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ORIGINAL ARTICLE

Immunohistochemical localization of D-aspartate oxidase in porcine peripheral tissues

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Abstract D-Aspartate (D-Asp) is an endogenous substance in mammals. Degradation of D-Asp is carried out only by D-aspartate oxidase (DDO). We measured DDO activity in porcine tissues, and produced an anti-porcine DDO antibody to examine the cellular localization of DDO. All the tissues examined showed DDO activities, whereas the substrate D-Asp was not detected in kidney cortex, liver, heart, and gastric mucosa. In the kidney, intensive immunohistochemical staining for DDO was found in the epithelial cells of the proximal tubules. In the liver, the epithelial cells of interlobular bile ducts, liver sinusoid-lining cells with cytoplasmic processes, and the smooth muscle cells of arterioles were strongly stained for DDO. In the heart, cardiomyocytes and the smooth muscle cells of arterioles showed DDO-immunoreactivity. In the gastric mucosa, only the chief cells were DDO-positive. These newly identified DDO-positive cells seem to actively degrade D-Asp to prevent an excess of D-Asp from exerting harmful effects on the respective functions of porcine tissues.

Keywords D-Aspartate oxidase · D-Aspartate · Immunohistochemistry · Kidney · Liver · Gastric mucosa

Abbreviations

DDO	D-Aspartate oxidase
D-Asp	D-Aspartate
PBS	Phosphate-buffered saline
HRP	Horseradish peroxidase
DAB	3,3′-Diaminobenzidine
NMDA	*N*-Methyl-D-aspartate

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Introduction

D-Aspartate oxidase (DDO, EC 1.4.3.1) is a flavin-dependent enzyme that catalyzes the oxidative deamination of D-aspartate (D-Asp) to produce oxaloacetate, ammonia, and hydrogen peroxide. In mammals, DDO activity was discovered first in rabbit kidney and liver (Still et al. 1949), and then in porcine and bovine kidney (De Marco 1966; Rinaldi 1971). However, the physiological role of DDO in mammals has long been controversial, mainly because free D-Asp is believed to be a non-physiological substance in mammals. By examining the fate of D-Asp administered in drinking water to rats and mice, D'Aniello et al. (1993) obtained strong evidence that once ingested, D-Asp is rapidly absorbed and transported into various tissues including brain tissue, and that DDO metabolizes D-Asp in vivo as it does in vitro. A line of recent studies using targeted deletion of the DDO gene in mice has clearly demonstrated that tissue levels of D-Asp are mainly regulated by DDO (Errico et al. 2006, 2008a, b; Huang et al. 2006).

With the advent of methods to analyze D-amino acids, D-Asp has been found in varying amounts in mammalian tissues (for reviews, see Hamase 2007; Hashimoto and Oka 1997; Imai et al. 1996). In rats, a reciprocal relationship between the tissue localization of D-Asp and DDO activity has been discovered (Schell et al. 1997). For example, D-Asp is concentrated in the posterior pituitary and pineal glands, and no DDO activity is detected in these tissues by

enzyme histochemistry. In line with an increasing list of tissues/cells endogenously containing high levels of D-Asp, various physiological functions of D-Asp have been proposed (for recent reviews, see D'Aniello 2007; Homma 2007). In secretary glands such as the pituitary, adrenal, and pineal glands, it is suggested that D-Asp plays regulatory roles in the synthesis and secretion of hormones by these glands (Boni et al. 2006; D'Aniello et al. 2000a, b; Ishio et al. 1998; Pampillo et al. 2001, 2002; Takigawa et al. 1998; Wang et al. 2000).

To further elucidate the physiological role of DDO in mammals, it is important not only to understand the enzymatic properties of DDO (for bovine DDO, Negri et al. 1987, 1988; for porcine DDO, Yamamoto et al. 2007) but also to reveal the cellular localization of DDO in various tissues. The localization of DDO in the mammalian tissues has never been examined systematically, although the enzyme's distribution has been examined in some detail in the bovine kidney and liver (Zaar 1996), in the rat neuroendocrine gland and brain (Schell et al. 1997), and in the human and rat brain (Zaar et al. 2002). We have recently developed a specific and sensitive method of assaying DDO activity (Tanaka et al. 2007; Yamamoto et al. 2007). In the present study, we applied the method to measure the DDO activity in individual regions of porcine peripheral and brain tissues. A specific polyclonal antibody against porcine DDO was successfully prepared using the enzyme overexpressed in *Escherichia coli* (Yamamoto et al. 2007). The antibody enabled us to localize DDO-expressing cells in various porcine peripheral tissues.

