenge, and analyzed for testosterone by radioimmunoassay. In an in vitro study, rat testicular interstitial cells were isolated and incubated with digitoxin, hCG, 8-bromo-cAMP (8-Br-cAMP), digitoxin plus hCG, or digitoxin plus 8-Br-cAMP at 34°C for 1 h. The media were collected and analyzed for testosterone. For studying cAMP accumulation, testicular interstitial cells were incubated for 1 h in the medium containing isobutyl-1-methylxanthine (IBMX) and different doses of digitoxin with the absence or presence of hCG. After incubation, cells were processed for determining cAMP content. Intravenous injection of digitoxin decreased hCG-stimulated, but not basal, plasma testosterone levels. Administration of digitoxin in vitro resulted in an inhibition of both basal and hCG- as well as 8-Br-cAMP-stimulated release of testosterone. In addition, digitoxin diminished hCG-stimulated cAMP accumulation in rat testicular interstitial cells. Furthermore, digitoxin inhibited the activity of cytochrome P450 side chain cleavage enzyme (P450scc) but failed to affect the activities of other steroidogenic enzymes. Taken together, these results suggest that the acute inhibitory effect of digitoxin on the testosterone production in testicular interstitial cells involves, at least partly, an inefficiency of post-cAMP events, and a decrease of P450scc activity. J. Cell. Biochem. 74:74–80, 1999. © 1999 Wiley-Liss, Inc.

Key words: testosterone; digitoxin; rat interstitial cells

Digitoxin is one of the principal cardiac glycosides derived from the leaves of *Digitalis lanata* and *Digitalis purpurea* [Smith et al., 1984]. Digitalis such as digitoxin and digoxin are commonly used in the treatment of congestive heart failure and cardiac arrhythmias [Smith, 1988]. Digitalis drugs are known to exert the inotropic effects through inhibition of Na^+-K^+-ATPase, that maintains the normal high intracellular K^+ concentrations and low intracellular Na^+ concentrations in cardiac cells and other types of cells [Smith, 1973, 1988; Smith et al., 1984]. As compared to digoxin, digitoxin is highly lipid soluble and is completely absorbed from the gastrointestinal tract and possesses a longer half-life [Clark et al., 1992]. Overdose of either one of these compounds could produce the well-known phenomena that characterize digitalis toxicity by greater degrees of sodium-pump inhibition [Smith, 1988]. These symptoms produced by digitalis intoxication include cardiac symptoms-anorexia, nausea, and vomiting, and noncardiac symptoms-gastrointestinal complaint [Smith, 1973, 1988, Smith et al., 1984].

Several lines of evidence indicate that digitalis may play reproductive function. First, digitalis has been shown to have estrogen effect on male patients. Increased plasma estrogen [Tappler and Katz, 1979; Neri et al., 1987] and decreased androgen [Neri et al., 1987] has been observed in long-term (e.g., 2 years) digoxin-treated patients. Also, significant decreases in sexual desire, sexual excitement phase, and

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frequency of sexual relations were reported in male patients who received digoxin on a long-term basis [Neri et al., 1987]. In contrast, patients on short-term (35 to 210 days) digoxin therapy exhibit normal plasma estrogen or androgen levels [Kley et al., 1982, 1984].

Second, we demonstrated the inhibitory effects of bufalin, a kind of digitalis derived from the venom of Chinese *Bufo bufo gargarizans*, on the testicular function in male rats. This inhibitory effect of bufalin is associated in part with a decrease of testicular cAMP accumulation and luteinizing hormone (LH) response to gonadotropin-releasing hormone (GnRH) [Wang et al., 1997]. Third, we recently showed that digoxin inhibited testosterone production in rat testicular interstitial cells through attenuation of the activities of adenylyl cyclase and cytochrome P450scc [Lin et al., 1998]. In addition, digitoxin was shown to interfere the binding of dihydrotestosterone on rat ventral prostate [Pita et al., 1975] and estrogen on human uteri [Rifka et al., 1977]. Up to date, no report documents the effect of digitoxin on the testosterone production in male subjects. Therefore, the purpose of the present study was to investigate the effects of digitoxin on the basal and hCG-stimulated secretion of testosterone and cAMP level both in vivo and in vitro. Moreover, the inhibitory mechanism of digitoxin on the activities of the steroidogenic enzymes was also studied.

