Original Article

Systemic Distribution of Salusin Expression in the Rat

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Salusin-α and salusin-β are multifunctional bioactive peptides with hypotensive and bradycardic effects. They were originally identified from full-length human cDNAs by bioinformatics analyses. Salusin peptides are expressed in human tissues at the mRNA level, but no information is available about their systemic distributions in any species. We examined the distributions of preprosalusin mRNA and the salusin peptides in a variety of normal rat organs. Whereas preprosalusin mRNA was expressed ubiquitously, immunoreactive salusin-β was detected most strongly in the hypothalamus and posterior pituitary, and less abundantly in the anterior pituitary and gastrointestinal, immune, and hematopoietic systems. Salusin-β–positive cells appeared to be of either hematopoietic or endocrine origin, and many hematopoietic cells were also stained with anti-CD68, which specifically recognizes macrophages. Salusin-α–like immunoreactivity was not detected in any of the rat tissues. These results indicate that rat salusin is immunologically similar to human salusin-β and widely expressed, especially in the immune, gastrointestinal, and central nervous systems and mainly in endocrine- and hematopoietic-derived cells. (*Hypertens Res* 2007; 30: 1255–1262)

***Key Words*: salusin, immunohistochemistry, real-time quantitative polymerase chain reaction, expression, macrophage**

Introduction

Salusins are bioactive peptides that were originally identified using *in silico* analyses of a full-length human cDNA library (*1*). Salusin-β causes rapid and temporary decreases in blood pressure and heart rate in rats, while the hemodynamic effects of salusin-α are far less potent (*1*). Salusin-β's effects are mediated mainly by parasympathetic stimulation rather than direct suppression of cardiac contractility (*2*). Human salusins have been shown to promote cardiomyocyte hypertrophy (*3*) as well as the growth of vascular smooth muscle cells and fibroblasts (*1*), and to protect against apoptotic death of cardiomyocytes (*4*). However, the exact mechanisms underlying these actions by exogenous human salusin peptides are largely unknown, and salusin receptors have not yet been identified (*5*). Both salusins are considered to be concomitantly biosynthesized as a result of alternative splicing from the torsion dystonia–related gene, TOR2a (DYT1), with subsequent frameshift reading and processing at dibasic amino acids (*1*). We previously reported the distribution of preprosalusin mRNA in human tissues as well as the concomitant presence of immunoreactive salusin-α and salusin-β in human kidney tissues (*1*). In nonhuman species, salusin-β–like immunoreactivity (LI) was found to coexist in vasopressin-expressing neurons of the rat posterior pituitary and

