Sphingomyelinase Inhibits In Vitro Leydig Cell Function*†‡

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ABSTRACT

Activation of the immune system has profound effects on endocrine function which are mediated by cytokines including tumor necrosis factor-α (TNFα). In vitro, TNFα has been shown to directly inhibit Leydig cell testosterone (T) production, but the mechanism of this effect is still unclear. Recent studies using cultured human fibroblasts have shown that TNFα stimulates the activity of neutral sphingomyelinase (SMase) which hydrolyses sphingomyelin (SM) generating ceramide and changing membrane components including cholesterol. The cellular effects of increased SMase activity have been reproduced in vitro by the addition of exogenous SMase. In cultured fibroblasts, exogenous SMase decreases cholesterol synthesis. These findings led us to hypothesize that SMase might be important in the regulation of steroid hormone synthesis. To our knowledge, no previous studies have investigated this possibility. To test this hypothesis, rat Leydig cell enriched cultures were incubated in media containing SMase (0.1 to 100 mU/ml) or in control media. SMase significantly decreased basal and human chorionic gonadotropin (hCG) stimulated T production. SMase also decreased hCG binding and hCG stimulated

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SPHINGOMYELINASE INHIBITS IN VITRO LEYDIG CELL FUNCTION

adenosine 3':5'-cyclic monophosphate (cAMP). N-acetyl-sphingosine (0.1 to 10 μM), a water soluble ceramide, was used to determine whether or not the effects of SMase could be reproduced by ceramide addition. N-acetyl-sphingosine had only slight effects on basal T and cAMP, and no effect on hCG binding or hCG stimulated T or cAMP. These data suggest the metabolism of membrane sphingomyelin may be an important regulatory pathway in the control of Leydig cell function.

Introduction

Activation of the immune system has profound effects on endocrine function, the majority of which are mediated by cytokines.1-16 During acute major illness, the liberation of several cytokines, including tumor necrosis factor-α (TNFα) suppresses serum testosterone (T) levels. In vitro, TNFα has been shown to directly inhibit Leydig cell T production, but the mechanism of this effect is still unclear.12,17

Recent studies using cultured human fibroblasts have shown that TNFα stimulates the activity of neutral sphingomyelinase (SMase) which hydrolyses membrane sphingomyelin (SM) to generate ceramide.18,19 Ceramide is an important signalling compound which can directly increase the activity of nuclear transcription factor κB (NF-κB) or be further metabolized to sphingosine which regulates the activity of protein kinase c (pKc).20-23 In addition to generating ceramide, SMase has other direct effects on membrane components. Once activated, SMase depletes membrane SM content, in response to which cholesterol migrates out of the membrane and is metabolized to cholesterol esters.23-25 This shift in cholesterol is regulated by calcium, ceramide, and sterol carrier proteins,25 and is one of the early and potentially rate limiting steps in the synthesis of steroid hormones.26

The cellular effects of increased SMase activity including SM depletion, ceramide generation, and cellular differentiation have all been reproduced in vitro by the addition of exogenous SMase.20,27,28 In cultured fibroblasts, exogenous SMase decreases the activity of 3-hydroxy-3-methyl glutaryl coenzyme A reductase and the synthesis of cholesterol.27,28

These findings led us to hypothesize that SMase might be important in the regulation of steroid hormone synthesis by cytokines. To our knowledge, there have been no previous studies which have investigated such a role for SMase. The present study was designed to answer two questions: (1) Does SMase have any effect on hormone production by rat Leydig cells enriched cultures? and (2) Is ceramide generation important in mediating these effects? To answer the first question, exogenous SMase was directly added to Leydig cell cultures and the binding of human chorionic gonadotropin (hCG), and synthesis of cyclic nucleotides and T were determined. To answer the second question, N-acetyl-sphingosine (NACs), a water-soluble ceramide, was directly added to Leydig cell cultures, and similar measurements were performed.

Materials and Methods

APPROVAL

This study was approved by the Animal Research Committee and the Institutional Review Board of the Department of Clinical Investigation, Walter Reed Army Medical Center, Washington, DC.
PREPARATION OF LEYDIG CELLS

Adult Sprague-Dawley rat Leydig cell enriched cultures were prepared as previously described. Testes were aseptically removed, decapsulated, and digested with collagenase (0.25 mg/ml, 10 min, 37°C). The enriched Leydig cells were then separated from the tubular elements by filtration through a cell sieve. The cells (1 to 3 × 10^6, as indicated) were immediately used in incubations of 15 min to 24 hr in either control media [Media 199 containing bovine serum albumin (1 mg/ml), penicillin (50 units/ml), and streptomycin (0.05 gm/ml)] or control media with other additions as specified.

