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Effects of Dietary Cholesterol on Pyroglutamyl Aminopeptidase Activity in Mouse Frontal Cortex, Pituitary, and Adrenal Glands

Abstract

Pyroglutamyl aminopeptidase (pGluAP) is an omega peptidase that hydrolyzes biologically active peptides, such as thyrotropin-releasing hormone (TRH), with neuronal and extraendocrine functions. We analyzed the effects of a cholesterol-enriched diet on soluble and membrane-bound pGluAP activity in frontal cortex, pituitary and adrenal glands of male and female mice using fluorimetric assays. Significant increases were observed in soluble pGluAP activity in the frontal cortex and adrenal glands in males and in the pituitary in females. Membrane-bound pGluAP activity was increased in the frontal cortex and pituitary of males and females after the mice were fed a cholesterol-enriched diet. These increases may produce changes in the metabolism of endogenous substrates, including TRH, which may be related to alterations in its neuromodulator functions and to the possible relationship between TRH and other neurotransmitter systems.

Key words

Thyrotropin-Releasing Hormone Degrading Ectoenzyme · Dietary Lipids · Sex Differences · Pyrrolidon Carboxypeptidase

Introduction

Pyroglutamyl aminopeptidase (pGluAP) has been classified as an omega peptidase that is widely distributed in fluids and tissues and removes pyroglutamyl (pyroGlu) N-terminal residues from peptides, proteins and arylamide derivatives in a highly selective manner [1]. To date, three distinct forms of pGluAP have been observed in mammalian tissues. The first, pyroglutamyl aminopeptidase type I (pGluAP I), is principally localized in the cytosolic compartment [2], and has a broad pyroglutamyl-substrate specificity. This enzyme has been shown to cleave the N-terminal pyroGlu residue from a range of biologically active peptides that include thyrotropin-releasing hormone (TRH), acid TRH, gonadotropin-releasing hormone (GnRH), neurotensin, bombesin and anorexigenic peptide *in vitro* [2]. Pyroglutamyl aminopeptidase type II (pGluAP II) has been shown to be a membrane-bound metalloenzyme that displays a narrow substrate specificity restricted to the pyroGlu-His bond of TRH or very closely related peptides [3]. A third pyroglutamyl aminopeptidase type, thyroliberinase, has also been observed in the serum of various mammalian species, and displays biochemical characteristics remarkably similar to those of tissue pGluAP II, with a narrow substrate specificity restricted to TRH or closely related peptides [4].

Our laboratory has performed some *in vivo* and *in vitro* studies analyzing the influence of dietary fats on different aminopeptidase activities, including pGluAP [5,6]. Furthermore, the influence of dietary cholesterol has been described in several neuronal and neuroendocrine processes in recent years, such as the development and evolution of cerebral ischemic processes [7], myelinization [8], neurotransmitter release [9], and Alzheimer's disease [10]. The aim of this work was to study the effects of dietary cholesterol on pGluAP (as a regulator of TRH metabolism) in the frontal cortex, pituitary and adrenal glands of mice fed on a

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Received 13 February 2002 · **Accepted after revision** 18 April 2002

Bibliography
Horm Metab Res 2002; 34: 431–434 © Georg Thieme Verlag Stuttgart · New York · ISSN 0018-5043

cholesterol enriched diet, and to analyze possible gender differences. TRH is not only a putative neuromodulator or neurotransmitter in the central nervous system; several important functions have been also attributed to TRH as an autocrine or paracrine regulator in these endocrine glands.

Material and Methods

Twenty male and twenty female Balb/C mice were used in this study. The animals were housed at constant temperature (25 °C) and day length (12 hours). The experimental procedures for animal use and care were performed in accordance with the European Community Council Directive (86/609/EEC). All animals were allowed access to water and food *ad libitum*, and were fed as follows over a period of 15 days: ten male and ten female (control groups) with body weight 26.76 ± 1.014 g and 22.80 ± 1.34 g, respectively, were fed a standard diet containing 15.6% protein, 2.8% fat and 55% carbohydrate. The other animals with body-weight 27.096 ± 0.93 g (males) and 23.37 ± 0.65 g (females) were fed the same diet, but enriched with 1% cholesterol and 0.5% cholic acid (cholesterol groups).

