Inhibition of Bufalin on Pituitary and Testicular Function in Rats

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ABSTRACT

The effects of bufalin on the secretion of testosterone and luteinizing hormone (LH) and the accumulation of testicular adenosine 3′:5′-cyclic monophosphate (cAMP) were studied. Male rats were injected with bufalin, human chorionic gonadotropin (hCG), gonadotropin releasing hormone (GnRH), hCG plus bufalin or GnRH plus bufalin via a jugular catheter. Blood samples were collected at several intervals subsequent to the challenge. In the in vitro study, rat testis blocks were incubated with bufalin, hCG or both for 1 h. The anterior pituitary gland was incubated with bufalin, GnRH or both for 30 min. The media were analyzed for testosterone and LH. For studying cAMP accumulation, testicular blocks were incubated for 1 h with the medium containing isobutyl-1-methylxanthine. After incubation, tissues were extracted by ethanol before measuring cAMP concentration. A single intravenous injection of bufalin decreased the basal and hCG-stimulated levels of plasma testosterone. Administration of bufalin in vitro resulted in an inhibition of both basal and hCG-stimulated release of testosterone. Bufalin diminished cAMP accumulation in rat testes. However, the basal levels of plasma and medium LH were not altered by bufalin administration. Likewise, the LH response to GnRH was diminished by bufalin administration, both in vivo and in vitro. These results suggest that the inhibition of testosterone production by bufalin is partly caused by a decrease of testicular cAMP accumulation and LH response to GnRH in rats.

Bufalin is a cardiotonic steroid isolated from Chansu, a galenical preparation of the dried white venom of Chinese Bufo bufo gargarizans (Hong, et al., 1992; Panesar, 1994). It has been shown that bufalin blocks vasodilation, and increases vasoconstriction, vascular resistance and blood pressure via an inhibition of Na, K-ATPase (Bagrov et al., 1993; Eliades et al., 1989; Pamnani et al., 1991, 1994), despite the increase of sodium excretion (Yates and McDougall, 1993). This evidence indicates that bufalin is an endogenous digitalis-like factor. Meanwhile, bufalin has been demonstrated to be a more potent inhibitor of Na, K-ATPase than ouabain (Brownlee et al., 1990).

Digoxin is a cardiac glycoside purified from the plant Digitalis lanata, which has been used clinically in the treatment of congestive heart conditions for more than 200 years (Antman and Smith, 1985; Doherty et al., 1978; Heller, 1990; Rietbrock and Woodcock, 1985). Because of the similarity in the chemical structure between bufalin and digoxin, it is not surprising that bufalin has digoxin-like function (Panesar, 1994). It is well known that both bufalin and digoxin as well as ouabain are specific inhibitors of the sodium pump. (Tao et al., 1995). The digoxin-like immunoactivity has been observed in Chansu (Fushimi et al., 1990). The effects of bufalin can be blocked by an antidigoxin antibody (Bagrov et al., 1993).

Although treatment of digoxin in healthy men for 35 days did not alter plasma testosterone levels (Kley et al., 1982), administration of digoxin in male patients for 2 years decreased the plasma testosterone concentrations (Neri et al., 1987; Stoffer et al., 1973). The binding of dihydrotestosterone with its receptors is either hindered in the rat prostate (Pita et al., 1975) or unaffected in the human prostate (Rifka et al., 1977) by digitalis. Whether bufalin affects the production of testosterone in the gonads is yet unknown.

In this study, we examined the effects of bufalin on the basal and hCG-stimulated secretion of testosterone, and also on the basal and GnRH-stimulated secretion of LH in male rats. This study was supported by the grants from Chang Gung College of Medicine and Technology (CMRP 504), the Department of Health, the Executive Yuan (DOH85-CM-032) and National Science Council (NSC84–2331-B-182–101), Taiwan, R.O.C.

ABBREVIATIONS: cAMP, adenosine 3′:5′-cyclic monophosphate; hCG, human chorionic gonadotropin; LH, luteinizing hormone; RIA, radioimmunoassay; IBMX, isobutyl-1-methylxanthine; GnRH, gonadotropin releasing hormone; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid.
rats, both in vivo and in vitro. The effects of bufalin on the production of cAMP in rat testes were also evaluated to determine whether cAMP accumulation is involved in the regulation of testosterone secretion in rats by bufalin.

Methods

Animals

Male rats of the Sprague-Dawley strain weighing 300 to 350 g were housed in a temperature-controlled room (22 ± 1°C) with 14 h of artificial illumination daily (6:00 A.M. to 8:00 P.M.) and given food and water ad libitum.

In Vivo Experiments

Effects of bufalin on plasma testosterone. Male rats were catheterized in the right jugular vein (Hwang et al., 1990; Wang et al., 1994). Twenty hours later, they were injected with bufalin (1 \( \mu \)g/ml/kg, Sigma Chemical Co., St. Louis, MO), hCG (5 IU/ml/kg, Sigma) or hCG plus bufalin via the jugular catheter. Blood samples (0.5 ml each) were collected at 0, 30, 60, 120 and 180 min after the challenge. An equal volume of heparinized saline was injected immediately after each bleeding.

