Endokrynologia Polska / Polish Journal of Endocrinology
Tom/Volume 56; Numer/Number 6/2005
ISSN 0423-104X

Wpływ comiesięcznego podawania GHRH (fragment 1-29) za pomocą pompy osmotycznej na przedni płat przysadki szczura

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Streszczenie

Somatoliberyna (GHRH) jest głównym czynnikiem pobudzającym wydzielanie hormonu wzrostu (GH) i proliferację komórek somatotropowych, natomiast jej przewlekły wpływ na komórki przedniego płata przysadki nie jest tak jednoznaczny. Wiadomo, że u chorych z wyspiakiem trzustki wydzielającym GHRH dochodzi do rozwoju akromegalii i hiperplazji komórek przedniego płata przysadki. U myszy transgenicznych dla GHRH dochodzi natomiast do rozwoju gruczolaków somatotropowych. Celem naszej pracy była ocena wpływu przewlekłego podawania GHRH na aktywność wydzielniczą komórek somatotropowych, ich liczbę oraz proliferację komórek przedniego płata przysadki. Doświadczenie przeprowadzono na szczurach samcach szczepu Fischer 344 o masie ciała 200 ± 20 g. Zwierzęta podzielono na 2 grupy: grupa I – kontrola (13 zwierząt) otrzymywała rozpuszczalnik dla GHRH - 5% etanol w wodzie demineralizowanej; grupa II (10 zwierząt) otrzymywała GHRH (Growth Hormone Releasing Factor, fragment 1-29 amide) w dawce 5 µg/dobę. Substancje były podawane przez miesiąc za pomocą pomp osmotycznych firmy ALZET, które wszczepiono zwierzętom podskórnie w okolicę grzbietu w znieczuleniu ketaminą. Po 4 tygodniach zwierzęta uśmiercono przez dekapitację. Od wszystkich zwierząt pobrano krew celem oznaczenia stężenia szczurzego GH (rGH) (metodą RIA). W preparatach mikroskopowych przysadek barwionych metodą tetrachromową Herlanta oceniano morfologię przysadek, a w preparatach barwionych immunohistochemicznie - odsetek komórek somatotropowych i proliferację komórkową (indeks PCNA).

Wykazano, że przewlekłe podawanie GHRH powoduje znamienny wzrost średniego stężenia rGH w surowicy krwi zwierząt oraz odsetka komórek somatotropowych w przysadce, nie zmieniając wskaźnika proliferacji komórkowej w porównaniu z grupą kontrolną.

Podsumowując, miesięczne podawanie GHRH nie indukuje rozwoju gruczolaków somatotropowych, powoduje natomiast wzrost stężenia GH w surowicy krwi zwierząt, co przynajmniej częściowo wydaje się zależeć od wzrostu odsetka komórek somatotropowych.

(Endokrynol Pol 2005; 6(56): 927-932)

Słowa kluczowe: *przysadka, proliferacja, somatoliberyna, komórki somatotropowe*

Endokrynologia Polska / Polish Journal of Endocrinology
Tom/Volume 56; Numer/Number 6/2005
ISSN 0423-104X

Effect of monthly administration of GHRH (fragment 1-29) with osmotic pump on the rat anterior pituitary

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Abstract

Growth Hormone-Releasing Hormone (GHRH) is the main factor, which regulates GH secretion and somatotrope proliferation. However, its chronic effect on anterior pituitary gland is still unknown. It is known that excessive GHRH secretion in patients with gastroenteropancreatic tumors secreting GHRH results in acromegaly and somatotrope hyperplasia. In mice transgenic for GHRH somatotrope tumors develop. Thus, the aim of this paper was to examine the effect of GHRH chronic administration on somatotrope secretion, their percentage and cell proliferation in anterior pituitary gland in rats. The experiment was performed on male Fischer 344 rats weighing 200 ± 20 g. The animals were divided into two groups: group I – controls (13 rats) received solvent for GHRH (5% ethanol in demineralized water); group II (10 rats) received GHRH (Growth Hormone Releasing Factor, fragment 1-29 amide) at a dose of 5 μg/day. The substances were given for 1 month via osmotic pump (ALZET), which were implanted subcutaneously in the dorsal region under ketamin anesthesia. After 4 weeks all rats were decapitated and the blood was collected. In the microscopic preparations of anterior pituitary gland the morphology of pituitary (Herlant staining) and the percentage of somatotrope cells and proliferation index based on PCNA staining were assessed.