MATERIALS AND METHODS

Animals

Male rats of the Sprague-Dawley strain weighing 300–350 g were housed in a temperature controlled room (22 ± 1°C) with 14 h of artificial illumination daily (0600–2000 h) and given food and water ad libitum.

In Vivo Experiment

Male rats were anesthetized with ether and then catheterized *via* the right jugular vein [Wang et al., 1989, 1994; Tsai et al., 1996a]. Twenty hours later, the conscious rats were injected intravenously with vehicle (saline, 1 ml/kg), hCG (5 IU/ml/kg), digitoxin (1 μg/ml/kg), or hCG plus digitoxin, via the jugular catheter. Blood samples (0.5 ml each) were collected at 0, 30, and 60 min after the challenge. Plasma was separated by centrifugation at 10,000*g* for 1 min. The concentration of testosterone in each plasma sample was measured by radioimmunoassay (RIA) after ether extraction.

Preparation of Testicular Interstitial Cells

The method of collagenase dispersion of testicular interstitial cells followed the procedure described by Tsai et al. [1997]. Generally, each group contained cells from eight dispersions (n = 8). For each dispersion, five decapsulated testes from different rats were added to a 50 ml polypropylene tube containing 5 ml preincubation medium and 700 μg collagenase (Type IA, Sigma, St. Louis, MO). Preincubation medium were made up of 1% bovine serum albumin (BSA, Fraction V, Sigma) in Hank's balanced salt solution (HBSS), with N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES, 25 mM), sodium bicarbonate 0.35 g/l, penicillin-G 100 IU/ml, streptomycin sulfate 50 μg/ml, heparine 2550 USP K units/l, pH 7.3, and aerated with 95% O_2 and 5% CO_2. The tube was laid horizontally in a 34°C water bath, parallel to the direction of the shaking. Fifteen min after shaking at 100 cycles/min, the digestion was stopped by adding 35 ml of cold preincubation medium and inverting the tube several times. The tubes were allowed to stand for 5 min and the digest was then filtered through a four-layer fine nylon mesh. Cells were collected by centrifugation at 4°C, 100*g* for 10 min. The cell pellets were washed with deionized water to disrupt red blood cells (RBCs) and the osmolarity was recovered immediately with 10-fold HBSS. Hypotonic shock was repeated twice for disrupting RBCs and cell pellets were resuspended in preincubation medium (substitution of HBSS in preincubation medium with medium 199, and sodium bicarbonate 2.2 g/l). Cell concentration (1×10^6 cells/ml), viability (over 97%), and the number of sperm cells (less than 5%) were determined using a haemocytometer and the trypan blue method. The total cell proteins were determined by the method of Lowry et al. [1951]. To measure the abundance of Leydig cells in our preparation, the 3β-hydroxysteroid dehydrogenase (3β-HSD) staining method was used [Dirami et al., 1991; Krummen et al., 1994]. The cells (1×10^6 cells/ml) were incubated with a solution containing 0.2 mg/ml nitro blue tetrozolium (Sigma), 0.12 mg/ml 5-androstane-3β-ol-one (Sigma), and 1 mg/ml NAD^+ (Sigma) in 0.05 M PBS, pH 7.4 at 34°C for 90 min. Upon development of the flue formazan deposit sites of 3β-HSD activity, the abundance of Leydig cells was determined by use of a haemocytometer. Our preparation was

found to contain approximately 18 ± 2% Leydig cells.

