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Possible involvement of ceramide in the regulation of rat Leydig cell function

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Abstract

In the present study, a possible role of a ceramide-dependent pathway in the regulation of Leydig cell function was investigated. Intracellular ceramide levels were increased by: (a) adding ceramide analogs; (b) inhibiting ceramidase activity; and (c) adding sphingomyelinase (SMase). The cell-permeable ceramide analogs *N*-acetyl-, *N*-hexanoyl- and *N*-octanoylsphingosine (C2, C6 and C8) were used. As inhibitor of ceramidase activity 1*S*,2*R*-D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol (MAPP) was used. Shingomyelinase from *S. aureus* origin was utilized. Leydig cells were cultured for 3 or 24 h with or without the different drugs (10 μM) and SMase (0.3 U/ml) in the presence or absence of hCG (10 ng/ml). Basal testosterone production was not modified under any of the experimental conditions. A decrease in hCG-stimulated testosterone production was observed at 3 and 24 h in all cases. The inactive analog (*N*-hexanoyl dihydrosphingosine) did not produce inhibition in hCG-stimulated testosterone production. TNFα and IL1β, two possible inducers of sphingomyelin hydrolysis, produced similar effects on hCG-stimulated testosterone production. In experiments performed in the presence of C6, inhibition in hCG-stimulated cAMP production was observed. The inhibitory effect of ceramide was also observed in dbcAMP-stimulated cultures indicating that this pathway inhibits post-cAMP formation events. To study possible loci for the action of ceramide on the steroidogenic pathway, cells were incubated with C6 and MAPP in the presence of different testosterone precursors. The drugs inhibited testosterone produced from 22(*R*)-hydroxycholesterol (22*R*-OHChol), pregnenolone and 17α-hydroxyprogesterone (17OHP4) but not from androstenedione (Δ4). These results suggest that a ceramide-dependent pathway regulates hCG-stimulated Leydig cell steroidogenesis at the level of cAMP production and at post-cAMP events.

Keywords: Leydig; Ceramide; Testosterone; IL1β; TNFα; Sphingomyelinase

1. Introduction

The sphingomyelin pathway is a new signal transduction pathway that is initiated by activation of a sphingomyelinase (SMase) which hydrolyzes membrane sphingomyelin to ceramide and phosphorylcoline [1]. Ceramide functions as a second messenger that can stimulate a specific proline-directed Ser/Thr protein kinase (CAPK) [2], as well as a specific protein phosphatase (CAPP) [3]. In turn, ceramide is degraded through the action of ceramidase with the formation of sphingosine and free fatty acid [4]. The involvement of ceramide as an effector/mediator of cellular functions emerged by the demonstration that treatment with exogenous SMase or cell-permeable ceramide analogs as well as ceramidase inhibitors reproduced the effects of different agonists, which induce sphingomyelin hydrolysis in several cell types [5–7].

Testosterone biosynthesis in Leydig cells is primarily regulated by pituitary gonadotrophin LH through activation of a cAMP-pkA-dependent pathway [8]. The regulation of testosterone biosynthesis also involves numerous paracrine and autocrine factors produced in the testis [9]. Among these testicular factors are TNFα and IL1β which are produced by testicular macrophages and/or Leydig cells [10,11], and modulate Leydig cell testosterone production [12,13].

Previous studies in other cell types, have shown that TNFα and IL1β can affect cell function by the activation of the sphingomyelin pathway and ceramide pro-

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duction [14–16]. Three previous reports have dealt with the participation of the sphingomyelin/ceramide-dependent pathway on the regulation of Leydig cell steroidogenesis. Degnan et al. [17] have shown that ceramide analogs do not modify rat Leydig cell function while sphingomyelinase inhibits hGC-stimulated testosterone production. Utilizing MA-10 mouse tumour Leydig cells, Kwun et al. [18] have shown that ceramide increases steroid hormone production and Budnik et al. [19] have reported that ceramide inhibits testosterone production by decreasing StAR protein levels. The latter report has also shown, in rather elegant experiments, that TNFα is able to induce ceramide accumulation and its authors suggest that this sphingolipid may be considered as a transmitter of TNFα signals. Based on these controversial results we have decided to evaluate the role of sphingomyelin/ceramide-dependent pathway on the regulation of rat Leydig cell steroidogenesis focusing on the regulation of cAMP production and cholesterol metabolism.

