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Role of Metastin in the Release of Gonadotropin-Releasing Hormone From the Hypothalamus of the Male Rat

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ABSTRACT: Recent genetic analysis has suggested that the expression of the orphan receptor GPR54 is essential for the onset of puberty in both rodents and humans. Indirect evidence has suggested that this action is via gonadotropin-releasing hormone induction of luteinizing hormone release. The experiments described here were intended to provide direct evidence that metastin, the naturally occurring ligand for GPR54, was capable of stimulating GnRH secretion by examining GnRH release from an immortalized hypothalamic cell line (GT1-7) and from male rat hypothalamic explants. GT1-7 cells were treated for 2½ hours and overnight with the biologically active fragment of metastin, metastin(45–54), in amounts ranging from 0.1 nM to 1 μM. Hypothalamic fragments were obtained from infantile male rats and exposed to progressively increasing concentrations of metastin(45–54) (0.1 nM to 1 μM) for 1-hour periods. In both experiments, GnRH release was measured by radioimmunoassay (RIA). The release of metastin from hypothalami obtained from infantile and adult male rats was also determined. Explants were incubated for 6 hours, and the release of metastin into the media was determined by RIA. The results support the hypothesis that metastin stimulates GnRH secretion from the hypothalamus. The data indicate that an increase in the secretion of metastin, rather than the appearance of the receptor, is required for puberty onset. The results also suggest that metastin influences the GnRH-secreting neurons indirectly via an interneuron rather than acting directly on the GnRH-secreting neurons.

Key words: Puberty, sexual maturation, kisspeptin.
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The sexual maturation of the male is the result of a complex interplay among the hypothalamus, anterior pituitary, gonads, and secondary sexual organs (Nazian and Mahesh, 1980; Grumbach et al, 1990). At one time or another, a change in each of these components was considered to be the "cause" of the onset of puberty. Currently, most researchers stress that the change in the pulsatile secretion of luteinizing hormone (LH) is the critical event (Terasawa and Fernandez, 2001). Pulsatile release of LH is, in turn, principally driven by a pulsatile release of GnRH from the hypothalamus, which undergoes a corresponding alteration in pattern at puberty (Bourguignon et al, 1987, 1993). Thus, the current consensus would suggest that puberty is initiated by a change in hypothalamic function.

A genetic analysis was recently performed on a consanguineous family, some of whose members had idiopathic hypogonadotropic hypogonadism. Affected patients were homozygous for a mutation of the GPR54 gene (Seminara et al, 2003). This gene codes for a so-called orphan receptor that is known to be G protein coupled. A GPR54-deficient mouse model created as the result of this discovery appeared to have the murine equivalent of hypogonadotropic hypogonadism. Both humans and mice lacking the GPR54 gene responded to GnRH injections with both LH and follicle-stimulating hormone (FSH) release. As a result, these investigators suggested that the expression of this receptor was essential for puberty onset. The naturally occurring ligand for the GPR54 receptor appears to be the protein products of the KiSS-1 gene, a metastasis suppressor gene for melanoma cells (Kotani et al, 2001; Terao et al, 2004). This gene is present in a variety of tissues, including the hypothalamus, but is particularly abundant in the placenta (Dun et al, 2003; Horikoshi et al, 2003). Posttranslational processing of the human KiSS1 peptide results in a carboxy-terminally amidated 54 amino acid peptide that has been designated metastin. Rat and mouse metastins are quite similar but contain only 52 amino acids (Kotani et al, 2001; Terao et al, 2004). When examined for their ability to activate the GPR54 receptor, it became apparent that fragments containing only the final 10 amino acids, metastin(45–54), were active. Human metastin(45–54) and rodent

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metastin(45–54) are identical except for position 54, where the rodent form has a tyrosine substituted for phenylalanine (Terao et al, 2004).

Indirect evidence supports the hypothesis that metastin and the GPR54 receptor stimulate the onset of puberty by increasing LH secretion via induction of GnRH release. Treatment of either rhesus monkeys (Shahab et al, 2005) or rats (Irwig et al, 2004) with metastin resulted in an increase in serum LH. Pretreatment with a GnRH antagonist before the administration of metastin prevented this response; however, GnRH was not measured. The studies reported here were designed to test this hypothesis directly by examining the release of GnRH from a GnRH-secreting cell line and from rat hypothalamic explants treated with metastin.

