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Presence of immunoreactive salusin-α in human serum and urine

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ABSTRACT

Salusins, identified from a full-length enriched human cDNA library by bioinformatics analyses, show mitogenic, neuromodulatory and hemodynamic activities in rats. They are expressed in a wide variety of human tissues, but their precise structures and levels in human body fluids remain unknown. We developed a radioimmunoassay suitable for the detection of immunoreactive human salusin-α and characterized the molecular forms and concentrations of salusin-α in human serum and urine. The assay allowed for measurement of immunoreactive salusin-α concentrations as low as 1 fmol/tube after extraction of serum with an octyl-silica column, and the concentration required for 50% inhibition of binding was 40 fmol/tube. Cross-reactivities with salusin-β and other bioactive peptides were negligible. Salusin-α-like immunoreactivity in normal human serum and urine ranged from 11.0 to 40.4 pmol/l (mean ± S.D., 23.3 ± 8.1 pmol/l, $n = 31$) and from 18.6 to 367.3 pmol/l (mean ± S.D., 156.8 ± 95.8 pmol/l), respectively. Reverse-phase high performance liquid chromatography coupled with radioimmunoassay detection revealed a major immunoreactive component that coeluted with authentic salusin-α. These data indicate the presence of salusin-α in human serum and urine, thereby verifying the initially predicted processing sites for salusin-α in humans.

1. Introduction

Using bioinformatics analyses of a full-length cDNA library, we predicted the presence of two salusin peptides with potent biological activities [15]. They are considered to be biosynthesized from an alternative-splicing product of the torsion dystonia-related gene, TOR2a (DYT1), after frameshift reading and digestion at dibasic amino acids [10]. Salusins are widely expressed throughout human tissues, including the blood cells, central nervous system and kidneys [15], while salusin-β has very recently been shown to coexist in vasopressin-containing neurons of the rat posterior pituitary and hypothalamus, suggesting its neural secretion into the systemic circulation via axon terminals [16]. Salusin-β is responsible for causing rapid and profound decreases in blood pressure and heart rate in rats [15], while salusin-α is less potent, and the hypotensive and bradycardiac effects of salusin-β are mainly mediated by parasympathetic stimulation rather than direct suppression of cardiac contractility [6]. Salusins have been shown to promote the growth of cardiocytes [17] as well as vascular smooth muscle cells and fibroblasts [15]. Although we previously suggested the presence of immunoreactive salusins in human tissues and body fluids using gel filtration chromatography coupled with ELISA

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[15], the assays were not sensitive enough or sufficiently specific to estimate the processing sites for the biosynthesis of salusin peptides in humans.

In this communication, we report the establishment of a sensitive and specific radioimmunoassay (RIA) for measurement of immunoreactive salusin-α in normal human serum and urine. Reverse-phase high performance liquid chromatography (RP-HPLC) analysis of extracted serum and urine combined with RIA detection showed a major immunoreactive peak that coeluted with synthetic salusin-α. The results of these assays revealed the presence of salusin-α as well as its serum and urine concentrations in healthy volunteers, thereby verifying the predicted processing sites for prosalusin into salusin-α in humans.

2. Materials and methods

2.1. Sample collection and extraction

We collected 5 ml venous blood samples from healthy volunteers (16 males and 15 females) aged between 19 and 55 yr (males: 31.3 ± 11.3 yr; females: 23.4 ± 4.7 yr) into plastic tubes containing a procoagulant, EDTA, citrate or heparin. None of the subjects were receiving any medication. The serum was left for 30 min, and the plasma was quickly separated by centrifugation and stored at −30 °C until processing. A 2 ml aliquot of serum or plasma was acidified with a 0.1 vol.% of trifluoroacetic acid (TFA) and centrifuged (3000 rpm, 10 min) before the supernatant was applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, MA) that had been sequentially prewashed with 5 ml of 99.8% methanol, 5 ml of distilled water, and 5 ml of 0.1% TFA [14]. The materials adsorbed onto the cartridge were eluted with 1 ml of 50% acetonitrile in 0.1% TFA and evaporated to approximately 100 μl using a centrifugal concentrator (2000 rpm). Extracts were added to the assay buffer to 250 μl, vortexed and 100 μl was subjected to the RIA. Recovery was calculated from the amount of salusin-α measured in a serum extract compared with that in a serum extract to which synthetic salusin-α had been added. Recovery of labeled salusin-α was also obtained by counting buffer containing labeled salusin-α before and after the extraction procedure using various concentrations of acetonitrile. The recovery of ^{125}I-labeled salusin-α (20,000 cpm) using 15, 20, 25, 30, 50 and 100% acetonitrile was 0.7, 10.3, 56.8, 78.9, 84.6 and 87.6%, respectively, whereas the average recovery of unlabeled salusin-α added to human serum using 50% acetonitrile was 79.5% (n = 8). The between-assay variation was 10.8%. This value was used to correct the final concentration of salusin-α in known samples. Therefore, we employed an extraction procedure using 50% acetonitrile as the final extraction protocol. Urine samples (10 ml) were centrifuged at 1000 rpm for 1 min to remove any cellular debris, acidified with 0.5% 12N HCl and centrifuged at 3000 rpm for 5 min. The resulting supernatants were directly subjected to the RIA.

