Ornithine metabolism in male and female rat kidney: mitochondrial expression of ornithine aminotransferase and arginase II

Olivier Levillain,1,2 Annette Hus-Citharel,3 Sandra Garvi,4 Simone Peyrol,4 Isabelle Reymond,5 Mireille Mutin,6 and François Morel2

1Laboratoire de Physiopathologie Métabolique et Rénale, Institut National de la Santé et de la Recherche Médicale (INSERM) U 499. 2Centre Commun d’imagerie Laennec, and 3Laboratoire de Neurobiologie Expérimentale et Pathophysiologie, INSERM U 433, Faculté de Médecine Lyon R. T. H. Laennec, 69372 Lyon Cedex 08; 4Département de Physiologie, Centre Médical Universitaire, CH-1211 Geneva 4, Switzerland; and 5Laboratoire de Physiologie Cellulaire, Centre National de la Recherche Scientifique Unité de Recherche Associée 219, and 6Laboratoire de Pathologie Vasculaire et Endocrinologie Rénale INSERM U 36, Collège de France, 75231 Paris Cedex 05, France

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OAT and AII proteins overlapped in PST mitochondria, L-arginine-pressed more in female rat proximal tubules than in male. Because complete oxidation of ornithine. In conclusion, the OAT gene was expressed predominantly in proximal tubules. L-[1-14C]ornithine decarboxylation occurred in all tubules, but predominantly in proximal tubules. L-[1-14C]ornithine decarboxylation was enhanced when L-[1-14C]ornithine was given to tubules as the sole substrate. The use of L-[U-14C]ornithine demonstrated the complete oxidation of ornithine. In conclusion, the OAT gene was expressed more in female rat proximal tubules than in male. Because OAT and AII proteins overlapped in PST mitochondria, L-arginine-derived ornithine may be preferentially converted to L-glutamate, as proven by ornithine oxidation. However, the coexpression of ODC, glutamate decarboxylase, and glutamine synthetase in PST suggests that L-ornithine can also be metabolized to putrescine, GABA, and L-glutamine. The fate of L-ornithine may depend on the cellular context.

L-ornithine; L-arginine; proximal tubules; isolated nephron segments; Western blot analysis; immunofluorescence; electron microscopy; mitochondria; ornithine decarboxylase

THE AMINO ACID L-ORNITHINE is absent from food and is not a constituent of proteins; therefore, vertebrate organisms are dependent on its formation. In kidneys, four sources of L-ornithine can be considered: 1) L-ornithine is present in the blood and then transported across the basolateral membranes of a variety of renal cells (36, 40); 2) L-ornithine is filtered in glomeruli and reabsorbed by specific carriers located in the apical membrane of the proximal convoluted tubule (PCT) (43); 3) in PCT, the enzyme L-arginine:glycine amidotransferase (GAT; EC 2.1.4.1) metabolizes L-arginine in the presence of L-glutamate and produces guanidinoacetic acid and L-ornithine (42); and 4) in the whole proximal straight tubule (PST) of several mammals including rats, the extrahepatic arginase II isoenzyme (AII; EC 3.5.3.1) hydrolyzes L-arginine into urea and L-ornithine (23).

L-Ornithine is known to play a pivotal role in several metabolic pathways as precursor for l-proline, l-glutamate, l-glutamate, l-citrulline, and l-arginine biosynthesis. Interestingly, renal tubular cells contain l-ornithine-metabolizing enzymes. Indeed, ornithine decarboxylase (ODC; EC 4.1.1.17), the key enzyme in the polyamine pathway that converts L-ornithine into putrescine (Fig. 1) (31), is expressed in rat PCT, cortical and medullary proximal straight tubules (CPST and OSPST, respectively) (2, 6, 21). In another metabolic route, the reversible enzyme ornithine aminotransferase (OAT; EC 2.6.1.13) produces L-glutamate and glutamate-γ-semialdehyde in the presence of L-ornithine and α-ketoglutarate (Fig. 1) (37). The nuclear encoded mitochondrial OAT has been isolated and purified from male rat kidney, characterized and visualized in male rat proximal tubules by immunohistochemistry (14, 25). Finally, it is generally assumed that the genes encoding the enzymes that metabolize L-ornithine to l-citrulline and l-proline are expressed in the liver and the intestine but not in the rat kidney.

In kidneys, L-ornithine may be a source of energy when completely oxidized in the citric acid cycle or serves as a precursor for l-glutamate and GABA synthesis (Fig. 1). The glutamate-γ-semialdehyde produced during L-ornithine transamination by OAT is converted into L-glutamate by the enzyme pyrroline-5-carboxylate (P5C) dehydrogenase. To produce energy, L-glutamate is desaminated into α-ketoglutarate before entering in the citric acid cycle (Fig. 1). Alternatively, L-glutamate is converted into l-glutamine by glutamine syn-
Fig. 1. Catabolism of l-arginine and l-ornithine. 1. Nonenzymatic step; 2. pyrroline-5-carboxylate (P5C) dehydrogenase; 3. glutamine synthetase; 4. glutamate dehydrogenase; and 5. glutamate decarboxylase. AII, arginase II; ODC, ornithine decarboxylase; OAT, ornithine aminotransferase.

Western blot analysis. In addition, to establish a metabolic link between AII and the enzymes that process in metabolizing l-arginine-derived ornithine, the distribution of OAT and AII was analyzed by Western blotting in whole kidneys, renal zones, and isolated mitochondria, whereas ODC mRNAs were localized by in situ hybridization. Finally, to prove that l-ornithine could be metabolized to l-glutamate by OAT, l-ornithine oxidation was analyzed by incubating dissected tubules with l-[1-14C]ornithine or l-[U-14C]ornithine as sole substrates.

