Acta Histochemica 113 (2011) 436–441

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.de/acthis

Changes in bradykinin and bradykinin B_2-receptor during estrous cycle of mouse

Padmasana Singh[a], Amitabh Krishna[a,*], Rajagopala Sridaran[b]

[a] Department of Zoology, Banaras Hindu University, Varanasi 221005, India
[b] Department of Physiology, Morehouse School of Medicine, 720 Westview Dr. SW, Atlanta, GA 30310-1495, USA

ARTICLE INFO

Article history:
Received 18 January 2010
Received in revised form 23 March 2010
Accepted 24 March 2010

Keywords:
Bradykinin
Bradykinin B2-receptor
Ovary
Estrous cycle
Mice

ABSTRACT

The aim of the study was to investigate changes in the abundance of bradykinin and bradykinin B2-receptor in the ovary of mice during its estrous cycle. Changes in the abundance of bradykinin were correlated with changes in bradykinin B_2-receptor in order to determine the functional significance of this peptide for follicular development, ovulation and luteinization. Bradykinin immunoreactivity was mainly observed in the granulosa cells of antral follicles, especially around the oocytes and lining the antral cavity during proestrus and estrus phases of the cycle. Recently formed corpora lutea showed mild immunoreactivity for both bradykinin and bradykinin B_2-receptor. During diestrus 1 and diestrus 2, bradykinin and bradykinin B_2-receptor immunostaining was mainly found in the corpora lutea and mildly in the antral follicles. Immunoblot analysis for bradykinin and bradykinin B_2-receptor attained a peak during late evening in proestrus, which may be the time of the LH surge. Thereafter bradykinin and bradykinin B_2-receptor declined sharply during the estrus phase. When the concentration of bradykinin was correlated with bradykinin B_2-receptor throughout the estrous cycle, they showed strong positive correlation. Thus, this study indicates that the levels of bradykinin and bradykinin B_2-receptor both simultaneously regulate estrous cycle and are important components for the reproductive process.

Introduction

Bradykinin is a physiologically and pharmacologically active peptide of the kinin group of proteins, consisting of nine amino acids. The kinin-kallikrein system consists of kininogens, kallikreins, kinins, kininases, kallikrein inhibitors and kinin receptors (Bhoola et al., 1992). Kininogens are primarily synthesized in the liver. However, kininogen-like substances are also found associated with several cell types, suggesting that kininogen production may be a local event (Proud et al., 1981). Bradykinin is formed by the proteolytic cleavage of its preferred kininogen precursor, high-molecular-weight kininogen (HMWK) by kallikrein enzyme. Another kinin, Lys-bradykinin, also known as kallidin, is produced via the action of an enzyme kallikrein on low molecular-weight kininogen (LMWK). Nearly all cells express kinin receptors, which mediate the activities of both bradykinin and kallidin. The half-life of bradykinin in plasma is less than 1 min. Bradykinin mediates a variety of responses in tissues including smooth muscle contraction, vasodilation (Ludbrook et al., 1973), increased vascular permeability (Haddy et al., 1970), inflammation and pain (Bhoola et al., 1992).

Bradykinin is also known to play some specific roles in reproductive processes. Kinins have been detected in the follicular fluids of several species (Ramwell et al., 1969). Kihara et al. (2000) showed the presence of a component of bradykinin and the bradykinin-producing system in the porcine ovarian follicle, and suggested its role in early follicular development and ovulation. It has been demonstrated that bradykinin induces ovulation in perfused rat ovaries (Hellberg et al., 1991) and potentiates the action of LH during follicular rupture at the time of ovulation (Brännström and Hellberg, 1989). Also, a physiological role of bradykinin has been implicated in the LH surge (Shi et al., 1998). A recent study from our laboratory has demonstrated the presence of bradykinin and bradykinin B_2-receptor in various ovarian cells in several non-mammalian vertebrates (Singh et al., 2007).

Ohkura et al. (2003) detected bradykinin B_2-receptor mRNA in granulosa cells and corpora lutea at all stages of follicles in the mouse ovary, but the signal intensities differ from one follicle to another during proestrus. Therefore the aim of the present study was to localize the bradykinin and bradykinin B_2-receptor proteins in the ovary of mice during its estrous cycle and to determine

* Corresponding author.
E-mail address: akrishna_ak@yahoo.co.in (A. Krishna).

0065-1281/$ – see front matter © 2010 Elsevier GmbH. All rights reserved.
doi:10.1016/j.acthis.2010.03.008

the concentrations of the bradykinin and bradykinin B_2-receptor proteins during different phases of the estrous cycle which can be correlated with the ovulatory processes of the animal.

Materials and methods

Animals

All experiments were conducted in accordance with the principles and procedures approved by the Departmental Research Committee at Banaras Hindu University, Varanasi, India. Parkes strain mice were housed under controlled temperature (24 ± 2 °C), humidity and illumination (lights on between 07.00 and 20.00 h). Food and water were available *ad libitum*. Adult female mice (10–12 weeks old) were used in this study. Estrous cycles were monitored through daily examination of vaginal cytology. Mice that exhibited at least two consecutive regular estrous cycles were selected for the study. Animals were sacrificed by decapitation under mild anesthesia (anesthetic ether) at 10.00, 12.00, 14.00 and 19.00 h on proestrus, 10.00 and 14.00 h on estrus, diestrus 1 and diestrus 2 of the estrous cycle. The ovaries were immediately dissected out and one side of the ovary was snap-frozen and kept at −40 °C for immunoblotting analysis, whereas contralateral ovaries were fixed in Bouin's fixative for immunohistochemistry. The fixed tissues were dehydrated in ethanol, embedded in paraffin wax and sectioned at 5 μm.