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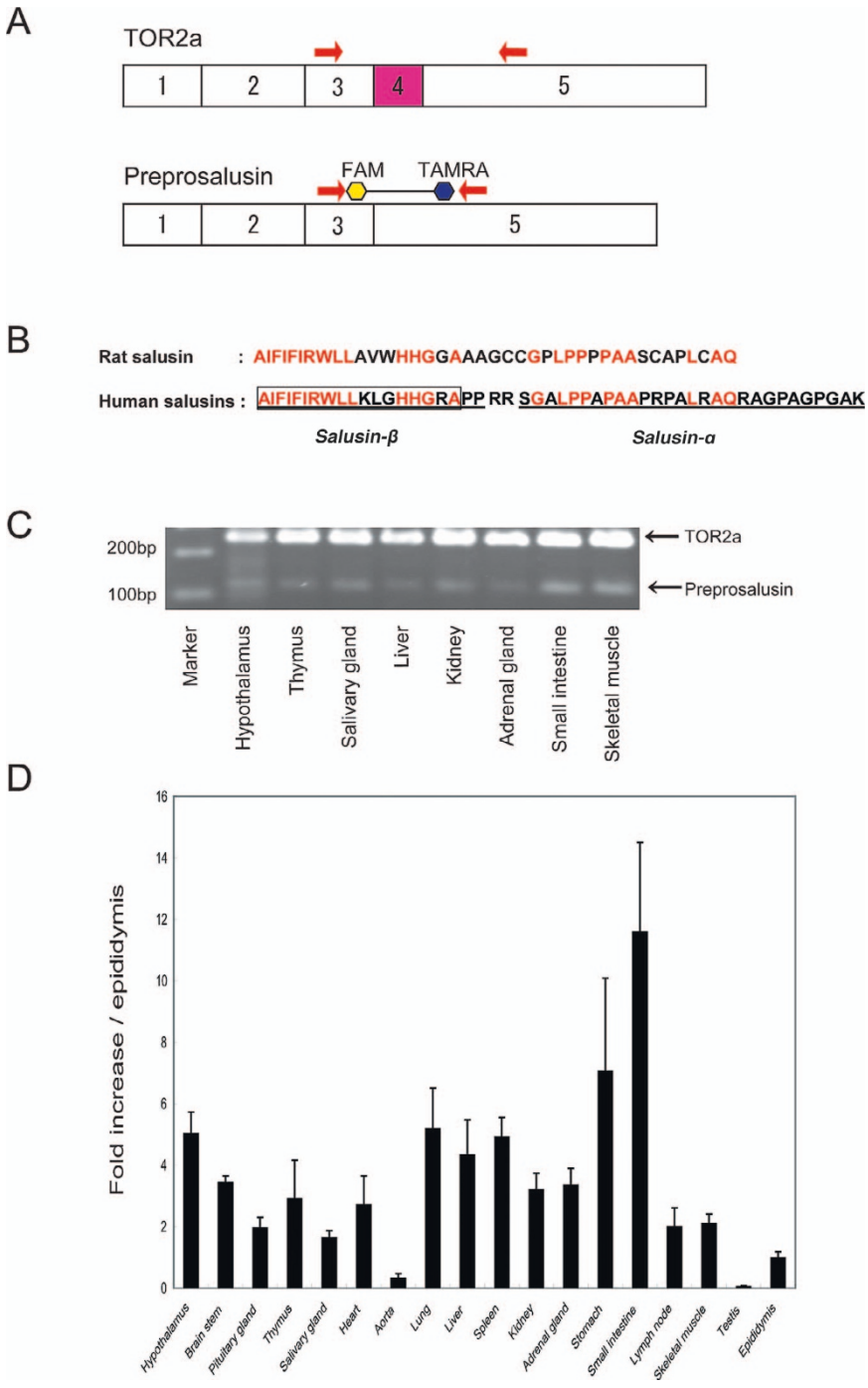

Fig. 1. *Structures and distribution of rat salusin. A: Schematic representation of TOR2a and preprosalusin mRNAs. Numbers indicate the exon numbers, and red arrows indicate the RT-PCR primer positions. Indicated between exons 3 and 5 is the position of the TaqMan probe, labeled with the fluorescent dye 6-carboxy-fluoroscein (FAM) at its 5′ end and quencher dye 6-carboxy-tetramethylrhodamine (TAMRA) at its 3′ end for use in real-time quantitative RT-PCR. B: Comparison of the putative rat salusin with the human salusin-β and salusin-α amino acid sequences. Identical residues are shown in red. The antigenic peptide sequence used to raise the polyclonal antibody is boxed. C: Conventional RT-PCR analysis for detection of preprosalusin expression in rat tissue samples. The arrows indicate the positions of bands corresponding to the sizes of TOR2A (250 bp) and preprosalusin (122 bp) after agarose gel electrophoresis. D: Distribution of preprosalusin transcripts in various rat tissues as measured by real-time quantitative PCR using a TaqMan probe. The quantified transcripts are expressed as copy numbers relative to the corresponding copy numbers in an epididymis extract. Each column with a bar represents the mean±SEM obtained from 8 independent rats.*

hypothalamus, suggesting its possible neural secretion into the systemic circulation *via* axon terminals (*6*). However, the systemic distributions of salusin peptides remain unclarified in any species, and preprosalusin gene expression in nonhuman species has not yet been investigated. Thus, it remains unknown whether or not the TOR2a gene undergoes alternative splicing, resulting in salusin peptide biosynthesis, in nonhuman species.

The present study was designed to determine the presence and distribution of rat preprosalusin transcripts and immunoreactive rat salusin in a variety of rat tissues. The predicted N-terminal amino acid residues were highly homologous to human salusin-β, thereby allowing efficient detection of immunoreactive rat salusin in rat tissue specimens.

Methods

Animals

Adult male Sprague-Dawley rats, weighing 250–300 g (Charles River Japan, Shiga, Japan), were used in all experiments. All procedures were performed in accordance with the Tokyo Medical and Dental University Guidelines for the Care and Use of Experimental Animals.