After this period, the animals were killed under equithesin anaesthesia (2 ml/kg body weight). Samples of frontal cortex, pituitary and adrenal glands were quickly removed and frozen on dry ice. To obtain the soluble fraction, tissue samples were homogenized in 10 volumes of 10 mM HCl-Tris buffer (pH 7.4) and ultracentrifuged at 100 000 × g for 30 min (4 °C). The resulting supernatants were used to measure soluble enzymatic activity and protein content, assayed in triplicate. To solubilize membrane proteins, the pellets were rehomogenized in HCl-Tris buffer (pH 7.4) plus 1% Triton X-100. After centrifugation (100 000 × g, 30 min, 4 °C), the supernatants were used to measure solubilized membrane-bound activity and proteins, also in triplicate. To ensure complete recovery of activity, the detergent was removed from the medium by adding to the samples adsorbent polymeric Biobeads SM-2 (100 mg/ml) (Bio-Rad, Richmond, CA) and shaking for 2 h at 4 °C.

Pyroglutamyl aminopeptidase activity was measured fluorimetrically using pyroGlu-β-naphthylamide (pyroGluNNap) as a substrate according to the modified method devised by Schwabe and McDonald [11]. Ten µl of each supernatant were incubated over 30 min at 37 °C with 1 ml of the substrate solution: 100 µM pyroGluNNap, 1.5 mM bovine serum albumin (BSA), 0.65 mM dithiothreitol (DTT) and 1.33 mM EDTA in 50 mM phosphate buffer at pH 7.4. The reactions were stopped by adding 1 ml of 0.1 M acetate buffer at pH 4.2. The amount of β-naphthylamine released as a result of the enzymatic activity was measured fluorimetrically at a 412-nm emission wavelength with an excitation wavelength of 345 nm. Proteins were quantified in triplicate using the method of Bradford [12], with BSA as a standard. Specific soluble and membrane-bound pGluAP concentration rates were expressed as pmol of pyroGluNNap hydrolysed per min per mg protein by using a standard curve prepared with the latter compound under corresponding assay conditions. The fluorogenic assay was linear with respect to time of hydrolysis and protein content.

Statistical analysis

To analyze the differences between control and cholesterol groups and possible gender differences, we used multiple analysis of variance (MANOVA) followed by the Newman-Keuls post-hoc test. All comparisons with p values below 0.05 were considered significant.

Results

Specific soluble and membrane-bound pGluAP activity in frontal cortex, pituitary and adrenal glands are shown in Figs. **1, 2, 3**.

In the frontal cortex, soluble pGluAP activity was significantly higher ($p < 0.01$) in males after having been fed the cholesterol-enriched diet, but no changes were observed for the corresponding AP activity in females. The membrane-bound form increased in both cholesterol groups in relation to control groups ($p < 0.05$).

In the pituitary, soluble and membrane-bound pGluAP activities were significantly higher in females ($p < 0.001$ and $p < 0.01$, respectively) after having been fed the cholesterol enriched diet. In males, soluble pGluAP did not change with cholesterol, but the results for membrane-bound activity showed a significant increase ($p < 0.001$) in the male cholesterol group (Fig. **2**).

In the adrenal, no changes were observed in the female groups in soluble and membrane-bound form, but in males, the cholesterol-enriched diet led to a significant increase in soluble pGluAP activity ($p < 0.01$). Membrane-bound pGluAP activity did not change in the male cholesterol group (Fig. **3**).

Discussion

In the present work, the effects of a cholesterol-enriched diet on soluble and membrane-bound pGluAP activity in frontal cortex, pituitary and adrenal glands of male and female mice were studied.