Briefly, OAT protein is constitutively expressed in a relatively ubiquitous fashion in normal male and female rat kidneys. The highest levels of OAT protein were detected in female compared with male kidneys, and in CPST and OSPST compared with other nephron segments. OAT protein expression overlapped with the distribution of AII and ODC in CPST and OSPST. l-Ornithine can be completely oxidized in the citric acid cycle. Because AII and OAT are coexpressed in mitochondria, l-arginine-derived ornithine may be preferentially a source of l-glutamate. The metabolic fate of l-ornithine may depend on the cellular context.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats from Ifla Credo (L’Arbresle sur Orge, France) had free access to tap water and standard laboratory food (Soulferrat, 20% protein, Genthon, France). Animals were housed in a room maintained at 20°C with a 12:12-h light-dark cycle. Six-week-old male (236 ± 4 g body wt (BW)) and female (168 ± 6 g BW) rats were used for Western blot analyses and histological studies. Nine-week-old male (295 g BW) and female (223 g BW) rats were used for Western blot analysis (see Fig. 4). Male rats (~150 g BW) were used for microdissection studies. Rats were anesthetized (ip) using 0.1 ml/100 g BW pentobarbital sodium (Nembutal, 6%, Clin Midy, Paris, France). The experiments were approved by the local Committee for Animal Experiments.

Kidney Preparation and Dissection of Renal Zones

Male and female rats were used in these experiments. In a first series of experiments designed to localize proteins by Western blot analysis, the right and left kidneys were rapidly removed, decapsulated, and cut along the corticopapillary axis to dissect six renal zones [superficial cortex (Cs), deep cortex (Cd), outer stripe of the outer medulla (OS), inner stripe of the outer medulla (IS), inner medulla (IM), and papilla (Pap)] at 4°C under a stereomicroscope. The dissected tissue was immediately placed in a sterilized Eppendorf tube, frozen in liquid nitrogen, and maintained at −80°C. In a second series of experiments, the whole right and left kidneys were rapidly removed, decapsulated, frozen in liquid nitrogen, and maintained at −80°C.

OAT and All Antibodies

Peptide-derived polyclonal antibodies to OAT and All were prepared by CovalAb (Oullins, France) and used for semiquantitative immunoblotting, immunohistochemistry, and immunogold labeling. To obtain a polyclonal antibody against OAT, three amino acid synthetic peptides corresponding to amino acids 27–41 (SVATK-TEQGPFPSEC), 43–56 (IFERESKYGAHNYHC), and 311–323 (CMLT1KPGHGSTGY) of the mouse and rat OAT sequences were synthesized on the basis of the sequence reported in SwissProt P29756 and P04182, respectively. To obtain a polyclonal antibody against All, one amino acid synthetic peptide corresponding to amino acids...
Concentrations were determined using the Bradford protein assay (3). Mitochondria was resuspended twice in the same buffer. Protein concentrations were determined in the supernatant using the Bradford protein assay (3). Fifty-microgram samples of mitochondrial proteins were subjected to a 12% PAGE containing 0.1% SDS and transferred for Western blotting as described above.

**Kidney Preparation and Microdissection of Nephron Segments**

Male rats were anesthetized, and the left kidney was prepared for microdissection as previously described (22). Briefly, the kidney was perfused with a complete medium (see below) containing 1.63 mg/ml collagenase and sliced along the corticopapillary axis. Small pyramids containing both cortical and medullary tissue were incubated at 30°C in complete medium containing 0.9 mg/ml collagenase. Glomeruli (Glm), PCT, CPST, OSPST, descending thin limb (DTL), distal convoluted tubule (DCT), ascending thin limb (ATL), medullary and cortical thick ascending limbs (MTAL and CTAL), cortical and outer medullary and inner medullary collecting ducts (CCD, OMCD, and IMCD, respectively) were identified and microdissected in the complete medium at 4°C using a stereomicroscope (16). Glomeruli and tubules were rinsed in the basal medium (see below), transferred onto siliconized BSA-coated, hollow glass slides with 0.5 μl basal or complete medium, and tightly sealed with a glass coverslip. The tubules were drawn under a microscope with a clear chamber for subsequent length measurement. Samples were maintained at 4°C until metabolic incubation.

Basal medium consisted of (in mM) 137 NaCl, 5 KCl, 0.44 KH₂PO₄, 1 MgCl₂, 0.8 MgSO₄, 0.33 Na₂HPO₄, 1 CaCl₂, and 20 HEPES, as well as 0.1% BSA, 1% vitamin mixture, and 6% dextan and was adjusted to pH 7.4 with NaOH. The osmolarity was 350 mosmol/kgH₂O. Complete medium consisted of basal medium supplemented with energy-providing substrates consisting of (in mM) 5 glucose, 5 lactate, 10 acetate, 1 pyruvate, and 2 glutamine. Before use, each medium was bubbled with O₂.

**Measurement of L-Ornithine Oxidation in Intact Nephron Segments**

In an initial series of four to five experiments, glomeruli and tubules were incubated in either basal or complete medium. In the former incubation medium, L-ornithine is the sole possible source of energy if OAT is expressed in glomeruli or tubules. In this case, L-ornithine-derived glutamate (Fig. 1) will be preferentially oxidized in the citric acid cycle to provide energy (ATP). In contrast, in complete medium, the contribution of L-ornithine in energy production is minor. Incubation was started by the addition of 0.5 μl of either basal or complete medium containing ~100 μM L-[1-¹⁴C]ornithine. The concentration of L-ornithine used is in the range of its plasma level (36).

In a second series of three experiments designed to prove the complete oxidation of L-ornithine, glomeruli and tubules dissected in the same experiments were divided into two groups and incubated with basal medium. Incubation was started by the addition of 0.5 μl of basal medium that contained ~100 μM of either L-[1-¹⁴C]ornithine or L-[U-¹⁴C]ornithine. If L-ornithine is completely oxidized, a fivefold increase in ¹⁴CO₂ production from L-[U-¹⁴C]ornithine compared with L-[1-¹⁴C]ornithine is expected because this amino acid is composed of five labeled carbons.