2.2. Anti-salusin-α antisera

Antisera were prepared as follows. Synthetic human Cys-salusin-α(10–28) (Sigma Genosis, Japan) was conjugated to bovine serum albumin (BSA) using carbodiimide and then dialyzed and emulsified with an equal volume of Freund's complete adjuvant, before being injected subcutaneously into two Japanese White rabbits. Each rabbit received Cys-salusin-α(10–28) via bi-weekly injections for 10 wk and was bled at 7 d after the injection. Antisera obtained on the last bleed from one rabbit were used in the assay.

2.3. Iodination of salusin-α(10–28)

A total of 1 μg of salusin-α(10–28)-Tyr (Sigma Genosis, Japan) was incubated with 0.5 mCi of Na^{125}I (specific activity 37 TBq/mmol; Amersham Biosciences, NJ) and 10 μg of Chloramine-T (Tokyo Kasei Kogyo, Japan) at room temperature for 25 s. The reaction was stopped by the addition of 40 μg of sodium pyrosulfite. The iodinated products were immediately separated by gel filtration chromatography using Sephadex G-10 (Amersham Biosciences) in a polypropylene column (1.5 cm × 12 cm, bed volume: 5 ml: Nippon Bio-Rad Laboratories, Japan) and 0.05 mol/l phosphate buffer pH 7.4 containing 0.5% BSA. The iodinated products were assessed for binding in the assay, and fractions corresponding to ^{125}I-labeled salusin-α were collected and stored at −20 °C until use in the assay.

2.4. Radioimmunoassay (RIA)

The radioimmunoassay was performed essentially as described [1,2,13], except for the following aspects. The assay buffer used to dilute the standards and antibodies and reconstitute the extracts was 0.05 mol/l phosphate buffer, pH 7.4, containing 0.01% BSA, 0.1% Triton X-100, and 0.05% sodium azide. Working standards of salusin-α were prepared by serial dilution (0.1–10,000 fmol/tube = 10^{-12} to 10^{-7} mol/l) of a stock standard with RIA buffer. Aliquots (0.1 ml) of samples or the standard were mixed with 0.1 ml of assay buffer and the anti-salusin-α(10–28) polyclonal antibody (final dilution: 1:3000). Incubation was carried out for 16–18 h at room temperature, followed by the late addition of 0.1 ml ^{125}I-salusin-α (specific activity: ∼1000 Ci/mmol, 20,000 cpm/tube) and further incubation for 48 h at 4 °C. The bound ligands were separated from free ligands by the addition of 0.1 ml of assay buffer containing 0.5 μl of goat anti-rabbit IgG (R-0881; Sigma, MO) and 0.1 ml of 15% polyethylene glycol, incubation for 20 h at 4 °C and centrifugation at 3000 rpm for 30 min at 4 °C. The radioactivity in the precipitate was counted in a RIASTAR 5410 Gamma Counter (Packard Instruments, Japan). The results were interpolated from a computer-fitted standard curve using ''Newcra for Windows'' software provided by the Japanese Society of Animal Reproduction.

2.5. Specificity

We measured the cross-reactivity of our salusin-α antiserum by assaying serial dilutions of the following standards: authentic salusin-α(1–28), salusin-α(1–24), salusin-α(10–28), salusin-α(16–28) and salusin-β. In addition, we assayed standards of other vasoactive hormones (human C-type natriuretic peptide, endothelin-1, angiotensin II, CGRP and urotensin II).