Materials and methods

Tissue collection

Small organ parts (1–5 g) were removed from three prepuberal female pigs (4–6 months old) sacrificed at a local slaughterhouse. Five-millimeter blocks were made from the individual parts and immediately fixed in phosphate-buffered saline (PBS) containing 2.5% glutaraldehyde and 2% paraformaldehyde for 4 h at room temperature. The remaining portions were immediately frozen in liquid nitrogen, and then stored at −80°C until use.

Assay of DDO activity

Each porcine tissue (0.1–0.5 g) was homogenized on ice with 4 volumes of 50 mM sodium pyrophosphate, pH 8.3, containing 1:50 (v/v) protease inhibitor cocktail (Sigma-Aldrich, Inc.) using a small glass homogenizer. A standard assay mixture (100 μL) contained 50 mM sodium pyrophosphate, pH 8.3, 50 mM D-Asp, 50 μM FAD, and 10 μg of catalase (Boehringer-Mannheim, Germany). The reaction was initiated by the addition of a 5-μL aliquot of each homogenate, run at 37°C for 30 min, and then stopped with the addition of 40 μL of 12.5% trichloroacetic acid. After the addition of 5 nmol of 2-oxoglutaric acid (5 μL of 1 mM stock solution in H_2O) as an internal standard, the mixture was centrifuged at 10,000×*g* for 10 min at 4°C. As we described previously (Tanaka et al. 2007; Yamamoto et al. 2007), an aliquot of the supernatant (100 μL) was treated with 3-methyl-2-benzothiazoline hydrazone hydrochloride to convert the oxaloacetate into the azine derivatives of pyruvate, which were then quantified by HPLC on a Cosmosil 3C18 column (Nacalai Tesque, Japan). One unit of DDO activity was defined as the amount of enzyme producing 1 μmol of oxaloacetate per min at 37°C under the standard assay conditions.

Determination of D-aspartate

The D-Asp contents were quantified according to a method described by D'Aniello et al. (2000b) except the sample treatment with D-aspartate oxidase. Briefly, an aliquot of the tissue homogenate (10–50 μL) prepared as described above was immediately mixed with 4 volumes of methanol and 25 nmol of L-homocysteic acid (2.5 μL of a 10 mM stock solution in H_2O). After a 10-min centrifugation at 12,000×*g* and 4°C, an aliquot of the supernatant (60 μL) was dried in an 1.5-mL Eppendorf tube under reduced pressure at 60°C, and the resultant residue was dissolved with 20 μL of 20 mM sodium borate, pH 8.0. After the insoluble material was removed by centrifugation, an aliquot of the supernatant (10 μL) was mixed with 0.5 μL of 50% methanol containing 18.7 mM *o*-phthalaldehyde and 15.3 mM *N*-acetyl-L-cysteine, and then incubated at room temperature for 2 min. Five microliters of this mixture were immediately injected onto a Cosmosil 3C18 column (4.6 × 50 mm). The solvent system was composed of 0.1 M sodium acetate (pH 6.0, buffer A) and acetonitrile (solvent B). The amino acid derivatives were eluted by developing a linear gradient from 9%B to 16%B over 20 min. The flow rate was maintained at 1.0 mL/min. Because we did not confirm the disappearance of the D-aspartate peak by DDO treatment, the obtained D-Asp concentration may be higher than the actual value.

Preparation of polyclonal antibody against DDO

Porcine DDO was overexpressed in *E. coli* and purified as described previously (Yamamoto et al. 2007). According to the standard methods of Takara Bio Inc. (Shiga, Japan), a rabbit was immunized by the subcutaneous injection of an emulsion of the recombinant DDO (0.5 mg) and Freund's complete adjuvant. A booster immunization was repeated

three times at 2-week intervals using the DDO and Freund's incomplete adjuvant. An aliquot of the antiserum obtained from the rabbit (10 mL) was applied to a protein A Sepharose column (2.5 × 0.8 cm, GE Healthcare, Sweden) preequilibrated with 20 mM sodium phosphate, pH 7.0. After a wash with 20 mL of the equilibration buffer, immunoglobulins were eluted from the column using 0.1 M glycine buffer, pH 3.0. The eluate was immediately neutralized by the addition of 2 volumes of 1 M Tris–HCl buffer, pH 7.4, and stored at −80°C prior to use (IgG fraction).