Materials and Methods

GT1-7 Cells

An aliquot of the GT1-7 cell line was originally obtained from Dr Pamela Mellon (Mellon et al, 1990). After thawing pass 6 cells from liquid nitrogen, the cells were cultured and maintained as described earlier (Nazian et al, 1994). Before use, approximately 8×10^5 cells in 1 mL of maintenance media were incubated overnight in 12 well plates. The media was then removed and replaced with 1.0 mL of Opti-MEM (GIBICO Laboratories, Grand Island, NY) for 48 hours. On the day of the experiment, the media was removed and replaced with 0.5 mL of Opti-MEM containing 0.1% bovine serum albumin (BSA), gentomycin (100 mg/L), bacitracin (50 μM), and various amounts of synthetic human metastin(45–54) (Sigma-Aldrich, St Louis, Mo). Doses ranged from 0.1 nM to 1 μM. After 2½ hours of incubation, the media was removed and replaced with identical media and was incubated overnight. Control wells received media without added metastin, which was also changed at 2½ hours. The next day, the media was removed and each well was challenged with plain media containing 60 mM potassium chloride (KCl) for 40 minutes to confirm that these cells were capable of releasing GnRH in response to a depolarizing stimulus. Media was frozen (−70°C) until assayed for GnRH by radioimmunoassay (RIA).

Hypothalamic Incubation

Hypothalamic explants were incubated as described previously (Nazian, 1992; Nazian et al, 2000). Briefly, male rats were anesthetized with pentobarbital and the hypothalamus dissected out, bisected, and placed in an acid-washed incubation vial containing plain medium (Dulbecco's Modified Eagle Medium with 0.1% BSA, penicillin/streptomycin and bacitracin). Vials were placed in a Dubnoff shaking incubator at 37°C and gassed with a 95% oxygen and 5% carbon dioxide mix. Three 30-minute preincubation periods preceded the experimental use of the explants. At the end of each of these periods, media was removed, discarded, and replaced with fresh plain media. The hypothalamus was delimited by the lateral hypothalamic sulci, the mammillary bodies, a line 1 to 2 mm anterior to the rostral border of the optic chiasma and the dorsal border of the thalamus as visualized in the sagittal plane. The use of animals in the experiments described here was performed with the permission of the University of South Florida Institutional Animal Care and Use Committee.

The first hypothalamic experiment examined the ability of the bioactive fragment of human metastin to elicit GnRH secretion. Hypothalami were obtained from infantile rats (16 to 17 days old). They were incubated for 1 hour in plain media and then incubated in progressively increasing concentrations of human metastin(45–54) for 1 hour. The final incubation consisted of a 30-minute period in media containing 60 mM KCl. Time controls were incubated in plain media, changed hourly, and incubated in KCl for the final 30 minutes. Media was frozen until assayed for GnRH by RIA.

The second hypothalamic experiment examined the ability of hypothalami obtained from infantile (16 to 17 days old) and adult (>120 days old) male rats to release metastin into the media. Hypothalami were obtained as described above, and 2 hypothalamic equivalents (4 halves) were placed into each incubation vial and preincubated for three 30-minute periods. After 6 hours of incubation in plain media, the media was removed and replaced with media containing 60 mM KCl for 1 hour. Media was frozen at −70°C until assayed.

RIAs and Statistics

GnRH was measured by double-antibody RIA as described previously (Nazian, 1992; Nazian et al, 2000). Dr Allen Root provided the first antibody (Copeland et al, 1979; Rettig et al, 1981). Radioiodinated GnRH was prepared by the chloramine-T method as described elsewhere (Nett et al, 1973).

