Effects of arecoline on testosterone release in rats

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Wang S-W, Hwang G-S, Chen T-J, Wang PS. Effects of arecoline on testosterone release in rats. Am J Physiol Endocrinol Metab 295:E497–E504, 2008. First published June 17, 2008; doi:10.1152/ajpendo.00045.2008.—Arecoline is one of the major components of betel nuts, which have been consumed as chewing gum in Southeast Asia. In this study, the effects of arecoline on testosterone (T) secretion were explored. Male rats were injected with human chorionic gonadotropin (hCG, 5 IU/kg) or arecoline (1 µg/kg) plus hCG via a jugular catheter. Blood samples were collected at several time intervals subsequent to the challenge. Rat anterior pituitary was treated with gonadotropin-releasing hormone in vitro with or without arecoline, and then the concentrations of luteinizing hormone (LH) in the medium were measured. Rat Leydig cells were purified by Percoll density gradient centrifugation and incubated with arecoline, hCG, forskolin, 8-bromo-cAMP (8-Br-cAMP), nifedipine, nimodipine, or tetrandrine at 34°C for 1 h. A single intravenous injection of arecoline resulted in an increase of the hCG-induced level of plasma T. Administration of arecoline (10−8 to 10−6 M) in vitro increased T production in Leydig cells. The stimulatory effect of arecoline on T release in vitro was enhanced by hCG (0.001 IU/ml), forskolin (10−6 M), or 8-Br-cAMP (10−5 M). By contrast, nifedipine, nimodipine, or tetrandrine inhibited the increased T concentrations induced by arecoline. Western blot showed that arecoline increases steroidogenic acute regulatory (StAR) protein expression compared with vehicle. These results suggested that arecoline stimulates testosterone production by acting directly on Leydig cells via mechanisms involving an activation of L-type calcium channels, increasing the activity of 17β-hydroxysteroid dehydrogenase and enhancing the expression of StAR.

Arecoline; Leydig cell; L-type calcium channel; testosterone

Arecoline (1,2,5,6-tetrahydrol-1-methyl-3-pyridinecarboxylic acid methyl ester) (Fig. 1) is an alkaloid extracted from betel nuts (Areca catechu L.). Four major alkaloids are found in betel nut: arecoline (7.5 mg/g wt), arecaidine (1.5 mg/g wt), guvacoline (2.0 mg/g wt), and guvacine (2.9 mg/g wt) (56). Epidemiological studies showed a strong correlation between oral cancer and betel nut-chewing habit (16, 22, 50). Arecoline and/or its alkaloids have been shown to be characterized by carcinogenicity (16, 34, 45), immunotoxicity (42), genotoxicity (30, 46), and teratogenicity (46) in animal model systems. In addition, arecoline has been shown to be mutagenic in mammalian cells, especially in oral mucosal fibroblasts (5, 44). The increased frequency of micronucleated cells, chromosomal aberrations, and sister chromatid exchanges in exfoliated cells of the buccal mucosa was observed in areca-nut consumers (9, 11). In addition, study showed that arecoline enhances the frequency of chromosomal aberrations and micronuclei in mouse bone marrow cells in vivo (12).

Arecoline mimics the actions of acetylcholine and exerts its effects at both muscarinic and nicotinic receptors (3). Studies showed that arecoline readily penetrates the blood-brain barrier (35) and exerts its excitatory action by binding to M2-muscarinic receptors on the cell membrane of neurons of the locus coeruleus (61). Calogero et al. (4) showed that the cholinergic agonist arecoline stimulates the hypothalamic-pituitary-adrenal (HPA) axis in the rat, and this effect is mediated mainly by the release of endogenous corticotropin-releasing hormone (CRH). Arecoline, injected intravenously to catheterized, freely moving male Sprague-Dawley rats stimulated plasma ACTH and corticosterone release in a dose-dependent fashion. Arecoline stimulates the HPA axis via secretion of CRH.