2. Materials and methods

2.1. Materials

Human chorionic gonadotrophin (hCG-CR-127) was obtained from the National Hormone and Pituitary Program, NIDDK. Tissue culture media were purchased from Grand Island Biological Co. (Grand Island, NY, USA). The membrane-permeable analogs of ceramide *N*-acetylsphingosine (C2), *N*-hexanoylsphingosine (C6), *N*-octanoylsphingosine (C8), the ceramidase inhibitor (1S,2R)-D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol (MAPP) and the inactive analog *N*-hexanoyldihydrosphingosine (C6i) were obtained from Biomol Research Laboratories (Plymouth Meeting, PA, USA). All other drugs and reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Leydig cell isolation

Adult male Sprague–Dawley rats were killed by decapitation, and testes were removed and placed in ice-cold PBS, pH 7.4. Leydig cells were isolated according to the method described by Dufau et al. [20] and purified on a discontinuous Percoll gradient (21–26–40–60%) as described by Lefevre et al. [21]. 3βHSD immunohistochemistry showed a purity of approximately 95%. Purified Leydig cells were resuspended in an adequate medium and incubated at 34°C in a mixture of 5% CO_2: 95% air. Treatments were performed in triplicate incubations and all experiments were repeated at least three times.

2.3. Steroid hormone production

To determine the effects of ceramide on Leydig cell testosterone production in 3 h incubations, the cells were resuspended in medium 199 containing 0.1% bovine serum albumin and 0.125 mM 3-isobutyl-1-methylxanthine (10^6 cells/ml). For 24 h studies cells were cultured on 24-multiwell dishes (0.3×10^6 cells/ml) in medium consisting of a 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle medium, supplemented with 20 mM HEPES, 100 IU/ml penicillin, 2.5 μg/ml amphotericin B, 1.2 mg/ml sodium bicarbonate, 10 μg/ml transferrin and 5 μg/ml vitamin E. Leydig cells were incubated with the drugs and cytokines with or without the addition of variable doses of hCG (0.005–10 ng/ml). When indicated, incubations with different steroid substrates were performed. At the end of 3 h incubation period, cells were centrifuged at $1500 \times g$ for 10 min at 4°C and supernatants were saved at −20°C until testosterone determinations were performed. At the end of the 24 h incubation period most of the cells were attached to the plastic surface. 24 h incubation media was recovered from the wells, centrifuged at $1500 \times g$ for 10 min at 4°C and supernatants were saved at −20°C until testosterone determinations were performed. Testosterone was determined by RIA using a specific antibody. The RIA has a sensitivity of 6.25 pg per tube and intra- and interassay coefficients of variation are 8 and 15%, respectively. Results are expressed as ng testosterone/10^6 cells. A cell viability test (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega Corporation) was used to exclude toxic effects of the drugs after 3 or 24 h incubations.

2.4. cAMP production

To determine the effects of ceramide on Leydig cell cAMP production, cells incubated for 3 h were used. The effect of C6 and C6i on cAMP production was evaluated. At the end of the incubation period, cells were centrifuged at $1500 \times g$ for 10 min at 4°C and supernatants were saved at −20°C until cAMP determinations were performed. cAMP was determined by RIA using a specific antibody. The RIA has a sensitivity of 6.25 fmol/tube and intra- and interassay coefficients of variation are 9 and 17%, respectively. Results are expressed as pmol cAMP/10^6 cells per 3 h.

2.5. Statistical analysis

Statistical analysis was performed by analysis of variance followed by Tukey's protected *t* test for comparison of data from multiple groups using the GB-STAT version 4.0 statistical program (Dynamic Microsystems, Inc. Silver Spring, MD, USA). To analyze dose-re-

sponse curves and to obtain ED_{50} values, the computerized method described by De Lean et al. [22] was used.

3. Results

3.1. Effect of TNFα and IL1β on basal and hCG-stimulated testosterone production

Fig. 1 shows that a maximum dose of hCG (10 ng/ml) produced a 10 fold stimulation in testosterone secretion. This figure also shows that TNFα and IL1β treatments did not modify basal testosterone production while they inhibited hGC-stimulated testosterone production. The cytokines also inhibited hCG-stimulated testosterone production in 24 h cultured cells (data not shown).

