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Purification and identification of 25-hydroxycholesterol in a reptile: Seasonal variation and hormonal regulation

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ABSTRACT

The present *in vitro* study, for the first time, demonstrates the production of 25-hydroxycholestrol (25-HC) by testicular macrophages of a non-mammalian vertebrate. The ether extracts of testicular macrophage-conditioned medium (TMCM) were fractionated on a C18 reversed phase high-performance liquid chromatography (HPLC) column using methanol as the mobile phase. The mass spectrometry (MS) fragmentation pattern of HPLC-purified 25-HC was found to be identical to that of authentic 25-HC. Further, a significant seasonal variation in 25-HC concentration was observed with maximal level in regressed and minimal during breeding phase. To understand the hormonal control of 25-HC production, testicular macrophages from regressed phase testes were incubated with 0.5 μg/ml of ovine follicle stimulating hormone (FSH) and 0.1, 1 and 10 μg/ml of testosterone (T). FSH considerably enhanced 25-HC production by testicular macrophages. In contrast, T markedly inhibited 25-HC production in a dose-dependent manner. In addition, T significantly inhibited FSH-induced 25-HC production, though pre-treatment with T was more effective as compared to post-treatment with T to FSH. Our findings on production, seasonal variation and hormonal control of 25-HC suggest the functional significance of 25-HC in the testis of reptiles.

1. Introduction

Testicular macrophages in mammals are shown to produce an oxysterol, 25-hydroxycholesterol (25-HC) by action of 25-hydroxylase (Chen et al., 2002; Lund et al., 1998) or auto-oxidation (Smith, 1996). 25-HC is directly transported to the side chain cleavage system on inner mitochondrial membrane to yield pregnenolone (Lukyanenko et al., 1998) without the involvement of steroid acute regulatory protein (StAR) that is needed for transport of cholesterol from outer to inner mitochondrial membrane (Iida et al., 1989; Stocco et al., 2005). 25-HC has been demonstrated to be utilized as an alternate substrate for testosterone biosynthesis in Leydig cells (Chen et al., 2002; Hutson, 2006; Lukyanenko et al., 2001; Nes et al., 2000). Further, in immature rat Leydig cells, 25-HC has been shown to enhance the activity of 3β-hydroxysteroid dehydrogenase enzyme which plays key role in testosterone production (Lukyanenko et al., 2002). Intra-testicular administration of 25-HC has resulted in increase of testicular testosterone level in adult rats (Nes et al., 2000). From these studies, it is evident that 25-HC produced by testicular macrophages influences testosterone biosynthesis in Leydig cells as an alternate substrate and/or through modulating steroidogenic enzyme activity.

In wall lizards, testicular macrophage-conditioned medium (TMCM) is reported to increase testosterone production by Leydig cells raising the possibility that 25-HC produced by testicular macrophages might have been utilized for testosterone biosynthesis in Leydig cells. In addition, ovine follicle stimulating hormone (FSH)-activated TMCM was more effective than non-activated TMCM in stimulating testosterone production by Leydig cells of wall lizards (Khan and Rai, 2008a), indicating the role of FSH in production of steroidogenic factors by testicular macrophages. A similar effect of TMCM/FSH-activated TMCM on testosterone production is demonstrated in rat Leydig cells (Yee and Hutson, 1985a). In contrast, testosterone has been shown to decrease 25-hydroxylase and consequently 25-HC production in rat testicular macrophages (Lukyanenko et al., 2002). However, no report is available in non-mammalian vertebrates that demonstrates 25-HC production by testicular macrophage and its hormonal regulation. In view of this, purification, identification, seasonal variation and hormonal regulation of 25-HC produced by lizard testicular macrophages were undertaken in the present study.

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2. Materials and methods

2.1. Animals

The testicular cycle of wall lizards, *Hemidactylus flaviviridis*, is divided into regressed (late May-July), recrudescent (August-October) and spermatogenically active (November-April) phases. Adult male wall lizards were procured locally in June, October and March to study the seasonal variation of 25-HC production by testicular macrophages. They were acclimated to laboratory conditions for a week prior to 25-HC assay. Wall lizards were maintained in well ventilated wooden cages and provided with food and water *ad libitum*. The guidelines of Institutional Animal Ethics Committee were followed in executing the experiments.

2.2. Reagents and culture media

Dulbecco's modified Eagle's medium (DMEM/F-12, 1:1 mixture), 25 hydroxycholesterol (25-HC), ovine follicle stimulating hormone (FSH), testosterone propionate (T) and dihydrotestosterone (DHT) were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). The fetal calf serum (FCS) was procured from Biological Industries, Kibbutz Beit Haemek, Israel. Gentamycin, streptomycin and penicillin (Ranbaxy fine chemicals Ltd., Navi Mumbai, India) were bought from local medical store. High performance liquid chromatography (HPLC) grade methanol, cholesterol and other routine chemicals were obtained from Merck India Ltd. (Navi Mumbai, India). The culture medium, DMEM/F-12 (1:1) was supplemented with 40 μg/ml gentamycin, 100 μg/ml streptomycin and 100 IU/ml penicillin and pH was adjusted to 7.2. Heat-inactivated FCS (1%) was added to the culture medium prior to use.