The incubation chamber was sealed with a glass coverslip containing a 2-μl droplet of KOH (350 mosmol/kgH₂O). Samples were incubated in a water bath at 37°C for 70 min. The KOH droplet containing the ¹⁴CO₂ was removed, and the amount of radioactivity was estimated by liquid scintillation counting. Control or blank samples contained either L-[1-¹⁴C]ornithine or L-[U-¹⁴C]ornithine but no tubules. Results are means ± SE expressed in femtomoles of ¹⁴CO₂ produced per minute and per millimeter tubular length.

**Indirect Immunofluorescence**

Kidneys were rapidly removed, cut along the corticopapillary axis, immersed in Bouin fixative, and embedded in paraffin. Tissue sections

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F729

ORNITHINE METABOLISM IN RAT KIDNEY

318–332 (1ASSFGQTREGGHIEC) of the human, mouse, and rat AII sequences was synthesized on the basis of the sequences reported in SwissProt P78540, O08691, and O08701, respectively. Each synthetic peptide was analyzed by HPLC and conjugated to keyhole limpet hemocyanin via covalent linkage to cysteine. For each antibody, one female New Zealand rabbit was immunized three times with the conjugates using complete Freund’s adjuvant. OAT and AII antibody titer was determined by ELISA and immunopurified using a column on which the relevant synthetic peptide was immobilized.

**Protein Extraction and Western Blot Analyses of OAT and All Proteins**

Each frozen kidney and renal zone were mixed at 4°C with a Turrax in the proportion of 100 mg tissue/2 ml lysing buffer (18) containing 1 mM protease inhibitor cocktail, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine and then centrifuged at 10,000 g for 30 min at 4°C. Protein concentrations were determined in the supernatant using the Bradford protein assay (3). Microgram samples of soluble proteins were subjected to a 10% PAGE containing 0.1% SDS using 6 W/gel and transferred to a polyvinylidene difluoride membrane (0.45 μm, Immobilon-P, Millipore) at 150 mA for 90 min. Proteins were fixed on the membrane with Ponceau S solution for 15 min. Immunoblots were washed twice for 15 min in 1× Tris-buffered salt + 0.15% Tween 20 (TBST) and immersed twice in a blocking solution consisting of 5% fat-free milk powder in 1× TBST for 30 min.

The blots were incubated with the following primary antibodies in 5% milk-1× TBST: a purified polyclonal rabbit anti-OAT (dilution 1:1,000); a purified polyclonal rabbit anti-AII (dilution 1:500); a polyclonal rabbit anti-αT-casein (dilution 1:1,000) (34); a rabbit polyclonal anti-core 2 (mitochondrial complex III core protein 2; dilution 1:20,000) (19); a mouse monoclonal anti-iron sulfur protein (ISP; dilution 1:80,000) (19); a mouse monoclonal anti-α-subunit F1-ATP synthase (dilution 1:5,000) (27); a polyclonal rabbit anti-mouse aldose reductase (AR; dilution 1:3,000); a monoclonal mouse anti-β-actin (dilution 1:2,000); and a monoclonal mouse anti-gluceraldehyde-3-phosphate dehydrogenase (G3PDH; dilution 1:170). The blots were washed three times for 20 min in 1× TBST and incubated for 60 min with either peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG secondary antibodies (dilution 1:10,000) in 5% milk-1× TBST. Blots were washed three times for 10 min in 1× TBST, and antibody binding was revealed using an ECL Western blotting kit and Kodak X-MAT films. Exposures were made with a broad range of exposure times to allow us to choose films for analysis in which the band densities were below saturation. Films were scanned, and the intensity of the bands was estimated using the ImagEMaster Total Lab v1.00 program. CSD, a 55-kDa protein (SwissProt Q64611, rat CSD: 55,248 Da), was used as a marker of Cd and OD because it is specifically located in CPST and OSPST (34), whereas AR, a 35-kDa protein (SwissProt P07943, rat AR: 35,666 Da), was used as a marker of IM and PAP because its distribution is restricted to the inner medullary and papillary collecting ducts (35). α-Subunit F1-ATP synthase, a 55-kDa protein, core 2, a 45-kDa protein, and ISP, a 25-kDa protein, were used as specific mitochondrial markers and belong to the respiratory chain.

**Preparation of Mitochondrial Fraction**

Renal cortex + OS of two female rats were dissected at 4°C under a microscope and rapidly immersed in a buffer composed of (in mM) 250 sucrose, 10 Tris-HCl, and 1 Na₂EDTA, pH 7.6, maintained at 4°C. The tissue was homogenized gently using a Potter-Elvehjem glass homogenizer PTFE pestle (Belco Glass, Vineland, NJ). The homogenate was centrifuged at 600 g for 10 min. The supernatant was centrifuged at 9,000 g for 10 min. The pellet that contained the mitochondria was resuspended twice in the same buffer. Protein concentrations were determined using the Bradford protein assay (3).
of 4–7 μm were glassed on collect slides (ChemMate, Dako), deparaffinized three times in methylocyclohexane (toluene substitute) for 5 min at room temperature (RT), step-rehydrated in 100% ethanol for 5 min, twice in 95% ethanol for 5 min, and rinsed with doubly distilled water and then with PBS (pH 7.4). Nonspecific sites were coated with PBS containing 100 mM phosphate buffer, 150 mM NaCl, supplemented with 0.3% Triton X-100 and 1% BSA (PBST-BSA) for 120 min at RT and incubated overnight at 4°C with purified primary rabbit anti-OAT antibody (dilution 1:100) in PBST. The slides were rinsed three times in PBST for 5 min and incubated for 120 min at RT with secondary Alexa Fluor 546-conjugated goat anti-rabbit IgG (dilution 1:1,000; Interchim, Montluçon, France). Slides were washed twice for 5–10 min in PBST, once in PBS for 5–10 min, and incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; 0.1 μg/ml) in PBS for 30 min at RT. Slides were washed thrice in PBS and mounted with Fluoroprep (Biomérieux, France). Tissue sections were examined with a fluorescent microscope.