It has been confirmed by the dose-dependent ability of arecoline to cause hypothalamic CRH secretion in vitro and its failure to elicit ACTH and corticosterone secretion by dispersed anterior pituicytes and adrenocortical cells in culture (4). Arecoline at concentrations of 0.2–2 µM significantly elevated the number of micronucleated cells in a dose-dependent manner (24). In addition, significant prolongation of cell cycles was observed by treatment with arecoline (~2 µM) in Chinese hamster ovarian cells (24). Moreover, morphology abnormality and unscheduled DNA synthesis were observed in the arecoline-treated germ cells of the mouse (47). Intravenous arecoline administration (3 mg/person) was followed by the increases in plasma epinephrine and ACTH, which were maximal at 10 and 20 min, respectively (32). Although there is no evidence proving that arecoline affects the thyroid function, the speed of induction of rapid eye movement sleep induced by arecoline had a modestly significant negative correlation with free thyroxine (T4) index (20). Panda and Kar (29) showed that a low dose of betel leaf extract was found to increase the triiodothyronine (T3) concentration and T3-to-T4 ratio along with a decrease in T4 concentration. By contrast, increased T4 and decreased T3 and T3-to-T4 ratios were observed in higher-dose betel leaf extract. In Alzheimer’s patients, elevated plasma ACTH and cortisol were observed in acute high-dose but not in low-dose arecoline administration (2). These studies indicated that arecoline might affect the endocrine system. However, it is still unclear whether arecoline affects reproductive function.

To examine the possible physiological effects of arecoline, we studied the acute effects of arecoline on endocrine systems. The purpose of this study was to 1) determine the effect of arecoline on human chorionic gonadotropin (hCG)-stimulated

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testosterone production in rat Leydig cells, and to determine the mechanisms of this effect at the molecular level. In addition, we also investigated the direct effects of arecoline on purified Leydig cells in vitro. The data presented here demonstrate that arecoline stimulates both basal and hCG-evoked testosterone production in rat Leydig cells. The data also indicate that arecoline stimulates the protein expression of StAR protein.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats weighing 300–350 g were housed in a temperature-controlled room (22 ± 1°C) with 14 h of artificial illumination daily (0600–2000) and food and water ad libitum. The use of the animals was approved by the Institutional Animal Care and Use Committee of the National Yang-Ming University. All animals received humane care in compliance with the principles of laboratory animal care and the Guide for Care and Use of Laboratory Animals published by the National Science Council (Taiwan, Republic of China).

Effect of arecoline on plasma testosterone in rats. Male rats were divided into four groups with seven to eight rats in each group. Each animal was anesthetized with ether and catheterized via the right jugular vein (53, 57). After 20 h, they were infused with 1 ml of saline, arecoline (1 µg/kg; Sigma Chemical), hCG (5 IU/kg), or hCG + arecoline via a jugular catheter. Blood samples (0.3 ml each) were collected from the jugular catheter at 0, 30, 60, 120, 180, 240, 300, and 360 min after infusion.

Plasma was separated by centrifugation at 10,000 g for 1 min and stored at −20°C. To measure the concentrations of testosterone, 0.1 ml plasma was mixed with 0.5 ml diethyl ether, agitated for 20 min, centrifuged at 1,000 g for 5 min, and then quick-frozen in a mixture of acetone and dry ice. The organic phase was collected, dried, and reconstituted in a buffer solution containing 0.1% gelatin in PBS (pH 7.5). The concentrations of testosterone in the reconstituted extracts were measured by RIA.

Effect of arecoline on luteinizing hormone release in vitro. Male rats were decapitated, and the anterior pituitary (AP) glands were removed (6). The procedure used for preparing Leydig cells has been previously described (6). Testicular interstitial cells were isolated with the collagenase dispersion method as previously described (6). The procedure used for preparing Leydig cells has been previously described (6). Testicular interstitial cells were isolated with the collagenase dispersion method as previously described (6). The procedure used for preparing Leydig cells has been previously described (6).

Effects of arecoline on the activities of calcium channels in rat Leydig cells. Rat Leydig cells were incubated for 1 h with or without arecoline (10−7 M) in the presence of immediate precursors of testosterone, androstenedione (10−7 M) and 10−5 M). At the end of incubation, the media were collected for testosterone RIA.

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The membrane was incubated with or without arecoline (10^{-4} M). At the end of incubation, the media were collected for pregnenolone RIA.

