Okadaic Acid Remarkably Suppresses Testosterone Production in Murine Leydig Cells

Shin Asada, Motokazu Koga, Kenji Nagao, Masako Tabata, and Ken Takeda*

Department of Hygiene Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162–0826, Japan

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Okadaic acid, a tumor promoter derived from a black sponge, dose-dependently suppressed human chorionic gonadotropin (hCG)-stimulated testosterone production in primary cultured mouse Leydig cells. Okadaic acid at 10 nM caused a 70% reduction in testosterone production, and this inhibition was not due to cell damage. Okadaic acid did not affect basal testosterone production. Our results suggest that okadaic acid may inhibit the hCG-stimulated signaling pathway. We found that okadaic acid suppressed the expression of mRNA for two steroidogenic enzymes, cytochrome P450 cholesterol side-chain cleavage (P450scc) and 3β-hydroxysteroid dehydrogenase (3β-HSD), which catalyze cholesterol conversion to testosterone. It is interesting to analyze the mechanisms by which okadaic acid, which inhibits protein phosphatase 1 and 2A, suppressed steroidogenesis.

Key words ——okadaic acid, testosterone production, P450scc, tumor promoter, steroidogenic enzyme

INTRODUCTION

A number of chemicals with the capacity to disrupt endocrine and reproductive systems are now widespread in the environment.[1] These include many man-made chemicals[2] and phytoestrogens. There are many reports about assay methods and the evaluation of endocrine-disrupting chemicals that have focused on the agonistic and antagonistic activity of estrogen.[3,4] However, few studies have evaluated androgen production with respect to endocrine disrupting chemicals.

*To whom correspondence should be addressed: Department of Hygiene Chemistry, Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12 Ichigaya Funagawara-machi, Shinjuku-ku, Tokyo 162–0826, Japan. Tel.: +81-3-3260-4272 (Ext. 5076); Fax:+81-3-3260-5856; E-mail: takeda@ps.kagu.sut.ac.jp

Leydig cells, located in the interstitial tissue of the testis, are the site of testosterone biosynthesis and secretion. Testosterone biosynthesis in Leydig cells is primarily regulated by luteinizing hormone (LH).[5] Testosterone is required for normal development of the male reproductive tract.[6] Synthesis of testicular steroid hormones requires four enzymes: cholesterol side-chain cleavage (P450scc), 17α-hydroxylase/C17-20lyase (P450c17), 3β-hydroxysteroid dehydrogenase Δ^5-Δ^4isomarase (3β-HSD) and 17β-Hydroxysteroid dehydrogenase (17β-HSD).

Previous studies have shown that tumor necrosis factor (TNF), a cytokine secreted by activated macrophages, modulates Leydig cell steroidogenesis.[6] 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a tumor promoter, has been shown to influence gonadal steroidogenesis *in vitro*.[7] Okadaic acid, isolated from the black sponge *Halichondria okadai*, is a different type of tumor promoter.[8] TPA is known to activate protein kinase C (PKC),[9] whereas okadaic acid inhibits protein phosphatase 1 and 2A (PP-1 and PP-2A).[10]

In this study, we attempted to establish an assay system for testosterone production with primary cultured mouse Leydig cells, and to examine the effect of okadaic acid on testosterone synthesis in the Leydig cells of mice.

MATERIALS AND METHODS

Isolation and Culture of Leydig Cells —— Mice (Tokyo Laboratory Animals Co., Ltd., Tokyo, Japan) were killed by cervical dislocation, and the testes were removed aseptically. Testes were decapsulated and dispersed in 25 mM hepes buffer (Nakalai, Kyoto, Japan) containing 1.0 mg/ml collagenase (Wako Pure Chemical Industries, Osaka, Japan), 1.0 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, MO, U.S.A.), and 0.07% bovine serum albu-

min (BSA) (Nakalai), and were subjected to vigorous shaking for 20 min at 34°C. All other procedures were carried out under sterile conditions. Isolation of Leydig cells was performed by a method modified from Schumacher *et al.*[11]

Nine parts Percoll (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, U.K.) were mixed with 1 part 10-fold concentration Earle's balanced salt solution (EBSS) (GIBCO, New York, NY, U.S.A.) containing 0.7% BSA and 250 mM hepes buffer, pH 7.4, to give an osmolality of 300 mOsmol/kg. Three density gradients (12, 25 and 65%) were prepared by diluting the Percoll solution with isotonic EBSS containing 0.07% BSA. Crude cell suspensions were layered on top of the gradient and centrifuged for 20 min at 2050 rpm (room temperature). Two visible bands of testicular cells were obtained. Highly purified Leydig cells were found in the second band corresponding to a Percoll concentration of 38–52% (v/v). This band was isolated, added to 3 volumes of EBSS containing 0.25% BSA, and sedimented at 1150 rpm (5 min, room temperature). The sedimented cells were washed with EBSS with 0.25% BSA at 1150 rpm (5 min, room temperature).