**Immunogold Labeling for Transmission Electron Microscopy**

Dissection of Cs, Cd, and OS was performed at 4°C. Each renal zone was cut into small pieces (<1 mm³) and fixed for 60 min at 4°C in PLP composed of 10 mM metaperiodate, 75 mM lysine, and 2% paraformaldehyde in 37 mM Sorensen phosphate buffer containing 0.2% glutaraldehyde. Pieces of tissue were washed three times in 50 mM Sorensen’s phosphate buffer, 200 mM sucrose, and 100 mM lysine monochlorhydrate for 20 min at 4°C, dehydrated through a series of washes with 30, 50, 70, and 95% ethanol maintained previously at 4°C for 5 min, infiltrated with London Resin White (LRWhite, EMS) using 1:1 LRWhite and 4°C absolute ethanol for 60 min followed by pure LRWhite for 4°C for three periods of 60 min, and finally embedded in pure LRWhite in gelatin capsules for polymerization at 50°C for 48 h.

Ultrathin sections (~80 nm thick) were cut on a RMC MTX ultramicrotome (Exelience), mounted on 200-mesh nickel grids coated with 1:1,000 polylysine, and stabilized for 1 day at RT. Immunogold labeling was performed by flotation of grids on drops of reactive media. Nonspecific sites were coated with 1% BSA and 1% normal goat serum in 50 mM Tris-HCl, pH 7.4, for 60 min at RT. Thereafter, incubation was carried out overnight at 4°C in a wet chamber with the purified rabbit-OAT primary antibody diluted 1:100 in 1% BSA, 50 mM Tris-HCl, pH 7.4. Tissue sections were successively washed three times in 50 mM Tris-HCl, pH 7.4 and pH 8.2 at RT, dehydrated in a series of washes with 30, 50, 70, and 95% ethanol for 45 min at RT in 1% BSA, 50 mM Tris-HCl, pH 8.2, for 20 min at RT, labeled with 10 nm gold-conjugated goat anti-rabbit IgG (Tebu) diluted 1:25 in 1% BSA, 50 mM Tris-HCl, pH 8.2, successively washed three times in 50 mM Tris-HCl, pH 8.2 and 7.4, and three times in filtered distilled water. Sections were contrasted with aqueous saturated 3% uranyl acetate for 45 min in darkness and lead citrate for 5 min and observed on a transmission electron microscope (Jeol 1200EX, Tokyo, Japan) equipped with a MegaViewII digital camera and AnalySIS software (Éloise).

**In Situ Hybridization**

**Preparation of the probes.** Sense and antisense RNA probes were synthesized in vitro from mouse ODC cDNA as previously described (20). In vitro transcription reactions were carried out using 1 μg of DNA and 30 μCi of 32P[UTP. After phenol-chloroform extraction and ethanol precipitation, transcripts were subjected to a limited alkaline hydrolysis to obtain fragments of ~400 nucleotides.

**Tissue preparation and hybridization.** Control male and female rats were anesthetized, and their kidneys were immediately fixed and frozen in isopentane at ~70°C. Cryostat sections (16 μm thick) were mounted on silanized slides (Dako, Trappes, France). The sections were dipped in acetone for 5 min at 4°C, air-dried, and fixed with 4% paraformaldehyde in 100 mM PBS (pH 7.4) for 15 min at 4°C. The tissue sections were acetylated in 100 mM triethanolamine buffer (pH 8.0) containing 0.25% acetic anhydride for 10 min at RT and treated with 1% SSC containing 50% formamide for 10 min at 55°C. After ethanol dehydration and air-drying, the tissue sections were hybridized overnight at 52°C in a solution containing (in m) 1 Na2HPO4, 20 Tris-HCl, 5 EDTA (pH 6.8), 300 NaCl, and 10 DTT as well as 50% formamide, 10% dextran sulfate, 1× Denhardt’s solution, 0.5 mg/ml yeast transfer RNA (tRNA), and 8 ng either sense- or antisense-labeled riboprobes. The incubating medium contained 5% 3C (50 μl per slide)–1.

**Posthybridization and detection.** The sections were rinsed in 1× SSC containing 50% formamide for 30 min at 55°C, 2× SSC for 10 min at RT, and then incubated with 20 μl/ml RNAse A in an RNAse buffer containing (in m) 500 NaCl, 10 Tris, and 5 EDTA, pH 7.5, for 20 min at 37°C. They were then washed twice in 1× SSC containing 50% formamide for 60 min at 55°C, rinsed with 0.1× SSC for 10 min at 55°C, and dehydrated in ethanol and air-dried. Slides were initially exposed to X-ray films for 2–3 days to provide an indication of the intensity of the hybridization signal. They were then coated with Ilford Nuclear Research emulsion diluted 1:1 with water. The sections were exposed for 2–3 wk at 4°C in sealed dark boxes. After developing in Kodak D19, they were fixed in Ilford Hypam and washed abundantly with water. They were counterstained with toluidine blue to allow morphological identification.

**Chemicals and Radioactive Tracers**

Salts, chemicals, phenylmethylsulfonyl fluoride, benzamidine, Ponceau S solution, monocalon mouse anti-β-actin and anti-G3PDH primary antibodies, peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibody, X-MAT films, and 3,3′ diaminobenzidine were purchased from Sigma. Collagenase from Clostridium histolyticum (CLS II, 151 U/mg) was purchased from Worthington (Freehold, NJ). Vatides (100X MBE VT 00) were from Eurobio (Paris, France) and Triton X-100 from Prolabo (Vaulx-en-Velin, France). L-[1-14C]ornithine (1.85 GBq/mmol = 50 mCi/mmol), L-[U-14C]ornithine (9.25 GBq/mmol = 250 mCi/mmol), liquid scintillation counting mixture (Aqueous Counting Scintillant ACS II), ECL Western blotting kit, and the ImagerMaster Total Lab v1.00 program were purchased from Amersham International. The cocktail of protease inhibitors and DAPI were purchased from Boehringer Mannheim.

**Statistical Analyses**

Where appropriate, statistical differences were assessed using the Kruskal-Wallis and/or the Mann-Whitney tests. Differences were considered when P ≤ 0.05 and were calculated using the StatView II SE+Gr 1.04 VP program.

