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Orchidectomy Upregulates While Testosterone Treatment Downregulates the Expression of Ornithine Aminotransferase Gene in the Mouse Kidney

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1. Introduction

Ornithine aminotransferase (L-ornithine: 2-oxoacid aminotransferase, OAT, EC 2.6.1.13) plays crucial physiological roles in amino acid metabolism because this enzyme is at the crossroad of several pathways including those of L-arginine, L-ornithine, L-glutamate, L-glutamine, and L-proline. Specifically, OAT catalyzes the transamination of L-ornithine in the presence of α-ketoglutarate to produce one molecule of L-glutamate and the unstable compound glutamate-γ-semialdehyde that is spontaneously converted into Δ^1-pyrroline-5-carboxylate. This latter molecule is further metabolized by the enzyme pyrroline-5-carboxylate dehydrogenase into a second molecule of L-glutamate (Wakabayashi, 2004). The enzyme is expressed in many mammalian tissues including the liver, the kidney, and the intestine which exhibit the highest OAT activities (Peraino & Pitot, 1963; Herzfeld & Knox, 1968; Sanada et al., 1970; Kasahara et al., 1986; Alonso & Rubio, 1989; Levillain et al., 2007; Ventura et al., 2009). These enzymes may not only display diverse tissue-specific physiological roles, but demonstrate marked sexual differences in expression and activity.

In rat kidneys, estrogen dramatically increased the expression of OAT and is responsible for the higher levels of OAT in female than in male rat kidney (Herzfeld & Knox, 1968; Lyons & Pitot, 1977; Mueckler & Pitot, 1983; Mueckler et al., 1984; Levillain et al., 2004). The presence of thyroid hormone is required for estrogen induction. These hormones exert a synergistic effect on the expression of OAT gene (Mueckler & Pitot, 1983). The expression of OAT gene during the rat postnatal development strongly supports the sexual dimorphism of OAT in kidney, but not in liver (Herzfeld & Knox, 1968; Herzfeld & Greengard, 1969). Taken together, the expression of OAT gene in the female rat kidney is naturally upregulated in the presence of estrogen.

The expression of OAT gene in the mouse kidney has been reported by different authors who independently measured OAT mRNA and protein levels or OAT activity (Alonso & Rubio, 1989; Natesan & Reddy, 2001; Yu et al., 2003; Levillain et al., 2005; Manteuffel-Cymborowska et al., 2005; Levillain et al., 2007; Ventura et al., 2009). Strong evidences

support a marked sexual dimorphism in the expression of OAT gene in the mouse kidney with three-fold higher levels in females than in males. A detailed study of the renal expression of OAT gene during the postnatal development of male and female mice revealed that puberty is the starter responsible for this sexual dimorphism (Levillain et al., 2007). In addition, the level of OAT protein has been inversely correlated with the plasma level of testosterone (Levillain et al., 2005).

The present study was designed to explore the mechanismes involved in the sexual dimorphism of the expression of OAT gene in the mouse kidney. We shall determine by *in vivo* studies at which level testosterone regulates the expression of OAT gene. To answer this question, male mice were subjected to orchidectomy and testosterone replacement. The renal expression of OAT gene was analyzed at the transcriptional, translational, and post-translational levels. The possible involvement of the eukaryotic initiation factor eIF4-E in the control of OAT gene expression was analyzed. We also explored the delay required for testosterone to induce a decrease in the expression of OAT gene in orchidectomized mice. For this, a time-course study was performed over a period of 32 hrs following a single injection of testosterone to castrated male mice. Finally, to explain the physiological role of testosterone on the expression of OAT specifically in the mouse kidney, we searched to identify androgen response elements (ARE) in the promoter of the murine OAT gene.

2. Material and methods

2.1 Animals and treatment

Eight- to nine-week-old adult male (35-40 g body weight) OF-1 Swiss (IOPS Caw) mice, from either Charles River Laboratories (L'Arbresle-sur-Orge, France) or Janvier (Le Genest-saint-Isle, France) had free access to tap water and standard food (2018 Teklad Global 18% Protein Rodent Diet, Harlan, Gannat, France). Animals were housed in a controlled environment maintained at 21 ± 1°C with a 12-h light, 12-h dark cycle, lights on at 0600 h.

Twenty-four thirty-day-old male mice were subdivided into four groups of six mice: nonoperated (group I, control), sham-operated (group II) and two groups of orchidectomized mice. Eleven days later (*i.e.* 41 days after birth), mice of groups I, II, and III were sacrified, whereas mice of group IV were sacrified seven days latter (*i.e.* 18 days after orchidectomy).