**Hormone RIAs.** Testosterone concentrations in media were determined by RIA as described previously (53, 57). The sensitivity of the testosterone RIA was 2 pg/assay tube. The intra-assay and interassay coefficients of variation were 4.1% (n = 6) and 4.7% (n = 10), respectively.

The concentrations of pregnenolone were determined by RIA, as described previously (7). The sensitivity of the pregnenolone RIA was 16 pg/assay tube. The intra-assay and interassay coefficients of variation were 2.3% (n = 6) and 3.7% (n = 4), respectively.

The concentrations of LH were determined by RIA as described previously (7). The sensitivity of the LH RIA was 0.1 ng/assay tube. The intra-assay and interassay coefficients of variation were 2.0% (n = 6) and 4.5% (n = 8), respectively.

Western blot analysis. The Western blotting method has been described elsewhere (6, 25). Leydig cells (1 x 10^6 cells/ml) were incubated with or without arecoline (10^{-7} to 10^{-5} M) for 1 h. At the end of incubation, cells were washed two times with ice-cold saline and then homogenized, and the protein was extracted in 50 μl of homogenization buffer (1.5% sodium lauroylsacrosine, 2.5 mM Tris base, 1 mM EDTA, and 0.1% phenylmethylsulfonyl fluoride, pH 7.8). The cell extract was centrifuged at 12,000 g for 10 min at 4°C. The supernatant was separated, and protein concentration was determined by modifying the Bradford protein assay method (62). SDS-PAGE sample buffer (0.06 M Tris base, 2% SDS, 0.0005% bromphenol blue, 6% sucrose, and 50 μM dithiothreitol) was added to the samples, and, after boiling for 5 min, the samples were stored at −20°C until they were used.

Samples (20 μg) were electrophoresed on a 12% minigel by standard SDS-PAGE procedures, along with prestained molecular weight markers (Bio-Rad, Hercules, CA). Gels were electrophoresed at 75 V for 15 min and then 150 V for 30 min. The protein bands were transferred to polyvinylidene difluoride membranes (NEN Life Sciences Products, Boston, MA) with a semidry transfer cell (Bio-Rad) for 45 min in a blotting solution. The membrane was washed in 0.8% NaCl, 0.02 M Tris base, and 0.3% Tween 20 (pH 7.6; TBS-T buffer) for 5 min and blocked by a 120-min incubation in blocking buffer (TBS-T buffer containing 5% nonfat dry milk). The membrane was then incubated overnight with anti-STAR (1:1,000), anti-P450scc (1:2,000), and anti-β-actin (1:8,000). After three washes for 5 min each with TBS-T buffer, the membranes were incubated for 2 h with horseradish peroxidase-conjugated secondary antibody (1:6,000). Specific signals were detected by enhanced chemiluminescence (ECL Western blotting detection reagents; Amersham Life Science Limited).

Statistical analysis. All values were given as the means ± SE. The treatment means were used for homogeneity by one-way ANOVA, and the differences between specific means were tested for significance by Duncan’s multiple-range test or by paired Student’s t-test (48). A difference between two means was considered statistically significant when P < 0.05.

**RESULTS**

Effect of arecoline on the concentration of plasma testosterone. The basal level of plasma testosterone was 0.4–0.5 ng/ml (Fig. 2, top). A single intravenous injection of arecoline (1 μg/kg) gradually increased (P < 0.01) the concentration of plasma testosterone. A plateau of plasma testosterone concentration (0.9–1.0 ng/ml) was reached 5–6 h following injection of arecoline.

A single intravenous injection of hCG (5 IU/kg) stimulated the concentration of plasma testosterone by 3.7-fold at 60 min following challenge (Fig. 2, bottom). The concentration of plasma testosterone returned to the basal level 4 h following hCG challenge. After administration of arecoline plus hCG (1 h), the secretion of testosterone increased markedly by 7.2-fold compared with the basal level. The peak and postpeak levels of plasma testosterone were significantly higher (P < 0.01) in rats treated with arecoline plus hCG than in hCG-treated animals (Fig. 2, bottom).

Effect of arecoline on LH release in vitro. Incubation of rat AP with 10 nM GnRH increased (3.2-fold, P < 0.01) the concentration of plasma testosterone by 3.7-fold at 60 min following injection of hCG plus arecoline plus hCG than in hCG-treated animals (Fig. 2).

