Differential Release of Polyamines by Cultured Rat Sertoli Cells

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Cellular and media concentrations of polyamines in Sertoli cell cultures were determined by fluorescent spectroscopy of dansylated compounds after separation by high-performance liquid chromatography. In spite of low cellular levels of putrescine, the Sertoli cells released relatively large amounts of putrescine and spermidine even after several media changes. The inclusion in the culture media of cortisol, insulin, and thyroxine significantly elevated cellular polyamine levels, altered the spermidine to spermine ratio, and enhanced putrescine release by 3- to 4-fold. No spermine, however, was detected in the media under any of the conditions studied. The polyamine concentrations in cultured Sertoli cells from 13-day-old rats and the pattern of polyamine release by these cells differed significantly from those in the Sertoli cells from 46-day-old rats. These data demonstrate the differential release of polyamines by cultured rat Sertoli cells. The profiles of polyamine secretion appear to be age-dependent, and the significance of this phenomenon is discussed.

Key words: Sertoli cells, polyamines, putrescine, spermine, spermidine.

J Androl 1985; 6:348–352.

The polyamines (putrescine, spermidine, and spermine) are small, aliphatic, nitrogenous bases that function as the organic cations of a cell, and are found in various quantities in all living cells. An increase in polyamine synthesis and the stimulation of related metabolic enzyme activities have been associated with cell proliferation and differentiation (Tabor and Tabor, 1976; Williams-Ashman and Canellakis, 1979; Sunkara et al, 1979; Heby, 1981; Oka et al, 1981, 1982; Cohen, 1982; Luk et al, 1982).

Spermatogenesis is a process that involves both germ cell proliferation and differentiation. To date, the involvement of polyamines in spermatogenesis has only been studied indirectly. Accumulation of spermine is closely associated with germ cell differentiation (MaCindoe and Turkington, 1973). The testicular activities of ornithine decarboxylase and S-adenosylmethionine decarboxylase, enzymes involved in polyamine biosynthesis, are increased greatly by gonadotropins (MaCindoe and Turkington, 1973; Reddy and Villee, 1975). Spermidine and spermine levels in newt testes are low in the winter, increase in the spring, and reach a peak in the summer during active spermatogenesis (Matsuzaki et al, 1981). The above observations suggest that testicular polyamines may play an important role in spermatogenesis in mammals and amphibians; however, the cellular sources of polyamines, and the cells that respond to them have not been defined.

This paper describes measurements of intracellular polyamine concentrations in cultured Sertoli cells, and presents the first observations on the differential release of polyamines by these cells into the medium. The preliminary data show that concentrations of polyamines in Sertoli cells and the amounts of polyamines released into the media appear to be age-dependent.

Materials and Methods

Materials

Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, and 1,10-diaminodecane were obtained from Sigma Chemical Co. (St. Louis, MO); tributylamine from Eastman Kodak Co. (Rochester, NY); and dansyl chloride from Pierce Chemical Co. (Rockford, IL). Acetonitrile was obtained from Burdick and Jackson Laboratories Inc. (Muskegon, MI). All solvents were HPLC grade. Eagle's minimum essential medium (MEM) and

Supported partially by NIH grants HD 14453 and HD 19019.
Submitted for publication October 9, 1984; revised version received April 24, 1985; accepted for publication June 6, 1985.
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vitamins, penicillin, streptomycin, and Fungizone were purchased from Gibco (Grand Island, NY). Cortisol was obtained from Steraloids (Wilton, NH), insulin from Lilly (Indianapolis, IN), thyroxine from Flint Laboratory (Deerfield, IL), transferrin from Sigma (St. Louis, MO), and vitamin A from USV Pharmaceutical Corp. (Tuckahoe, NY). Sprague-Dawley rats were obtained from Timco (Houston, TX).