Twenty-four thirty-day-old male mice were subdivided into four groups of six mice: sham-operated (group V, control), 11-*day* orchidectomized (group VI), 11-*day* orchidectomized treated with sesame oil (group VII), and 11-*day* orchidectomized treated with testosterone + sesame oil (group VIII). Mice subjected to oil or testosterone treatment were injected subcutaneously with 150 µL vehicule or testosterone propionate (3.1 mg/mL in sesame oil, i.e. approximately 15 µg/g BW or 0.55 mg per mouse). All mice were sacrified forty-eight hrs after the treatment.

Twenty thirty-day-old male mice were subjected to orchidectomy (11-*day*) and subdivided into five groups of four mice: 11-*day* orchidectomized (group IX, control) and four groups of mice treated with testosterone as described above (0.55 mg per mouse). Mice were sacrified 8 hrs (group X), 24 hrs (group XI), 28 hrs (group XII), or 32 hrs (group XIII) after the treatment (Fig. 1). For these three protocols, half of the mice were purchased from Charles River Laboratories and the other from Janvier. Mice were equally distributed in the different experimental groups. Orchidectomy was carried out by the suppliers. Mice were anesthetized (ip) using 0.1 mL/30 g BW pentobarbital sodium (Nembutal 6%, Clin Midy, Paris, France) diluted 1:2 in 0.9% NaCl solution.

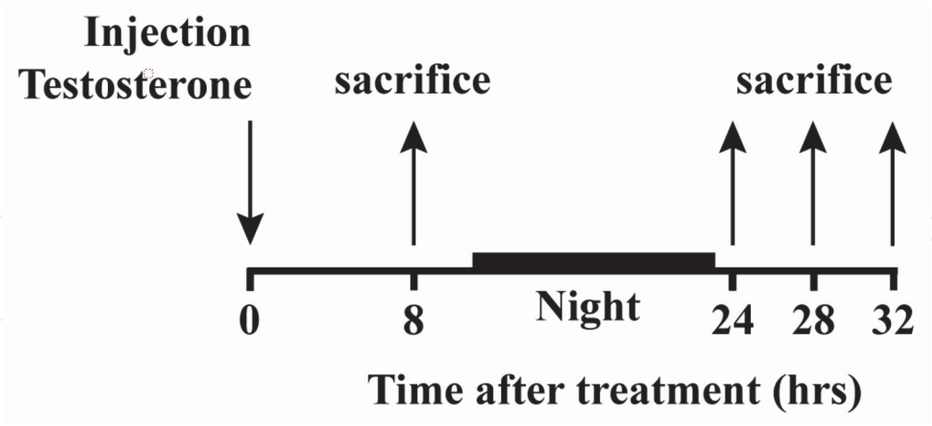

Fig. 1. Schema of the time-course study of testosterone effect on the expression of OAT gene.

Animal care complied with French regulations for the protection of animals used for experimental and other scientific purposes and with European Community regulations (Council of Europe N° 123, Strasbourg, 1985). The author (O. Levillain) is authorized by the "Direction Départementale des Services Vétérinaires" (authorization no. 69-33 and 69266391) and the local Animal Care Committee to use animals for these experiments (authorization no. 299 revised and no. BH 2009-15).

2.2 Sampling of kidneys and blood

The renal pedicle of each kidney was clamped and the kidney was rapidly removed, decapsulated; the blood contained in each kidney was removed with blotting paper (free-blood). The kidney was placed in a sterilized Eppendorf tube, frozen, and conserved at -80°C.

Blood was collected in the vena cava of all mice with a 25-gauge needle (Neolus, VWR, Limonest, France) mounted on a 1-mL syringe (Terumo, VWR) prealably heparinized (Heparin, Roche, Meylan, France). Blood was immediately transferred in a cold BD Vacutainer tube, centrifuged at 4,000 x *g* for 20 min at 4°C. Plasma was frozen and stored in liquid nitrogen until testosterone and corticosterone measurement.

2.3 RNA extraction and semiquantitative RT-PCR

The steady state levels of OAT and cyclophilin A transcripts were estimated by semi-quantitative polymerase chain reaction (PCR) and quantitative PCR (qPCR) as described further in the text.

2.3.1 RNA extraction

Total RNA was extracted from whole kidney by using Trizol® according to the supplier's procedure. Briefly, kidneys were mixed in the proportion of 150 mg tissue per 1 mL Trizol® at 4°C with a Ultra-Turrax T10 (VWR, Fontenay-sous-Bois, France). RNAs were extracted with chloroform, purified by isopropanol precipitation, and washed with 70% ethanol. RNA pellets were resuspended in sterilized water (Eurobio, Courtaboeuf, France) and stored

frozen (-80°C). RNA concentration was determined from the absorbance at 260 nm and RNA purity the absorbance ratio at 260 and 280 nm (ratio ≈ 2) using a BioPhotometer (Eppendorf France S.A.R.L., Le Pecq, France). RNA integrity was checked by electrophoresis on 1% agarose gel (Eurobio).