It was found that the chronic treatment with GHRH caused a statistically significant increase in serum rGH concentration and in percentage of somatotropes, but did not change proliferation index and did not induce pathological changes in the morphology of the anterior pituitary gland when compared to the control group.

Summing up, monthly GHRH administration did not induce somatotrope adenomas but it caused serum GH level elevation, what seems to depend partially on the increase of somatotrope number.

(Pol J Endocrinol 2005; 6(56): 927-932)

Key words: *pituitary gland, proliferation, somatorelin, somatotrope*

Aknowledgments
This work was supported by the statutory funds of Medical University of Łódź number 503-184-3 and 503-584-1.

Introduction

Somatorelin – GHRH (Growth Hormone Realising Hormone) is a 44 amino acid neurohormone stimulating synthesis and secretion of growth hormone (GH) in the pituitary gland and is produced in hypothalamic nuclei and other tissues. It was isolated in 1982 by 2 independent groups of researchers: Guillemin et al. [1] and Rivier et al. [2] from pancreatic islet-cell tumors causing acromegaly. The first isolation and structural characterization of somatorelin from human hypothalami was carried out by Ling et al. in 1984 [3]. Somatorelin regulates not only the function of somatotropes, but also their proliferation [4]. This hormone acts through its specific G-protein coupled receptor (GHRH-R), which activates the adenylyl cyclase to generate cAMP [5]. Beside GHRH, the secretion of growth hormone is also controlled by other factors like somatostatin, ghrelin and somatomedin C (IGF-I). Somatorelin stimulates cell proliferation in several non-pituitary tissues, both normal and neoplastic, acting indirectly trough GH and IGF-I and directly as an auto- and paracrine factor [6]. The same conclusions result from the studies with GHRH antagonists (in vitro and in vivo), which inhibit tumor growth through the a.m. mechanisms [7-9]. Recently many other functions of GHRH were suggested. This hormone is involved in sleep regulation, especially the non-REM phase [10]. The role of GHRH in the counteraction of aging is also discussed [11].

Although the number of publications concerning the effect of GHRH on somatotrope proliferation is limited, the experimental studies of animal models with genetically modified hypothalamo-pituitary GH axis provide some additional information. The animals with diminished production of GHRH (*Tgr* rat [12], TH-hGH mouse [13], *Gsh-1* gene disrupted mouse [14], GHRHKO mouse [15]) and with impaired GHRH signaling ("little" (*lit*) mouse [16-17], "dwarf" (*dw*) rat [18], CREB-mutant transgenic mouse [19]) are characterized by the hypoplastic pituitary caused by the decreased number of somato-

tropes. Several mutations in the GHRH-R gene were also identified in children with growth retardation and reduced pituitary size observed in the MRI pictures, probably connected with hypoplasia of somatotropic cells [20-21]. On the other hand, the experiments with transgenic mouse overexpressing GHRH (metallothionein – human growth hormone-releasing hormone (MT-hGRH) transgene) [22-26] and clinical observations in patients with ectopic GHRH secretion [27-33] give information about the chronic effect of supraphysiological level of endogenous somatorelin. Hyperplasia of somatotropic cells or even somatotrope tumor development observed in a.m. studies suggest the role of somatorelin in the control of pituitary cell proliferation and pituitary tumorigenesis.

All these findings indicate the importance of GHRH not only in the proper development and function of pituitary, but also in pathogenesis of somatotrope adenoma.