Further purification of the anti-DDO antibody was performed as follows. A HiTrap NHS-activated HP column (2.5 × 0.7 cm, GE Healthcare, Sweden) was incubated with 15 mg/mL of recombinant DDO in 1 mL of 20 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl for 1 h at room temperature. The column was washed with 5 mL of 20 mM sodium phosphate, pH 7.4, containing 3.0 M KSCN until the fixed DDO became colorless. An aliquot of the IgG fraction (1 mL) was dialyzed against 1 L of 20 mM sodium phosphate, pH 7.4, overnight at 4°C, and then applied to the denatured DDO-fixed column preequilibrated with 20 mM sodium phosphate, pH 7.4, containing 0.15 M NaCl. The column was washed with 20 mL of the equilibration buffer. Anti-DDO immunoglobulins were eluted from the column with 1 mL of 0.2 M glycine buffer, pH 2.8. The eluate was dialyzed against 1 L of PBS overnight at 4°C. The dialyzed anti-DDO antibody (0.4 mg/mL stock) was stored at 4°C.

Western blot analysis

Aliquots of the total tissue homogenates prepared for the DDO assay were incubated in the presence of 2% SDS and 5% 2-mercaptoethanol at about 80°C for 3 min, and then separated by SDS-PAGE using a 5–20% gradient gel (ATTO, Tokyo, Japan). The protein contents of the tissue homogenates were determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA). The proteins were transferred to a Clear Blot Membrane-P using a semi-dry blotter (ATTO). All subsequent procedures were carried out at room temperature. The membrane was rinsed twice by incubation with 100 mL of PBS containing 0.1% Tween 20 (Buffer A) for 5 min, and then incubated with PBS containing 5% (w/v) skim milk and 0.1% Tween 20 for 1 h. The purified anti-DDO antibody (0.4-mg/mL stock) was diluted 500-fold with Buffer A containing 1% bovine serum albumin. The blocked membrane was incubated with the diluted antibody for 1 h. The membrane was washed three times with Buffer A, and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:20,000, diluted with Buffer A) (Amersham ECL Plus Western Blotting Reagent Pack; GE Healthcare, UK) for 1 h at room temperature. After five washes with Buffer A, antibodies were detected using Amersham ECL Western Blotting Detection Reagents (GE Healthcare, UK). The specificity of the anti-DDO antibody was confirmed by performing negative controls using the diluted antibody (1:500) pre-incubated overnight with 2 mg/mL of the recombinant DDO at 4°C. As a control for quantification, the antibody used for the detection of the DDO antigen was removed by incubation with 2.0 M glycine buffer, pH 2.8 overnight at 4°C, and then the membrane was re-probed with anti-β-actin monoclonal antibody.

Immunohistochemistry

The fixed tissues were embedded in paraffin. Three-micrometer thick paraffin sections were made and placed on silane-coated glass slides, and then deparaffinized by incubation for 10 min in xylene. The sections were rehydrated through consecutive incubations in 100, 90, 80, and 70% ethanol, and finally washed with PBS. The sections were first incubated in 3% H_2O_2 in methanol for 20 min at room temperature to inhibit endogenous peroxidase. Then, the sections were washed with PBS and incubated overnight at 4°C with the anti-DDO antibody diluted in PBS containing 1% BSA [1:1000 for kidney; 1:500 for liver, heart (right atrium), and stomach (corpus)]. After removing unbound antibodies by washing with PBS, the sections were incubated with a HRP-labeled goat antibody against rabbit IgG [Histofine Simple Stain Max PO (MULTI); Nichirei Bioscience Inc., Tokyo, Japan] twofold diluted with PBS containing 1% BSA. The sections were washed with PBS, and then peroxidase activity was developed by incubation with 3,3′-diaminobenzidine (DAB) using a Simple Stain DAB Solution (Nichirei Bioscience Inc.). Counterstaining was performed with hematoxylin. Thereafter, the sections were dehydrated in ethanol, cleared with xylene, coverslipped with Crystal/Mount (Biomeda, Pittsburg, PA), and observed with a Nikon Microphoto-FXA (Nikon Co., Tokyo, Japan). The specificity of the immunohistochemical procedures was evaluated with the following negative controls: (1) omission of the primary anti-DDO antibody or the secondary antibody, and (2) overnight pre-incubation of the primary antibodies with the recombinant DDO (2 mg/mL) at 4°C.