2.3.2 Reverse transcription

Total RNA (1 µg) was denatured for 5 min at 65°C and mRNAs were reverse-transcripted (60 min at 37°C) in 25 µL final volume containing 5 µL of M-MLV RT 5X buffer, 100 U moloney murine leukemia virus reverse transcriptase (Invitrogen, France), 15 nmoles of deoxynucleotide triphosphate and 1 µg oligo dT (PCR) or 100 ng random primer (qPCR, Invitrogen). After RT, inactivation step was 15 min at 70°C. RT products were frozen (-30°C).

2.3.3 Polymerase chain reaction for PCR

The cDNA from 2.5 µL of RT product were amplified by PCR in 47.5 µL of PCR mixture containing 5 µL of 10X Taq PCR buffer, 100 nmoles $MgCl_2$, 15 nmoles of deoxynucleoside triphosphate (Promega), 2.5 U Taq DNA polymerase, 15 pmoles corresponding to forward and reverse primers. Gene-specific oligonucleotide primers (20-22 nucleotides) were selected from the cDNA sequences of mouse OAT and rat cyclophilin A. Forward and reverse primers were designed using Primer3 software (v. 0.4.0) (http://frodo.wi.mit.edu/primer3/) and are shown in Table 1. Sequence identity between rat and mouse cyclophilin A was 91% for forward primer and 95% for reverse primer. The PCR program consisted of a denaturation step (2 min at 94°C), X cycles (see Table 1) and a final elongation step (10 min at 72°C). Each cycle included denaturation (1 min at 94°C), primer annealing (1 min at 60°C), and elongation (1 min at 72°C). Preliminary experiments were performed to determine the linear range of PCR amplication for each target mRNA. RT and PCR reactions were performed using a thermocycler (Mastercycler® Personal, Eppendorf, Le Pecq, France).

Gene	Accession number	Primer	Sequences (5′ -> 3′)	PCR product size (pb)	Cycle number
OAT	NM_016978.2	Forward	TCCAGGATACCTGACGGGAGTT	330	27
		Reverse	ATCTTGTCTGCGTTTTCAGCAA		
eIF4-E	NM_007917.3	Forward	AGCAGAGTGGACTGCACTGA	394	27
		Reverse	GCAAGGACAATGCTGTGAAA		
Cyclophilin A	NM_017101	Forward	GTGGCAAGTCCATCTACGGAG	265	24
		Reverse	CCACAGTCGGAGATGGTGATC		

Abbreviation: ornithine aminotransferase (OAT) and eukaryotic initiation factor eIF4-E (eIF4-E).

Table 1. Gene-specific sequences of PCR primers, predicted PCR products, and parameters for PCR analyses

2.3.4 Quantification of RT-PCR products

Fourteen µL of each PCR products containing 14% of a gel loading buffer (Blue juice 10X) were separated by electrophoresis on a 4% (w/v) agarose (Eurobio) gel prestained with

0.01% ethidium bromide. For quantitation of band intensities, pictures were taken with a Camera DC120 (Kodak) and intensities of the bands were determined with Kodak Digital Science 1D 2.0 (Kodak Scientific Imaging System). The housekeeping rat cyclophilin A gene was used as an internal control to normalize the target gene expression as previously reported (Bitoun et al., 2001).

2.3.5 Polymerase chain reaction for qPCR

Real-time PCR was performed in a MyiQ thermal cycler (Bio-Rad, Marnes La Coquette, France) using IQ SYBR Green Supermix (Bio-Rad). Primers specific to the mouse sequence of OAT and HPRT were designed using Primer3 software. The following qPCR conditions were used: 3 min at 95°C, followed by 40 cycles of denaturation for 10 s at 95°C and annealing/extension for 45 s at 60°C, according to the manufacturer's instructions. All samples were run in duplicate along with dilutions of known amounts of target sequence to quantify the initial cDNA copy number (Concentration = Efficiency$^{\Delta Ct}$). The results are expressed as the ratio of the target gene over HPRT mRNA concentration which was verified to exhibit non-significant variation between the different groups of cDNAs.

Gene	Accession number	Primer	Sequences (5′ -> 3′)	qPCR product size (pb)
OAT	NM_016978.2	Forward	GGGCTCTTGTGAAACTCTGC	195
		Reverse	AGATGGGTCCGTTTCTCCTT	
HPRT	NM_013556	Forward	GTAATGATCAGTCAACGGGGGAC	177
		Reverse	CCAGCAAGCTTGCAACCTTAACCA	

Abbreviation: ornithine aminotransferase (OAT) and hypoxanthine guanine phosphoribosyl transferase (HPRT).