3.2. Effect of ceramide on basal and hCG-stimulated testosterone production

In order to evaluate a possible role of ceramide on Leydig cell testosterone production, cells were incubated with different cell-permeable ceramide analogs (C2, C6, C8), the ceramidase inhibitor MAPP and SMase with or without hCG for 3 h. Drugs did not modify basal testosterone production. Fig. 2 shows a dose-response inhibition of hCG-stimulated testosterone production for one of the cell-permeable ceramide analog utilized (C6). This figure also shows that the inactive analog C6i did not inhibit hCG-stimulated testosterone production. Fig. 3 shows that MAPP and other ceramide analogs (C2, C8) inhibited submaximal (0.025 ng/ml) and maximal (10 ng/ml) hCG-stimulated testosterone production to a similar extent to that observed with C6. Dose-response curves to hCG (0.005–10 ng/ml) obtained with or without C2 showed that there is no significant change in ED_{50} (Control: 0.092 ± 0.004 ng hCG/ml vs C2: 0.081 ± 0.007 ng hCG/ml, mean $\pm$ SEM, $n = 3$). An inhibition of testosterone production with increasing doses of SMase was also observed (Fig. 4). Similar results were obtained in rat Leydig cells cultured for 24 h (data not shown). Utilizing a cell viability test, we observed that these treatments did not modify cell viability in 3 or 24 h incubations.

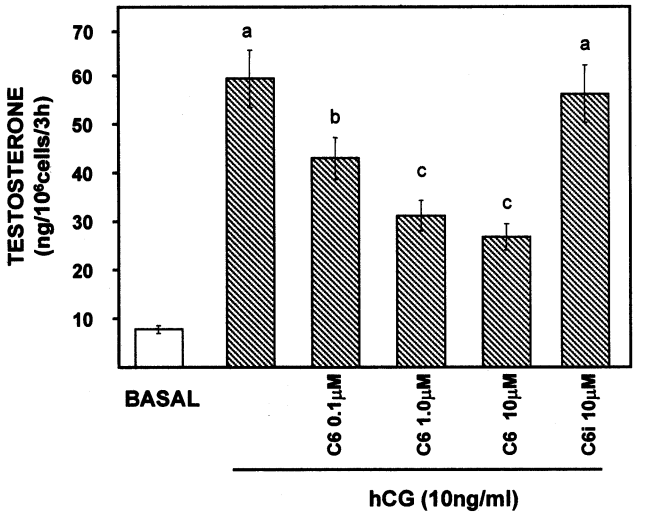

Fig. 2. Effect of different doses of C6 on hCG-stimulated testosterone production. Purified Leydig cells were incubated without drugs or with different doses of C6 (0.1, 1 and 10 μM) and C6i (10 μM) in the presence of hCG (10 ng/ml) for 3 h. Results are expressed as mean ± SD of triplicate incubations of one experiment representative of three. Different superscripts indicate statistically significant differences ($P < 0.01$).

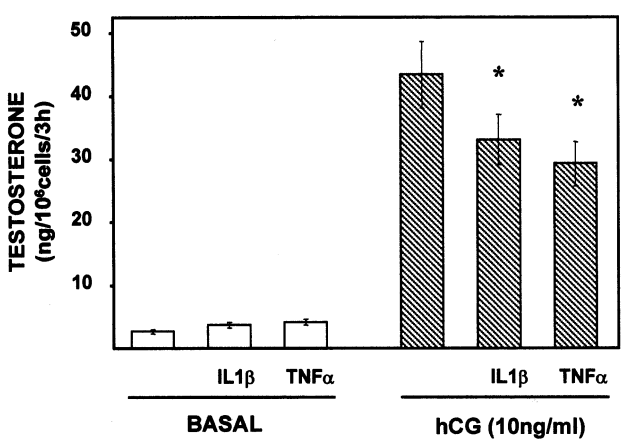

Fig. 1. Effect of IL1β and TNFα on basal and hCG-stimulated testosterone production. Purified Leydig cells were incubated with or without cytokines (10 ng/ml) under basal or hCG-stimulated conditions (10 ng/ml) for 3 h. Results are expressed as mean ± SD of triplicate incubations of one experiment representative of three. * $P < 0.01$ vs. hCG.