**RESULTS**

**Expression of OAT Protein in Male and Female Rat Kidneys**

In rat kidneys, our specific anti-OAT antibody revealed a 48-kDa protein that corresponds to the expected size of the OAT polypeptide (rat: 48,332 Da; Fig. 2) and a single OAT polypeptide after Western blotting on soluble proteins extracted from mouse kidney and separated by two-dimensional gel electrophoresis (data not shown). The level of OAT protein was about fourfold higher in female compared with male kidneys (Mann-Whitney, z = 2.61, P < 0.009). As expected, β-actin (42 kDa) and G3PDH (35 kDa) protein levels were similar and did not statistically differ between the two groups of rats (Fig. 2).

Inasmuch as female rat kidneys contain much higher OAT protein levels than male rat kidneys, we attempted to elucidate
whether the regional OAT distribution within the kidney differs between female and male rats. To localize OAT protein in their kidneys, soluble proteins of the different renal zones were analyzed by Western blotting, and the relative OAT protein levels were quantified. Figure 3 shows representative immunoblots probed with our purified anti-OAT antibody. The single band of 48 kDa, which corresponds to OAT protein, was detected in all female (Fig. 3A, left) and male (Fig. 3B, left) renal zones. The intensity of this band increased from Cs toward OS, where it reached its highest level. Longer exposure allowed detection of a low level of OAT protein in IM and Pap. Densitometric analyses of the bands monitored allowed us to calculate its relative abundance and to depict its renal distribution pattern. As shown in Fig. 3A, right, in female rat kidney, ≈20% of OAT protein was localized in Cs, 35% in Cd, and 40% in OS. In contrast, in male rat kidney, <10% of OAT protein was found in Cs, 30% in Cd, and ≈55% in OS (Fig. 3B, right). Because the main gender difference in OAT renal distribution lay in Cs (Fig. 3), we verified by Western blotting whether the absolute level of OAT protein differed between female and male Cs, Cd, and OS zones. Figure 4 shows that the level of OAT protein was significantly much higher in Cs than in Cd and OS.

Fig. 2. Western blot analysis of OAT, AII, β-actin, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) levels in the kidney of female (F) and male (M) rats. Each lane corresponds to 1 rat and a 50-μg sample of soluble proteins subjected to 10% SDS-PAGE. Representative immunoblots probed with rabbit anti-OAT, rabbit anti AII, mouse anti-β-actin, and mouse anti-G3PDH were revealed by ECL. After exposure to X-ray film, OAT, AII, β-actin, and G3PDH protein levels were quantified by densitometry. β-Actin and G3PDH were used as controls of protein loading and transfer. Values are means ± SE; n = 5 rats. *P < 0.009 (Mann-Whitney test).

Fig. 3. Western blot analysis of cysteine sulfinic decarboxylase (CSD), OAT, AII, and aldose reductase (AR) levels in the main renal zones of female (A) and male (B) rats. Each lane corresponds to 1 rat, except for IM, where n = 4 rats. Fifty-microgram samples of soluble proteins were subjected to 10% SDS-PAGE. Left: representative immunoblots were probed with rabbit anti-CSD, OAT, AII, and AR antibodies revealed by ECL and exposed to X-ray film. Right: OAT protein levels were quantified from X-ray films by densitometry. CSD and AR were used as specific markers of renal zones. Values are means ± SE of the relative OAT distribution. Cs, superficial cortex; Cd, deep cortex; OS, outer stripe of the outer medulla; IS, inner stripe of the outer medulla; IM, inner medulla; Pap, papilla.
(4.6-fold), Cd (2.9-fold), and OS (2.2-fold) in female compared with male rats (Mann-Whitney, \( P < 0.004 \) in each case). These results reveal a higher differential expression of OAT protein in female than male rat cortex and OS.

**Histological Localization of OAT Protein in Male and Female Rat Kidneys**

In female rat kidney, OAT was clearly detected in PCT and in medullary rays, but not in glomeruli, CTAL, DCT, and CCD (Fig. 5A). OAT immunostaining was intense in CPST and OSPST (Fig. 5A) but was not detected in tubules localized in IS and IM (not shown). At a higher magnification, nuclei were devoid of OAT, whereas almost all of the labeling was observed in the cytoplasm and was precisely more concentrated near the basal membrane of OSPST, an area rich in mitochondria (Fig. 5B). In male rat kidney, the renal distribution of OAT strongly resembled that in female rat kidney, as shown in Fig. 5C. Indeed, PCT were weakly immunostained, whereas CPST and OSPST were strongly positive.

Fig. 4. Comparative levels of OAT and AII levels in Cs, Cd, and OS of male and female rats. Kidneys of 2 female and male rats (9 wk old) were used. One hundred-microgram samples of soluble proteins were subjected to 10% SDS-PAGE. A: representative immunoblot probed with rabbit anti-OAT and anti-AII antibodies, revealed by ECL, and exposed to X-ray film. B: OAT and AII protein levels quantified by densitometry. Values are means ± SE; \( n = 6 \) films for OAT and \( n = 10 \) films for AII. *\( P < 0.004 \) (Mann-Whitney test).