Plasma was separated by centrifugation at 10,000 \( \times g \) for 1 min. The concentration of testosterone in each plasma sample was measured by RIA according to Wang et al. (1994).

Effects of bufalin on plasma LH. Male rats were injected with bufalin (1 \( \mu \)g/ml/kg), GnRH (2 \( \mu \)g/ml/kg, Sigma) or GnRH plus bufalin via the jugular catheter. Blood samples were collected at 0, 15, 30, 60 and 120 min after the challenge. The concentration of LH in each plasma sample was measured by RIA according to Wang et al. (1994).

In Vitro Experiments

Male rats were decapitated. The testes were decapsulated and cut into eight equal pieces before preincubation for 90 min with Locke’s solution containing 10 mM glucose, 0.003% bacitracin and 0.05% HEPEs at 34°C (Tsai et al., 1996; Wang et al., 1994). Each piece was placed in a flask containing 2 ml medium. The medium was aerated with 95% O2 and 5% CO2. The testes blocks were then incubated with bufalin (0–10 \( \mu \)M) at 37°C for 30 min. At the end of incubation, the tissue was immediately after each bleeding.

Effects of a Single Intravenous Injection of Bufalin on Testosterone and LH Secretion

The post-hCG levels (1.69 ± 0.22 to 2.62 ± 0.25 ng/ml, \( n = 8 \)) of plasma testosterone were significantly (\( P < .01 \)) greater than the value (0.44 ± 0.09 ng/ml, \( n = 8 \)) at 0 min (fig. 1). Intravenous injection of bufalin did not alter the level of plasma testosterone until 120 min. From 120 to 180 min after bufalin injection, the mean concentration of plasma testosterone dropped by 84% (0.080 ± 0.004 ng/ml at 180 min, \( n = 8, \) \( \bar{v} = 0.49 ± 0.14 \) ng/ml at 0 min, \( n = 8, \) \( P < .05 \)).

The levels of plasma testosterone from 0.5 to 2 h after coinjection of bufalin and hCG were significantly (\( P < .01 \)) greater than the basal level. Although the plasma testosterone levels were increased, coinjection of bufalin and hCG caused significantly lower levels of plasma testosterone at 30 (0.81 ± 0.11 ng/ml), 60 (1.13 ± 0.14 ng/ml) and 120 min (1.65 ± 0.20 ng/ml), than of those induced by hCG alone (1.69 ± 0.22, 2.62 ± 0.25, 2.55 ± 0.17 ng/ml, \( n = 8 \), respectively).

The mean basal levels of rat plasma LH ranged from 6.63 to 8.19 ng/ml (fig. 2). A single intravenous injection of GnRH resulted in a significant (\( P < .01 \)) increase of plasma LH at 15

Results

The concentration of plasma LH was determined by RIA as described previously with anti-LH serum PW11–2 (Hwang et al., 1990; Wang et al., 1994). The rat LH-I-6 used for iodination and the rat LH-RP-3 which served as standard preparation were provided by the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Rockville, MD. The sensitivity was 0.1 ng for LH RIA. The intra- and interassay coefficients of variability were 3.8% (\( n = 4 \)) and 6.6% (\( n = 5 \)), respectively.

RIA of cAMP

The concentration of testicular cAMP was determined by RIA as described previously with anti-cAMP antiserum no. CV-27 pool provided by the NIDDK (Lu et al., 1996; Tsai, et al., 1996). The synthetic tyr-cAMP (Sigma) was used for radioiodination. The sensitivity was 2 fmol per tube for the cAMP RIA.

Materials

Chemicals used in the study included bufalin (Sigma), GnRH (Sigma) and hCG (Sigma). Chemicals were prepared as stock solutions solubilized in twice deionized H2O, and prepared daily. The doses of drugs are expressed as unit weight per body weight in vivo, e.g., IU/ml or \( \mu \)g/ml; concentrations of drugs for the in vitro experiment are expressed in their final molar concentrations in the flask.

Statistical Analysis

All values are given as the mean ± S.E.M. For analyzing the effect of bufalin at each indicated time, the treatment means were tested for homogeneity by the analysis of variance, and the difference between specific means was tested for significance by Duncan’s multiple-range test (Steel and Torrie, 1960). The differences of hormone levels between 0 min and the indicated time or 0 M and the indicated dose of bufalin were analyzed by a one-way analysis of variance. A difference between two means was considered statistically significant when \( P < .05 \).
and 30 min after the challenge. The basal level of plasma LH was not altered by the administration of bufalin. However, the plasma LH in response to GnRH was completely diminished by bufalin administration.