In general, soluble and membrane-bound pGluAP activity increased after cholesterol treatment in both males and females. Higher increases were observed in the pituitary with less prominent changes in adrenal glands. However, the results showed a differential effect on soluble, but not membrane-bound pGluAP activity in male vs. female animals. Thus, changes were detected in soluble pGluAP activity in male but not female frontal cortex and adrenal glands, whereas changes were observed in female but not male soluble pGluAP in the pituitary. Previous studies performed by us *in vitro* have demonstrated the influence of sex steroids on aminopeptidase activities [13,14]. Bauer [15] and Schomberg and Bauer [16] have also reported on the activity and the mRNA levels of pGluAP rapidly decreasing after estradiol injection (but not after injection of other steroid hormones) and, conversely, after ovariectomy, an increase in the enzymatic activity occurs. The present results corroborate the hypothesis that aminopeptidase activity may be regulated by hormonal status leading to gender differences in the metabolism and degradation of various neuromodulator substances. Furthermore, it has been described that cholesterol can be converted to steroid hormones

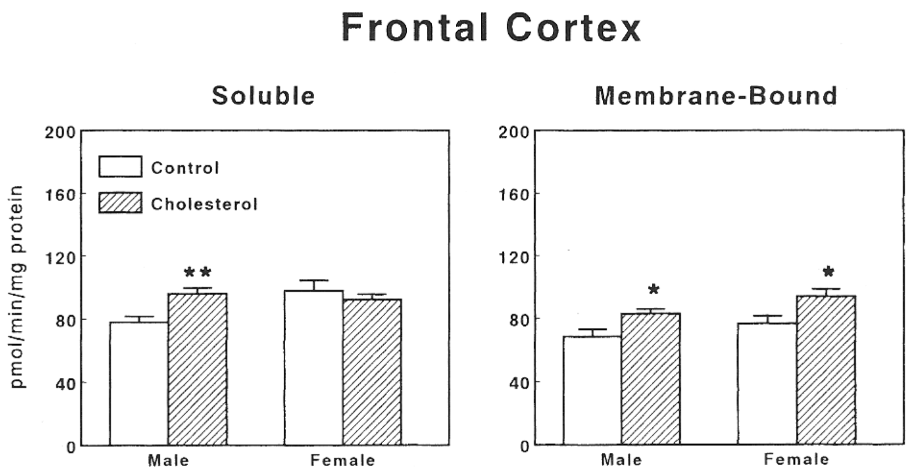

Fig. 1 Specific soluble and membrane-bound pyroglutamyl-aminopeptidase activities in frontal cortex of control and cholesterol groups (Mean ± SEM) (*$p < 0.05$; **$p < 0.01$).

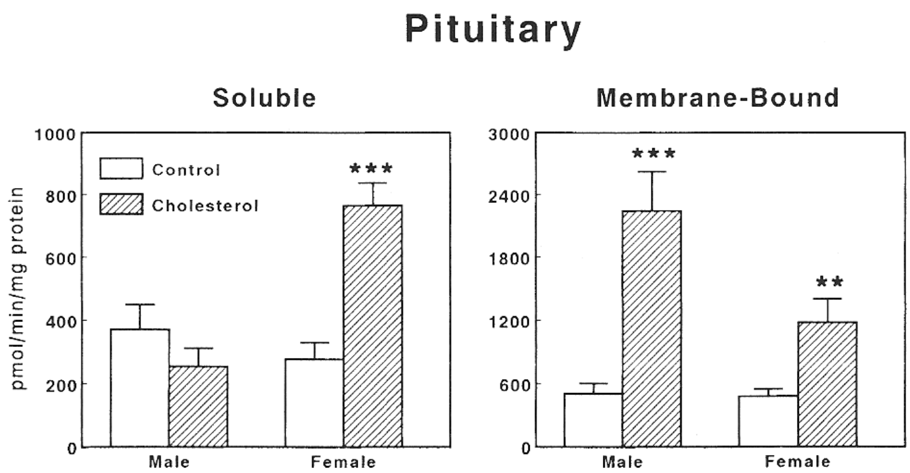

Fig. 2 Specific soluble and membrane-bound pyroglutamyl-aminopeptidase activities in pituitary of control and cholesterol groups (Mean ± SEM) (**$p < 0.01$; ***$p < 0.001$).

