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Histochemical distribution of endothelin-converting enzyme subtypes in *Podarcis sicula* (Squamata, Lacertidae) tissues

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Abstract

The distribution of endothelin-converting enzyme (ECE) in the lizard *Podarcis sicula* was investigated immunohistochemically using antibodies against endothelin-converting enzyme ECE-1 and endothelin-converting enzyme ECE-2 homologues. In all the tissues examined, immunoreactivity for both antibodies was found, although the distribution and degree of expression varied. Strong immunoreactivity was found in the endothelial cells and chromaffin tissue for both enzymes, whereas other tissues such as nervous tissue, renal tissue and hepatocytes display distinct patterns. Current knowledge does not allow correlation of these distribution patterns to specific functions but the data suggest that, in reptiles as in mammals, ECE is probably involved in physiological functions such as paracrine activity through endothelins and/or other substrates.

Key words ECE-1; ECE-2; histology; immunohistochemistry; reptiles.

Introduction

Endothelin-converting enzyme (ECE) is a key enzyme in the biosynthesis pathway of endothelins (ET), a family of potent vasoconstrictor peptides, which includes endothelin-1 (ET-1), originally isolated from cultured porcine aortic endothelial cells (Yanagisawa et al. 1988), endothelin-2 (ET-2) and endothelin-3 (ET-3).

After purification and cDNA cloning studies ECE was revealed as a highly glycosylated protein, integrated into the membrane (Ikura et al. 1994; Xu et al. 1994). ECE has been characterized as a membrane-bound phosphoramidon-sensitive metalloproteinase (Shimada et al. 1994). Two homologues have been cloned and characterized: ECE-1 and ECE-2 (Shimada et al. 1994; Emoto & Yanagisawa, 1995). ECE-1 is a membrane protein, which displays a single transmembrane stretch separating a short N-terminal cytoplasmic tail from a large C-terminal intraluminal/extracellular domain (Valdenaire et al. 1999). The existence of four isoforms has been shown, with similar enzymatic properties, identical efficiency but different N-terminal cytoplasmic domains and distinct subcellular localization (Valdenaire et al. 1999). ECE-2 shares 59% identity with ECE-1, has a similar pharmacological profile, converts ET with the same efficiency as ECE-1, but whereas ECE-1 is a transmembrane enzyme, ECE-2 seems to act as an intracellular enzyme working in an acidic environment (Emoto & Yanagisawa, 1995; Mzhavia et al. 2003). ECE-1 exists as a disulphide-linked homodimer (Schmidt et al. 1994; Shimada et al. 1996) and has been localized by immunohistochemical analysis in a variety of tissues and several cell lines, including of endothelial, neuronal and glial strain (Barnes et al. 1996, 1997). In the rat lung immunoreactivity was observed in the endothelial cells of the arteries and veins (Takahashi et al. 1995). Several studies report the localization of ECE in a variety of animals: this enzyme has been found in *Caenorhabditis elegans* (Sarras et al. 2002) and *Hydra vulgaris* (Zhang et al. 2001), and an endopeptidase with significant sequence identity to ECE has been recognized even in *Streptococcus parasanguis* (Froeliger et al. 1999); it is

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noteworthy that in most invertebrates ECE was found to act as a monomer (for a review see Macours & Hens, 2004). Together, these data suggest that ECE is highly conserved during evolution and that it appeared early in metazoans (Sarras et al. 2002). The presence and influence of ET system components in the regulation of neural, adrenal and renal activities has been widely investigated in mammals (Mortensen, 1999). The crucial involvement of the ET system in embryogenesis, particularly with respect to the development of neural crest-derived tissues such as chromaffin tissue of the adrenal glands, was recently demonstrated (Valdenaire et al. 1999).

In contrast to that from mammals, little information about the ET system is available in lower vertebrates, although ET-1 is known to act on the frog adrenal gland through an ETa receptor subtype (Cartier et al. 1997). As previously demonstrated in the lizard *Podarcis sicula*, ET-1 plays a role in the secretion of catecholamines and steroids from chromaffin and steroidogenic tissue of the adrenal gland, respectively (De Falco et al. 2001); furthermore, it also influences thyroid gland activity in this reptile (Virgilio et al. 2003) and an ETb receptor is present in *P. sicula* tissues (De Falco et al. 2002). These findings suggest the presence of an endothelin system in lower vertebrates as well as in mammals, even if nothing is known about the presence and localization of ECE enzymes in these animals. In the present study we investigated the distribution of ECE-1 and ECE-2 in certain tissues from *P. sicula*, the liver, adrenal glands, brain and kidney, because of their involvement in basic physiological functions.