2.3. Isolation of testicular macrophage

a) Isolation of testicular interstitial cell: Testicular Interstitial cells were isolated following the protocol of Mayerhofer et al. (1992) modified by Khan and Rai (2007). In brief, lizards were sacrificed, testes were excised and transferred to cold culture medium. After washing, testes were decapsulated, chopped, resuspended in cold culture medium and shaken gently to disperse the tubules. Thereafter, suspension was kept on ice for 20 min at unit gravity to sediment the tubules. The supernatant containing interstitial cells including macrophages was decanted. This process was repeated thrice. The pellet of interstitial cells was obtained following centrifugation at 200*g* for 5 min at 4 °C. The pellet was resuspended in culture medium to give the final concentration of 1.6×10^6 cells/ml.

b) Preparation of macrophage monolayer: One milliliter of testicular interstitial cell suspension (1.6×10^6cells/ml) was added to each well of 24-well culture plate. Testicular macrophages were allowed to adhere by incubating at 25 °C (±0.1) in a CO_2 incubator for 2 h. Non-adherent interstitial cells (Leydig cells) were washed off with medium. Adhered testicular macrophages were used for purification, identification, seasonal variation and hormonal regulation of 25-HC. After incubation, medium was collected from each well of 24-well culture plate and pooled to make one sample. Five such plates (5 replicates) were made for each set of experiments, and each experiment was repeated three times. Samples were processed for steroid extraction followed by high performance liquid chromatography (HPLC).

2.4. Seasonal changes in 25-HC production

Macrophage monolayer prepared from regressed, recrudescent and spermatogenetically active testis was incubated in medium containing cholesterol (50 μg/ml) for 12 and 24 h. Macrophages incubated in medium alone (without cholesterol) for respective durations served as controls. After incubation, medium was collected and processed for 25-HC assay.

2.5. In vitro effect of FSH and testosterone on 25-HC production

The effect of hormones on 25-HC production by testicular macrophages was studied during regressed phase. The concentration of FSH (0.5 μg/ml) was decided based on earlier *in vitro* study in wall lizards (Khan and Rai, 2004), while a pilot experiment was performed to determine testosterone concentrations (0.1, 1 and 10 μg/ml) following *in vitro* study in rats (Lukyanenko et al., 2002). Cholesterol (50 μg/ml) was supplemented in medium as substrate to be utilized for the production of 25-HC. Testicular macrophages were incubated individually either with FSH (0.5 μg/ml) or different concentrations of testosterone (0.1, 1 and 10 μg/ml) for 24 h. The monolayer incubated without hormone was considered as control. An experiment was also performed using DHT (10 μg/ml) to verify the specificity of testosterone effect on 25-HC production. After incubation, medium was collected and processed for assay of 25-HC. A separate experiment was performed to investigate the effect of FSH and testosterone in combination. On the basis of dose-dependent effect of testosterone, 10 μg/ml concentration was selected for further *in vitro* experiment. FSH (0.5 μg/ml) and T (10 μg/ml) were used in following combinations for a total duration of 24 h: (a) After 12 h incubation with FSH, T was added in culture medium (0 h FSH + 12 h T); (b) T was supplemented 12 h prior to FSH (0 h T + 12 h FSH); (c) after 12 h incubation in medium (M), FSH/T was supplemented (0 h M + 12 h FSH/T); (d) incubated either with FSH or T for 24 h (0 h FSH/0 h T); (e) cultured in medium for 24 h. In each set of experiment, medium contained 50 μg/ml of cholesterol. Comparable volume of the solvent (ethanol) in which testosterone was dissolved was added wherever required. After treatment, monolayer was washed thrice and then incubated in medium containing cholesterol (50 μg/ml) for another 24 h. After completion of culture, medium was collected and processed for 25-HC assay.

2.6. Steroid extraction

The TMCM was extracted with three volumes of diethyl ether and repeated three times. The ether phase was collected, evaporated, dried and stored at −20 °C till HPLC (Nagahama and Adachi, 1985).

2.7. Purification and characterization of 25-HC

Lyophilized extracts were reconstituted in HPLC grade methanol (200 μl/sample) and processed for purification and characterization of 25-HC by HPLC following the method of Lukyanenko et al. (2001). A Perkin Elmer series 200 HPLC system with two pumps, system controller and an ultraviolet (UV) detector (190 – 370 nm range) was used for HPLC. The system was operated with TcNAV-HPLC series software. Samples were run in a reversed phase C18 column (150 × 4.6 mm, id, 5 μm, 100 Å Luna, Phenomenex) connected to a guard column and absorbance was measured at 206 nm. Methanol (100%) was used as mobile phase with flow rate adjusted at 1 ml/min for run time of 15 min. Different concentrations (two sets: 1–100 μg/ml, volume 20 μl) of the standard in pentaplicate injected into the column showed a linear response curve for concentration versus peak area with a minimum detectable limit at 125 ng/ml and the inter- and intra-assay variations were 8.8% and 7.6% respectively. For percentage recovery analysis, different concentrations of 25-HC standard were processed for steroid extraction in the same manner as TMCM (as described above) and were injected into the column. The percentage recovery for 25-HC-

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