Results

Distribution of DDO activity in porcine tissues

As shown in Fig. 1, the highest DDO activity was found in the kidney cortex (17.9 ± 2.6 U/g protein), followed by the choroid plexus (2.33 ± 0.21 U/g protein), heart (right atrium, 2.19 ± 0.40 U/g protein) and liver (1.46 ± 0.20 U/g

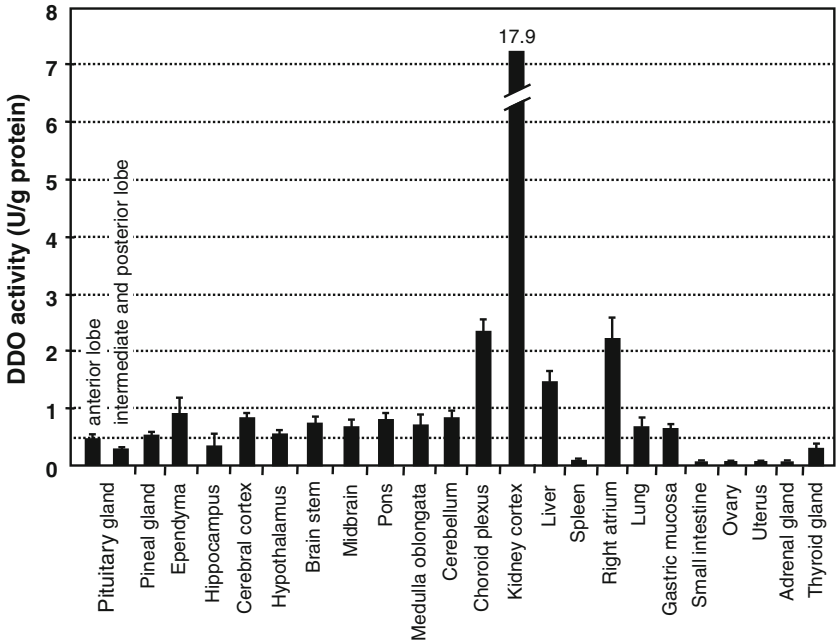

Fig. 1 Distribution of DDO activity in porcine tissues. Values are the mean ± SD determined for three female pigs. The kidney cortex exhibited an extremely high level of DDO activity, 17.9 ± 2.6 U/g protein

protein). We could detect only trace levels of DDO activity in the spleen, small intestine, ovary, uterus, and adrenal gland (<0.05 U/g protein). The remaining tissues showed low to moderate DDO activity ranging from 0.4 to 0.9 U/g protein. D-aspartate was not detected in the kidney cortex, choroid plexus, liver, right atrium, and gastric mucosa.

Western blot analysis

Schell et al. (1997) reported the successful visualization of DDO activity in rat adrenal and brain sections using *N*-methyl-D-aspartate (NMDA) as a substrate. However, it was difficult for us to localize DDO activity even in sections of porcine kidney cortex where the highest DDO activity was found. Therefore, we developed an anti-DDO antiserum in a rabbit, and purified an anti-DDO antibody from the serum using a denatured DDO-fixed affinity column. The specificity and sensitivity of the resultant antibody were examined in a Western blot analysis of total homogenate of porcine tissue. A distinct signal was observed for all the tissues examined at a molecular weight corresponding to that of purified DDO (Fig. 2). When the antibody was pre-absorbed with 2 mg/mL of recombinant porcine DDO, staining was completely blocked (data not shown). The strength of the signal correlated well with the tissue levels of DDO activity (Figs. 1, 2).