However, some diagnostic difficulties, such as problems with distinction between hyperplastic and adenomatous changes and the lack of sufficient amount of material to examine, contribute to the problems with defining the role of GHRH and other hypothalamic factors in the pituitary adenoma formation.

The effects of chronic GHRH administration in animals and humans remain still unknown. Therefore, the aim of this paper was to examine the chronic (monthly) effect of GHRH on somatotrope secretion, their percentage and cell proliferation in the anterior pituitary gland in rats.

Materials and methods

The experiment was performed on 50 male Fisher 344 rats weighing 200 ±20 g. The animals were divided into 2 groups: group I – controls (C) (13 rats) received solvent for GHRH (5% ethanol in demineralized water); group II (10 rats) received GHRH (Growth Hormone Releasing Factor, fragment 1-29, ICN Biomedicals Inc.) at a dose of 5µg/day. The substances were given for 1 month via osmotic pumps (ALZET, model 2004), which were implanted subcutaneously in the dorsal region of rat under ketamin anesthesia (Ketanest; PARKE-DAVIS; at a dose 120 mg/kg b.w. intraperitoneally). The model 2004 of Alzet pump is able to deliver the given dose of examined substance during 28 days.

After 4 weeks all rats were decapitated and the blood was collected to measure the serum rat GH (rGH) concentration using the Rat Growth Hormone (rGH) [^{125}I] Biotrak Assay System with Magnetic Separation (Amersham Biosciences). The pituitaries were removed, fixed in Bouin-Holland's solution, weighed and underwent the routine histopathological procedure. The paraffin-embedded sections were stained according to tetrachrome Herlant's technique to assess the gland morphology. The paraffin-embedded sections were also used for immunohistochemistry. The antibodies against rGH (Growth Hormone, Sheep Polyclonal Antibody, ICN Biomedicals Inc.) were applied to detect somatotropes and the antibody against Proliferating Cell Nuclear Antigen (PCNA) (Mouse Anti-Proliferating Cell Nuclear Antigen, PC10; DAKO) were used to evaluate proliferation. The sections with the primary antibodies were incubated for 24 hours at the temperature of 4ºC. The primary antibodies for rGH were then detected using the appropriate biotinylated secondary antibodies. The immunoreaction was visualized by the using of the streptavidin-peroxidase complex (StreptABC/HRP, Dako) and 3, 3'-diaminobenzidine. The immunoreaction for PCNA was visualized using En Vision System AP (Dako). The number of somatotropes (GH-positive cells) was calculated per 400-500 pituitary cells from randomly selected sections and was expressed in percentages. The number of PCNA-positive cells was calculated per 1000 cells of anterior pituitary and was expressed in promilles.

Results

The results were showed in Figures 1-4.

It was found that monthly administration of GHRH to rats caused a small, statistically insignificant, increase in body weight in comparison with the average body weight of animals in the control group (Fig. 1). In our experiment somatorelin did not change the weight of pituitary gland (Fig. 2), but it caused an increase in mean rGH serum concentration compared to the control group (Fig. 3). However, it should be emphasized that the rGH serum concentration in the group treated with GHRH varied considerably from animal to animal (17.9 ng/ml - 169.69 ng/ml), while it did not occur in the control group (Table I).

Chronic administration of GHRH led to the increase in somatotrope percentage (Fig.4-6) without the enhancement of total proliferation index (based on PCNA staining) of the anterior pituitary gland (Fig.7). Only a small, statistically insignificant rise in the number of PCNA-positive cells was stated in the group treated with GHRH. There were not found any pathological changes in morphology of the anterior pituitary gland in the GHRH treated group except foci of increased vascularisation in 2 of 10 pituitaries.