Conventional Reverse Transcription–Polymerase Chain Reaction for Detecting TOR2a/Preprosalusin Transcripts

After homogenization of the tissues, total RNA was extracted using QIAzol (Qiagen, Valencia, USA), which includes a DNase incubation step. RNA was quantified by spectrophotometry (Biochrom Ltd., Cambridge, UK), and diluted to 50 μg/mL for use in reverse transcription (RT)–polymerase chain reaction (PCR) assays. First-strand cDNA was synthesized using a Quantitect® Reverse Transcription kit (Qiagen) as described (*7*). Specific primers used for amplification and located in exon 3 (5′-ATCGCAAAGCCATTTTCATC-3′) and exon 5 (5′-ACACAGTGGCGCACATGAT-3′), and that detect both unspliced TOR2a (250 bp) and spliced preprosalusin (122 bp) (Fig. 1A), were synthesized by FASMAC Co. Ltd. (Atsugi, Japan). The amplified products were subjected to agarose gel electrophoresis.

Real-Time RT-PCR for Quantifying Rat Preprosalusin Transcripts

A TaqMan probe (5′-TATCAGATGGCTTCTGGCG-3′) labeled with a fluorescent dye, 6-carboxy-fluoroscein (FAM), at the 5′ end and a quencher dye, 6-carboxy-tetramethylrhodamine (TAMRA), at the 3′ end, and that was designed to recognize a sequence between exons 3 and 5 (Fig. 1A), was synthesized by FASMAC. After RT for 15 min at 42°C, the reaction mixture was denatured at 92°C for 5 min followed by 50 cycles of PCR at 92°C for 30 s, 59°C for 30 s, and 72°C for 30 s in the presence of the labeled oligonucleotide probe. The RT-PCR reactions were performed, recorded, and analyzed using a Chromo4™ Multicolor Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA).

Anti–Salusin-β Antibody

Polyclonal antibodies were raised in rabbits by immunization with synthetic human salusin-α-[Cys^0] (10–28) and human salusin-β-[Cys^0] (1–18), each of which was coupled to maleimide-activated mariculture keyhole limpet hemocyanin (*1*) and affinity-purified for use in immunohistochemistry. The initial 18 amino acids of human salusin-β had high homology with the estimated N-terminal sequence of rat salusin (Fig. 1B). The affinity-purified antibody recognized rat salusin in the hypothalamo-pituitary system, and the specificity of the salusin-β staining was assessed by preabsorption of the antibody with the full-length human salusin-β peptide, which completely abolished salusin-β staining as previously reported (*6*).

Immunohistochemistry for Salusin-β

The animals were anesthetized with ether and decapitated. The tissues were avulsed, quickly transferred to a fixative comprising 10% neutral-buffered formalin solution (Muto Pure Chemicals, Tokyo, Japan), dehydrated through a graded ethanol series, and embedded in paraffin wax. Tissue sections (4 μm thick) were placed on silane-coated slides, dewaxed, rehydrated, and subjected to antigen unmasking by treatment with citric acid buffer (10 mmol/L, pH 6.0) in a microwave oven at 600 W for 5 min. Endogenous peroxidase activity was blocked with 1% hydrogen peroxide in methanol for 30 min. Nonspecific binding sites were blocked by incubation in normal horse serum diluted 1:10 in phosphate-buffered saline for 60 min. Next, the sections were incubated with a primary antibody (1:2,400 dilution) overnight at 4°C, followed by treatment with a labeled polymer anti-rabbit IgG (Nichirei Bioscience, Tokyo, Japan) for 30 min at room temperature. The antibody-peroxidase complex was visualized using diaminobenzidine (Nichirei Bioscience), and the sections were counterstained with hematoxylin.

Double Immunostaining

Using a labeled polymer system (Nichirei Bioscience), immunoreactivity for salusin was first visualized with diaminobenzidine, which produced brown staining. After the sections were heated in a microwave oven for 15 min, mouse monoclonal anti–rat CD68 antibody ED1 (1:200 dilution; Serotec, Oxford, UK) was immunostained by the streptavidin-biotin method using Vector red (Vector Laboratories, Burlingame, USA), which produced red staining. ED1 is known to specifically detect murine macrophages expressing CD68 on their cell surface (*8*, *9*).