Rabbit anti-human metastin(45–54), radioiodinated human metastin(45–54), and rat metastin were obtained from Phoenix Pharmaceuticals (Bebmont, Calif) and used in a double-antibody RIA. Assay buffer consisted of 0.1% BSA and 0.1% Triton-X 100 in phosphate-buffered saline (PBS) (pH = 7.5). Standards or incubation media samples were first diluted with sufficient buffer to bring the volume to 0.5 mL. First antibody was diluted to 1:3000 with 1:400 normal rabbit serum (NRS) in EDTA (0.05 M)-PBS with 200 μL added to each tube. Standards and unknowns were allowed to incubate overnight in the refrigerator, after which 100 μL of trace in assay buffer (~12 000 counts per minute [CPM]) was added, resulting in a final tube dilution of first antibody of 1:12 000. Second antibody was diluted appropriately with assay buffer and was added 24 hours later and allowed to incubate for 72 hours. All assay incubations were performed in the refrigerator. Three milliliters of cold PBS was added to each tube, followed by centrifugation in a refrigerated centrifuge at approximately $1500 \times g$ for 35 minutes. The supernatant was aspirated, and an additional 4 mL of cold PBS was added to each tube, followed by a final 35-minute centrifugation. The following preparations were examined for cross-reactivity in this assay: human metastin(45–54), rat metastin(45–54), rat metastin, incubation media, incubation media with added rat metastin,

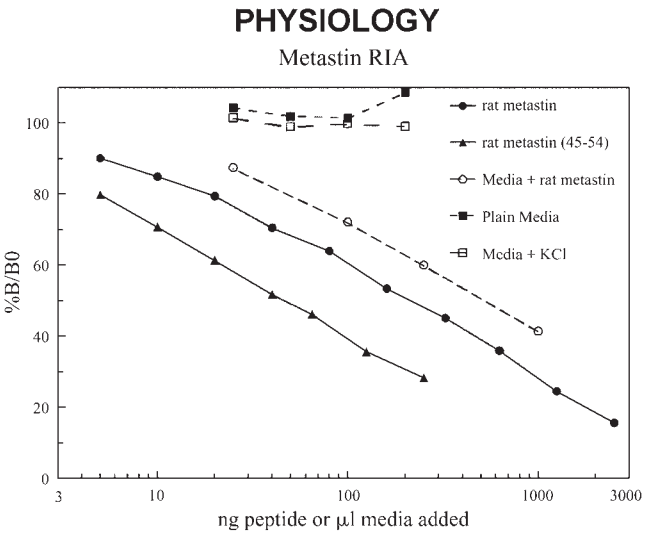

Figure 1. Standard curve and cross-reactivity results of the rat metastin radioimmunoassay. Rat metastin(45–54) in assay buffer and rat metastin in either incubation media showed parallel displacement curves to rat metastin in assay buffer. Incubation media alone, with or without 60 mM KCl, did not cross-react under these assay conditions.

and incubation media containing 60 mM KCl. For both GnRH and rat metastin, all samples from a given experiment were run in the same assay and at the same volume of unknown.

The data obtained in these studies are illustrated as mean ± SEM. Data were evaluated and significance was determined by an analysis of variance followed by the Student-Newman-Keuls test for multigroup comparisons. Student's *t* test was used for all comparisons between the 2 groups.

Results

Metastin RIA

A representative standard curve and cross-reactivity results are shown in Figure 1. Under these conditions, approximately 50% of the radioiodinated rat metastin(45–54) added to the assay was bound in the absence of unlabeled rat metastin. The minimum detectable dose (defined as the dose displacing 5% of the bound radioactivity, ie, the 95% bound point) averaged 2.8 ng per tube during the last 3 assays. Rat metastin either in buffer or incubation media, rat metastin(45–54), and human metastin(45–54) showed significant cross-reactivity in this assay, whereas plain incubation media or media containing 60 mM KCl did not.

GnRH Secretion From GT1-7 Cells

Incubation of GT1-7 cells for 2½ hours with doses of metastin(45–54) ranging from 100 pM to 1 μM did not significantly alter the secretion of GnRH into the media