Fig. 5. Immunofluorescent detection of OAT in the kidneys of female (A and B) and male (C) rats. Red represents OAT immunoreactivity, and blue represents nuclei revealed by 4',6-diamidine-2-phenylindole dihydrochloride (DAPI). A: strong intense immunoreactivity was detected in cortical and medullary proximal straight tubules (CPST and OSPST, respectively), whereas tubules located in IS were unlabeled. Glom, glomerulus; MR, medullary ray; PCT, proximal convoluted tubule. Magnification: \( \times 25 \). B: at a higher magnification, immunoreactivity was more intense at the basolateral than the apical pole of OSPST cells, suggesting mitochondrial localization of OAT. Magnification: \( \times 200 \). C: weak OAT immunoreactivity was observed in male rat PCT, whereas more intense labeling was found in CPST and OSPST. Magnification: \( \times 50 \).
Because the basolateral pole of proximal tubular cells is rich in mitochondria (32) and was strongly immunostained with OAT antibody, these organelles might be the functional and physiological site of this protein. Indeed, OAT amino acid sequencing predicts internalization within mitochondria. However, to our knowledge, the precise renal subcellular localization of OAT has never been documented. This was first attempted by localizing OAT in female rat OS and cortex using immunogold labeling and transmission electron microscopy. Figure 6A shows representative control mitochondria of OSPST incubated in the absence of anti-OAT antibody but with secondary gold-conjugated goat anti-rabbit IgG antibody. In these conditions, 90% of 375 analyzed mitochondria were devoid of immunogold particles. In the remaining 10% of mitochondria, gold particles were heterogenously distributed within the tubular cells. In contrast, when OS was incubated with our purified anti-OAT antibody, 80% of 980 analyzed mitochondria located in OSPST contained gold particles, and 80% of 4,084 gold particles were visualized within the mitochondria and sometimes linked to the mitochondrial membranes (Fig. 6, B–D). In MTAL incubated in the absence of anti-OAT antibody, 90% of 310 analyzed mitochondria were devoid of immunogold particles. Surprisingly, in MTAL incubated with our anti-OAT antibody, 77% of 434 analyzed mitochondria were devoid of gold particles and 61% of 395 particles were located in the cytosol but not in mitochondria (data not shown). Only a few gold particles were observed in PCT mitochondria (data not shown). These data revealed a predominantly mitochondrial localization of OAT protein in OSPST, which contrasts with its predominantly cytosolic location in MTAL. In OS, 90% of the gold particles (4,084/395) were located in OSPST mitochondria compared with 10% in MTAL mitochondria.

In the second approach, mitochondria were isolated from cortex and OS of two female rats and analyzed by Western blotting. Mitochondria were characterized by the presence of the following three specific proteins: α-subunit F1-ATP synthase (55 kDa) and core 2 (45 kDa) are detected in the mitochondrial matrix, whereas ISP (25 kDa) is located at the opposite side of the inner membrane. Core 2 and ISP are two proteins belonging to complex III of the respiratory chain (Fig. 7). OAT protein was clearly detected and abundant in the mitochondrial fraction (Fig. 7).
**ORNITHINE METABOLISM IN RAT KIDNEY**

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**L-Ornithine Oxidation in Dissected Nephron Segments**

In the presence of OAT, L-ornithine is metabolized to L-glutamate, which can be further desaminated before entering the citric acid cycle for its complete oxidation (Fig. 1). Experiments were conducted to demonstrate the functionality of OAT and the complete oxidation of L-ornithine in isolated viable tubules. The results show that male rat glomeruli, proximal tubules, DTL, thick ascending limbs, distal tubules, and collecting ducts incubated in the presence of energy-providing substrates metabolized L-[1-14C]ornithine and released 14CO2 (Fig. 8, filled bars). The level of 14CO2 produced varied from one tubule to another. Indeed, the highest rates of L-[1-14C]ornithine decarboxylation occurred in the proximal tubules (PCT, CPST, and OSPST), followed by DCT, CCD, and OMCD, whereas the lowest were in DTL, thick ascending limbs, and IMCD (Kruskal-Wallis, P < 0.0002). As 14CO2 is simultaneously released during L-[1-14C]ornithine oxidation and L-[1-14C]ornithine decarboxylation by renal ODC activity, it is impossible to identify the origin of 14CO2. To resolve this point, three approaches can be considered. First, the selective ODC inactivator difluoromethylornithine (DFMO) can be used to abolish L-[1-14C]ornithine decarboxylation. As published in a previous paper, DFMO sharply reduced the production of 14CO2, and the remaining fraction of 14CO2, which probably originates from the citric acid cycle, represented 22% in PCT, 29% in CPCT, and 60% in OSPST of male rats (21). Unfortunately, in this protocol, DFMO can compete with L-[1-14C]ornithine to enter the cells, and DFMO exerts nonspecific effects (38). Second, gabaculine is a potent suicide inhibitor of OAT activity (13, 20). Here, we chose a third approach, which consists of incubating tubules with L-[1-14C]ornithine as the sole substrate to shift its catabolism toward the oxidative route, to increase its consumption, and to produce ATP during its decarboxylation in the citric acid cycle.

Glomeruli and tubules were incubated in a medium that contained only 100 μM L-[1-14C]ornithine, and the levels of 14CO2 produced (Fig. 8, hatched bars) were compared with those obtained when glomeruli and tubules were incubated in the presence of several energy-providing substrates (Fig. 8, filled bars). The results show a drastic increase in 14CO2 production in each subsegment of the proximal tubules (Fig. 8, hatched bars, Mann-Whitney, P < 0.0001), with the highest rate in OSPST (Kruskal-Wallis, P < 0.05 followed by Mann-Whitney test, 0 < 0.0009). L-[1-14C]ornithine decarboxylation was enhanced in thick ascending limbs and IMCD (Fig. 8, hatched bars, Mann-Whitney, P < 0.0001) and moderately increased in glomeruli, DTL, CCD, and OMCD (Fig. 8, hatched bars, Mann-Whitney, P < 0.025 or less). Data obtained for ATL and DCT were not analyzed because too few samples could be dissected. These results indicate that in the absence of several energy-providing substrates, a lot of L-[1-14C]ornithine was consumed, presumably to sustain the cellular energetic needs and indirectly prove that the OAT gene was expressed in glomeruli and in all tubules tested. The difference in 14CO2 production between the two incubation conditions, 1) medium containing energy-providing substrates (Fig. 8, filled bars) and 2) medium with L-[1-14C]ornithine as the sole substrate (Fig. 8, hatched bars), is represented in the inset in Fig. 8. This profile represents the calculated physiological OAT activity within the male rat nephron and, furthermore, strongly resembles the regional distribution of OAT protein in male rat kidney (Fig. 3).