Purified Leydig cells were plated in a collagen-coated dish (IWAKI, Chiba, Japan) and maintained in Dulbecco's Modified Eagle Medium/Ham's F-12 (DMEM/F12) (GIBCO) with 100 IU/ml Penicillin and 100 mg/ml streptomycin without serum at 34°C in a humidified atmosphere of 95% air–5% CO_2.

Leydig cell preparations were checked for purity by histochemical staining for 3β-HSD using a method described by Levy *et al*. and Cooke *et al*.[12,13] Purity of the Leydig cells was about 90%.

Leydig cells, primarily divided from murine testes, were cultured for 24 hr with okadaic acid at the indicated concentration and then with 1IU/ml human chorionic gonadotropin (hCG).

RNA Extraction —— Total cellular RNA was extracted from Leydig cells with Isogen (Nippon Gene) according to the manufacturer's directions. Treatment of DNase was performed with total RNA, 2 ml of 10 × DNase buffer, 1 ml RNasin® ribonuclease inhibitor (Promega Corporation, Madison, WI, U.S.A.) and 1 ml RQ1 RNase-free DNase (Promega Corporation) for 60 min at 37°C. Inactivation of these enzymes was carried out for 5 min at 95°C. The extracted RNA was reverse transcribed to synthesize cDNA using a First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, UK, Ltd.) according to the manufacturer's directions.

Quantitative Reverse Transcription (RT)-PCR —— Amplification reactions (50 μl) contained a DNA sample, 10 × PCR buffer II (5 μl) 200 μM dATP, dCTP, dGTP, and 400 μM dUTP, 4 μM $MgCl_2$, 1.25 units Ampli Taq DNA polymerase, 0.5 units AmpErase uracil *N*-glycosylase (UNG), 3 pmole of specific primer and 6.2–6.8 pmole of Taqman probe. Frosted MicroAmp Optical Tubes (PE Applied Biosystems, Norwalk, CN, U.S.A.) were used to prevent light refraction. The tube caps were similar to MicroAmp Caps, but they were specially designed to prevent light scattering.[14,15] All PCR consumables were supplied by PE Applied Biosystems, except the primers, which were synthesized at Genentech, Inc. Briefly, the probe Tm should be at least 5°C higher than the annealing temperature used during thermal cycling; primers should not form stable duplexes with the probe. The thermal cycling was initiated at 2 min at 50°C and 10 min at 95°C. Thermal cycling proceeded at 45 cycles of 95°C for 0.5 min and 60°C for 2 min. All reactions were performed in the Model 7700 Sequence detector (PE Applied Biosystems). Reaction conditions were programmed on a Power Macintosh (Apple Computer) linked directly to the sequence detector.

RESULTS

Effects of Okadaic Acid on Basal and hCG-Stimulated Testosterone Production

As shown in Fig. 1A, treatment with 0.3, 1, 3, and 10 nM okadaic acid caused a significant and dose–dependent inhibition of hCG-stimulated testosterone production in primary cultured mouse Leydig cells.

As shown in Fig. 1B, okadaic acid had no effect on basal testosterone production by Leydig cells not stimulated by hCG.

Effect of Okadaic Acid on Steroidogenic Enzyme mRNA Expression

The effects of okadaic acid on the mRNA expression of steroidogenic enzymes P450scc, P450c17, 3β-HSD, and 17β-HSD are shown in Fig. 2. hCG (1IU/ml) induced the expression of mRNA of P450scc (5-fold) and of 3β-HSD (13-fold). Okadaic acid at 10 nM suppressed hCG-induced P450scc mRNA expression to 30% that of control values, and hCG-induced 3β-HSD mRNA expression to approximately the control level. P450c17 and 17β-HSD mRNA, however, were not affected by