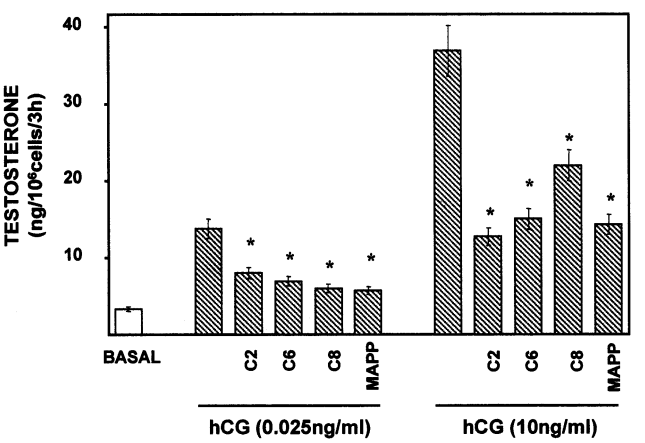

Fig. 3. Effect of C2, C6, C8 and MAPP on hCG-stimulated testosterone production. Purified Leydig cells were incubated without drugs or with C2 (10 μM), C6 (10 μM), C8 (10 μM) or MAPP (10 μM) in the presence of 0.025 and 10 ng/ml hCG for 3 h. Results are expressed as mean ± SD of triplicate incubations of one experiment representative of three. * $P < 0.01$ vs. hCG.

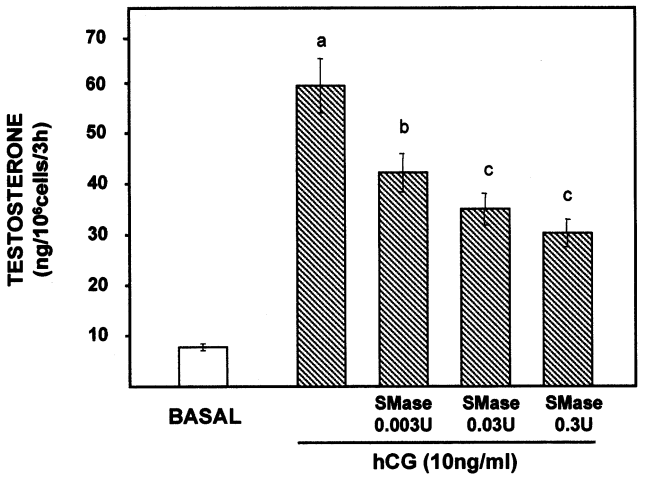

Fig. 4. Effect of different doses of SMase on hCG-stimulated testosterone production. Purified Leydig cells were incubated under basal conditions or with different doses of SMase (0.003, 0.03 and 0.3 U/ml) in the presence of hCG (10 ng/ml) for 3 h. Results are expressed as mean ± SD of triplicate incubations of one experiment representative of three. Different superscripts indicate statistically significant differences ($P < 0.01$).

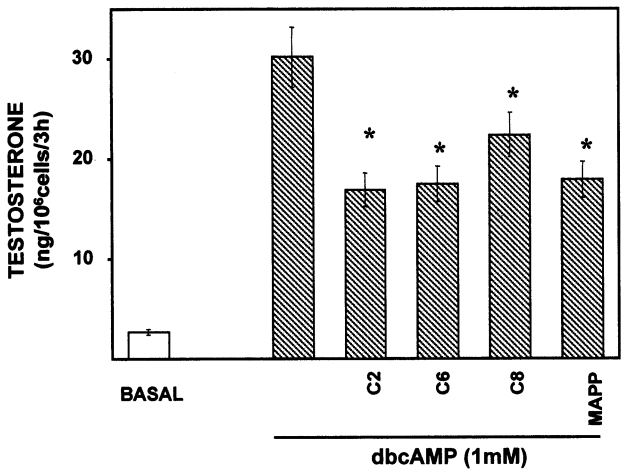

Fig. 5. Effect of C2, C6, C8 and MAPP on dbcAMP-stimulated testosterone production. Purified Leydig cells were incubated without drugs and with C2 (10 μM), C6 (10 μM), C8 (10 μM) or MAPP (10 μM) in the presence of dbcAMP (1 mM) for 3 h. Results are expressed as mean ± SD of triplicate incubations of one experiment representative of three. * $P < 0.01$ vs. dbcAMP.