Materials and methods

Animals and housing conditions

Twenty adult *Podarcis sicula* lizards were housed in a temperature-controlled room with a 12-h light–dark photoperiod (lights on from 06:00 to 18:00 h) and fed *ad libitum* for at least 1 week. The experiments were approved by committees established by the Italian Ministry of Health and were organized to minimize the number of animals used (n = 20).

Immunolocalization

The adrenal glands, kidneys, livers and brains were collected and fixed in Bouin's solution (71% picric acid, 5% acetic acid and 24% formaldehyde) at room temperature for 2–24 h depending on the thickness of the sample, then dehydrated and embedded in Paraplast (Carlo Erba). Five-micrometre-thick sections were dewaxed and hydrated. Antigen unmasking was performed with citrate buffer, pH 6.0, twice for 10 min in the microwave at 96 °C. Immunolocalizations were carried out with two rabbit antibodies, anti-ECE-1 and anti-ECE-2 kindly provided by Professor Yanagisawa (Howard Hughes Medical Institute, Dallas, Texas, USA), corresponding to the C-terminal of bovine ECE-1 and ECE-2, respectively, diluted 1 : 1000 in 0.1 M phosphate buffer, pH 7.4, revealed by a goat anti-rabbit secondary antibody, conjugated with biotin, and revealed with an ABC system (Pierce) using DAB as chromogen. The immunostained sections were dehydrated and mounted with Histovitrex (Carlo Erba), the immunocytochemical signal was analysed with an Axioskop System (Zeiss) and images were acquired by using KS300 software (Zeiss).

Controls for both antibodies were performed by (1) replacing specific antiserum with normal rabbit serum, (2) omitting the primary antibody and (3) pre-absorbing primary antiserum with 10 nmol of antigen per millilitre of optimally diluted serum. All samples were processed under the same conditions. To estimate the degree of labelling, three observers separately evaluated, using KS300 software, the staining pattern of each enzyme on each tissue to obtain the proportion (%) of immunopositive cells. The level of concordance, expressed as the percentage of agreement between the observers, was 93%.

Results

The degree of expression for each tissue is represented in Table 1; all tissues express both ECE-1 and ECE-2 but there was considerable variation among different organs and marked differences between cells in the same organ.

Brain

The localization of immunoreactivity for both ECE enzymes in the brain is summarized in Fig. 1 and Table 1.

The brain of *P. sicula* shows stronger immunoreactivity for ECE-1 than for ECE-2. The main regions of expression are located in the diencephalon: in particular, ECE-1 marks the preoptic periventricular nuclei both in cell bodies and in hypothalamic nerve fibres (Fig. 2g,h); labelling occurs also in magnocellular periventricular

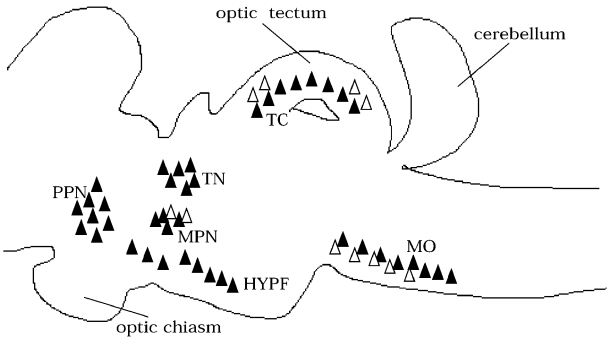

Fig. 1 Schematic parasagittal section through lizard brain depicting the distribution of ECE immunoreactivity. Black triangles represent ECE-1-immunoreactive cell bodies and fibres, white triangles ECE-2 immunoreactivity. Abbreviations: hypothalamic fibres (HYPF); thalamic nuclei (TN); tectal commissure (TC); magnocellular periventricular nuclei (MPN); periventricular parvocellular nuclei (PPN); medulla oblongata (MO).