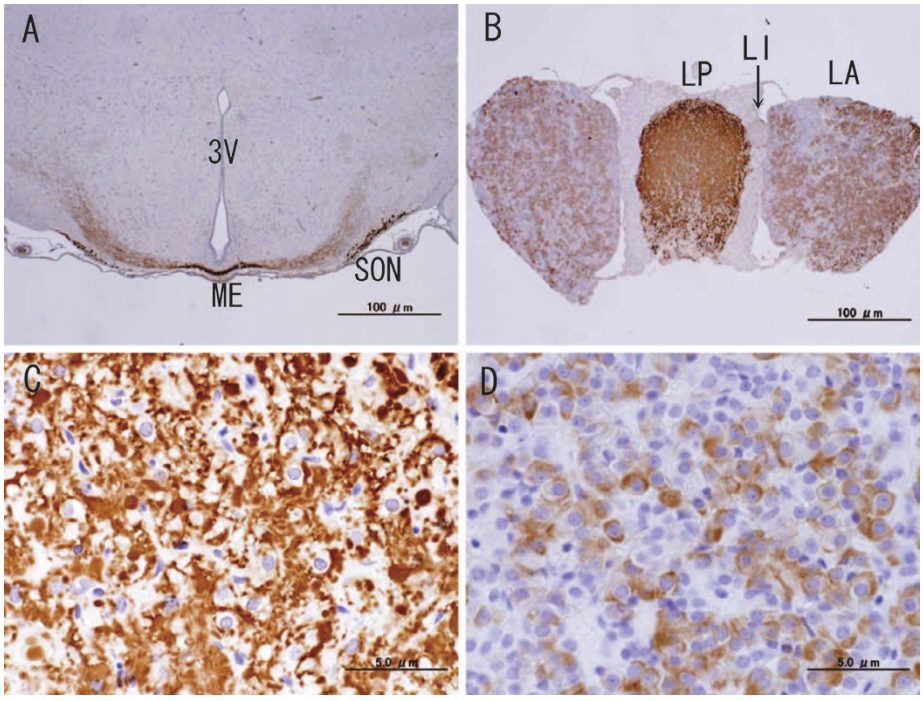

Fig. 2. *Localization of salusin-β-LI in the rat brain. Rat hypothalamus and pituitary sections were stained with an anti–salusin-β antibody at 1:2,400 dilution. Coronary sections of the hypothalamus (A) and pituitary (B) are shown. SON, supraoptic nucleus; ME, median eminence; 3V, third ventricle; LA, anterior lobe; LI, intermediate lobe; LP, posterior lobe. Higher magnification views of the posterior (C) and anterior (D) lobes of the pituitary gland are shown.*

Salusin-α Radioimmunoassay

Excised rat tissues were homogenized in 2 mL of lysis buffer (50 mmol/L Tris-HCl pH 7.5, 300 mmol/L NaCl, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1% Triton X-100) at 4°C using a Polytron homogenizer (Kinematica, Lucerne, Switzerland) and centrifuged at 3,000 rpm for 30 min at 4°C. The mixture was then acidified with 0.1 vol % of trifluoroacetic acid (TFA) and centrifuged at 3,000 rpm for 10 min. The resulting supernatant was applied to a Sep-Pak C18 cartridge (Waters Associates, Milford, USA) that had been sequentially prewashed with 5 mL of 99.8% methanol, 5 mL of distilled water, and 5 mL of 0.1% TFA. 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 (2,000 rpm). The extracts were added to the assay buffer to a final volume of 250 μL and vortexed, after which 100 μL of the solution was subjected to the salusin-α radioimmunoassay as reported previously (*10*).

Results

Conventional RT-PCR of 8 different rat tissue samples and subsequent agarose gel electrophoresis revealed a faint but distinct band and a thick band, corresponding to rat preprosalusin and TOR2a, respectively (Fig. 1C). Similar to human preprosalusin, rat preprosalusin transcripts appeared to be expressed at quantitatively lower levels than TOR2a transcripts. However, since the faint preprosalusin RT-PCR band suggested only low amplification of the final product and did not necessarily indicate a low frequency of alternative splicing events (*1*), we analyzed the preprosalusin transcripts in various tissues of adult male rats by real-time quantitative PCR (Fig. 1D). A high level of expression was detected in the small intestine, stomach, lung, hypothalamus, spleen, and liver, whereas moderate transcript levels were detected in the brain stem, adrenal gland, kidney, thymus, heart, skeletal muscle, lymph node, pituitary gland, and salivary gland. There were small numbers of transcripts in the epididymis and aorta, with negligible levels in the testis.