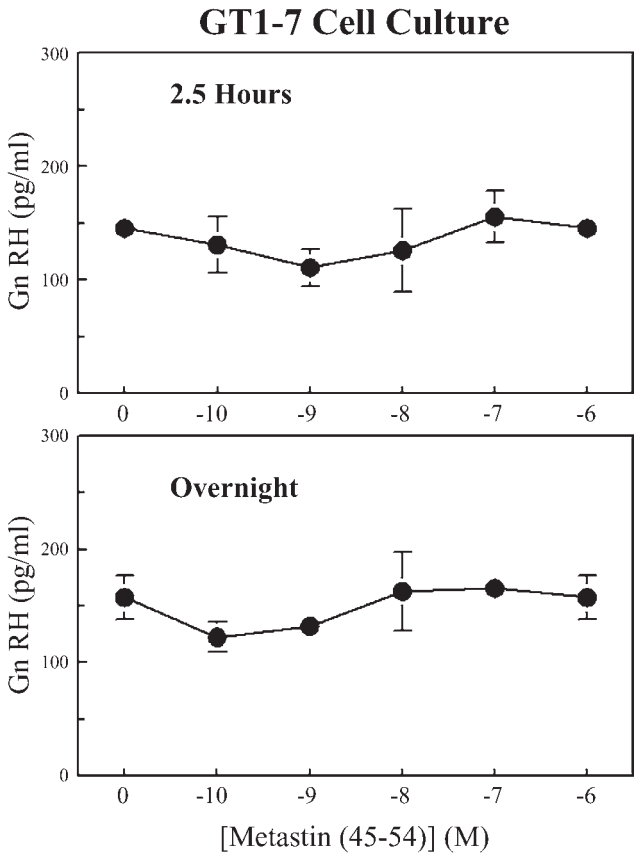

Figure 2. Secretion of GnRH (pg/mL) into the media from GT1-7 cells after 2½ hours (upper panel) or overnight (lower panel) incubation with plain media (0) or metastin(45–54) in concentrations ranging from 0.1 nM to 1 μM. Each point represents mean ± SEM of 3 to 4 wells.

compared with controls that were incubated with plain media (Figure 2, upper panel). Continuing the incubation overnight had no additional effect (Figure 2, lower panel). All wells responded to KCl with an increase in GnRH secretion (data not shown). When corrected to secretion per milliliter per hour, wells challenged with KCl typically released 100% more GnRH compared with incubation with metastin(45–54). The poorest response was a 39% increase; the strongest response was a 300% increase.

GnRH Secretion From Hypothalamic Explants

Infantile hypothalami incubated for 6 hours in plain media maintained a relatively constant release of GnRH from hour to hour (Figure 3). All hypothalami significantly increased their release of GnRH in response to 60 mM KCl treatment at the end of the experiment, indicating that all hypothalami were viable throughout the experiment. When exposed to increasing amounts of metastin(45–54), hypothalamic fragments

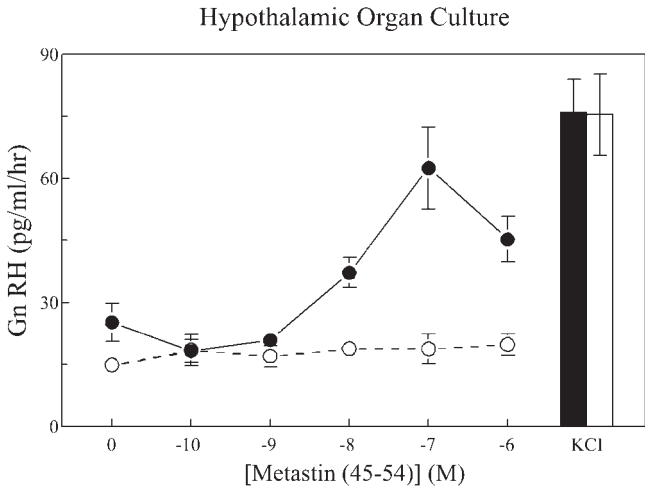

Figure 3. Secretion of GnRH (pg/mL/h) from infantile rat hypothalami exposed for 1 hour to progressively increasing concentrations of metastin(45–54) starting with plain media (0) and subsequently ranging from 0.1 nm to 1 μm and followed by 30-minute incubation with 60 mM KCl (solid circles and bar). Time controls (open circles and bar) received media changes of plain media every hour. Each point represents the mean ± SEM of 14 (metastin treated) or 6 (time control) bisected hypothalami. $*P < .05$; $**P < .01$ vs time control.

released increasing amounts of GnRH (Figure 3). Treatment with 100 nM or 1 μM of metastin(45–54) resulted in a significant ($P < .05$) increase in the amount of GnRH released into the media when compared with the initial incubation in plain media. When compared with the appropriate time control (incubated in plain media throughout), hypothalami exposed to 10 nM, 100 nM, or 1 μM metastin(45–54) released significantly ($P < .01$) more GnRH. All hypothalami significantly increased their release of GnRH in response to 60 mM KCl.