Discussion

To assess the chronic effect of GHRH on the somatotrope secretion, their percentage and cell proliferation in anterior pituitary gland in rats, we applied the osmotic pump. The pumps assure an optimal way of chronic administration of the investigated

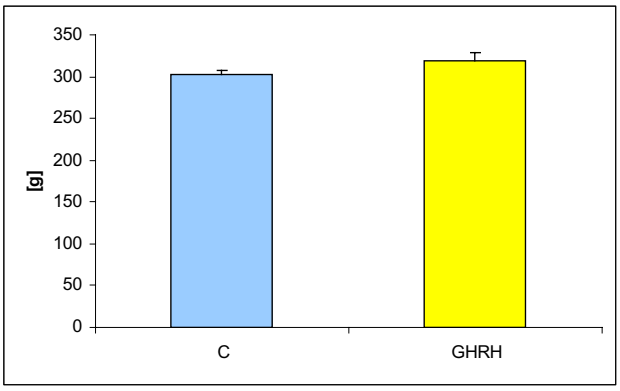

Fig. 1 *Effect of GHRH on rat body weight (X ± SEM)*

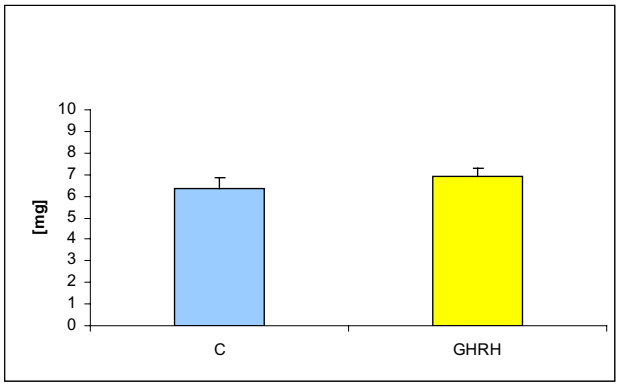

Fig. 2 *Effect of GHRH on rat pituitary weight (X ± SEM)*

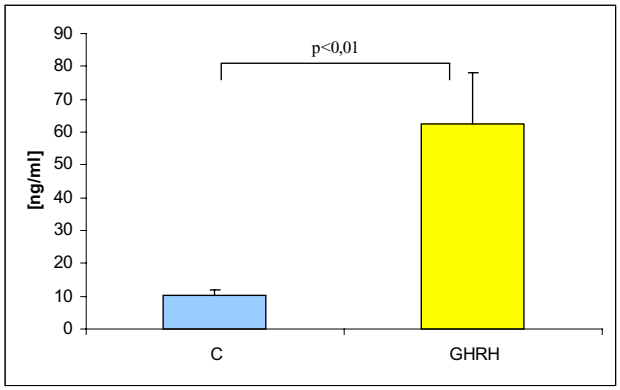

Fig. 3 *Effect of GHRH on rGH serum concentration (X ± SEM)*

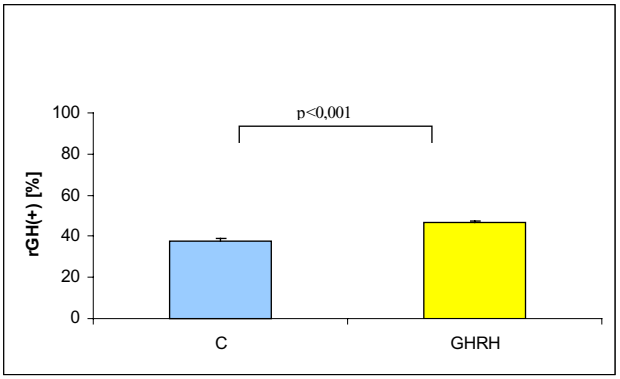

Fig. 4 *Effect of GHRH on the percentage of somatotropes (X ± SEM)*

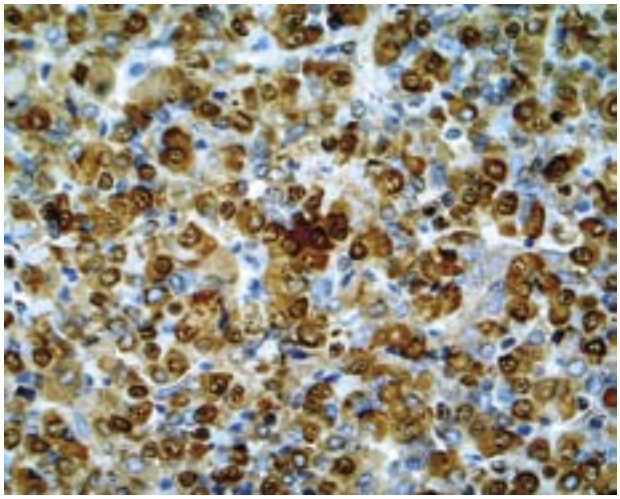

Fig. 5 *Microphotograph of pituitary gland from the rat treated with GHRH immunostained for rGH. (x400)*

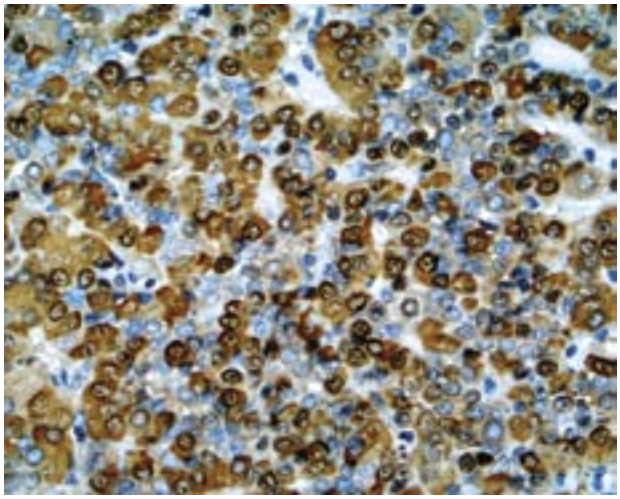

Fig. 6 *Microphotograph of pituitary gland from the rat of control group immunostained for rGH. (x400)*

