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Immunohistochemical localization of osteoblast activating peptide in the mouse kidney

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ABSTRACT

Osteoblast activating peptide (OBAP) is a newly discovered peptide detected in the rat stomach, which has a major role in osteogenesis. Recently, we revealed its localization in the parietal cells of the rat stomach. There have been no data regarding OBAP expression in the kidney, despite its role in calcium reabsorption in renal tubules. The current study aimed to inspect the expression of OBAP in the kidney of twelve 10-week-old male C3H/HeNJc1 mice using immunohistochemistry, and immunoelectron microscopic localization. The immunohistochemical investigation revealed an OBAP positive reaction mainly in the medulla, which was stronger than the cortex of the kidney and was concentrated in the distal convoluted tubules (DCT), connecting tubules (CT), and the thick limbs of the loop of Henle (HL). Moreover, we clarified that the OBAP was co-distributed with ghrelin and calbindin (markers of the DCT). Interestingly, immunoelectron microscopy demonstrated that OBAP was concentrated in the mitochondrial inner membrane of the DCT and CT. Based on these results, it was concluded that the mitochondria of the DCT, CT, and HL of the mice kidney generate OBAP. Furthermore, our results suggest that OBAP might have a role in the regulation of calcium reabsorption by the renal tubule; however, further investigations are required to clarify this potential role.

1. Introduction

Mitochondria have a pivotal role in calcium sequestration/signaling, energy production, apoptosis, oxidative stress, and metabolism (Drago et al., 2011). Mitochondrial-derived peptides (MDP) were recently reported to be encoded by the mitochondrial genome and have various functions inside the body (Kim et al., 2017). These include humanin, which has an important role in Alzheimer's disease (Hashimoto et al., 2001) and the novel mitochondrial open reading frame of the 12S rRNA-c (MOTS-c), which organizes metabolic homeostasis and insulin sensitivity (Lee et al., 2016). Recently, osteoblast-activating peptide (OBAP) was discovered in the rat stomach and showed a significant role in stimulating the expression of osteoblast differentiation markers including osteocalcin and alkaline phosphatase (Fukushima et al., 2010). We recently revealed that OBAP was located in the mitochondria of the parietal cells of the rat stomach (Noreldin et al., 2016) and OBAP sequence is a part of mitochondrial NADH dehydrogenase (ubiquinone) flavoprotein 3 protein encoded on murine chromosome 7 far from mitochondrial genome (Guerrero-Castillo et al., 2017) so, it is not classical MDP but may be considered as a new class of MDP.

Additionally, it has been demonstrated that the kidney has a vital role in calcium metabolism inside the body through calcium reabsorption by renal tubules (Peng et al., 2000). calcium reabsorption varies from the passive transport of calcium in the proximal convoluted tubules (PCT) to active transportation of calcium via the distal convoluted tubules (DCT), and connecting tubules (CT) by calbindin 28 kDa (Hoenderop et al., 2005). Calbindin 28 kDa is a vitamin D-

Abbreviations: CaSR, calcium-sensing receptor; CT, connecting tubules; DCT, distal convoluted tubules; ECaC, epithelial calcium channels; HL, thick limbs of the loop of Henle; IHC, immunohistochemistry; IF, immunofluorescent; MDP, mitochondrial-derived peptides; MOTS-c, mitochondrial open reading frame of the 12S rRNA-c; OBAP, osteoblast activating peptide; PB, phosphate buffer; PBS, phosphate buffer saline; PCT, proximal convoluted tubules; PFA, paraformaldehyde

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dependent calcium-transporting protein detected in the intestinal mucosa of chicken by Wasserman and Taylor (1966). Detection of the 28 kDa calbindin protein was mainly limited to the enterochromaffin-like cells of the stomach (Buffa et al., 1989) and the renal DCT (Hoenderop et al., 2005) where the active transportation of calcium is regulated by calcitonin and parathyroid hormones (Hemmingsen, 2000).

The peptide hormone, ghrelin, was detected in human and rat stomachs (Kojima et al., 1999). Ghrelin is secreted into the bloodstream mainly by X/A-like cells of the stomach fundus (Date et al., 2000; Sakata et al., 2002). Ghrelin acts to control the functions of releasing growth hormones from the pituitary gland, as well as being involved in appetite stimulation (Kojima et al., 1999; Kojima and Kangawa, 2002; Nakazato et al., 2001). Furthermore, ghrelin has been reported to be an effective candidate for treating osteoporosis (Kojima et al., 1999). Ghrelin administration has also been linked to an increase in bone mineral density and calcium accumulation in the bone matrix, where a direct relationship between bone formation and ghrelin has been demonstrated (Fukushima et al., 2005). Moreover, ghrelin has also been isolated from the kidney (Mori et al., 2000) where it is localized at the DCT and CT (Yabuki et al., 2006).

The distribution of OBAP in parietal cells (Noreldin et al., 2016) suggested the possibility of a direct role of parietal cells in calcium metabolism. In this study, we investigated the distribution of OBAP in the kidney suggests a role for OBAP in the coordination of calcium metabolism.

2. Materials and methods

2.1. Animals and tissue preparation

All the investigations were performed in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and all experiments were carried out according to the protocols accepted by the Ethics Animal Care Committee of Tottori University, Japan (Approval No. 13-T-19).

Twelve 10-week-old male C3H/HeNJc1 mice (15–18 g; Japan Clea Inc., Tokyo, Japan) were used in this study. Following ether inhalation, the animals were euthanized by cervical dislocation. Then kidneys were immediately isolated and were divided into two groups: 1) transverse kidney slices, including the hilum, were preserved in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) overnight at 4 °C for immunofluorescence (IF) and immunohistochemistry (IHC) studies, and 2) small specimens from the renal cortex were fixed in 0.5% glutaraldehyde and 4% PFA 0.1 M PB (pH 7.2) overnight at 4 °C for immunoelectron microscopy.