Metastin Secretion From Infantile and Adult Hypothalami

When incubated for 6 hours in plain media, hypothalamic explants obtained from adult male rats released significantly ($P < .01$) more metastin into the media than did hypothalami obtained from infantile rats (Figure 4). Upon challenge with 60 mM KCl, all hypothalami were capable of significantly ($P < .01$) increasing their release of metastin. The response to KCl was somewhat larger in hypothalami obtained from infantile rats than from adult hypothalami ($P < .05$).

Discussion

Hypothalami obtained from 15- to 16-day-old rats showed a clear dose related response to metastin(45–54). These results provide direct evidence that, in the rat, metastin acts to stimulate gonadotropin secretion by

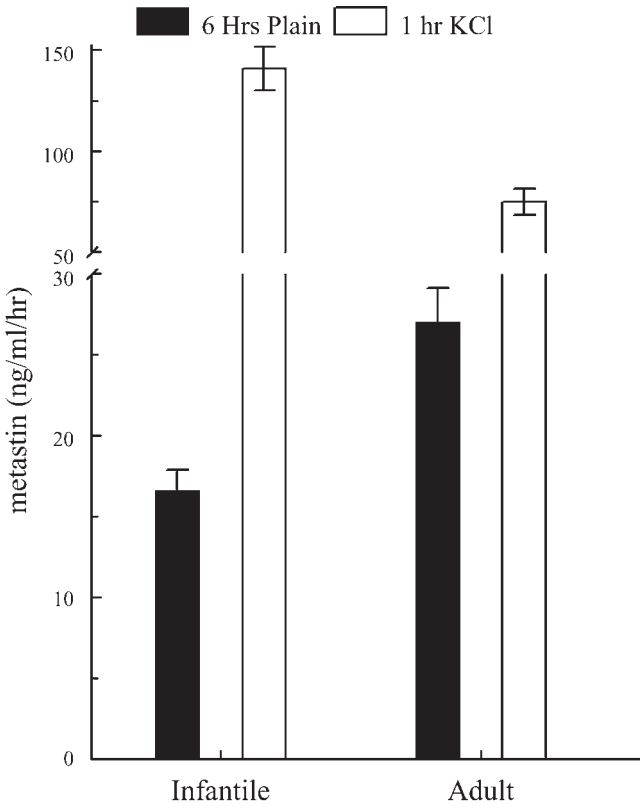

Figure 4. Secretion of metastin (ng/mL/h) by hypothalami obtained from infantile or adult male rats for 6 hours (solid bars) followed by 30-minute incubation in media containing 60 mM KCl (open bars). Bars represent mean ± SEM of 10 vials containing 2 infantile hypothalami or 7 vials containing 2 adult hypothalami.

acting on the hypothalamus to increase GnRH secretion. This is supported by observations in the sheep that a single dose of metastin(45–54) infused directly into the third ventricle resulted in an increase in GnRH in the cerebrospinal fluid (Messager et al, 2005).

It was originally hypothesized (Seminara et al, 2003) that the expression of the GPR54 protein was the initiating, or at least an essential, component of the onset of puberty. The results reported here indicate that the expression of this receptor cannot be the initiating event. The experiment shown in Figure 3 clearly indicates a response to metastin and thus the presence of a receptor. The donors of the hypothalami used to produce these data were clearly infantile. Puberty onset by any of a number of systemic criteria would not have occurred for a number of weeks (Nazian and Mahesh, 1980; Grumbach et al, 1990; Nazian and Cameron, 1992, 1999; Nazian et al, 2000). Even from a hypothalamic standpoint these donors were prepubertal, for the earliest reported alterations in pulsatile release of GnRH from isolated rat hypothalami is 23 days of age (Bourguignon and Franchimont, 1984). The idea that expression of the GPR54 receptor is required for sexual

maturation is supported by the observations that mice and humans without the gene for this receptor have primary hypogonadotropic hypogonadism (Seminara et al, 2003). Thus, these data suggest that an increase in the secretion of metastin may be the initiating event for puberty in the male rat. Precise correlation of any such rise with the demonstrable events of puberty onset (eg, initiation of spermatogenesis, peripubertal testosterone rise) will be required to corroborate this suggestion. A similar situation may also hold for the rhesus monkey, where metastin administration to juvenile monkeys also results in an increase in LH secretion (Shahab et al, 2005).