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**Fig. 1.** Chemical structure and molecular weight of arecoline.

**Fig. 2.** Effects of arecoline on the basal (top) and human chorionic gonadotropin (hCG)-stimulated (bottom) level of plasma testosterone in male rats. Rats were given a single intravenous injection of vehicle arecoline, hCG, or hCG + arecoline via a right jugular catheter and bled at different time intervals after injection. The concentration of testosterone was measured by RIA. **P < 0.01 compared with vehicle or hCG group. P < 0.05 (+) and 0.01 (++) compared with time 0 min. Each value represents the mean ± SE.
release of LH in vitro (Fig. 3). Neither basal nor GnRH-stimulated release of LH was significantly altered by the administration of arecoline ($10^{-7}$ to $10^{-6}$ M).

**Effect of arecoline on the basal and cAMP-evoked release of testosterone in vitro.** Administration of arecoline at the doses ranging from $10^{-8}$ M to $10^{-6}$ M dose dependently increased the basal release of testosterone by rat Leydig cells. (Fig. 4).

Incubation of rat Leydig cells with 8-Br-cAMP (a permeable analog of cAMP, $10^{-5}$ M), forskolin (an adenylyl cyclase activator, $10^{-6}$ M), or hCG (0.001 IU/ml) significantly increased ($P < 0.001$) the release of testosterone in vitro. Coincubation of arecoline ($10^{-7}$ M) increased not only the basal but also the 8-Br-cAMP, forskolin, and hCG-evoked production of testosterone ($P < 0.01$, Fig. 5).

**Effect of arecoline on the activity of 17β-HSD.** Incubation of rat Leydig cells with $10^{-7}$ and $10^{-5}$ M of androstenedione (the immediate precursor of testosterone) dose dependently increased the production of testosterone (Fig. 6). Administration of $10^{-7}$ M arecoline significantly enhanced the release of testosterone in response to androstenedione (Fig. 6).

**Effect of arecoline on testosterone release in response to calcium channel blockers.** Although the spontaneous release of testosterone was not significantly altered by the administration of tetrandrine (a blocker of L-type and T-type calcium channels, $10^{-5}$ M), nifedipine (an L-type calcium channel blocker, $10^{-5}$ M), or nimodipine (an L-type calcium channel blocker, $10^{-5}$ M), the arecoline-evoked production of testosterone was significantly reduced by all of these calcium channel blockers ($P < 0.01$, Fig. 7).

**Effect of arecoline on the activity of P450scc.** To examine the activity of P450scc (an enzyme converts cholesterol into pregnenolone), an inhibitor of 3β-HSD (an enzyme converts pregnenolone into progesterone), trilostane, was employed to incubate with rat Leydig cells. Incubation of trilostane ($10^{-6}$ M) with Leydig cells significantly increases the production of pregnenolone ($P < 0.01$, Fig. 8). Administration of $10^{-7}$ M arecoline increased both basal and trilostane-evoked levels of pregnenolone released by rat Leydig cells ($P < 0.01$; Fig. 8). In addition, increased pregnenolone release from Leydig cells treated with different concentrations of 25-OH-C with trilostane only or trilostane plus arecoline was observed ($P < 0.05$; Fig. 9).

**Protein expression of StAR and P450scc.** Bands at 54 kDa (P450scc) and 30 kDa (StAR) were detected in rat Leydig cells (Fig. 10A). The β-actin signal (45 kDa) was employed as an internal control. Six replications showed the similar pattern. The Leydig cells were challenged with $10^{-7}$ or $10^{-6}$ M arecoline for 1 h, and then the protein expression of StAR was increased by the addition of arecoline (Fig. 10B). No difference in the protein expression of P450scc was observed after the addition of arecoline (Fig. 10, A and B).