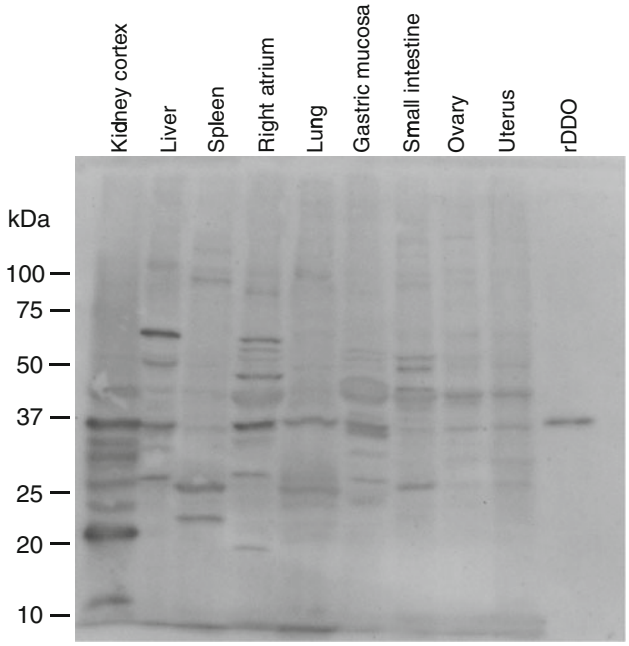

Fig. 2 Western blot detection of DDO in whole tissue homogenates with the anti-DDO antibody. Aliquots of whole tissue homogenates (containing 20 μg of protein except for the kidney cortex, 10 μg) were loaded on a 5–12% SDS-PAGE gel and consequently transferred to a PVDF membrane. The immunoblotting was done using the antibody (1:500, final concentration of 0.8 μg/mL). rDDO is recombinant DDO (0.5 μg)

Immunohistochemical localization of DDO

The cellular distribution of DDO was revealed with the use of DAB as a substrate for the final HRP reaction, which resulted in a brown stain. Tissues were counterstained with hematoxylin (blue stain). No immunoreactivity was detected in parallel incubations of control sections when the anti-DDO antibody was omitted or pre-absorbed with 2 mg/mL of recombinant DDO (data not shown).

Kidney cortex

Strong staining for DDO was found only in the renal proximal tubules (Fig. 3a). No significant staining was found in the glomeruli and renal distal tubules.

Higher magnification revealed coarse granular staining in the cytoplasm of the epithelial cells of the proximal tubules (arrows in Fig. 3b). The specific granular staining is consistent with the peroxisomal localization of DDO reported for bovine and human kidney cortex (Zaar 1996; Zaar et al. 2002).

Gastric mucosa

In porcine gastric mucosa, only chief cells, which produce pepsinogen, showed strong cytoplasmic staining for DDO (Fig. 3c, d). No significant staining was observed for parietal cells and mucous neck cells. The surface epithelium and submucosal tissues were not stained either.

Liver

Some hepatocytes were strongly stained for DDO and others were weakly stained, resulting in a mosaic staining pattern of the liver lobules (Fig. 3e–g). Irrespective of the difference in staining intensity, all hepatocytes exhibited granular reactivity in the cytoplasm (Fig. 3g). There was no significant correlation between staining intensity and the position in the lobule of individual hepatocytes.

Intriguingly, intensely stained sinusoid-lining cells with long cytoplasmic processes were scattered throughout the lobule (arrows in Fig. 3g). Diffuse dense staining of the cytoplasm including the processes was observed. These cells may be possibly hepatic stellate cells (Van Rossen et al. 2009).

In interlobular connective tissues, the smooth muscle cells of arterioles and the epithelial cells of bile ducts showed strong granular staining for DDO in the cytoplasm (Fig. 3f). Interlobular veins showed no signal.

Right atrium of heart

All myocytes showed punctate cytoplasmic staining for DDO (Fig. 3h, i). Intense staining was found in the smooth muscle cells of the heart arterioles (Fig. 3h, j).

Discussion

In this study we measured biochemically the distribution of DDO activity and its substrate D-Asp in porcine tissues. Interestingly, most of the porcine tissues showed similar DDO activity. In all the porcine tissues examined, DDO immunoreactivity was found only in a subgroup of the constituent cells. The restricted expression of DDO seems to be physiologically important to keep the D-Asp concentration low in small defined areas of the relevant tissues.

Porcine kidney cortex showed the highest DDO activity, and only the cytoplasm of epithelial cells of the proximal tubules was strongly stained for DDO. These results are the same as that found for bovine and human kidney cortex (Zaar 1996; Zaar et al. 2002). We could not detect D-Asp in the kidney cortex. Because amino acids in the glomerular filtrate are reabsorbed by the proximal tubule epithelial cells, a physiological role of DDO in the kidney may be to remove D-Asp from the circulating blood to keep plasma levels of D-Asp low.