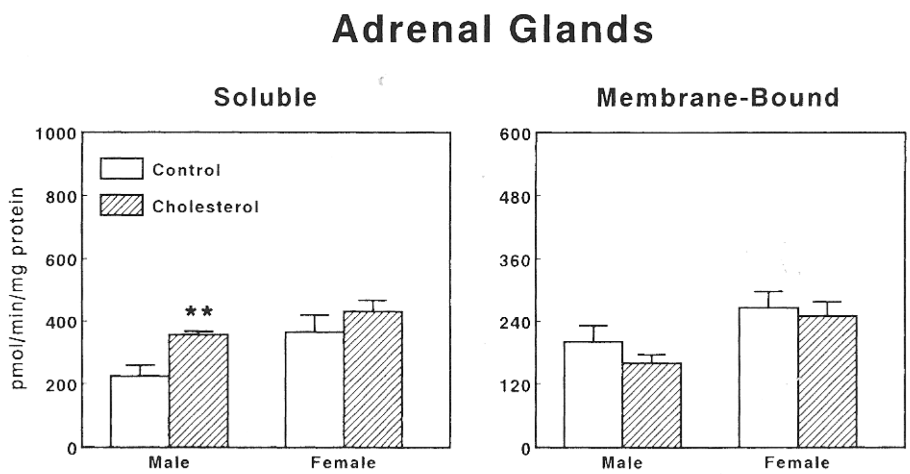

Fig. 3 Specific soluble and membrane-bound pyroglutamyl-aminopeptidase activities in adrenal glands of control and cholesterol groups (Mean ± SEM) (**$p < 0.01$).

[17,18]. Thus, this may lead to the differences observed between males and females.

Our results also showed changes in membrane-bound pGluAP activity after a cholesterol-enriched diet, although gender differences were not found. It is established that cholesterol is involved in many functions of the cellular membranes, and also that cholesterol decreases the membrane fluidity index [19], which influences the activity of membrane-bound enzymes, ion channels and receptor functions [20].

In previous studies performed in our laboratory, we had already demonstrated that an evoked release of pGluAP activity was calcium-dependent [21]. Oner et al [9] established a relationship between hypercholesterolemic diets and Ca^{2+}-ATPase activity. These authors reported on an inhibition of ATPase after the administration of high cholesterol. Taken together, these findings suggest that pGluAP released to the medium may be inhibited, while the tissue pGluAP activity may be increased. The present results are consistent with that hypothesis in the frontal cortex and pituitary, where this release may occur. Therefore, the changes observed in pGluAP levels may produce modifications in the endogenous substrates of this enzyme, mainly TRH.

On the other hand, many clinical and epidemiological investigations have linked low levels of cholesterol with alterations of other neurotransmitter systems such as the serotoninergic system [22]. Moreover, it is known that various neuropeptides are neuronally co-localized with biogenic amines, such as the coexistence of serotonin and TRH [23], although a possible relation between the serotonergic neurotransmission and the release of pituitary hormones has also been described [24].

In conclusion, our results could corroborate this hypothesis, as the administration of a cholesterol-enriched diet may inhibit the release of pGluAP into the medium, thereby increasing intracellular levels of the enzyme. It is therefore possible that TRH levels may be increased; thus, the peptide-receptor interaction will be enhanced and the neurotransmitter and neuromodulator effects will be increased. In agreement with these results, it has recently been demonstrated that TRH administration has antidepressant and euphoriant effects [25] since TRH interacts with the serotoninergic system potentiating serotonin effects. In any case, it is of a great interest that dietary manipulation might induce functional modifications in the action of pGluAP activity with a consequent impact on the activity of TRH since it is well known that this enzyme is a very important physiological mechanism for TRH inactivation. Therefore, some pathologies associated with this hormone may be due to a failure or alteration in pGluAP release.

Acknowledgements

Supported by Junta de Andalucía through PAI CVI-221.

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