STEROID HORMONE PRODUCTION

To determine the effects of SMase and NAcS on testosterone (T) production, cells (1 × 10^6/2 ml, n = 5 for each condition) were incubated 2 to 24 hr in either control media, media containing SMase (0.1 to 100 mU/ml), or media containing NAcS (0.1 to 10 μM). Following incubation, cells were centrifuged (1000 g, 10 min, 4°C), and the media were removed and frozen for T determination (direct radioimmunoassay).* To determine the effects of SMase and NAcS on either human chorionic gonadotropin (hCG) or dibutyryl-adenosine 3':5'-cyclic monophosphate (dBcAMP) stimulated cells, either hCG (0.0001 to 0.1 U/ml) or dBcAMP (10^-3 M) was added at the beginning of each incubation.

CYCLIC NUCLEOTIDE PRODUCTION

The production of cAMP was determined using basal and hCG stimulated cultures. To determine the effects of SMase and NAcS on cAMP production, cells (3 × 10^6) were incubated 15 min in control media, or media containing either SMase (10 to 100 mU/ml) or NAcS (1 to 10 μM). Where indicated, hCG (0.1 U/ml) was also added. Following incubation, cells were centrifuged (1000 g, 10 min, 4°C) and extracted (ethyl alcohol, 2 ml, 95%, 0°C, 1 hr). The extracts were evaporated to dryness, and reconstituted in radioimmunoassay buffer. The cAMP content was then determined by specific radioimmunoassay.†

SPECIFIC HCG BINDING

Maximal specific hCG binding was determined using intact Leydig cell cultures. To determine the effects of SMase and NAcS on hCG binding, 1 × 10^6 Leydig cells were incubated 1 hr in either control media, media containing SMase (1 to 100 mU/ml) or media containing NAcS (1 to 10 μM). There were 20 cultures prepared for the control and for each concentration of added SMase or NAcS. After 1 hr, cultures were divided into two sets of 10 each and used for determination of total and non-specific binding. Total hCG binding was determined by incubating cells with [125I]-hCG (2 ng/ml, 37°C, 1 hr). To determine non-specific binding, unlabelled hCG (0.1 μM) was also added. After incubation, cells were extensively washed with Hank's Balanced Salt Solution (HBSS, 0°C). The radioactivity in each cell pellet was quantified, and specific binding was calculated as the difference between total and non-specific binding.

Additionally, separate studies were performed to determine the effect of SMase on the affinity of hCG binding to intact Leydig cells. For these experiments, Leydig cells (1 × 10^6) were incubated 1 hr in either control media, or media containing SMase (10 mU/ml).

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There were 50 cultures prepared for both the control and for SMase treated cells. After 1 hr, cultures from both control and SMase treated cells were divided into groups of five each. These were used for the determination of maximal binding (\(^{125}\text{I})\text{-hCG, 2 ng/ml}\), non-specific binding (\(^{125}\text{I})\text{-hCG, 2 ng/ml plus unlabelled hCG, 0.1 \mu M}\), and binding in the presence of increasing concentrations of unlabelled hCG (\(^{125}\text{I})\text{-hCG, 2 ng/ml plus unlabelled hCG, 0.0089 to 4.58 pmol/ml}\). After incubation, cells were extensively washed and specific binding at each concentration was calculated as above.

**CELL VIABILITY**

Cells were collected after each incubation and the viability was determined by Trypan Blue exclusion.

**STATISTICAL ANALYSIS**

Statistical significance for the difference between groups was determined by analysis of variance. For hCG binding studies, Scatchard analysis could not be employed because the hCG and \(^{125}\text{I})\text{-hCG preparations were less than 100\% biologically active.**

**CHEMICALS AND REAGENTS**

Sphingomyelinase (S. aureus),‡ N-acetyl-sphingosine,§ and \(^{125}\text{I})\text{-hCG (87.1 Ci/gm)}\)† were obtained and used within two weeks of receipt. All other chemicals and reagents were of reagent quality.

**Results**

Cell viability was greater than 95\% following all incubations. There did not appear to be any effect of either SMase or NAcS on Leydig cell viability.

The effects of SMase and NAcS on basal and hCG stimulated T production are shown in figure 1. SMase significantly decreased both basal and hCG stimulated T production. In contrast, NAcS, only at a concentration of 1 \mu M, increased basal T but had no effect on hCG stimulated T production.

As shown in figure 2, SMase (10 mU/ml) had no effect on T production between 2 and 8 hr, but significantly reduced T at 24 hr (p < 0.005 vs control). In addition, as shown in figure 3, the effect of SMase appeared to be greatest when lower hCG concentrations were used to stimulate T production (40\% reduction in T vs 17\% reduction in T).