Salusin-β-LI was most densely localized in the hypothalamo-neurohypophyseal tract (Fig. 2) as reported previously (*6*). Intense salusin-β-LI was detected in neuronal cells in the supraoptic nucleus and median eminence (Fig. 2A), as well as in the posterior lobe of the pituitary gland (Fig. 2B, C). In the positive cells, the cytoplasm was uniformly stained while the cell nuclei were negative. Positively stained cells were also found in the anterior lobe of the pituitary gland, but not in the intermediate lobe (Fig. 2B, D).

Salusin-β-LI was detected in macrophages of the hematopoietic and immune systems, such as those in the splenic red

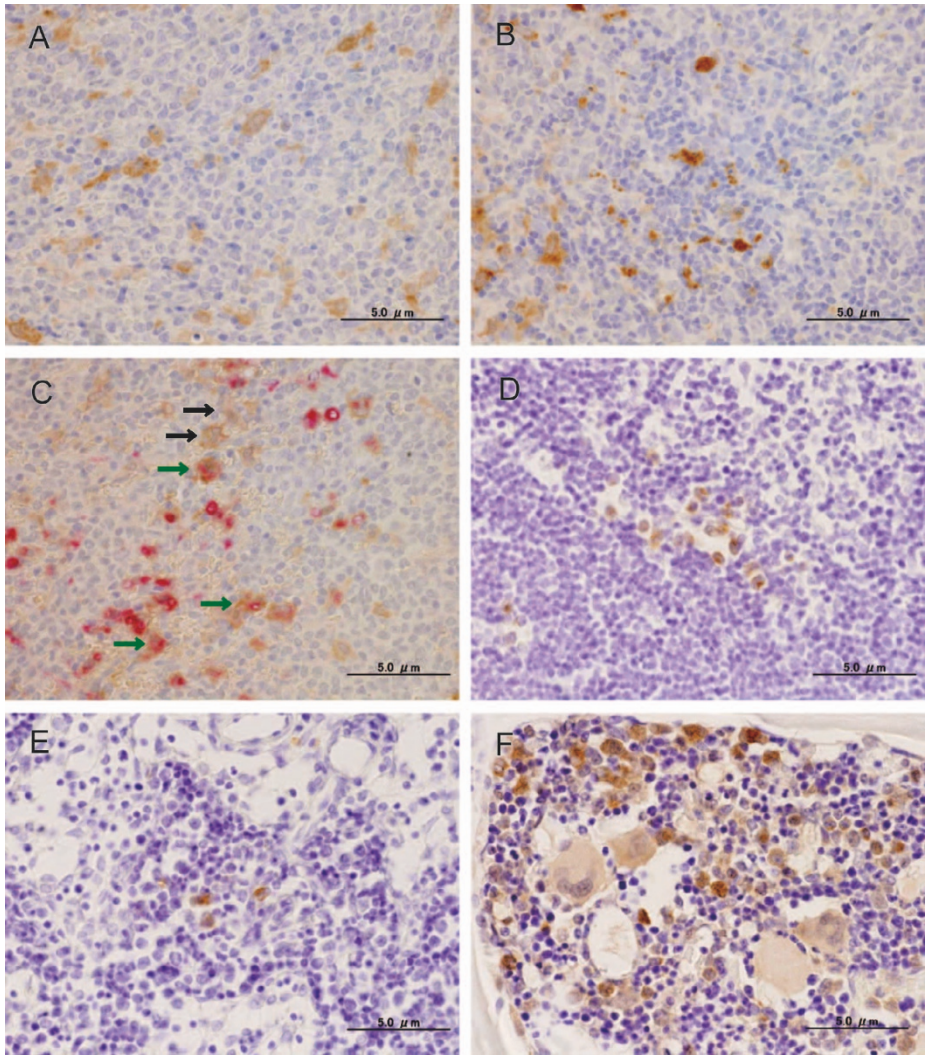

Fig. 3. *Distribution of salusin-β-LI in the hematopoietic and immune systems. Salusin-β-LI is detected in the splenic red (A, C) and white (B) pulps, thymus (D), lymph node (E), and bone marrow (F). In C, the splenic red pulp is double-stained with the anti–salusin-β antibody (brown staining) and an anti-CD68 antibody, ED1 (red staining). The black arrows show splenic cells stained with the anti–salusin-β antibody alone, while the green arrows show salusin-β–positive cells coexpressing CD68.*