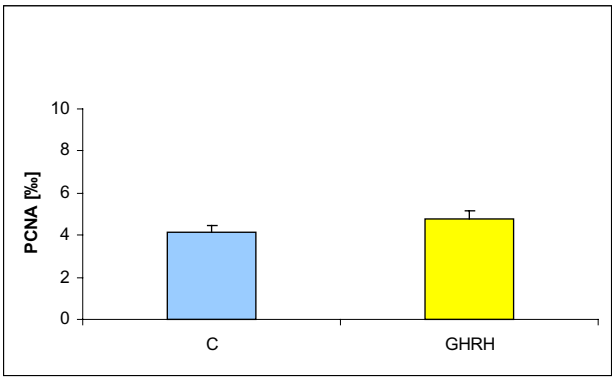

Fig. 7 *Effect of GHRH on rat anterior pituitary cell proliferation (X ±SEM)*

substances. They were implanted subcutaneously in dorsal region and guaranteed a steady release of compounds, great repeatability of doses, which resulted in the constant level of the examined

Tab. I *r-GH serum concentration [ng/ml]*

	controls	GHRH
	9,42	45,22
	14,25	169,69
	5,18	69,64
	11,65	37,78
	19,17	34,17
	7,86	89,15
	6,66	68,17
	10,27	31,89
	6,64	17,9
	5,57	
	16,75	
mean	10,31	62,62
sd	4,69	45,99

substance in the blood and tissues. Such way of application is especially important in experiments with compounds of short plasma half-time such as GHRH (T1/2 for the 200ug dose is 7.6min) [34]. The use of osmotic pump eliminates the fluctuations of substance concentration in the body and reduces stress as compared to the typical multiple injections.