DISCUSSION

Betel is a masticatory substance, and betel chewing is a popular oral habit in Southern Taiwan. Numerous studies showed a significant correlation of betel chewing with the incidence of oral cancer or oral submucous fibrosis (16, 17, 31, 36, 43, 51, 58). Arecoline is one of the major ingredients in betel nuts. The other three are arecaidine, guvacoline, and isoguvacine (55, 56). Each chewer in Taiwan consumed on average 14–23 betel quid a day (47), and each betel quid contains 7.5 mg/g of arecoline (55). Although there is no report concerning the plasma arecoline concentration, in vitro study showed that the arecoline cytotoxicity to gingival keratinocytes was observed at concentrations of 0.8–1.2 mM (18). Therefore, chewing betel quids is thought to be a bad habit associated with malignant cancer, especially the oral cancer. Although a number of studies showed betel chewing is strongly related to oral cancer, betel has been used for the treatment of diarrhea, edema, throat inflammations, and tapeworm (51). In this study, we demonstrated that arecoline, with or without hCG, significantly stimulated testosterone secretion both in vivo and in vitro.
Our results reveal a dose-dependent effect of arecoline, one of the major components in betel nuts, on the changes of testosterone concentration in male rats. In addition, the effects of arecoline on the increased testosterone secretion from isolated Leydig cells were due to, at least in part, the increased steroidogenesis. This conclusion is supported by the following observations. 1) Significantly increased plasma testosterone in rats was observed 60 min after arecoline injection. 2) The hCG-induced plasma testosterone could be enhanced by arecoline at 60 min. 3) Arecoline did not alter the GnRH-induced LH secretion in vitro. 4) Calcium channel blocker blunted the stimulatory effect of arecoline on the secretion of testosterone in vitro. 5) An increase of intracellular StAR protein was observed after arecoline treatment.

In this study, arecoline did not alter the LH secretion from the bisected AP gland treated with GnRH. However, the secretion of testosterone from Leydig cells was stimulated significantly by arecoline alone. We note that there has been no study of plasma concentration of arecoline in patients with betel chewing. Also, no investigation has shown the plasma concentration of testosterone in men who chew betel nuts. Recent studies also indicated that arecoline, with or without LH, enhances either the testosterone production in mouse interstitial cells (60) or plasma testosterone concentration in rats (41). Therefore, the stimulatory effect of arecoline on testosterone secretion was LH independent. In addition, we are
the first, as we know, to demonstrate that arecoline stimulates testosterone secretion from isolated Leydig cells. However, it is still unknown at present whether the action of arecoline on Leydig cells is through muscarinic and nicotinic receptors expressed in other cells and tissues (8, 19, 59).

In Leydig cells, testosterone is synthesized by several metabolic steps, collectively known as steroidogenesis. We examined the effects of arecoline on the activities of 17β-HSD in Leydig cells by challenging the cells in vitro with the immediate precursor of testosterone, androstenedione (\(\Delta_4\), substrate of 17β-HSD). Because androstenedione can be converted to testosterone by 17β-HSD, and production of testosterone stimulated by androstenedione was further enhanced in the presence of arecoline (Fig. 6), we suggested that the activity of 17β-HSD was increased by the administration of arecoline.

It is well known that the conversion of cholesterol into pregnenolone is catalyzed by P450scc enzyme (13). In addition, trilostane is an inhibitor of the 3β-HSD enzyme system with no inherent steroidal activity, and it inhibits the production of progesterone and results in an accumulation of pregnenolone, which is a sensitive index for the activity of 3β-HSD (40, 54). In the presence of trilostane, administration of arecoline increases the production of pregnenolone, suggesting that the activity of P450scc was increased by arecoline (Figs. 8 and 9). In addition, the stimulatory effect of arecoline was observed at both lower (10\(^{-7}\) M) and higher (10\(^{-5}\) M) concentrations of androstenedione (Fig. 6) and other testosterone precursors (data not shown). Also, the accumulation of pregnenolone was observed in the media treated with arecoline. These results indicate that arecoline affects the activities of several enzymes, at least including P450scc and 17β-HSD, required for the steroidogenesis.