and white pulps, cortex of the thymus, lymph nodes, and bone marrow (Fig. 3). Double-staining with the anti–salusin-β antibody and an anti-CD68 antibody revealed that a portion of the macrophages in the splenic red pulp co-expressed salusin-β-LI (Fig. 3C). Salusin-β–positive cells were also present in gastrointestinal tissues, most abundantly in the fundic gland of the stomach and the upper and lower small intestines (Fig. 4A–C), less abundantly in the colon. No salusin-β–positive cells were detected in the proventriculus (data not shown). Kupffer cells in the liver were also positive for salusin-β (Fig. 4D). Many of the CD68-negative salusin-β–positive cells in the neck of the stomach and small intestine appeared to be endocrine cells of nonhematopoietic origin. Salusin-β-LI was weakly detectable in the adrenal medulla (Fig. 4F), but not in the adrenal cortex (data not shown). Faint immunostaining was detected in renal tubules (Fig. 4E), whereas salusin-β-LI was barely detected in the heart, aorta, skeletal muscle, lung, testis, epididymis, and salivary gland (Fig. 5).

In contrast to the systemic expression of salusin-β, none of the rat tissues were stained with an anti–salusin-α-IgG (data not shown). Rat hypothalamus, thymus, spleen, and small intestine tissue extracts as well as serum were subjected to sensitive and specific salusin-α radioimmunoassays. However, no salusin-α-LI was detected, despite the abundant expression of preprosalusin and salusin-β-LI.

Discussion

The present study has demonstrated the expression of rat salusin through three lines of evidence. First, RT-PCR cou-