Sertoli Cell Culture

The Sertoli cells were isolated from 13-, 19-, 27-, or 46-day-old Sprague-Dawley rats, as described by Steinberger et al (1975), and cultured in 100-mm plastic dishes at 34 C for 5 to 6 days in serum-free Eagle's MEM, *p*H 7.2 to 7.4, containing penicillin (100 U/ml), streptomycin (100 μg/ml), Fungizone (5 μg/ml), and vitamins (MEM). In some experiments, the MEM was supplemented with CIT (28 mM cortisol, 100 mIU/ml insulin, 0.1 μM T_4 thyroxine: Sato, 1975; Wu and Sato, 1978) or CITTA (CIT plus 5 μg/ml transferrin and 12 IU/ml vitamin A: Lamb et al, 1981). The culture media were changed after 3 days or as indicated, and, in some cases, Sertoli cells were treated hypotonically to eliminate the remaining germ cells (Galdieri et al, 1981; Wagle et al, 1984) prior to media replenishment. The collected media were clarified by centrifugation (800 × g, 15 minutes) and stored at −40 C for subsequent HPLC analysis. The culture dishes were rinsed twice with Tris-saline (10 mM Tris-HCl, *p*H 7.4, 0.85% NaCl) and the cells were scraped wth a Teflon® spatula in 1.5 ml of Tris-saline. The cell suspensions were stored at −40 C.

Extraction and Dansylation

The procedures used are described elsewhere (Lin et al, 1982; Costa et al, 1982). Each cell suspension was brought to 0.4 M $HClO_4$ and homogenized with a Polytron PT10. An aliquot of the homogenate was mixed with a 2.5 μg 1,10-diaminodecane (internal standard, in 30 μl) and centrifuged (1500 × g, 20 minutes). The supernatant was removed, and the sediment was hydrolyzed in 0.5 M Perchloric acid for DNA assay (Burton, 1956). The supernatant was neutralized with 40% KOH and clarified by centrifugation (1500 × g, 20 minutes). A 0.2-ml aliquot of the neutralized supernatant, 50 μl saturated Na_2CO_3 and 2.5 mg dansyl chloride (25 mg/ml in acetone) were mixed and heated at 70 C for 30 minutes. The mixture was evaporated to dryness, redissolved in 0.5 ml water, and extracted with 2.5 ml toluene. After centrifugation, the organic portion was removed, evaporated, redissolved in 400 μl acetone, and an aliquot of 25 μl was used for HPLC analysis. To determine polyamine levels in the media from Sertoli cell cultures, a 1-ml aliquot was concentrated in the presence of 0.85 μg 1,10-diaminodecane by evaporation to dryness. Dansylation and further treatment of the sample were performed as described above.

Polyamine Quantitation

For polyamine quantitation, a Waters high-performance liquid chromatographic system with M-45 solvent delivery units and a 660 solvent programmer, a Schoeffels GM970 monochromator fluorescence detector and a 300 × 4 mm id μ-Bondapak C18 column were used. Solvent A and solvent B were composed of acetonitrile, water, glacial acetic acid, and tributylamine in the proportions of 40:60:0.02:0.005 and 95:5:0.02:0.005, v/v/v/v, respectively. Elution was performed at a flow rate of 1 ml/min with a gradient starting at 80% solvent A and 20% solvent B, and increasing in B solvent linearly to 85% in 15 minutes, followed by 10 minutes in 85% solution B, as described by Lin and colleagues (Lin et al, 1982; Costa et al, 1982). All data were expressed as mean ± SE and analyzed by one way ANOVA and Duncan's multiple range test.

Results

Polyamine Levels of Cultured Sertoli Cells

At a signal-to-noise ratio equal to 5, the limits of detection for putrescine, spermidine, and spermine

TABLE 1. Concentrations of Polyamines in Sertoli Cells and Spent Media*

Age of Rats (Days)	Polyamine Source	μg/mg DNA					
		Putrescine		Spermidine		Spermine	
		Exp A	Exp B	Exp A	Exp B	Exp A	Exp B
13	Cells	ND†	ND	2.4	1.7	11.7	17.1
	Media						
	1st	44.9	55.3	44.9	53.0	—	—
	2nd	15.7	13.5	30.9	23.5	—	—
	Ratio						
	(2nd/1st)	0.35	0.24	0.69	0.44	—	—
46	Cells	ND	ND	12.3	15.3	53.9	83.5
	Media						
	1st	4.4	6.3	66.5	72.4	—	—
	2nd	13.6	19.4	39.4	38.5	—	—
	Ratio						
	(2nd/1st)	3.1	3.1	0.59	0.54	—	—

*Sertoli cells were cultured in MEM for 5 days with media change at 72 hours (1st) after original plating. The cells and media were then collected 48 hours (2nd) after the media change. Data represent means of duplicates.