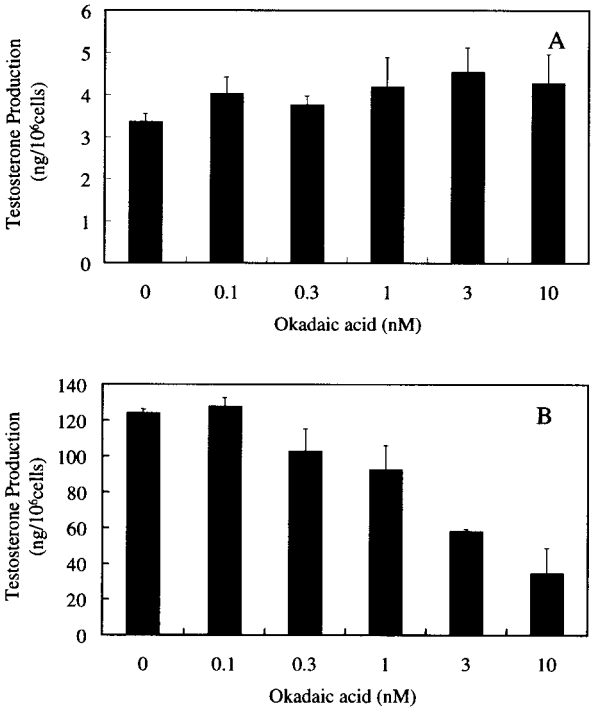

Fig.1. Effect of Okadaic Acid on Testosterone Production

Cells were cultured for 24 hr before treatment with 0.1, 0.3, 1, 3, or 10 nM of okadaic acid for 24 hr. Cells were treated with 1 IU/ml hCG for 6hr (B), or without (A). Testosterone production was measured by radioimmunoassay as described in *Materials and Methods*.

okadaic acid.

DISCUSSION

Okadaic acid inhibited hCG-stimulated testosterone production in murine Leydig cells. The inhibitory effect of okadaic acid might not be the result of damage to the cells, since viable cell numbers did not change during the experiments (data not shown). However, okadaic acid did not cause the suppression of basal testosterone production. The data suggest that okadaic acid might inhibit the hCG-stimulated signaling pathway.

It has been reported that okadaic acid activates macrophages and that activated macrophages secrete TNF.[16] Yuting *et al*. showed that TNF almost completely suppresses hCG-stimulated testosterone production at a concentration of 1 ng/ml.[6] We examined the possibility that TNF induced by okadaic acid might inhibit testosterone production. However, TNF production was not detected in the cultured medium by ELISA assay. In addition treatment with BB94,