Effects of Digitoxin on Testosterone and cAMP Production

Aliquots (1 ml) of cell suspensions (1×10^6 cells/ml) were preincubated with incubation medium in polyethylene tubes for 1 h at 34°C under a controlled atmosphere (95% CO_2 and 5% O_2), shaken at 100 cycles/min. The supernatant fluid was decanted after centrifugation of the tubes at 100*g* for 10 min. For studying the accumulation of cAMP in response to digitoxin, aliquots (1 ml) of cell suspensions (1×10^6 cells/ml) were primed for 30 min with 1 mM 3-isobutyo-1-methylxanthine (IBMX, a phosphodiesterase inhibitor, Sigma). Digitoxin (10^{-7} to 10^{-4} M), ouabain (10^{-7} to 10^{-4} M), hCG (0.05 IU/ml), hCG plus digitoxin, or hCG plus ouabain in 200 µl fresh medium in the presence or absence of IBMX was then added to the tubes. After 1 h of incubation, 2 ml ice-cold PBSG buffer (0.1 % gelatin in 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.5) was added to stop the incubation. The spent medium was centrifuged at 100*g* and stored at −20°C until analyzed for testosterone by RIA. In the presence of IBMX, the cell pellets were mixed with 1 ml of 65% ice-cold ethanol, homogenized by polytron (PT3000, Kinematica Ag., Luzern, Switzerland), and centrifuged at 1,500*g* for 15 min. The supernatant fluid was lyophilized in a vacuum concentrator (Speed Vac, Savant, Holbrook, NY) and stored at −20°C until analyzed for cAMP by RIA.

Effects of Digitoxin on cAMP-Related Testosterone Secretion

Cell suspensions were preincubated for 1 h and then incubated for 1 h with digitoxin in the presence of 8-Br-cAMP (a membrane-permeable analog of cAMP, 10^{-4} M, Sigma). At the end of the incubation, 2 ml ice-cold PBSG buffer were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

Effects of Digitoxin on the Biosynthesis Pathway of Testosterone

Cell suspensions were preincubated for 1 h and then were incubated for 1 h with or without digitoxin at 10^{-4} M in the presence or absence of five steroidal precursors. These precursors included 25-hydroxy-cholesterol (a membrane-permeable cholesterol, 25-OH-C), pregnenolone (Δ_5P), progesterone (P), 17α-hydroxy-progesterone (17α-OH-P), and androstenedione (Δ_4). At the end of the incubation, 2 ml ice-cold PBSG buffer were added and immediately followed by centrifugation at 100*g* for 10 min at 4°C. The supernatant fluid was stored at −20°C until analyzed for testosterone by RIA.

RIA of Testosterone and cAMP

The concentrations of testosterone in extracted samples (recovery 60–65%) were determined by RIA as described previously [Wang et al., 1994; Tsai et al., 1996a]. With anti-testosterone serum No. W8, the sensitivity of testosterone RIA was 2 pg per assay tube. The intra- and interassay coefficients of variation (CV) were 4.1% (n = 6) and 4.7% (n = 10), respectively.

The concentrations of cAMP were determined by RIA as described elsewhere [Tsai et al., 1996b; Lu et al., 1996; Chen et al., 1997]. With anti-cAMP serum No. CV-27 pool, the sensitivity of cAMP was 2 fmol per assay tube. The intra- and interassay coefficients of variation were 6.9% (n = 5) and 11.9% (n = 5), respectively.

Statistical Analysis

All values are expressed as the mean ± SEM. In some cases, the means of treatment were tested for homogeneity by a two-way analysis of variance, and differences between specific means was tested for significance by Duncan's multiple-range test [Steel and Torrie, 1960]. In other cases, Student's *t*-test was employed. A difference between two means was considered statistically significant when $P < 0.05$.

RESULTS

Effects of Digitoxin on the Plasma Testosterone Level

A single intravenous injection of hCG increased the level of plasma testosterone gradually from 30 to 60 min following the injection ($P < 0.01$, Fig. 1). Injection of digitoxin (1 µg/kg) did not alter the basal level of plasma testosterone, but suppressed the stimulatory effect of hCG on testosterone secretion.