†ND = too low to permit accurate determination under the experimental conditions of this study.

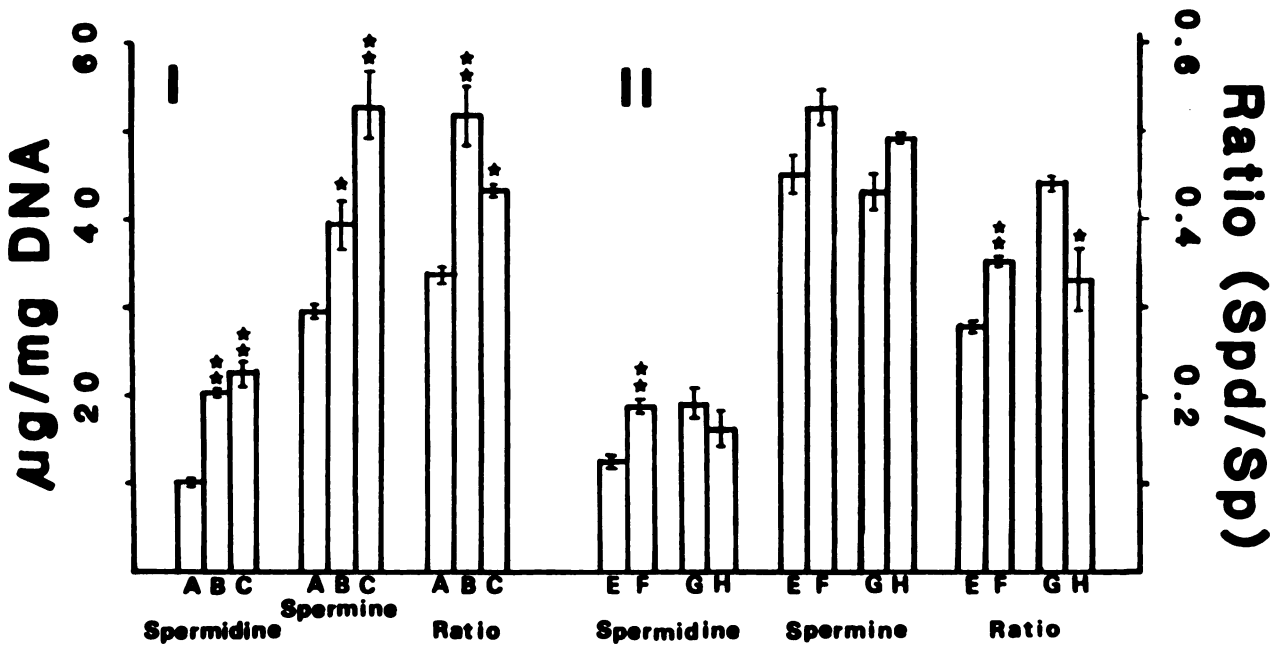

Fig. 1. Effect of hormones, vitamin A and transferrin on polyamine levels in cultured Sertoli cells. Sertoli cells were cultured from 19-day-old rats. Experiment I: Sertoli cells were cultured in MEM (A), MEM + CIT (B), or in MEM + CITTA (C). Media were changed at days 3 and 5. The cells were collected 6 hours after the last media change. Experiment II: Cells were cultured in MEM (groups E and F) or in MEM + CIT (groups G and H) with media changes at days 3 and 5. On day 5, media in groups E and H were replaced with MEM and those in groups F and G with MEM + CIT. The cells were collected 24 hours later. The data represent mean ± SE, n = 3. Ratio represents spermidine/spermine. *, $P<0.05$; **, $P<0.01$, groups B or C versus group A, group F versus group E, and group H versus group G. CIT = cortisol + insulin + thyroxine. CITTA = CIT + transferrin + vitamin A.

by the HPLC method were 1.0, 1.6 and 2.2 ng, respectively. Each dish of cultured Sertoli cells usually contained 40 to 60 μg DNA. The putrescine level in Sertoli cell extracts was too low to permit accurate determination. Repeated measurements of concentrations of spermidine and spermine in a given Sertoli cell sample (from 27-day-old rats) were reproducible within a 2.2% standard error (9.5 ± 0.08 and 57.8 ± 1.3 μg/mg DNA, respectively, n = 5). Due to the inherent variability of the primary culture, absolute values among cultures may vary by a slightly greater amount. Furthermore, in addition to the use of a specific retention time for the identification of each polyamine, each polyamine in Sertoli cell extracts and medium was also identified by coelution with dansylated authentic putrescine, spermidine or spermine.