Immunohistochemistry

The ovarian sections were processed through standard protocols of immunohistochemistry (Singh et al., 2007). The sections were deparaffinized, rehydrated in graded ethanol and endogenous peroxidase was quenched with 0.3% H_2O_2 in methanol. The sections were rinsed in 0.05 M Tris–HCl −0.15 M NaCl (TBS, pH 7.4). Background blocking was performed with 10% normal horse serum in PBS for 1 h. The tissue sections were then incubated for 1 h at room temperature either with bradykinin antibody (dilution 1:1500, Peninsula Labs Inc., San Carlos, CA, USA) or with bradykinin B_2-receptor antibody (dilution 1:25, BD Transduction Labs, San Jose, CA, USA) diluted in phosphate buffered saline (PBS). Following three rinses in PBS for 15 min, the slides were then incubated with secondary antibody. Detection system used was ABC universal staining kit (sc-PK-6200; Vector Labs., Burlingame, CA, USA). Peroxidase activity was revealed with 0.03% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma–Aldrich, St. Louis, MO, USA) in 0.05 M Tris, pH 7.6 and 0.1% H_2O_2 for 10 min. Sections were rinsed in buffer. Nuclei were counterstained with Ehlrich's hematoxylin, prior to the sections being dehydrated and mounted with DPX. To test the specificity of the immunoreactions, in the control section the primary antiserum was replaced by: (1) 1% normal horse serum; (2) bradykinin antiserum with bradykinin antigen (200 ng/ml). For preadsorption, the antigens were added to diluted antisera (at the same dilution as used for localization), incubated overnight at 4 °C, centrifuged and then the supernatant was used.

Slot blot

The ovaries were collected during each phase of the estrous cycle and homogenized for protein extraction (Singh et al., 2007). Equal amounts of protein, as determined by Lowry's method (Lowry et al., 1951), were adjusted to equal volume with PBS and 10 μl of these samples were loaded on nitrocellulose membrane using Millipore slot blot apparatus. Non-specific sites were blocked with 5% non-fat dried milk in PBS, 0.02% Tween 20. Membranes were then incubated for 1 h at room temperature with bradykinin antibody (dilution 1:3000) in PBS. Membranes were washed in PBS-Tween 20 buffer and then incubated for 30 min with anti-rabbit IgG conjugated horseradish peroxidase (1:500, v/v). Immunoreactive bands were detected with enhanced chemiluminescence (ECL, Bio-Rad, Hercules, CA, USA). Experiments were performed in triplicate. Equal loading was confirmed with Ponceau S staining and β-actin expression. Validation of the slot blot for bradykinin was performed using serially diluted ovarian protein samples. The sensitivity of the blot was 0.1 μg.

Western blot

Equal amounts of protein as determined by Lowry's method (Lowry et al., 1951) were loaded on 10% SDS PAGE for electrophoresis. Separated proteins were then transferred on nitrocellulose membrane (Sigma–Aldrich, St. Louis, MO, USA). Non-specific sites were blocked with 5% non-fat dried milk in PBS, 0.02% Tween 20. The membrane was then incubated for 1 h at room temperature with bradykinin B_2-receptor antibody (dilution 1:250) in PBS. Immunodetection was performed with secondary antibody conjugated with horseradish peroxidase (1:500 v/v). Finally, immunoreactive bands were detected with enhanced chemiluminescence (Bio-Rad, Hercules, CA, USA). All experiments were performed in triplicate. Equal loading was confirmed with Ponceau S staining and β-actin expression.

Densitometry

The relative intensities of different signals were quantified using a computer-assisted image-analysis system (Image J ver.1.36, NIH, Bethesda, MD, USA). The system was calibrated to have a constant parameter for illumination (intensity and area of light beam) throughout the experiment. Absorbance was expressed as normalized density value. Each measurement was repeated three times. The data are presented as the ±S.E.M. of the normalized density value of three blots of each peptide.

Statistics

Cyclic changes were analyzed by one-way ANOVA followed by Duncan's multiple range post hoc tests. Correlation studies were performed by using SPSS software 12 for window (SPSS Inc., Chicago, IL, USA) to compare the data from different phases of estrous cycle.

Results

Immunohistochemistry for bradykinin and bradykinin B_2-receptor in the ovaries of mice during the estrous cycle

Changes in the relative concentration of bradykinin and bradykinin B_2-receptor proteins expression in the ovaries of mice were studied during different phases of the estrous cycle using immunohistochemical analysis. The results are summarized in Fig. 1.

Bradykinin immunoreactivity was mainly observed in the granulosa cells of antral follicles during proestrus (Fig. 1A) and estrus phases (Fig. 1C) of the cycle. A moderate to intense immunoreactivity for bradykinin was observed in the granulosa cells located around the oocytes and lining the antral cavity. Recently formed corpora lutea showed mild immunoreactivity for bradykinin (Fig. 1C). Interstitial cells and thecal cells of antral follicles also showed moderate immunoreactivity for bradykinin. After the gonadotropin surge during late proestrus, bradykinin immunoreactivity increased in the granulosa cells and interstitial cells. Immunoreactivity declined during estrus and diestrus