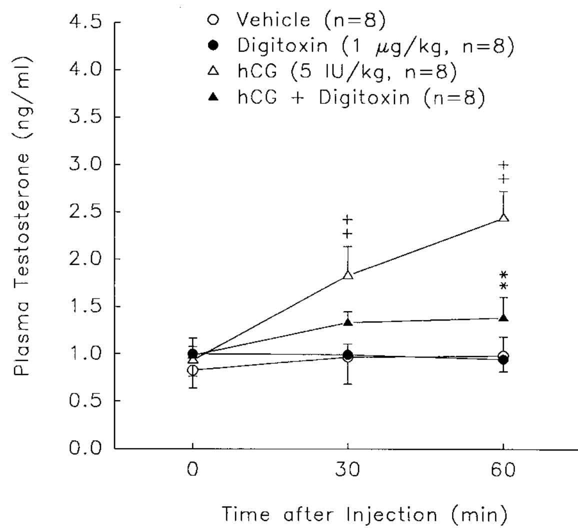

Fig. 1. Effects of digitoxin on the basal and hCG-stimulated concentrations of plasma testosterone in male rats. Rats were given a single intravenous injection of vehicle, digitoxin (1 μg/ml/kg), hCG (5 IU/ml/kg), or hCG plus digitoxin via right jugular vein. Blood samples were collected via the jugular catheter at time indicated after injection. Each value represents mean ± SEM, $^{**}P < 0.01$ compared with hCG injected animals at the same time point; $^{++}P < 0.01$ compared with the value at 0 min.

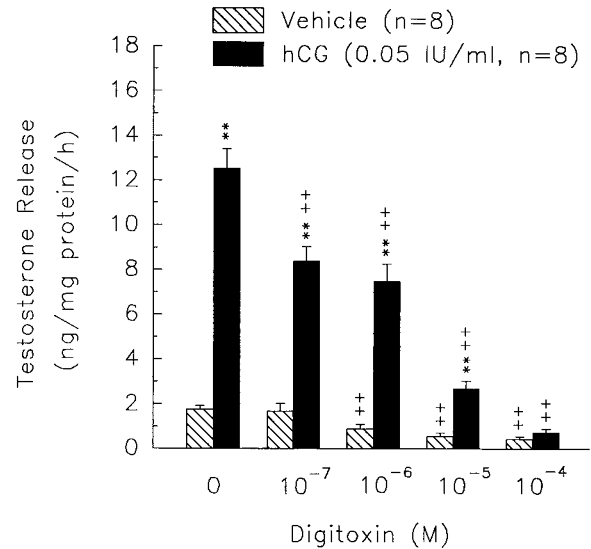

Fig. 2. Effects of digitoxin (10^{-7}–10^{-4} M) on testosterone release in vitro from rat testicular interstitial cells pretreated with vehicle or hCG (0.05 IU/ml). Each column represents mean ± SEM. $^{**}P < 0.01$ compared with vehicle group. $^{++}P < 0.01$ compared with digitoxin at 0 M.

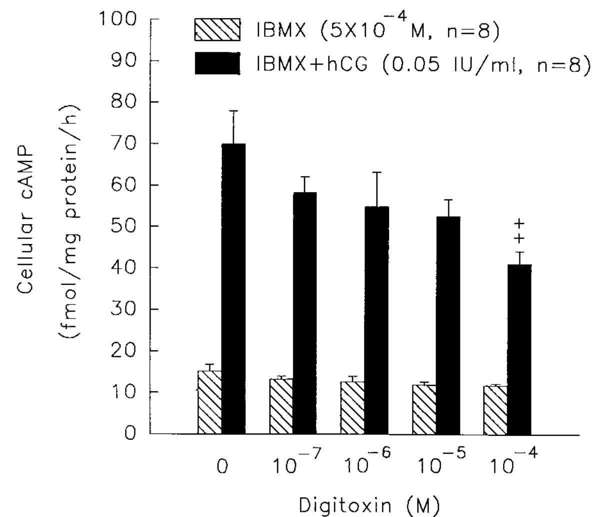

Fig. 3. Effects of digitoxin (10^{-7}–10^{-4} M) on the accumulation of cAMP in rat testicular interstitial cells pretreated with IBMX (5×10^{-4} M), or IBMX + hCG (0.05 IU/ml). Each column represents mean ± SEM. $^{++}P < 0.01$ compared with digitoxin at 0 M.