Table 1 shows the polyamine concentrations (μg/mg DNA) in 5-day cultured Sertoli cells from 13-day and 46-day old rats and in their media from the first 72 hours of culture (first) and the final 48 hours of culture (second). Although spermidine levels were low and putrescine levels were too low to be measured accurately in cultured cells, relatively large

TABLE 2. Effect of Supplementary Hormones on the Levels of Cellular Polyamines and the Quantities of Released Polyamines*

Group	Source	μg/mg DNA (Mean ± SE, n = 3) Putrescine	Spermidine	Spermine
A	Cells	ND†	9.86 ± 0.2	29.4 ± 1.0
	media (MEM)	4.9 ± 0.1	6.7 ± 1.1	—
B	Cells	ND	20.3 ± 0.1[a]	39.3 ± 2.7[b]
	media (MEM + CIT)	17.7 ± 1.4[a]	8.9 ± 0.1[b]	—
	ratio: media B/media A	3.77	1.33	—

*Sertoli cells were isolated from testes of 19-day-old rats and cultured in MEM (Group A) or MEM + CIT (Group B). The media were changed after 3 days and 5 days. Cells and media were collected 6 hours after the last media change.

†ND = too low to permit accurate determination. Each polyamine from cells or media compared with Group A: a = $P<0.01$; b = $P<0.05$.

quantities of spermidine and putrescine were present in the media. Spermine was not detectable in the media.

Table 1 also lists the ratios of the polyamine concentrations in the second compared with the first media for each Sertoli cell culture. It is of great interest to note that in cultures of Sertoli cells from 13-day-old rats, the second-to-first media ratio of putrescine concentration ranged from 0.24 to 0.35, while in the cultures from 46-day-old rats, the ratio (second-to-first) was 3.1. The second-to-first media ratios of spermidine concentrations, however, were almost equal at both ages.

The Effect of Medium Modification on Sertoli Cell Polyamine Levels

Although the requirements for Sertoli cell cultures have not been systematically defined, cortisol, insulin, and thyroxine (CIT) have been included in the chemically defined serum-free media for several cell lines to improve cell growth (Sato, 1975; Wu and Sato, 1978). When these hormones (CIT) were included in the media, both cellular spermidine and spermine contents of Sertoli cells increased. The spermine level was further increased when transferrin and vitamin A were added along with CIT (Fig. 1, experiment I). The cellular levels of polyamines and the spermidine-to-spermine ratio increased 24 hours after the addition of CIT, while removal of CIT from the media at 24 hours prior to cell collection did not significantly alter the levels (Fig. 1, experiment II). Furthermore, the inclusion of CIT also enhanced the release of putrescine by Sertoli cells 3- to 4-fold, while the cell content of putrescine remained at a level too low to permit accurate measurement (Table 2). Spermidine release, compared with putrescine, was not affected as markedly by the inclusion of CIT.

The Release of Polyamines by Sertoli Cells after Hypotonic Treatment

Hypotonic treatment prior to medium change was used to eliminate the remaining germ cells attached to the adhered Sertoli cell monolayers after 3 days of culture. Table 3 demonstrates that, in MEM, the rates of accumulation of putrescine and spermidine in the media were different. Although in the initial 24 hours the cells released more spermidine than putrescine, by 72 hours there was more putrescine in the media.