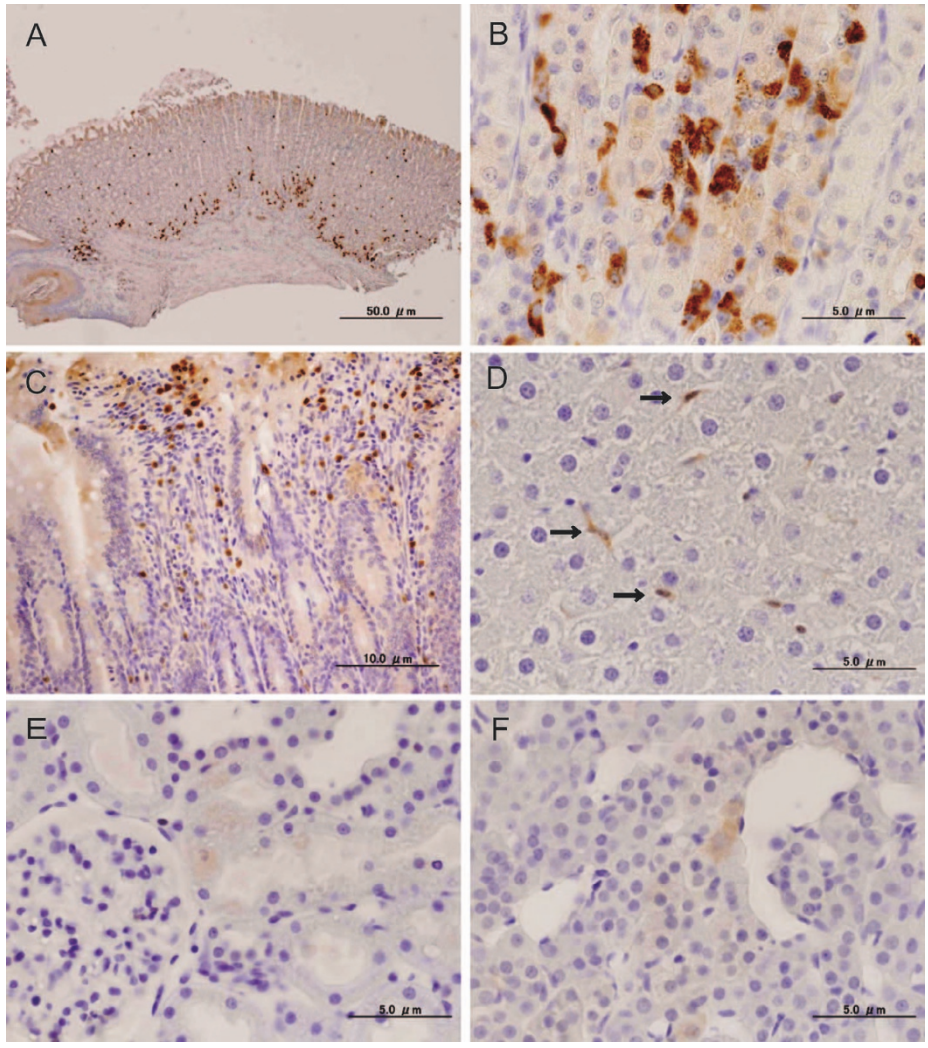

Fig. 4. *Photomicrographs showing positive salusin-β-LI in rat visceral organs. Salusin-β-LI is detected in the stomach (A, B), small intestine (C), liver (D), kidney (E), and adrenal medulla (F). The arrows in D indicate salusin-β–positive hepatic Kupffer cells.*

pled with agarose gel electrophoresis revealed a distinct band corresponding to preprosalusin, a spliced product of the TOR2a gene. Second, using a real-time RT-PCR TaqMan probe format that specifically detected the spliced site between exons 3 and 5 of the TOR2a gene, we detected systemic distribution of preprosalusin mRNA in a variety of rat tissues. Third, using a specific antibody raised against the N-terminal 18 amino acid residues of human salusin-β, which are highly homologous to the putative rat salusin, we demonstrated the presence of salusin-β-LI in many peripheral rat tissues, such as those in the immune system and gastrointestinal tract as well as the hypothalamo-neurohypophyseal tract.

Among nonhuman species, salusin-β-LI has previously been demonstrated only in the hypothalamo-neurohypophyseal tract of rats, especially in vasopressin-expressing neurons, by using the same polyclonal antibody employed in the present study (*6*). Our present data support these findings and further show that salusin-β-LI is also present in the anterior pituitary. We could not characterize the nature of the salusin-positive cells in the anterior pituitary, since antibodies that fully recognize rat anterior pituitary hormones are not yet available. However, the present data at least demonstrate that the expression of rat salusin in the brain is not restricted to the hypothalamus and posterior pituitary. On the other hand, real-time RT-PCR analyses revealed that preprosalusin expression is far lower in the pituitary tissues than in the hypothalamus, despite the dense staining of salusin-β-LI in the posterior pituitary and positive staining in many anterior pituitary cells. These results are consistent with the previously raised hypothesis that neurons of the supraoptic nucleus and paraventricular nucleus synthesize and axonally transport rat salusin to the posterior pituitary, to be released into