Table 1 Degree of expression of ECE-1 and ECE-2 in *Podarcis sicula* tissues

Tissue	ECE-1	ECE-2
Diencephalon		
periventricular nuclei	++	–
magnocellular nuclei	+	+/–
thalamic nuclei	++	–
hypothalamic fibres	+	–
Mesencephalon		
tectal commissure	++	+/–
Rhombencephalon		
Medulla oblongata	++	+
Liver	+	–
Kidney		
renal tubules	+++	+/–
medullary collecting ducts	–	++
Adrenal		
cortex	–	–
medulla	++	+++
Endothelial tissue	+++	+++

–, undetectable; +/–, very low expression level (> 0% up to 25% of positive cells); +, low/medium expression level (> 25% up to 50% of positive cells); ++, medium/high expression level (> 50 up to 75% of positive cells); +++, high expression level (> 75% up to 100% of positive cells).

nuclei (Fig. 2f). In the mesencephalon, ECE-1 is present in the commissure of the optic tectum and in ependymal cells of the third ventricle (Fig. 2e); in the rhombencephalon, neurons in medulla oblongata are immunoreactive for ECE-1.

Conversely, ECE-2 immunoreactivity is weaker and restricted in localization to the optic tectum (Fig. 3d) and magnocellular periventricular nuclei (Fig. 3c). No immunolabelling is present in thalamic nuclei or hypothalamic nerve fibres. In the rhombencephalon, the medulla oblongata also shows ECE-2 immunoreactivity.

Adrenal glands

All adrenal control sections show no labelling for ECE-1 (Fig. 2a). Endothelial cells of adrenal blood vessels show immunoreactivity for anti-ECE-1 (Fig. 2b). Chromaffin cells are also selectively labelled; in these cells labelling is confined to the cell border near the plasma membrane (Fig. 2c). Steroidogenic tissue is not labelled for anti-ECE-1 (Fig. 2d).

The ECE-2 labelling pattern differs somewhat from that of ECE-1; chromaffin cells are strongly labelled throughout their cytoplasm (Fig. 3b). Endothelial cells are immunoreactive and steroidogenic cells are weakly labelled (Fig. 3b).

Liver

Hepatocytes express weak immunoreactivity to ECE-1 in the peripheral portion of cytoplasm near the plasma membranes (Fig. 2i). Endothelial cells surrounding blood vessels are also immunoreactive (Fig. 2i). The ECE-2 antibody stains exclusively endothelial cells of blood vessels and arterioles, and no immunostaining was detectable in hepatocytes (Fig. 3e).

Kidney

Strong cytoplasmic immunoreactivity against ECE-1 is present within renal tubules, particularly in the apical portion of renal tubular cells (Fig. 2j). No other structures are labelled. No immunostaining for anti-ECE-2 was detectable in proximal tubular cells (Fig. 3g), but medullary collecting duct cells are highly labelled for this antibody near the luminal side of cells (Fig. 3f).

Discussion

We established the presence and localization of the ECE subtypes in various tissues of the lizard *Podarcis sicula* in order to advance our knowledge of the differential tissue-specific expression of this enzyme and to correlate its distribution with the physiological roles