Effects of Digitoxin on Testosterone and cAMP Production In Vitro

Incubation of rat testicular interstitial cells with hCG (0.05 IU/ml) for 1 h caused a significant increase in testosterone production in vitro ($P < 0.01$, Fig. 2). Administration of digitoxin (10^{-6}–10^{-4} M) significantly decreased both basal and hCG-stimulated release of testosterone in testicular interstitial cells in a dose-dependent manner.

In the presence of IBMX (a phosphodiesterase inhibitor), administration of hCG produced a significant increase in cAMP accumulation in testicular interstitial cells ($P < 0.01$, Fig. 3). Administration of digitoxin did not alter the basal level of cAMP, while a high dose of digitoxin (10^{-4} M) significantly decreased the hCG-induced rise of cAMP accumulation.

The mean basal levels of medium testosterone in response to different doses of ouabain were 1.80 ± 0.48 ng/mg protein/h (at ouabain = 0 M, i.e., control, n = 8), 1.99 ± 0.55 ng/mg protein/h (at ouabain = 10^{-7} M, n = 8), 1.79 ± 0.22 ng/mg protein/h (at ouabain = 10^{-6} M, n = 8), 1.57 ± 0.23 ng/mg protein/h (at ouabain = 10^{-5} M, n = 8), and 1.79 ± 0.17 ng/mg protein/h (at ouabain = 10^{-4} M, n = 8). The hCG-stimulated levels of testosterone release in response to different doses of ouabain were 13.37 ± 0.55 ng/mg protein/h (at ouabain = 0 M, i.e., control, n = 8), 13.93 ± 0.67 ng/mg protein/h (at ouabain = 10^{-7} M, n = 8), 15.98 ± 1.06 ng/mg protein/h (at ouabain = 10^{-6} M, n = 8), 14.37 ± 0.79 ng/mg protein/h (at ouabain = 10^{-5} M, n = 8), and 13.09 ± 0.90 ng/mg protein/h (at oua-

bain = 10^{-4} M, n = 8). Neither basal nor hCG-stimulated production of testosterone in rat interstitial cells was altered by the administration of ouabain.

Effects of Digitoxin on cAMP-Induced Testosterone Production in Vitro

Administration of 8-Br-cAMP (a membrane-permeable analog of cAMP, 10^{-4} M) resulted in a significant increase of testosterone production by rat testicular interstitial cells ($P < 0.01$, Fig. 4). Digitoxin (10^{-4} M) markedly decreased the testosterone release in response to 8-Br-cAMP by 70%.

Effects of Digitoxin on the Biosynthesis Pathway of Testosterone in Vitro

At the doses of 10^{-7} M and 10^{-5} M, each of the five testosterone precursors tested increased the production of testosterone by rat testicular interstitial cells (Fig. 5). Digitoxin at 10^{-4} M decreased ($P < 0.01$) the production of testosterone stimulated by 25-OH-C in testicular interstitial cells. However, digitoxin did not affect the production of testosterone induced by any of the other four precursors tested.

DISCUSSION

This study examines the effect of digitoxin on the testicular function both in vivo and in vitro. Our results show that digitoxin inhibits hCG-stimulated plasma concentration of testosterone in vivo and testosterone release from cultured testicular interstitial cells in vitro through an inhibition on the post-cAMP events. Moreover, the activity of P450scc, one of the key enzymes for steroidogenesis in the mitochondria, is also inhibited by digitoxin.