2.2. Antibodies

The following 3 primary antibodies were used for immunohistochemistry: 1) polyclonal anti-rabbit OBAP antibody was acquired from a custom antibody producing service (1:800 for IHC and 1:500 for IF; Operon Biotechnologies, Tokyo, Japan) and immunospecific reaction of anti-OBAP antibody was validated in supplementary data provided by Noreldin et al. (2016), 2) polyclonal rabbit anti-human calbindin antibody (detecting the 28 kDa subtype, 1:500; Spring Bioscience, Cat: E10340, Pleasanton, CA, USA) as a marker for DCT cells and CT cells (Bindels et al., 1991a). Its specificity of anti-calbindin antibody for mice tissues has already validated by Hashimoto et al. (2017), and we confirmed immunospecificity of this antibody by using cerebellum tissue of C3H/HeN strain (Supplementary data 1) and 3) polyclonal goat anti-rat ghrelin antibody (1:250; Abcam, catalog No.: ab104307, Cambridge, UK).

2.3. Immunohistochemistry

Three 2-μm-thick serial paraffin sections were cut and deparaffinized by xylene and rehydrated in graded alcohols for immunostaining of OBAP, calbindin, and ghrelin. For antigen retrieval of calbindin and ghrelin, the sections were heated in 10 mM citrate buffer (pH 6.0) for 20 min at 105 °C; OBAP was not subjected to antigen retrieval. Briefly, the sections were covered with 0.5% TritonX-100 (Nacalai, Kyoto, Japan) in phosphate buffer saline (PBS) for 20 min. Endogenous peroxidase signals were blocked with 3% hydrogen peroxide in methanol for 5 min at room temperature. Non-specific background staining was blocked with 5% bovine serum albumin (BSA: Sigma-Aldrich, Cat: A9647) diluted in 0.1 M PBS (pH 7.2) for 1 h, and incubated with anti-OBAP, anti-ghrelin, and anti-calbindin antibodies overnight at 4 °C. In negative control sections, PBS substituted the primary antibody-containing solution. Next, the sections were rinsed in PBS, and incubated with biotinylated goat anti-rabbit IgG antiserum (Histofine kit, Cat: 424032, Nichirei, Tokyo, Japan) with anti-OBAP and anti-calbindin or biotinylated rabbit anti-goat IgG or biotinylated rabbit anti-goat IgG antiserum (Histofine kit, Cat: 414012, Nichirei) with anti-ghrelin for 30 min at room temperature. After rinsing with PBS, sections were incubated with streptavidin-peroxidase conjugate for 30 min at room temperature. Antibody binding with streptavidin-biotin complex was detected by peroxidase/3,3′-diaminobenzidine (DAB) (peroxidase/DAB ChemMate Detection Kit; Dako, Cat: K5007, CA, USA). Lastly, the nuclei were counterstained lightly with Mayer's hematoxylin. The representative micrographs were captured with a digital camera (DP-71, Olympus, Tokyo, Japan) connected to a microscope (IX71, Olympus, Tokyo, Japan).

2.4. Immunofluorescence

IF for OBAP was carried out to detect the types of OBAP-immunopositive cells. The sections were covered with 0.5% TritonX-100, incubated with donkey serum (GeneTex, Cat: GTX30972, Irvine, CA, USA) for 30 min, and then incubated at 4 °C overnight with the anti-OBAP primary antibody. The detection of antibody binding was achieved by incubation for 30 min with fluorochrome-conjugated donkey anti-rabbit IgG Northern Lights 557 secondary antibodies (1:200 dilution; R&D Systems, Inc., Cat: NL004, Minneapolis, MN, USA). The sections were counterstained with Hoechst 33342 (1:10,000; Invitrogen, Cat: H3570, Eugene, OR, USA) for 2 min. IF signals were detected using an IX71 microscope (Olympus). Fluoromount was removed where necessary, and the sections were washed and stained with hematoxylin and eosin (H&E) for conventional light microscopy.

2.5. Immunoelectron microscopy

Kidney tissues were washed with 0.1 M PB and then dehydrated in a graded ethanol series and infiltrated with a mixture of ethanol and Lowicryl K4 M (Polysciences, Cat: 15923-1, Eppelheim, Germany) followed by pure Lowicryl K4 M overnight at 4 °C, and lastly were embedded in Lowicryl K4 M and stored at −20 °C for 4 days, according to the previously published protocol (Noreldin et al., 2016). Ultra-thin sections of 70–80 nm in thickness were obtained by a PT-X Power Tome ultramicrotome (RMC, Boeckeler Instruments, Tucson, AZ, USA) and mounted on coated nickel grids (T300H-Ni, Electron Microscopy Sciences, Cat: T300H-Ni, Fort Washington, PA, USA). Non-specific reactions were blocked with a mixture of 1% normal goat serum, 1% BSA, 0.1% Tween 20, and 0.1% sodium azide (pH 8.2) for 2 h at room temperature, followed by anti-OBAP antibody (1:400 in previous blocking mixture) incubation of the ultra-thin sections at 4 °C overnight. After the grids were rinsed by PBS drops, the sections were incubated with a secondary antibody, goat anti-rabbit IgG conjugated with 15 nm diameter gold particles (BBI solutions, Cat: EM.GAR15, Cardiff, UK; dilution of 1:50 in blocking mixture) for 1 h. All procedures

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