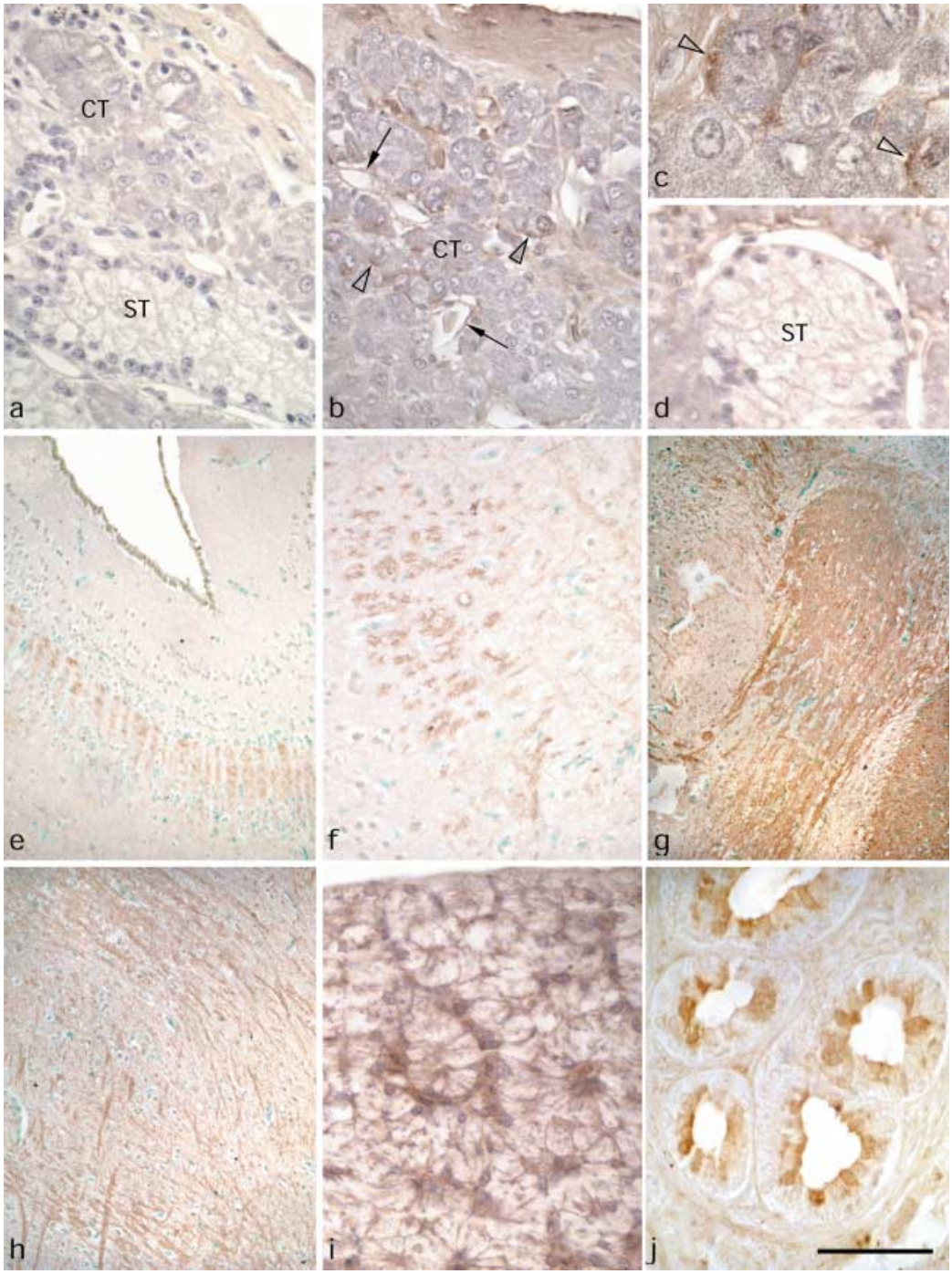

Fig. 2 Localization of ECE-1 enzyme in *Podarcis sicula* tissues. Scale bars = (a,b,d) 16.5 μm, (c) 6.5 μm, (e,g,h) 67 μm, (f,i) 25 μm, (j) 12.5 μm. (a) Adrenal gland control section obtained by pre-incubating anti-ECE-1 with its antigen. Cellular nuclei counterstained with Mayer's hemallum. No signal is detectable either in steroidogenic tissue (ST) or in the chromaffin tissue (CT). (b) Adrenal section treated with anti-ECE-1 showing immunoreactivity of some chromaffin cells (arrowhead) and endothelial cells surrounding blood vessels (black arrow). (c) Detail of chromaffin cells where labelling is evident in the internal side of the cell membrane (arrowhead). (d) Steroidogenic tissue (ST), treated with anti-ECE-1, does not show labelling for ECE-1. (e) Brain section labelled for ECE-1. Immunoreactivity arises from tectal commissure and from ependymal layer of mesencephalic ventricle. (f) Magnocellular periventricular nuclei show immunoreactivity to anti-ECE-1. Methyl-green counterstains nuclear portion of neurons. (g) Preoptic nuclei are strongly immunoreactive to ECE-1. (h) In the posterior hypothalamic area nerve fibres show labelling for ECE-1. (i) Anti-ECE-1 immunohistochemistry on liver section. Labelling is evident in the endothelium and weakly in the hepatocytes. (j) In the kidney, tubular cells are intensely marked in the cytoplasm.