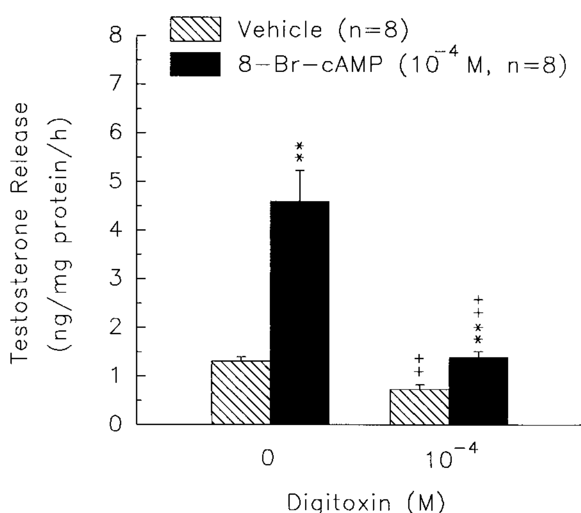

Fig. 4. Inhibitory effects of digitoxin (10^{-4} M) on the testosterone release in vitro from rat testicular interstitial cells pretreated with vehicle or 8-Br-cAMP (10^{-4} M). Each column represents mean ± SEM. $^{**}P < 0.01$ compared with vehicle group. $^{++}P < 0.01$ compared with digitoxin at 0 M.

The phenomena that characterize digitalis toxicity by greater degrees of sodium-pump inhibition was observed in the patients with overdose treatment [Smith et al., 1984]. Digitoxin is predominantly metabolized by the liver, which undergoes enterohepatic circulation. Because of its slow degradation, toxic effects of digitoxin persist for a long time after discontinuation of treatment as compared to digoxin [Clark et al., 1992; Smith et al., 1984; Smith, 1973]. Our results indicated that digitoxin have the inhibitory effect on the testicular function both in vivo and in vitro. This effect may partly result from the increased secretion of atrial natriuretic peptide (ANP) from atrial myocytes, and ANP can exert a potent direct inhibitory effect on testicular function [Mukhopadhyay et al., 1986; Foresta et al., 1993]. In addition, this study showed that digitoxin can act directly on the testicular interstitial cells to inhibit the secretion of testosterone (Fig. 2). Since the release of testosterone increased dose-dependently in response to the administration of pregnenolone, progesterone, 17α-hydroxy-progesterone, and androstenedione, despite the presence or absence of digitoxin (Fig. 5), the reduction of testosterone production by digitoxin should not be due to cellular toxicity or death.

Digitalis has a positive inotropic effect on the heart. These cardiac glycosides bind to and partially inhibit Na^+, K^+-ATPase. A chain event occurs that ultimately makes more calcium available to contractile elements, thus enhancing the force of myocardial contraction. Most studies showed that the positive inotropic effects of digitalis glycosides result from altered mechanism of excitation-contraction coupling. The calcium ion has been shown to be the major trigger that allows coupling of the electrical depolarization of heart-muscle cells to the mechanical contractile event [Smith, 1973, 1988; Smith et al., 1984]. Our study showed that, in the presence of 8-Br-cAMP, digitoxin inhibited testosterone production by 70% in testicular interstitial cells. Also, digitoxin inhibited hCG-stimulated cAMP accumulation in the presence of IBMX. These findings suggest that the inhibi-