To confirm that L-ornithine oxidation occurs in viable nephron segments, dissected tubules were divided into two groups and were incubated with either L-[1-14C]ornithine or L-[U-14C]ornithine as the sole substrate. The results presented in Fig. 9 show that both glomeruli and tubules oxidized L-ornithine, because the production of 14CO2 was higher with L-[U-14C]ornithine compared with L-[1-14C]ornithine (Mann-Whitney, P < 0.02 or less); 2) L-[U-14C]ornithine was completely oxidized because 14CO2 production was enhanced about fivefold compared with L-[1-14C]ornithine; and 3) L-ornithine oxidation occurred preferentially in proximal tubules. These results indicate that OAT plays a key role in driving L-ornithine to the oxidative pathway for energy production and that the OAT gene was differentially expressed within the rat nephron. These metabolic studies are in good agreement with the histological localization of OAT in rat kidneys (Fig. 6) and with the regional distribution of OAT protein (Fig. 3).

**Overlapping Expression Pattern of All Protein and ODC mRNA in Male and Female Rat Kidneys**

We examined the expression pattern of AII and ODC as well as their relationship with OAT. The kidney contains very...
active AII because its specific activity is only threefold lower than that of the hepatic arginase I isoenzyme (33). Two AII isoenzymes have been reported in the male rat kidney, whereas no data are available for the female rat kidney (8). For these reasons, the level of AII protein was quantified in whole kidneys of male and female rats, and its distribution was studied in the different renal zones using a specific AII antibody.

In soluble proteins extracted from whole male and female rat kidneys and analyzed by Western blotting, our specific anti-AII antibody revealed a 38-kDa protein that corresponds to the predicted size of the AII polypeptide (rat AII: 38,640 Da; Figs. 2-4). AII protein is constitutively expressed in male and female rat kidneys. The level of AII protein did not statistically differ in female compared with male rat kidneys. The results presented in Fig. 3 depict representative immunoblots of the different renal zones probed with our anti-AII antibody. The 38-kDa protein corresponding to AII was detected in C, OS, IS, IM, and to a lesser extent in Pap of female (Fig. 3A) and male (Fig. 3B) rats. The highest levels of AII protein were detected in IM and OS. High amounts of AII protein were also found in C. In Fig. 4, proteins extracted from whole female and male rat C, C, and OS and probed with our anti-AII antibody clearly show that the level of AII protein in C, C, and OS was independent of gender because no statistical difference was detected.

In the kidney, L-ornithine is metabolized not only by OAT but also by ODC to produce putrescine. We verified whether ODC and OAT are colocalized in the same renal zones and tubules, as they could then compete for the same substrate. The precise distribution of ODC within the male and female rat kidney was investigated by in situ hybridization. The results presented in Fig. 10 show that sense ODC cRNA did not hybridize on renal sections of male rats. A similar result was observed in sections from female rats (Fig. 10). In contrast, very dense labeling with antisense 35S ODC cRNA was observed in C and OS of male and female rats. Microscopic observations revealed a great density of silver grains exclusively in the pars recta of the proximal tubule (data not shown). In conclusion, ODC and OAT are coexpressed in the CPST and OSPST of male and female rats.

**DISCUSSION**

The present study was conducted to establish the metabolic fate of L-arginine-derived ornithine and, as a consequence, to identify the physiological role of OAT and AII in male and female rat kidneys. Our hypothesis is that, in PST, high AII activity intracellularly hydrolizes L-arginine and produces L-ornithine to supply cellular and metabolic needs locally. In the kidney, catabolism of L-arginine-derived ornithine is restricted to the production of either L-glutamate, after the transamination step controlled by OAT, or putrescine, after its decarboxylation by ODC (Fig. 1). However, the metabolic fate of L-ornithine depends on several parameters, including 1) characteristic metabolic needs of each nephron segment, 2) subcellular localization, 3) hormonal control, 4) rhythmic circadian cycles of the enzymes that share L-ornithine as a substrate (5), and 5) expression of the enzymes that metabolize L-ornithine-derived glutamate.

In this study, our attention was first focused on the expression and the localization of the key enzyme OAT, which has been partially studied in male but not in female rat kidneys. Our results reveal that the physiological level of OAT protein is about fourfold higher in female than in male rat kidneys. This gender difference in OAT gene expression may be attributed to sex hormones and could be explained by the production of 17β-estradiol in female rats. The promoter of the human and rat OAT gene contains numerous AGGTCA-like motifs related to the binding site for estrogen (39). In addition, administration of 17β-estradiol (50 μg/100 g BW) to male Sprague-Dawley and Holtzman rats induces a progressive increase in renal OAT mRNA, protein, and activity (24, 29). Finally, two estrogen receptor (ER) subtypes that belong to the superfamily of nuclear receptors, ER-α and ER-β, are expressed in several mouse and rat tissues where they mediate the effects of estrogens. However, ER-α but not ER-β was found in rat kidney (17). ER has been localized in the nucleus of rat PCT cells by immunoelectron microscopy (7). In a recent study, the effects of 17β-estradiol were analyzed by gene chip microar-
rays in wild-type, 129-strain female mice and Sprague-Dawley rat tissues. This study showed that 26 genes were induced and 4 were repressed (12). Renal in situ hybridization of the 17β-estradiol-induced genes revealed a predominant localization of their mRNAs in OS, medullary rays, and sometimes in cortical structures. Altogether, these results strongly suggest that ER-α gene is abundantly expressed in the PCT, CPST, and OSPST of female rats. Because the OAT gene is expressed more in female than male rats, this enzyme is likely to play a pivotal role in female rat renal metabolism.