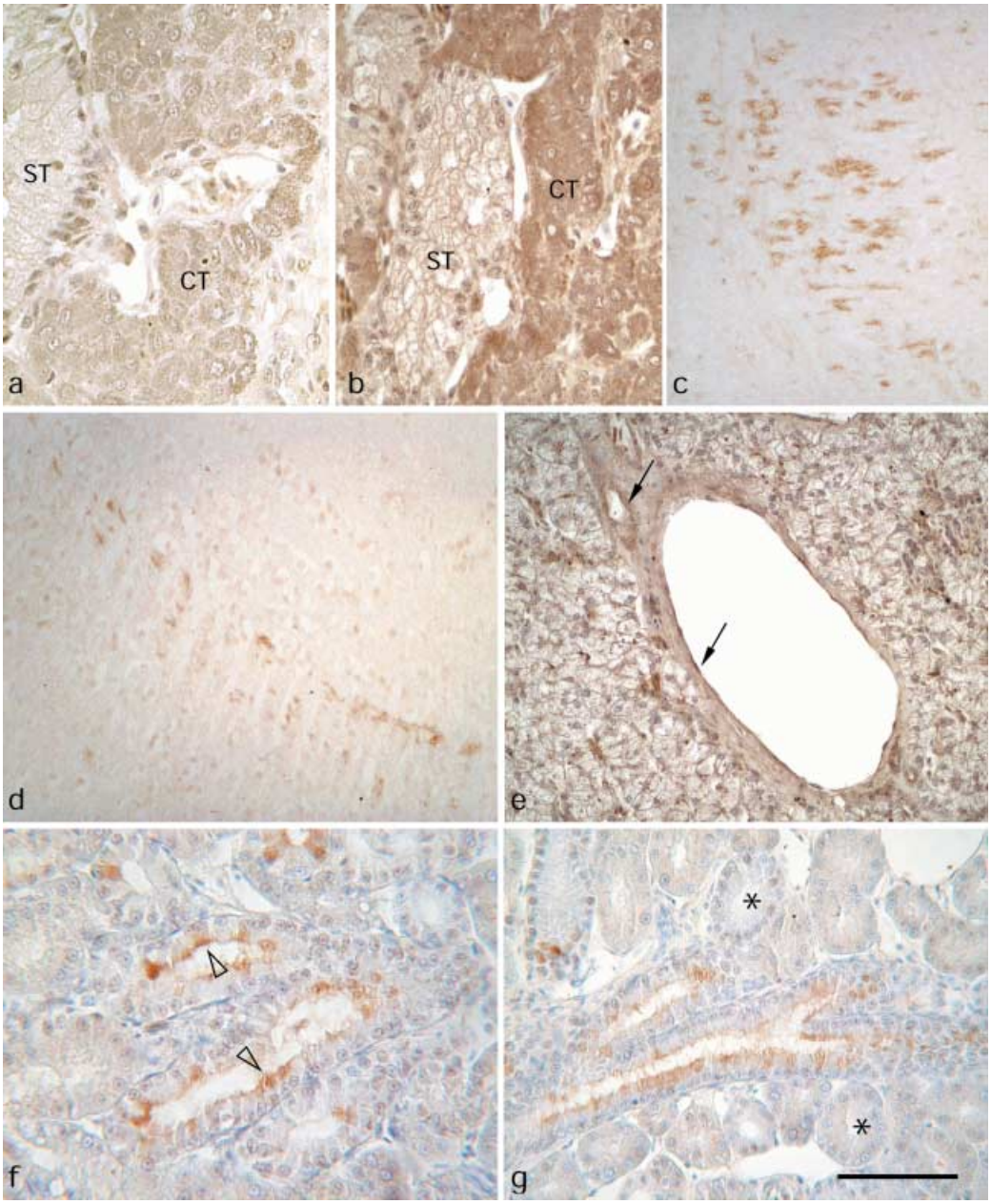

Fig. 3 Localization of ECE-2 immunoreactivity in *Podarcis sicula* tissues. Scale bar = (a–d) 16.5 μm, (e,g) 50 μm, (f) 12.5 μm. (a) Pre-incubating anti-ECE-2 with its antigen, signal in adrenal gland control section is undetectable. (b) Consecutive section of adrenal gland showing a strong cytoplasmic immunoreactivity to ECE-2 antiserum in chromaffin tissue (CT), while steroidogenic tissue (ST) shows weak labelling. (c) Magnocellular periventricular nuclei are weakly immunolabelled for ECE-2. (d) Faint immunoreactivity against ECE-2 in tectal commissure. (e) Cross-section of a blood vessel in the liver in which the endothelium shows clear labelling (black arrow). Hepatocytes are not immunoreactive. (f) Immunoreactivity of kidney collecting ducts (arrowhead). Not all cells express the same degree of labelling. (g) Low-power view of kidney showing the main labelling is in the collecting ducts while tubular cells and other structures show no labelling (*).

of ET previously found (De Falco et al. 2001). ECE was present widely, although its distribution was different in each tissue examined. Endothelial cells of all tissues were immunoreactive with both anti-ECE-1 and anti-ECE-2; this is not surprising considering the pivotal role of these enzymes in the production of ET. It is also significant that in this lower vertebrate ECE is associated with the ET role in the vasoconstriction process, suggesting early and strong evolutionary pressure acting on vertebrates to preserve this important function of ECE.