The biosynthesis of steroid hormones in the testes, in response to the steroidogenic stimuli including hCG, starts with the transfer of cholesterol from the outer to the inner mitochondrial membrane, which is facilitated by StAR (28, 49). Moreover, the P450scc enzyme is located in the inner mitochondrial membrane (13, 27). Subsequent steroid hormones, including progesterone, testosterone, and estrogen, are synthesized in the endoplasmic reticulum. Therefore, inhibition of StAR activity is associated with the transfer of cholesterol and the inhibition of the steroidogenic response. Our results showed that the expression of StAR protein was enhanced by

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**Fig. 8.** Effects of arecoline on the basal and trilostane-stimulated pregnenolone release by rat Leydig cells. The concentration of pregnenolone was measured by RIA. \(P < 0.01\) compared with 0 M arecoline (**) and compared with the vehicle group (+ +). Each value represents the mean ± SE. 

**Fig. 9.** Effects of arecoline (10\(^{-7}\) M) on 25-OH-cholesterol (25-OH-C)-stimulated pregnenolone release by Leydig cells in the presence of a 3β-hydroxysteroid dehydrogenase (3β-HSD, an enzyme for converting pregnenolone to progesterone) blocker, trilostane (10\(^{-5}\) M). \(*P < 0.05\) compared with the trilostane group. UD, undetectable.

**Fig. 10.** Effects of arecoline on cytochrome \(P-450_{\text{scc}}\) (\(P450_{\text{scc}}\); A) and steroidogenic acute regulatory (StAR) protein (B) expression in rat Leydig cells. Leydig cells were treated with arecoline for 1 h. The expressions of \(P450_{\text{scc}}\) and StAR protein were analyzed by Western blot. 

\(*P<0.05\) compared with the vehicle group.
arecoline, although this protein production might not be responsible for the immediate increase of testosterone release from Leydig cells.

It is well known that the stimulation of LH on the steroidogenesis in gonad cells (e.g., Leydig cells, granulosa cells, or theca cells) is through the binding of specific receptors, activation of adenylate cyclase, formation of cAMP, protein kinase A activation, phospholipid turnover, inositol trisphosphate formation, and the increase of intracellular calcium (1, 14, 26, 37, 39). The increase of cytoplasmic Casup+sup+, either released from endoplasmic reticulum or transported from calcium channel in the cell membrane, is involved in the enhancement of testosterone production in rat Leydig cells (14, 26, 39). However, few studies provided the information about specific calcium signals in primary Leydig cell cultures. Kumar et al. (23) showed that hCG induced a slow-onset and sustained monophasic intracellular Ca2+ concentration ([Ca2+]i) plateau that was dependent on extracellular Ca2+. By contrast, no change in [Ca2+]i was observed in Leydig cells after the exposure to either LH or hCG (52). In addition, a consistently transient, not sustained, rise in Ca2+ was observed in Leydig cells treated with thapsigargin and cyclopiazonic acid (CPA), two sarcoendoplasmic reticulum calcium adenosine triphosphatase inhibitors, in Ca2+-free medium (39). This rise in Ca2+ indicated that thapsigargin and CPA are able to empty the intracellular Ca2+ pool and to activate Ca2+ influx (39). This is consistent with the capacitative Ca2+ entry model or store-operated Ca2+ influx proposed in 1986 (33). However, the influx of external Ca2+ induced by the emptying of internal stores in Leydig cells could occur through Na+-dependent depolarization of the plasma membrane (38), not through voltage-activated Ca2+ channels (VACCs), since VACCs are not expressed in rat Leydig cells (52). Although the level in intracellular calcium was not detected in this study, our results showed that the increase of testosterone secretion from rat Leydig cells treated with 10−7 M arecoline was significantly reduced by administration of L-type calcium channel blockers, nifedipine and nimodipine, and a blocker of L-type and T-type calcium channel, tetrandrine. These data suggested that the activation of L-type calcium channel in rat Leydig cells is involved in the increase of testosterone production stimulated by arecoline.

Our data have demonstrated that arecoline stimulates testosterone production by acting directly on rat Leydig cells. Although further investigations are required to reveal more mechanisms of arecoline actions, the present findings indicate that moderate consumption of betel nuts may be beneficial only for male sexual ability, whereas excess intake has been proven harmful at least from the immunological function and carcinogenesis point of views. Taken together, the results suggest that arecoline stimulates testosterone production by rat Leydig cells via an enhancement of the expression of StAR as well as the functioning of cAMP and the activation of L-type calcium channels.

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