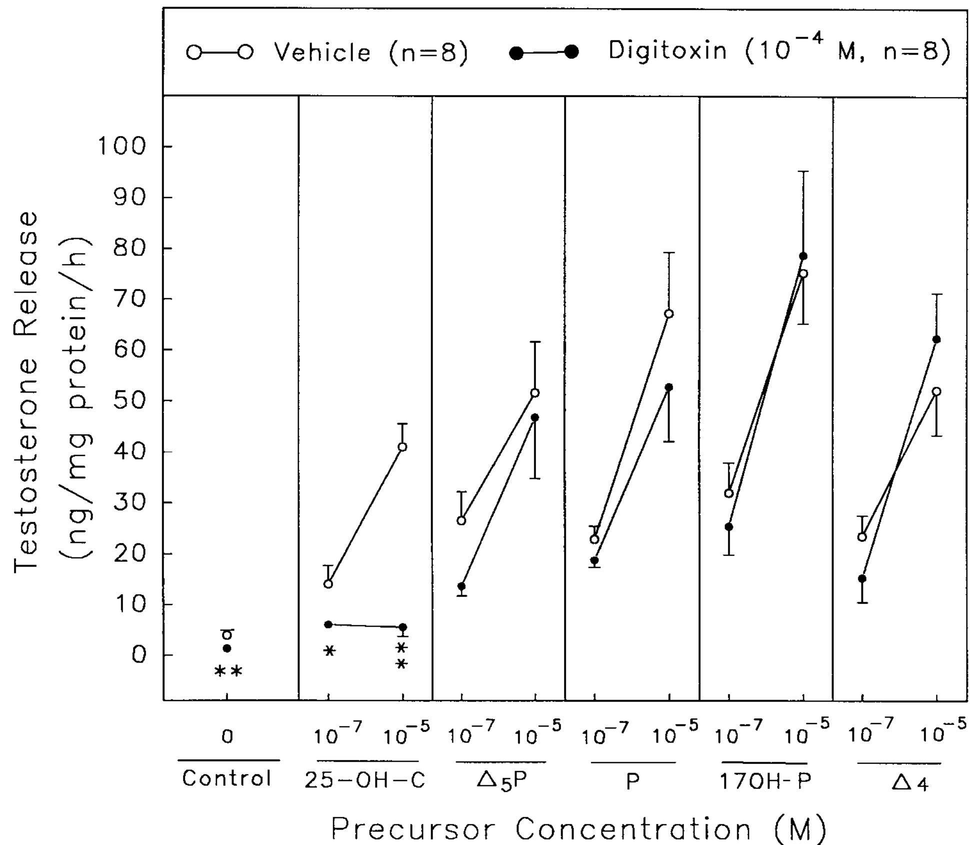

Fig. 5. Effects of digitoxin (10^{-4} M) on the testosterone release in vitro in rat testicular interstitial cells pretreated with vehicle or precursors of steroidogenesis. The precursors included 25-hydroxy-cholesterol (25-OH-C), pregnenolone (Δ_5P), progesterone (P), 17α-hydroxy-progesterone (17α-OH-P), and androstenedione (Δ_4). Each column represents mean ± SEM. $^*P < 0.05$ and $^{**}P < 0.01$ compared with vehicle group.

tory effect of digitoxin on testicular function involves at least partly through cAMP production as well as post-cAMP events. Interestingly, however, ouabain, another Na^+-K^+-ATPase inhibitor used in treatment of heart failure, failed to inhibit the testosterone production. Taken together, the findings that inhibition of testicular cAMP production by digitoxin and no effect of ouabain on testosterone production, suggested that digitoxin may exert its inhibitory effect unrelated to the ion transporter.

The biosynthesis of steroid hormones in the testes, in response to the steroidogenic stimuli including hCG, starts with the formation of pregnenolone from cholesterol in the inner mitochondria. This reaction is catalyzed by the cytochrome P450scc. Subsequent steroid hormones, including progesterone, testosterone, and estrogen are synthesized in the endoplasmic reticulum [Stocco and Clark, 1996]. A recent study indicated that the steroidogenic acute regulatory (StAR) protein is responsible for the acutely regulated transfer of cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane [Stocco and Clark, 1997]. The present study showed that digitoxin exerts its acute inhibitory effect on the P450scc activity which is the rate-limiting enzyme for the testosterone biosynthesis. In contrast, the activities of other steroidogenic enzymes for the subsequent steroid hormones were not affected by digitoxin. Whether digitoxin could exert its effect on the StAR protein remains to be elucidated.

In summary, these findings suggest that digitoxin inhibited the spontaneous secretion of testosterone from rat testes via a mechanism associated with an inefficiency of post-cAMP events and a decrease of P450scc activity. Physicians who use digitoxin for treatment of congestive heart failure and cardiac arrhythmias should be aware of its gonadotoxic effects. The appearance of hypogonadism is a designation for searching an alternative therapy.

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