TABLE 3. Accumulation of Polyamines by Cultured Sertoli Cells after Hypotonic Treatment*

Source	Time (h)	μg/mg DNA		
		Putrescine	Spermidine	Spermine
Cells	24	ND†	10.8 ± 1.2	86.0 ± 3.1
	48	ND	10.0 ± 0.5	62.6 ± 1.5
	72	ND	11.7 ± 1.4	58.6 ± 7.0
Media	24	4.0 ± 0.2[a]	13.4 ± 0.6[a]	—
	48	17.3 ± 1.5[b]	21.8 ± 1.2[b]	—
	72	61.0 ± 2.6[c]	42.5 ± 3.7[c]	—

*Sertoli cells from 27-day-old rats were cultured in MEM for 3 days. On day 3, fresh MEM was refurnished after hypotonic treatment. Cells and media in triplicate dishes were collected separately 24 hours, 48 hours, and 72 hours after media change.

†ND = too low to permit accurate determination. For each polyamine, values with different superscripts differ from each other at $P < 0.01$. Values are mean ± SE; n = 3.

Discussion

MaCindoe and Turkington (1973) reported that nearly all testicular ornithine decarboxylase activity was associated with the cell fraction in which the majority of cells were Sertoli cells, and no activity was detected in fractions rich in other cell types, including spermatids. Although its short half life might account for the lack of ornithine decarboxylase activity in most testicular cell types after the prolonged method of cell separation used by MaCindoe and Turkington, the speculation that germ cells might utilize putrescine synthesized and provided by other cell types could not be excluded. In contrast, ornithine decarboxylase activity has been demonstrated in the late spermatids of the rooster (Oliva et al. 1982).

The present study has established the Sertoli cell as a site of synthesis and secretion of testicular polyamines. Both putrescine and spermidine, but not spermine, were found in Sertoli cell culture media after several media changes and after hypotonic treatment which preferentially lyses the germ cells. In addition, polyamine synthesis and putrescine release were enhanced by the addition of CIT to the media. Thus, the differential release of polyamines by Sertoli cells is not an artifact. It is important to emphasize that not all cell types are capable of releasing polyamines. For example, cultured Chinese hamster ovary cells contain polyamines, but no polyamines are detected in the media (Russell and Haddox, 1981). It is, therefore, possible that putrescine released by Sertoli cells may serve as a precursor for polyamine synthesis in other testicular cell types, such as differentiating germ cells, in which ornithine decarboxylase is purportedly absent (MaCindoe and Turkington, 1973).

The data summarized in Table 2 show that the average rate of putrescine release by Sertoli cells

from 13-day-old rats and that of spermidine release by Sertoli cells from either 13-day-old or 46-day-old rats were apparently decreased during the second 48 hours of culture relative to the first 72 hours. These data may reflect the adaptation of Sertoli cells to culture conditions or the loss of certain cellular activity due to prolonged cell cultivation. However, the second media collection from Sertoli cells of 46-day-old rats contained more putrescine than the first media. Since more round and late spermatids were present in the first 72 hours of culture than in the second period of this primary culture, the lower level of putrescine in the first media may result from utilization of this compound by some contaminating germ cells. These speculations are consistent with the fact that germ cells are in close contact with Sertoli cells, and that gap junctions exist between these two cell types (Russell, 1980). Alternatively, the presence of germ cells may influence the patterns of polyamine metabolism in cultured Sertoli cells. Additional studies are necessary to explore these possibilities.

On the other hand, the extremely low cell levels of spermidine and spermine in Sertoli cells from 13-day-old rats and the high amount of putrescine present in their media, as compared with the levels of these compounds in Sertoli cells isolated from 19-day-old, 27-day-old, and 46-day-old rats, suggest that the conversion of putrescine to spermidine, and particularly from spermidine to spermine, may be limited in these cells and may result in the secretion of excess cellular polyamine. If this is the case, the induction of the enzymes responsible for the conversion of putrescine to spermidine and spermine may be used as markers of Sertoli cell maturation during testicular development.

In conclusion, these results present the first demonstrations that Sertoli cells synthesize all three polyamines, and release putrescine and spermidine but not spermine. Both the cellular polyamine concentrations and the polyamine release profiles of cultured Sertoli cells appear to be age-dependent.

Acknowledgment

The authors thank Dr. Anna Steinberger for the use of her tissue culture facilities and for her expert advice on tissue culture. Excellent technical assistance was provided by Ms. T-P. F. Wang in the polyamine determinations and by Ms. J. Gerst in the preparation of Sertoli cells.

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