Significant DDO expression was recently confirmed in the mouse stomach (Errico et al. 2006). In the present study, we demonstrated for the first time that in the porcine gastric mucosa only the chief cells, which produce and secrete pepsinogen, had intense cytoplasmic immunoreactivity for DDO.

In porcine liver, a small number of perisinusoidal cells with long thin processes exhibited the most intense cytoplasmic staining for DDO. The observed staining pattern of these cells very closely resembled that observed for human hepatic stellate cells stained for vinculin and cellular retinol-binding protein-1 (Van Rossen et al. 2009). Liver damage activates hepatic stellate cells into activated myofibroblast-like cells that have important roles in the pathogenesis of liver fibrosis (for recent review, see Parola et al. 2008). If the DDO-positive sinusoidal cells are identical to the hepatic stellate cells, DDO would be a good marker for this special group of cells.

The porcine hepatocytes were stained for DDO in varying intensity. There was no clear relationship between the staining intensity and the cellular location within a particular lobule. In the case of D-amino acid oxidase, the peripheral hepatocytes show stronger staining than the central ones in a particular lobe of rat liver (Horiike et al. 1985). D-Amino acid oxidase degrades most D-amino acids other than those with a carboxyl group at the side chain (D-aspartate, NMDA, and D-glutamate). The difference in the substrate preference between D-amino acid oxidase and DDO may have some relation to their different expression patterns.

The epithelial cells of the interlobular biliary ducts exhibited strong cytoplasmic staining for DDO. The apical side of the cytoplasm stained stronger than the basal side. In relation to these new findings, it is interesting that porcine small intestine, where amino acids from foods are absorbed, exhibited very low levels of DDO activity and contained low but significant levels of D-Asp. These results suggest that the absorbed D-Asp is first taken up by hepatocytes, which degrade part of it and secrete the rest into