**Effects of Bufalin on Testosterone and cAMP Production in Vitro**

The administration of $10^{-7}$ or $10^{-5}$ M bufalin significantly decreased $(31.85 \pm 5.42, 24.65 \pm 5.04$ pg/mg testis/h, $n = 6$, vs. basal level $46.00 \pm 4.22$ pg/mg testis/h, $P < .05$ and $P < .01$, respectively) testosterone release (fig. 3). Combination of hCG with bufalin $(10^{-5} \text{M})$ resulted in a 78% inhibition of the hCG-stimulated release of testosterone (hCG plus bufalin: $59.02 \pm 7.98$ pg/mg testis/h vs. hCG-alone treated group: $271.06 \pm 27.78$ pg/mg testis/h, $n = 6$, $P < .01$).

In the presence of IBMX, administration of hCG significantly increased the accumulation of cAMP in rat testes by 3-fold (hCG-treated group $14.05 \pm 3.27$ fmol/mg protein/h, $n = 8$, vs. control group $3.54 \pm 0.56$ fmol/mg protein/h, $n = 8$, $P < .01$) (fig. 4). Bufalin doses ranging from $10^{-9}$ to $10^{-5}$ M decreased the content of cAMP in rat testis blocks (0.34 ± 0.06 to 0.37 ± 0.05 fmol/mg protein/h, $n = 8$, $P < .01$).

**Effects of Bufalin on LH Release in Vitro**

The basal release of LH in vitro was not altered by the administration of bufalin (fig. 5). GnRH increased LH release from rat anterior pituitary glands by 50% ($P < .05$). The LH release in response to GnRH in vitro was completely diminished by bufalin at $10^{-5}$ or $10^{-4}$ M.

**Discussion**

The present results indicate that the administration of bufalin in rats diminished the pituitary response to GnRH stimulation for LH release and the secretion of testosterone, both in vivo and in vitro, and the accumulation of testicular cAMP.

It has been reported that the level of plasma testosterone in healthy men is not altered by the administration of digoxin for 35 days (Kley et al., 1982). Whereas digoxin therapy for 2 years in male patients with cardiac function capacity in late
Bufalin Inhibits Testosterone Production

A single injection of bufalin diminished both the basal and hCG-stimulated levels of plasma testosterone in rats. The injection of bufalin completely abolished the stimulatory effect of GnRH on LH secretion, although the basal level of plasma LH was not altered by bufalin. These data reflect that the inhibitory effects of bufalin on testosterone secretion might be caused by the direct effect of bufalin on the testes, and the inhibition of bufalin on pituitary gonadotropin response to GnRH. This aspect was demonstrated by our in vitro data in which both the spontaneous and hCG-stimulated release of testosterone from the testicular tissue and the GnRH-stimulated release of LH from the anterior pituitary gland was diminished by bufalin at the dose of $10^{-5} \text{ M}$.

In the presence of IBMX, hCG stimulated testicular cAMP production 4.3-fold (fig. 4). Our finding of the inhibition of cAMP accumulation by bufalin in the testes after incubation with IBMX suggested that the production of testicular cAMP was associated with the antiandrogenic activity of bufalin. Another possibility that bufalin decreased the binding affinity and/or the number of gonadotropin receptors as well as the possibility of competitive binding of bufalin and gonadotropin receptors in Leydig cells should not be overlooked.

Our in vitro data indicate that bufalin is a potent inhibitor in the production of cAMP in rat testes. The lower dose of bufalin (e.g., $10^{-9} \text{ M}$) decreased the accumulation of testicular cAMP but not the release of testosterone. This may result from the effect of bufalin on the accumulation of testicular cAMP involving not only Leydig cells, but also Sertoli and other cells. Perhaps, the measurement of inhibin and/or androgen binding protein levels will be helpful in solving this problem eventually.

Although there is no direct evidence currently, the reduction of LH secretion both in vivo and in vitro in response to GnRH by bufalin revealed that the binding of GnRH with anterior pituitary glands might be altered by bufalin. Depression of the levels of the plasma testosterone and LH in male subjects has been correlated with a decrease in the cardiac index, and elevation was observed by long-term therapy of digoxin (Tappler and Katz, 1979). Whether the LH response to GnRH is altered is not known. It will be interesting to explore the role of bufalin in regulating the secretion of gonadotropin in both healthy men and cardiac patients in the future.

In summary, this study demonstrated that bufalin, an endogenous digoxin-like factor of amphibian origin, inhibited the spontaneous and gonadotropin-stimulated secretion of testosterone from rat testes via a mechanism associated with a decrease of cAMP production.

Acknowledgments

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References


Fushimi, R., Koh, T., Iyama, S., Yasuhara, M., Tachi, J., Kohda, K., Amino, N.

Fig. 4. Effects of bufalin (hatched columns) and hCG (solid column, 0.5 IU/ml) on the accumulation of testicular cAMP after in vitro incubation of rat testes with 0.5 mM IBMX, n = 8. * P < .01 compared with the control group. Each value represents mean ± S.E.M. Please note the log scale in y-axis.

Fig. 5. The in vitro release of LH from rat anterior pituitary glands at different doses of bufalin in the presence (●) or absence (○) of GnRH ($10^{-8} \text{ M}$), n = 8. * P < .05 compared with the control group. Each value represents mean ± S.E.M.


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