3.3. Effect of ceramide on cAMP production and dbcAMP-stimulated testosterone production

Table 1 shows that C6 inhibited hCG-stimulated cAMP production. Again, the inactive analog (C6i) did not mimic the effect of C6. To examine whether ceramide may interact with the cAMP-pkA pathway downstream of the cAMP generation, we evaluated the effect of ceramide analogs — C2, C6 and C8 — and the ceramidase inhibitor — MAPP — on dbcAMP-stimulated cells. Fig. 5 shows that these treatments also decreased dbcAMP-stimulated testosterone production.

3.4. Effect of ceramide on cholesterol, pregnenolone, 17α-hydroxyprogesterone and androstenedione metabolism

Cells were incubated with 22(*R*)-hydroxycholesterol (22*R*-OHChol) –a cholesterol substrate derivative that readily passes through cell membranes– or with different steroid substrates: pregnenolone (P5), 17OH progesterone (17OHP4) and androstenedione (Δ4) in the presence or absence of C6 and MAPP. Fig. 6 shows that both drugs inhibited testosterone produced from 22*R*-OHChol, P5 and 17OHP4, but not from Δ4.

Table 1
Effect of C6 and C6i on hCG-stimulated cAMP production[a]

	cAMP (pmol/10⁶cells/3 h)
HCG	28.0 ± 3.6
hCG+C6	14.2 ± 2.6[b]
hCG+C6i	26.4 ± 2.1

[a] Leydig cells were treated without drugs or with C6 (10 μM) and C6i (10 μM) in the presence of hCG (10 ng/ml) for 3 h. Results are expressed as mean ± SD of triplicate incubations of one experiment representative of three.

[b] $P < 0.01$ vs hCG.

4. Discussion

Sphingolipids have important regulatory functions in signal transduction, cell differentiation, cell death and cell growth [4]. Some of these effects are mediated by the sphingomyelin hydrolysis product, ceramide. Evi-

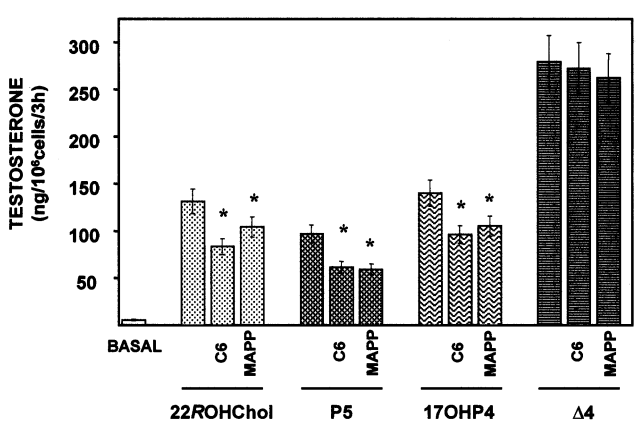

Fig. 6. Effect of C6 and MAPP on testosterone produced from 22*R*OHChol, P5, 17OHP4 and Δ4. Purified Leydig cells were incubated without drugs or with C6 (10 μM) or MAPP (10 μM) in the presence of 22*R*OHChol (20 μM), P5 (2 μM), 17OHP4 (2 μM) and Δ4 (2 μM) for 3 h. Results are expressed as mean ± SD of triplicate incubations of one experiment representative of three. * $P < 0.01$ vs steroid precursor.

dence of a role for ceramide as second messenger has come from studies showing that exogenous ceramide analogs induce biological responses in different cell types [23]. In addition, some of the cellular activities of ceramide have been mimicked by metabolic manipulation of endogenous ceramide levels. Thus, bacterial sphingomyelinase, which cleaves membrane sphingomyelin and causes accumulation of ceramide, has been shown to mimic at least some of the effects of exogenous cell permeable ceramides [24]. Moreover, treatment with synthetic ceramide analogs that inhibit ceramidase and elevate intracellular levels of ceramide [25] also results in cellular activities which are similar to those obtained with ceramide analogs [7].