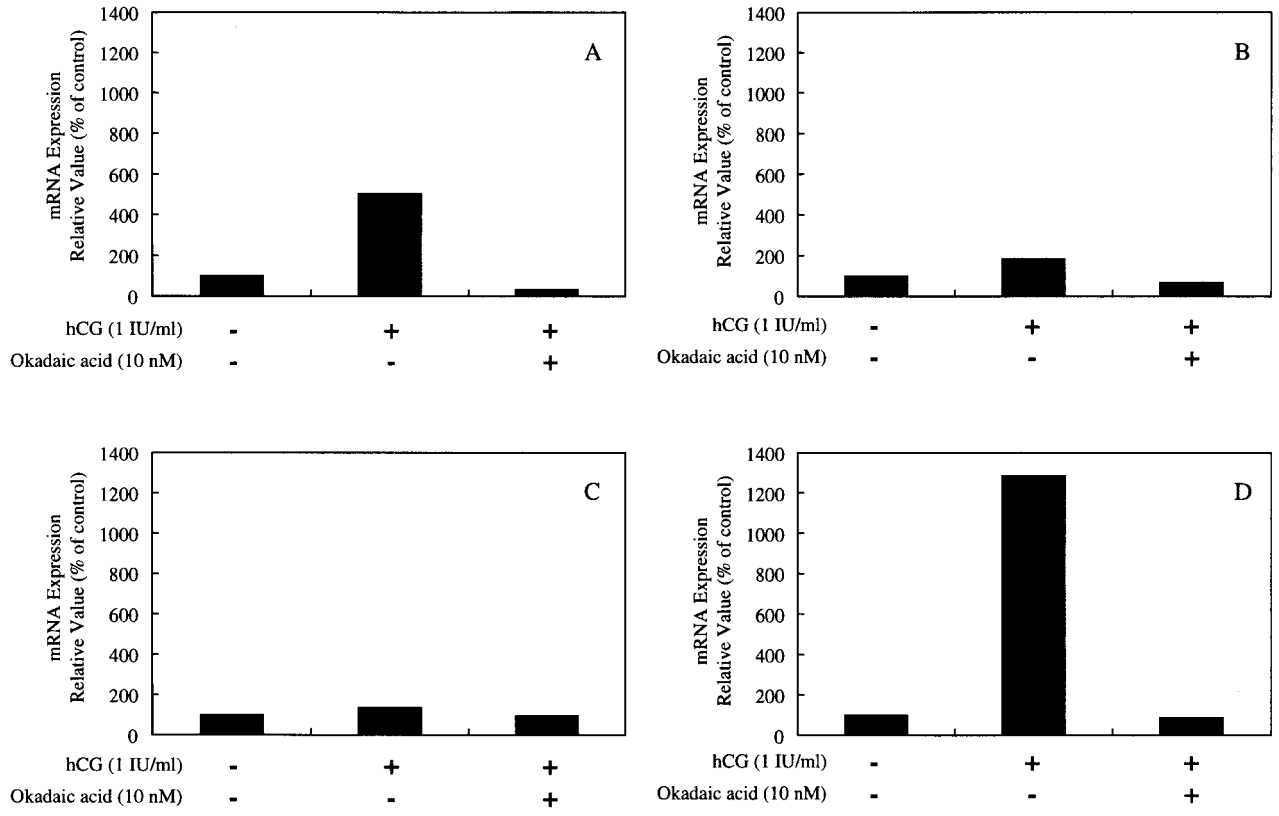

Fig. 2. Effects of Okadaic Acid on Steroidogenic Enzyme mRNA Expression

Cells were treated with 10 nM okadaic acid for 24 hr and for 6 hr with 1 IU/ml hCG. RNA from Leydig cells was subjected to quantitative RT-PCR. Cells were treated with or without hCG and okadaic acid, respectively. (A) P450scc, (B) P450c17, (C) 17β-HSD, and (D) 3β-HSD.

which almost completely inhibits the secretion of TNF,[17] did not alter the effects of okadaic acid on testosterone production (data not shown).

To analyze the mechanisms involved in the effects of okadaic acid upon steroid biosynthesis, the expression of steroidogenic enzymes P450scc, P450c17, 3β-HSD, and 17β-HSD was analyzed using a PRISM7700 sequence detector. In the murine steroidogenic pathway, conversion of cholesterol to pregnenolone catalyzed by P450scc is the first, rate-limiting and hormonally regulated step in the synthesis of all steroid hormones. 3β-HSD catalyzes the conversion of pregnenolone to progesterone. The steroidal enzyme P450c17 catalyzes the conversion of pregnenolone into the androstenedione, the direct precursors of estrogen and testosterone, respectively. The final step in the biosynthesis of testosterone is the reduction of androstendione to testosterone as catalyzed by the enzyme 17β-HSD.

The reduction of testosterone production in murine Leydig cells was accompanied by the reduced expression of P450scc and 3β-HSD mRNA, as suggested by our quantitative-RT-PCR data, consistent with the effect of okadaic acid. Quantitative-RT-PCR data reflect the steady state amounts of mRNA and do not distinguish between a decreased rate of transcription or changes in mRNA half-life. Although we report that okadaic acid inhibits testosterone production in mouse Leydig cells, it is still not clear at which level okadaic acid causes the inhibition of P450scc and 3β-HSD mRNA expression.

Phosphorylation of protein substrates by protein kinase A (PKA) is part of the mechanism by which hCG stimulates steroidogenesis,[18,19] and this process may be interfered with by other protein kinases or phosphatases. PP-1 and PP-2 are likely to be the enzymes that reverse the actions of PKC, and it is not surprising that a phosphatase inhibitor should act like PKC. Activation of PKC by TPA and TNF results in an increase of phosphorylated protein levels. The inhibition of PP-1 and PP-2A by okadaic acid results in inhibition of the dephosphorylation of phosphoprotein, which is previously phosphorylated by various protein kinases. Therefore, a PKC activator such as TNF or TPA and a PP inhibitor such as okadaic acid might cause a similar suppression of testosterone production.

Steroidogenic factor-1 (SF-1)/adrenal 4 binding protein (Ad4BP), a member of the nuclear hormone receptor superfamily, has been shown to regulate the expression of multiple genes involved in the steroidogenic pathway.[20] It has been reported that SF-1/Ad4BP can mediate the cAMP-dependent transcriptional activation of P450scc and 3β-HSD.[20] Therefore, we are interested in a nuclear hormone receptor family as a possible target of phosphatase regulated by okadaic acid.

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