The presence of ECE-1 and ECE-2 in adrenal medullary cells confirms a role for ET in the regulation of adrenal medullary functions. Several studies show that ET elicits catecholamine release in dog, bovine and rat (Hinojosa-Laborde & Lange, 1999; Nagayama et al. 2000); previous investigations on *P. sicula* showed that administration of ET-1 stimulates catecholamine release in a dose-dependent fashion (De Falco et al. 2001). We have also demonstrated the presence of endothelin receptor type B (ET_b) in *P. sicula* chromaffin cells (De Falco et al. 2002). Both A and B types of receptors were found in human adrenal glands (Davenport et al. 1996) and negative feedback of ET_b receptor on ECE-1 activity was previously demonstrated (Ehrenreich et al. 1999). Together, these observations suggest that local chromaffin cell production of ET, through ECE activity, can influence adrenal physiology in a paracrine manner, and not only through vasoconstriction of blood vessels as a result of endothelial cell ET secretion. Interestingly, chromaffin tissue possesses both variants of the ECE enzymes with a localization pattern similar to that of mammals (Emoto & Yanagisawa, 1995): ECE-1 near to chromaffin cell membrane and ECE-2 within the cytoplasm, suggesting that also in this lizard that ECE-2 could work at acidic pH values. ECE-immunoreactivity of steroidogenic tissue is in agreement with other findings in mammals (Korth et al. 1999) and may suggest that ECE is involved in the regulation of the steroidogenic function, as was previously demonstrated by ET-1 *in vivo* treatment which increased corticosterone secretion (De Falco et al. 2001). Furthermore, it is remarkable that both ECE-1 and ECE-2 cleave substrates other than endothelin such as pro-enkephalin and substance P (Mzhavia et al. 2003); our previous studies showed the presence and the influence of substance P in the chromaffin cells of *P. sicula* (Capaldo et al. 2003) and the presence of leu-enkephalin in *P. sicula* adrenal glands (De Falco et al. 2004). Together, this evidence might suggest a new role for ECE enzymes in the regulation of adrenal activity of *P. sicula* through the processing of regulatory peptides.

We have also demonstrated immunohistochemically the distribution of ECE enzymes in the central nervous system of *P. sicula*. The observed distribution of ECE subtypes is consistent with previous reports demonstrating expression of ECE throughout neuronal structures of several species (Mortensen, 1999). Furthermore, the finding of a specific distribution within the diencephalon, mainly the preoptic areas and in particular the periventricular nuclei, the same areas where ECE activity has been detected by other authors in the rat (Sluck et al. 1999), also suggests that in the nervous system of reptiles a physiological function for ECE is likely.

Further evidence of a mammalian-like pattern can be found in the kidney of *P. sicula*, where ECE distribution partially overlaps both ECE-1 and ET-1 expression in human kidney (Bruzzi et al. 1997; Pupilli et al. 1997). Immunoreactivity of renal tubular cells of *P. sicula* to ECE-1 is consistent with *in vitro* studies demonstrating that human tubular cells express constitutively the prepro-ET-1 gene (Bruzzi et al. 1997) and that both human and rat tubular cells express ET-1, ET-2 and ET-3 (Tereda et al. 1993; Ong et al. 1995). Moreover the presence of ECE-2 immunoreactivity exclusively in collecting ducts is consistent with the high ET-1 content of collecting ducts in the mammalian kidney (Kohan & Fiedorek, 1991). However, we are not yet able to elucidate the physiological reason for this different distribution between ECE-1 and ECE-2 in *P. sicula* kidney, even if it is essential to consider the fundamental significance of blood flow in renal physiology.

We detected ECE enzymes in the liver of *P. sicula*, where the presence of the ET_b receptor was previously shown (De Falco et al. 2002), with a spatial separation for the two subtypes investigated. In addition, we found endothelial cell immunoreactivity to both enzymes and ECE-1 labelling of hepatocytes exclusively: this is in agreement with previous findings (Korth et al. 1999).

In conclusion, all these findings confirm that in *P. sicula* ECE is involved in the regular vasoconstriction role of ET as it is for mammals, and they point to an as yet unidentified physiological function of ET also in lower vertebrates, such as the regulation of peptide and/or hormone secretion, suggesting a broader activity for this metalloprotease rather than the mere production of ET.

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