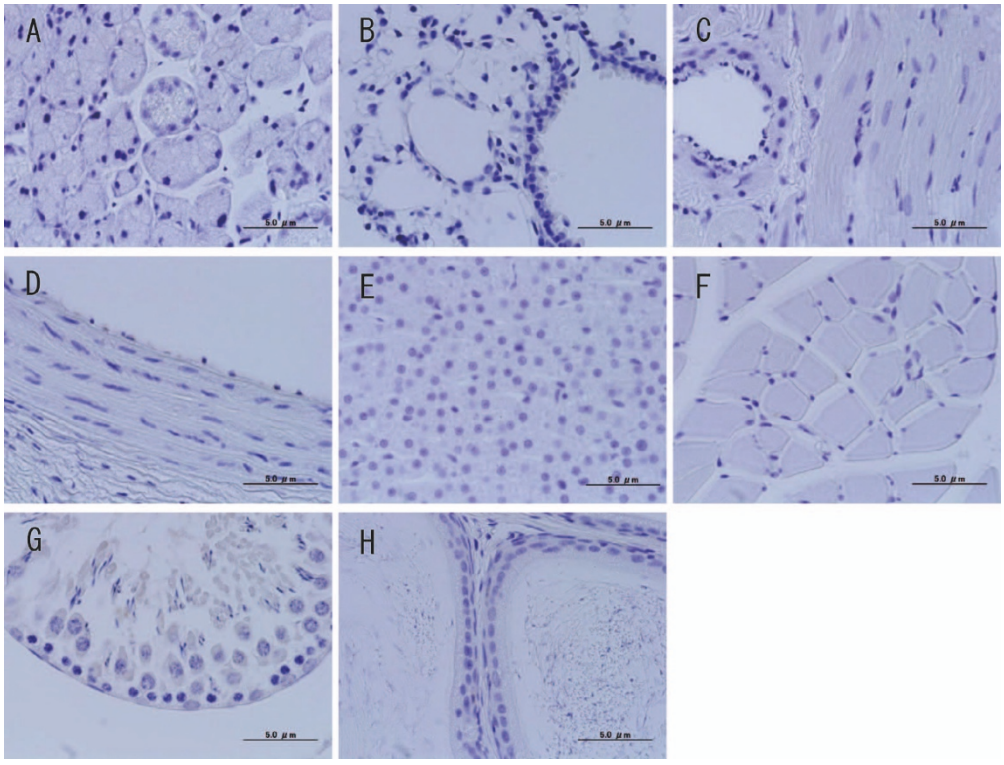

Fig. 5. *Photomicrographs showing barely detectable salusin-β-LI in rat organs. Salusin-β-LI is negative or faintly present in the salivary gland (A), lung (B), heart (C), aorta (D), adrenal cortex (E), skeletal muscle (F), testis (G), and epididymis (H).*

the bloodstream (*6*).

Another major finding of the present study is that salusin-β-LI is abundantly present in hematopoietic and immune system tissues, such as the spleen, thymus, lymph nodes, and bone marrow, as well as in gastrointestinal tissues, as they are distributed around the glandular tissues of the stomach and small intestine. Most salusin-β–positive cells in these tissues were of either hematopoietic or endocrine origin, and did not appear to be epithelial cells. A number of positive cells were double-stained with ED1, a specific antibody against CD68, which is a cell surface marker for macrophages. In the liver, only Kupffer cells derived from macrophages were positively stained with the anti–salusin-β antibody. These data are supported by our real-time quantitative RT-PCR analysis showing the presence of high levels of preprosalusin mRNA in tissues expressing salusin-β-LI. Taken together, the results indicate that salusin is produced in the gastrointestinal and immune systems, mostly by cells of hematopoietic or endocrine origin.

Our data revealed the ubiquitous expression of preprosalusin mRNA in rat tissues. However, compared with our previously reported salusin distribution in human tissues, there are some noteworthy discrepancies. The rat gastrointestinal tissues appeared to express comparatively more salusin, while the rat testis showed lower levels, than did the corresponding human tissues. These data were confirmed by our immunohistochemistry data showing abundant salusin-β-LI in the stomach and intestine, and little staining in the testis. In addition, rat kidney tissues were stained very weakly compared to human kidney tissues, which show dense staining in the renal tubular epithelial cells (*1*). Since salusin-α-LI was detected in normal human urine, it is conceivable that human renal tubules synthesize and release salusin peptides on their luminal side (*10*). The level of salusin peptide expression is thought to be influenced by the alternative splicing frequency in addition to transcriptional, translational, or post-translational regulation. However, we still have no information regarding the splicing mechanism of TOR2a into preprosalusin. Thus, it is reasonable to at least conclude that salusin expression is preserved in rat species.

The molecular form of rat salusin remains to be determined. In contrast to the high homology of the N-terminal 20 residues of the putative rat salusin to human salusin-β, the remaining C-terminal sequence shows limited homology to human salusin-α (Fig. 1B). Furthermore, dibasic amino acids between human salusin-β and salusin-α appear to be lost in the putative rat salusin. Thus, it remains unknown whether the salusin-β-LI detected systemically in the present study represents the putative rat salusin consisting of 40 amino acid residues or a shorter fragment produced by an unknown processing mechanism.

In summary, preprosalusin and a salusin peptide are widely

expressed throughout rat tissues, including neuronal cells of the hypothalamo-pituitary tract and hematopoietic and endocrine cells of the immune and gastrointestinal systems. However, the human salusin-α antibody does not cross-react with rat salusin.

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