The effects of SMase and NAcS on maximal specific hCG binding are shown in figure 4. SMase, but not NAcS, significantly decreased hCG binding at all concentrations tested.

As shown in figure 5, there was no shift in the half-maximal binding point of \(^{125}\text{I})\text{-hCG to the SMase treated cells compared to control. This suggests SMase had no effect on the affinity of hCG binding to the hCG receptor.**

The effects of both SMase and NAcS on basal and hCG stimulated cAMP production are shown in figure 6. SMase had no effect on basal cAMP production, but significantly reduced hCG stimulated cAMP production. In contrast, NAcS, at the highest concentration, slightly decreased basal cAMP levels but had no effect on hCG stimulated cAMP.

The effects of both SMase and NAcS on dBcAMP stimulated T production are shown in figure 7. Neither SMase nor NAcS had any effect.

**Discussion**

These results show that SMase, at concentrations ranging from 10 to 100 mU/ml significantly inhibits both basal and hCG stimulated T production by Leydig cell enriched cultures. Several hours of incu-

‡ Sigma, Chemical Co., St. Louis, MO.
§ Molecular Probes, Eugene, OR.
† New England Nuclear Corporation, Boston, MA.
bation with SMase appear to be required to inhibit T production. This is consistent with previously published observations that SMase inhibits the activity of 3-hydroxy-3-methyl glutaryl coenzyme A and the synthesis of cholesterol by cultured fibroblasts.\textsuperscript{27,28} It is possible that SMase could act similarly to inhibit cholesterol formation and, subsequently, steroid hormone synthesis by Leydig cells.

In addition, this study shows that SMase inhibits specific hCG binding and hCG stimulated cAMP production. These effects occur rapidly, such that
cAMP production is decreased after 15 min and hCG binding by 1 hr. Shorter incubations were not attempted, but, taken together, these observations suggest that SMase could have rapid effects on membrane components, or fluidity, which could result in decreased hCG binding and cAMP generation.\textsuperscript{23,24,25,30} Over a longer incubation, these changes could result in decreased T production.

Further analysis of the binding interaction between hCG and intact Leydig cells showed no shift in the half-maximal binding point for cells treated with...
SMase. This suggests that SMase decreases hCG receptor availability without changing receptor affinity. Such changes could result from alterations in membrane components which have been shown to occur following SMase exposure.23,24,25,30

The effect of SMase on T production was reversed by the addition of dBcAMP. This strongly suggests that the most important sites of SMase action on Leydig cell steroidogenesis may be the binding of hCG and the production of cAMP.

NACs, a water soluble ceramide, was used in similar studies to determine if the effects of SMase could be mediated through the generation of ceramide. NACs had only minimal effects on Ley-

![Graph](image-url)

**Figure 5.** The effect of sphingomyelinase (SMase) on percent of maximal hCG binding. Control cells, SMase (10 mU/ml) treated cells. Data represent the mean of 5 observations at each concentration. Half maximal binding is indicated by arrows.

![Graph](image-url)

**Figure 6.** The effects of sphingomyelinase (SMase) and N-acetyl-sphingosine (NACs) on basal and hCG stimulated cAMP production. Basal cAMP is shown on the left and hCG (0.1 U/ml) stimulated cAMP on the right. SMase concentrations ranged from 10 to 100 mU/ml (shown in double cross hatched bars). NACs concentrations ranged from 1 to 10 μM (shown in single cross hatched bars). Data represent the mean ± SEM with n = 5 for each concentration. **p < 0.005, #p = 0.06.
dig cell function. NAcS increased basal T production and decreased basal cAMP but had no effect on hCG binding, hCG stimulated T or cAMP production, or dBCAMP stimulated T production. These observations suggest that NAcS can not fully replicate the effects of SMase on Leydig cell cultures. There are several possible explanations for these differences. First, SMase is known to have direct membrane effects which are independent of ceramide generation. Second, NAcS, although a water soluble ceramide, might not gain effective access to Leydig cells. Third, NAcS might not be incorporated into the appropriate intracellular ceramide pool or be further metabolised to sphingosine (a potentially important signalling molecule in this system).

In summary, these observations suggest that SMase could be an important enzyme in the regulation of Leydig cell steroidogenesis. Further studies are necessary to determine: (1) the sequence of intracellular events which follow SMase, exposure to, (2) whether or not ceramide generation is important in this process, and (3) whether or not Leydig cells possess endogenous SMase activity which is regulated by any hormonal signals.

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References