OAT precursor is a 49-kDa nucleus-encoded mitochondrial protein composed of 439 amino acids. The first 35 amino acids (3.7 kDa) could be responsible for the importation of OAT into mitochondria, as the corresponding, chemically synthesized peptide is imported into the mitochondrial matrix by the same machinery as the precursors of mitochondrial proteins (30). In contrast, another group detected a 6-kDa peptide larger than the mature protein by SDS-PAGE (28). There is still some controversy concerning the location of the cleavage site of OAT precursor (11). This is supported by the different molecular masses of OAT found when analyzed by SDS-PAGE: 43 kDa [liver (28), kidney (14)], 45 kDa [liver (5)], and 48 kDa in the present study. Our observations suggest that the molecular mass of the mature OAT polypeptide is higher than 43–45 kDa because argininosuccinate synthetase, a 412-amino acid polypeptide, was clearly separated from OAT protein by 10–12% SDS-PAGE and was detected at its predicted (46 kDa) size below OAT polypeptide (Swiss-Prot: rat P09034, data not shown).

We have localized OAT protein in the mitochondria of OSPST and PCT in female rats (Figs. 5 and 6). Almost all OAT protein (80%) was localized in the mitochondrial matrix and near the mitochondrial membranes, whereas the remaining cytosolic fraction of OAT protein could correspond to the OAT precursor polypeptide not yet carried into the mitochondria. Interestingly, several reports indicate that, in contrast to the cytosolic hepatic arginase AI, the renal isoform AII contains a putative NH₂-terminal presequence for mitochondrial importation (10) and that AII activity is enriched in the mitochondrial fraction (15, 41). Here, we clearly observed that mitochondria isolated from female rat cortex and OS contained both OAT and AII proteins. Their colocalization in mitochondria of PST cells deeply favors the conversion of L-arginine into L-glutamate, as shown in Fig. 1. In contrast, if mitochondrial exportation of L-ornithine is more rapid than its transamination, or if OAT gene expression is low (5), L-ornithine becomes available in the cytosol and can be metabolized to putrescine by the cytosolic ODC (2). Our results clearly show that ODC is abundantly expressed in the PST (CPST and OSPST) of male and female rats together with OAT. At present, it can be assumed that the rate of ornithine produced by AII in female and male rat kidneys did not differ, because their level of AII protein was similar. The direction of L-ornithine in either the polyamine or the glutamate routes may depend on the cellular context. Because the level of OAT protein was about fourfold higher in female than in male kidneys, it is conceivable that, in females, a large fraction of L-arginine-derived ornithine turns preferentially into the glutamate route. Indeed, in a pilot experiment, PCT isolated from female rat kidney and incubated with [3-14C]ornithine and other substrates produced 70 ± 7 fmol 14CO₂·min⁻¹·mm⁻¹ (n = 7 samples). Ornithine decarboxylation is 40% higher than in male PCT (Fig. 8).

Finally, the differential level of AII/OAT protein content in female and male rat kidneys suggests a gender difference in L-ornithine metabolism. Further proteomic and biochemical analyses will be performed in isolated proximal tubules to clarify these points.

The metabolic fate of the L-glutamate produced during L-ornithine transamination and from PSC (Fig. 1) depends on the presence of those enzymes that use L-glutamate as a substrate: GAD, GS, and GLDH. First, GAD activity has been reported in the rat kidney (9). A suspension of proximal tubules exhibited twofold threefold higher GAD activity compared with the renal cortex and a suspension of glomeruli, respectively. Although, to our knowledge, the precise histological localization of GAD has not been performed in male and female rat kidneys, the renal content of GABA doubles between Cs and OS (9). The metabolic pathway and the physiological roles of GABA remain to be clarified in the rat kidney. However, γ-aminobutyric acid transaminase (EC 2.6.1.19), which catabolizes GABA, has been histochemically localized in the murine PST (44). Second, GS condenses L-glutamate and ammonia into L-glutamine (Fig. 1). High GS activity has been reported in the early and late PST of male Sprague-Dawley rats (4). Studies clearly reveal that the highest AII (23), OAT, and GS protein levels and activities are colocalized in the CPST and OSPST. Altogether, these findings strongly suggest that the unexplained physiological role of renal AII in these tubules may be to contribute to L-glutamine synthesis. Indeed, AII hydrolyzes L-arginine into L-ornithine, which is transaminated by OAT in the mitochondria. In the presence of ammonia, the L-ornithine-derived glutamate is locally metabolized to L-glutamine. Although L-glutamine synthesis in the kidney significantly contributes to ammonia detoxification, additional physiological roles have been attributed to this amino acid. L-Glutamine is a precursor of glutathione, which protects mitochondrial structure and function during oxidant injury (1) and cisplatin-induced lipid peroxidation damage (26), which also preserves cardiomyocyte viability and enhances recovery of contractile function after ischemia-reperfusion injury (46).

Because OSPST are sensitive to the low O2 level supplied by the ascending vasa recta, endogenous synthesis of L-glutamine may contribute to cellular protection. Third, L-ornithine-derived glutamate is desaminated by the mitochondrial enzyme GLDH to generate α-ketoglutarate, which is further decarboxylated in the citric acid cycle or reused for transamination (Fig. 1). In the rat kidney, the highest GLDH activities were reported in the three segments of the proximal tubule (PCT, CPST, and OSPST), and intermediate GLDH activities were found in thick ascending limbs and collecting ducts (47). In this study, the oxidative decarboxylation of L-ornithine was essentially found in the whole proximal tubule, but predominantly in OSPST. This result perfectly coincides with the distribution of the rate-limiting enzyme OAT. Because total oxidation of L-ornithine is associated with energy production via the citric acid cycle, L-ornithine can support cell viability in the absence of energy-providing substrates such as carbohydrates, fatty acids, and other amino acids. However, the relative contribution of L-ornithine to energy production, in vivo, remains to be clarified, because other preferential substrates sustain the energetic needs of the cells and solute transport (45).
In conclusion, OAT is widely and inequally distributed along the rat nephron. In rat kidneys, OAT protein is highly expressed in the PST, overlapped with the presence of All, ODC, GS, GLDH, and GAD. OAT is expressed more in female than male kidneys, but, in contrast, no gender difference was found in All protein expression. We propose that, depending on cellular needs, the physiological role of All in rat CPST and OSPST may be to supply L-ornithine for OAT and ODC, whereas the role of OAT may be to channel L-ornithine to L-glutamine, GABA, or the citric acid cycle to produce energy.

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REFERENCES