If this hypothesis is correct, then hypothalami obtained from adult animals should secrete more metastin than hypothalami obtained from animals before the onset of puberty. As shown in Figure 4, this is plainly the case. These data thus support the idea that it is an increase in metastin secretion and not an alteration in receptor function that is the critical event in the male rodent. The situation in females may be more complex than this, at least in primates. Hypothalami from midpubertal male rhesus monkeys contained more messenger RNA (mRNA) coding for metastin than did hypothalami from juveniles, though there was no change in the mRNA for GPR54. In females, by contrast, mRNA for both metastin and for the receptor increased with sexual maturation (Shahab et al, 2005).

The observation that hypothalami obtained from infantile rats responded to a depolarizing exposure to KCl with a greater release of metastin into the media than did hypothalami from adult rats was unanticipated. It is possible to speculate that this was due, in part, to a greater starting content of metastin in the hypothalami of immature male rats. In theory, 60 mM KCl should depolarize all cells present, resulting in the release of, in this case, all the metastin available for secretion. Because hypothalamic content was not determined in these experiments, this must remain a matter of speculation.

Previous studies using GT1-7 cells have indicated that they have many properties of neuronal cells. They express neuronal but not glial cell markers, develop long process that appear to "synapse" with adjacent cells, and produce and process an appropriate precursor into GnRH (Mellon et al, 1990; Liposits et al, 1991; Wetsel et al, 1991). They also appear to function in vitro in a fashion similar to that of the GnRH-secreting neuron in vivo. They release GnRH in a pulsatile fashion and respond appropriately to adrenergic, dopaminergic, and opioid treatment (Martinez de la Escalera et al, 1992a,b,c; Krsmanovic et al, 1993; Nazian et al, 1994). The inability of metastin(45–54) to influence GnRH secretion from these cells suggests the possibility that metastin does not act directly on the GnRH-secreting neuron but rather through an interneuron. In contrast, Messager et al (2005) have reported that the GRP54 receptor is found in cells that also contain GnRH. They examined mice lacking the GPR54 gene and followed a reporter gene found in these mice. Approximately half (55%) of the cells containing GnRH also expressed the reporter gene. In the rhesus monkey (Shahab et al, 2005), both mRNA coding for GnRH and mRNA coding for the metastin receptor were found in the arcuate nucleus. Although there appeared to be some overlap, the patterns of distribution were different. The apparent differences between these later observations and the results reported here may simply be attributed to the difficulties inherent in the use of an immortalized cell line, particularly GT1-7 cells (Martinez de la Escalera et al, 1992b; Nazian et al, 1994). One of these difficulties is demonstrated in Figure 2. Although clearly viable (as demonstrated by their ability to respond to KCl with substantial GnRH release), cells uniformly reduced their basal secretion of GnRH as the incubation time was prolonged. This may be due to an accumulation of GnRH in the media, for it has been demonstrated that GnRH agonists are capable of inhibiting GnRH secretion from both hypothalamic explants (Bourguignon et al, 1987, 1993) and GT1-7 cells (Krsmanovic et al, 1993).

On the basis of the results from this study, it seems likely that a necessary component of the initiation of sexual maturation in the male rat is a rise in the secretion of metastin from the appropriate hypothalamic neurons. Although not demonstrated here, it is presumed that this elevation stimulates, either directly or more likely indirectly via an interneuron, a corresponding increase in the pulsatile release of GnRH into the hypothalamo-pituitary portal system. This, in turn, leads to an increase in LH and FSH secretion. These increases then stimulate the maturation of the testes with subsequent initiation of spermatogenesis, the peripubertal rise in testosterone, and secondary sexual organ maturation.

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