It is well known that somatorelin enhances the synthesis and release of GH. However, to our knowledge its chronic effect has not been investigated so far. Our findings indicate that the monthly administration of GHRH caused an increase of mean rGH serum concentration compared to the control group. However, it should be stressed that the rGH serum concentration in the group treated with GHRH varied considerably from animal to animal, which might be attributed to the process of desensitization.

Although the proliferative effect of GHRH on somatotropes is widely accepted, it has not often been under investigation. The mitogenic influence of somatorelin on cultured somatotropes was showed in an in vitro study using autoradiographic detection of [3H]thymidine uptake and immunohistochemical identification of GH-producing cells. In this experiment somatorelin caused a substantial increase in the percentage of somatotropes labeled with [3H]thymidine compared to the control group and this effect seemed to be mediated by cAMP [4].

Other information about the proliferative effect of GHRH comes from observation of genetically modified animals. Mice transgenic for GHRH (metallothionein – human growth hormone-releasing hormone (MT-hGRH) transgene) are a classical model for investigations of the chronic effect of supraphysiological level of somatorelin. These animals are characterized by greater body weight and enlargement of pituitary gland with hyperplasia of somato- and/or lacto-and/or somatolacto- and/or thyreotrophs [22-26]. It was found that longer, 9-24 months lasting exposure to the great concentration of endogenous GHRH could lead not only to their hyperplasia, but also to somatotrope adenoma formation [22-23]. However, the modest increase in GHRH expression, as occurs in GH receptor gene disrupted mice (GHR-/- mice), is sufficient only to expand the somatotrope population but do not lead to the adenoma formation [24]. The appearance of adenoma preceded by somatotrope hyperplasia in animals generated from cross-breeding MT-hGHRH transgenic mouse with GHR-/- mice proved that the proliferative effect of GHRH on somatotropes is direct and independent of GH and IGF-I action [24].

The observations of patients with disorders of GHRH secretion or action confirme its proliferative effect. Patients with ectopic GHRH secreting tumors developed somatotrope hyperplasia [27-30] and in a few cases [31-33] somatotrope adenomas. From 30% to 40% of somatotrope adenomas are caused by an activating mutation in alfa subunit of Gs protein coupled with GHRH-R, which supports the role of GHRH in the control of pituitary cell proliferation [35].

The data imply that the duration of exposure to GHRH as well as its plasma concentration determine the variety of changes observed in the pituitary. In our experiment, the monthly administration of somatorelin induced only a rise in the percentage of somatotropes (immunohistochemical method) with small, statistically insignificant enhancement of the total pituitary cell proliferation (PCNA index) and without adenoma formation. Enhancement of GH synthesis resulting in the more visible immunoreactivity for GH could explain such result. It could also reflect the process of cytoredifferentation and transformation of other cells into GH producing cells. The increased somatotrope proliferation, although not statistically significant, cannot be excluded as a possible cause of increase of their percentage. The lack of changes in the weight of pituitary glands could be connected with imprecise measurements.

Surprising observations in our experiment were some foci of increased vascularisation in 2 of 10 pituitary glands in the group treated with GHRH. This result is the first mention in the literature concerning proangiogenic effect of chronic administration of somatorelin. These changes would not be noticed if it were not for the paralelly carried out studies with the newly synthesized GHRH analogs, which enhanced the angiogenesis considerably (unpublished observation from our laboratory). The studies with GHRH antagonists carried out in our laboratory seem to be in agreement with our observations of proangiogenic action of somatorelin. The GHRH antagonists exert antiangiogenic properties at least partially through the inhibition of vascular endothelial growth factor (VEGF) secretion and the suppression of endothelial cell proliferation [36]. It suggests an opposite proangiogenic action of GHRH.

The observed proangiogenic effect should be confirmed in further studies using the anti-CD 31

antibody in order to visualize immunohistochemically the endothelial cells in pituitary glands.

Conclusions

To sum up, monthly administration of GHRH caused serum GH level elevation, which seems to depend partially on the increase of somatotrope number. The role of GHRH in pituitary adenoma formation remains still unclear.

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