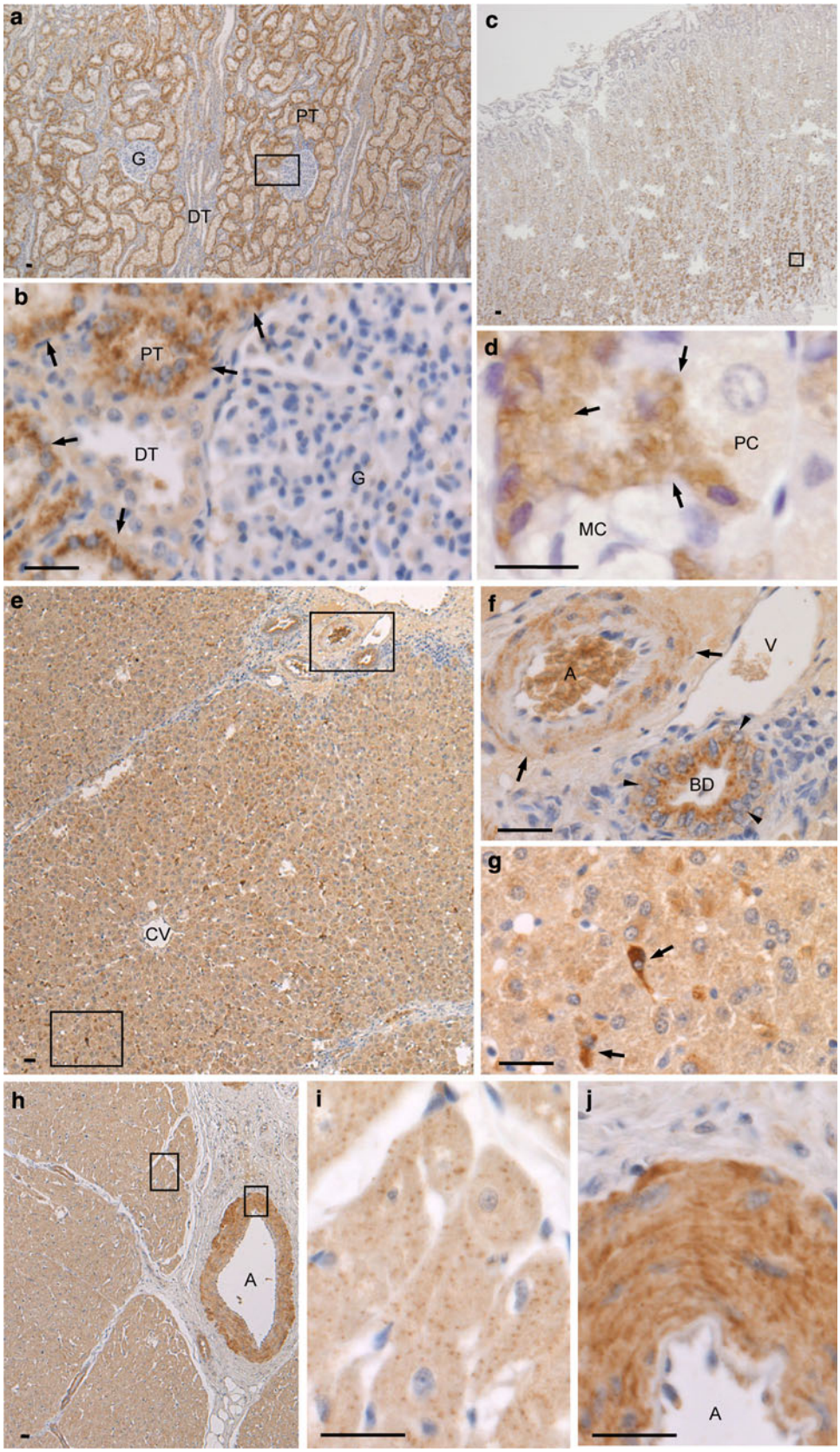

Fig. 3 Immunohistochemical detection of DDO in porcine peripheral tissues. The panels **d**, **f**, **g**, **i**, and **j** represent high magnification. **a**, **b** Kidney cortex. Strong staining was found only in the renal proximal tubules. High magnification shows granular staining in the cytoplasm of the epithelial cells (*arrows* in panel **b**). *G* glomerulus, *PT* proximal tubule, *DT* distal tubule. **c**, **d** Gastric mucosa. Only the chief cells showed strong cytoplasmic staining (*arrows* in panel **d**). *PC* parietal cells, *MC* mucous neck cells. **e**, **f**, **g** Liver. Some hepatocytes showed strong cytoplasmic staining and others showed weak staining, resulting in a mosaic pattern. A small population of perisinusoidal cells with long cytoplasmic processes exhibited intense staining (*arrows* in panel **g**). In the interlobular regions, granular cytoplasmic staining was found in the smooth muscle cells of arteries and the epithelial cells of bile ducts (*arrows* and *arrowheads* in panel **f**, respectively). *CV* central vein, *A* interlobular artery, *V* interlobular vein, *BD* interlobular bile duct. **h**, **i**, **j** Heart (right atrium). The myocytes showed coarse granular cytoplasmic staining (panel **i**). Strong positive staining was also observed in the smooth muscle cells of heart arterioles (panel **j**). *A* arteriole. Scale bars = 25 μm

bile ducts. The epithelial cells of biliary ducts might degrade actively the secreted D-Asp to prevent the D-amino acid from entering the enterohepatic circulation. D-Asp is an excitatory amino acid for the central nervous system and transported through the blood–brain barrier (Hosoya et al. 1999). Therefore, it seems to be important to prevent excess amounts of exogenous D-Asp from entering into the systemic circulation. The epithelial cells of the choroid plexus and ependyma stained intensely for DDO (data not shown). These cells seem to remove the excitatory D-amino acid from cerebrospinal fluid.

All the myocytes of the porcine right atrium of heart showed strong coarsely granular cytoplasmic staining for DDO. The heart muscle can use D-Asp as an energy source, and seems to facilitate the reduction of blood D-Asp levels. The smooth muscle cells of the interlobular arteries of the liver and the arterioles of the heart also contained high levels of DDO. These smooth muscle cells may play the same role in reducing the serum levels of D-Asp as the heart myocytes.

In conclusion, we examined the cellular localization of DDO in porcine peripheral tissues and found that the oxidase was selectively expressed in a defined subgroup of cells. The newly identified DDO-positive cells are (1) the epithelial cells of hepatic interlobular bile ducts, (2) smooth muscle cells of hepatic interlobular and heart arterioles, (3) heart myocytes, (4) the chief cells of the gastric mucosa, and probable (5) hepatic stellate cells. These findings suggest that DDO and/or D-Asp play yet unidentified roles in these various peripheral tissues.

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