It has been shown that IL1β and TNFα are produced by Leydig and interstitial macrophages in the testis [26]. For both cytokines, there is a large number of studies that demonstrate their influence on Leydig cell function. There is general agreement that IL1β and TNFα, either added exogenously or generated in vivo, are inhibitory to adult Leydig cell steroidogenesis [12,13,27–31]. However, at least three reports disagree on this conclusion [32–34]. In this study, we observed that the effects of IL1β and TNFα are inhibitory. It is well known that the effects of IL1β and TNFα are mediated, at least in part, by the activation of SMase and ceramide generation in many cell types like EL4 cells [15] and human dermal fibroblasts [5]. In cultured rat granulosa cells, Santana et al. [35] have shown that IL1β binding to its receptor induces sphingomyelin hydrolysis and that SMase and ceramide analogs reproduce the effects of IL1β on progesterone and PGE2 production. On the other hand, Santana et al. [6] have shown that TNFα also induces sphingomyelin hydrolysis in this cell type and suggested that ceramide mediates TNFα effects on granulosa cell P450 aromatase activity. More recently, Budnik et al. [19] have demonstrated that TNFα induces sphingomyelin hydrolysis and that ceramide regulates progesterone production by inhibiting StAR expression in MA-10 mouse tumour Leydig cells.

In the present study, evidence of a potential role of ceramide in the regulation of rat adult Leydig cell function is presented. Ceramide effects were studied by different strategies: utilizing different exogenous ceramide analogs or increasing intracellular levels of ceramide by adding SMase or by inhibiting ceramidase activity.

We have been able to demonstrate a dose response inhibition of hCG-stimulated testosterone production in the presence of C6 and a lack of effect of the inactive analog (C6i) in isolated rat Leydig cells. Also, a dose response inhibition of hCG-stimulated testosterone production in the presence of SMase was observed. No effects of the drugs were obtained under basal conditions. Similar effects were obtained with other ceramide analogs (C2 and C8) and the ceramidase inhibitor (MAPP). It can be claimed that the observed effects are related to non-specific effects of the drugs or to Leydig cell apoptosis produced by ceramide. Several lines of evidence argue against these possibilities: (1) different strategies were utilized to elevate intracellular levels of ceramide using drugs not structurally related and all of them produced similar effects; (2) neither cell viability nor cell morphology was altered as a consequence of these treatments; (3) dose response effects were observed; (4) no drug had effect on basal testosterone production; (5) C6i, a molecule structurally related to C6, had no effect. Ceramide analogs also inhibited hCG-stimulated cAMP accumulation, indicating that the first step to be modulated by ceramide is cAMP production. Additional steps in the regulation of the cAMP-pkA cascade are suggested by the inhibitory effects observed on dbcAMP-stimulated cultures.

As mentioned in the introduction, three previous reports have dealt with the participation of ceramide in the regulation of Leydig cell function. The results presented herein are partially in contrast with those obtained by Degnan et al. [17] in dispersed rat Leydig cells. In Degnan's study, SMase decreased hCG binding, cAMP generation and testosterone production but exogenously-added ceramide had no effect on hCG-stimulated testosterone production. The reason for these discrepancies is not clear but it can be accounted for by the different cell preparations used in both studies. Degnan et al. [17] used crude Leydig cell preparations and testosterone production in response to hCG was only slightly increased. In our study, interstitial cells were purified on a discontinuos Percoll gradient which yields a highly enriched Leydig cell preparation and a 10-fold increase in testosterone production in response to hCG was obtained. The second study, by Kwun et al. [18] showed that ceramide increases steroid hormone production in MA-10 Leydig cells. Neither SMase nor ceramide modified hCG specific binding or cAMP production. In this case, the discrepancies observed between their results and ours could be attributed to the different species or models utilized. However, despite different experimental models, our results agree with those recently reported by Budnik et al. [19]. They showed that ceramide and SMase inhibit progesterone production in MA-10 Leydig cells. They also showed that StAR protein expression is the possible site of regulation for the effect of ceramide on progesterone production.

In rat Leydig cells, TNFα and IL1β inhibition of steroidogenesis involves a decrease in 17α-hydroxylase/C17–20 lyase (P450c17) activity [12,13]. However, the participation of a ceramide-dependent pathway in this phenomenon has not been established. Our results show that the ceramide analog (C6) and the ceramidase inhibitor (MAPP) inhibit testosterone production from

22*R*OHChol, P5 and 17OHP4, but not from Δ4, suggesting that ceramide can inhibit also P450c17 enzyme activity.

In summary, our results show that ceramide can regulate rat Leydig cell testosterone production. At least two steps may be involved in this regulation: cAMP production and P450c17 enzyme activity. Altogether, our observations suggest that the ceramide-dependent pathway may be important in the regulation of rat Leydig cell steroidogenesis.

Acknowledgements

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