Table 2. Gene-specific sequences of PCR primers and predicted PCR products for qPCR analyses.

2.4 Protein extraction and western blot

Frozen kidneys were mixed at 4°C with a Turrax homogenizer at 100 mg frozen tissue per 2 mL of lysing buffer containing protease inhibitor (Laemmli, 1970). After centrifugation, the concentration of soluble proteins was determined using the Bradford protein assay (Bradford, 1976). Fifty or 100 µg samples of soluble proteins were subjected to 10% PAGE containing 0.1% SDS using 6 Watts per gel. Protein transfer and equal loading of proteins were visualized on the membranes with Ponceau S solution. Blocked immunoblots were incubated with primary polyclonal rabbit antibodies raised against rat/mouse-OAT (dilution 1:1,000), rat-aldose reductase (AR; EC 1.1.1.21, dilution 1:3,000) (Lambert-Langlais et al, 2009), and monoclonal mouse β-actin (dilution 1:2,000) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH; EC 1.2.1.12, dilution 1:170) antibodies, and then with a peroxidase-conjugated anti-rabbit or anti-mouse IgG secondary antibodies (dilution 1:10,000) as previously described (Levillain et al., 2004, 2005). Antibody binding was revealed using chemiluminescence (ECL) Western Blotting Kit. Details for ECL detection and quantitation of the bands have been mentioned earlier (Levillain et al., 2004, 2005).

2.5 Measurement of OAT activity

OAT activity was measured in whole kidneys. Samples were mixed at 4°C with a Turrax homogenizer in a buffer (0.33 M sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM DTT, and 0.5% Triton X 100). Homogenates were centrifuged 21,000 x *g* for 10 min at 4°C. The protein concentration was determined on the supernatant using the Bradford protein assay. OAT activity was determined using the enzyme assay previously described (Herzfeld & Knox, 1968; Peraino & Pitot, 1963). Briefly, the supernatant was incubated with a buffer composed of 75 mM potassium phosphate pH 8.0, 20 mM L-ornithine, 0.45 mM pyridoxal phosphate, 5 mM α-aminobenzaldehyde, and 3.75 mM α-keto-glutarate for 15 or 30 min at 37°C. Blanks did not contain α-keto-glutarate. The reaction was stopped by adding trichloroacetic acid 40%. Samples were centrifuged 21,000 x *g* for 3 min at 4°C and absorbance was measured on the clear supernatant at 440 nm on a Hitachi U-1100 spectophotometer (Meylan, France). Duplicate or triplicate samples and blanks were performed for all experiments.

2.6 Determination of testosterone and corticosterone levels

Testosterone was measured by radioimmunoassay after extraction by organic solvant and partition chromatography of the plasma samples as previously described (Dechaud et al, 1989). Corticosterone concentration was measured, after diethylether extraction, by radioimmunoassay using 1,2,6,7 [3H]-corticosterone and rabbit anti-corticosterone polyclonal antibody (Filipski et al, 2002).

2.7 Calculations and statistical analyses

The calculations were as follows: for each group of mice and for each protein, the mean intensity optical densitometry (IOD) of the bands was calculated. The mean IOD of the untreated (group I) and sham-operated (group V) mice were used as a reference (control). For each mouse, the IOD value of a given protein was divided by the mean IOD value of the control group. Consequently, this ratio value is 1 in each control group. Then, these ratios were related to those of β-actin and/or G3PDH.

OAT activity was expressed in absorbance per 15 min per mg soluble protein because we were unable to find a supplier to buy P5C as standart to convert the absorbance of P5C into molar unit. Depending on the material used or the physiological interest of expressing the data in other units, OAT activity is expressed in absorbance per 15 min per mg soluble proteins.

Results are expressed as means ± SE. Non-parametric statistical tests were used to analyze the data. Where appropriate, statistical differences were assessed using the Kruskal-Wallis test at level significance of 95% and this test was followed by the U-Mann-Whitney test at level significance of 95% (StatView SE+Gr). For correlation analyses, the correlation coefficient r^2 was calculated with Microsoft Excel, and *P* was determined from tables at the 95% level of significance.

2.8 Chemicals

Salts and most chemicals, Ponceau S solution, secondary anti-IgG antibodies, Kodak X-MAT film were purchased from Sigma (S^t Quentin Fallavier, France). Protease inhibitor cocktail were purchased from Boehringer Mannhein (Strasbourg, France). Agarose Seakem GTG was purchased from TEBU (Le Perray-en-Yvelines, France). ECL Western Blotting Kits, and ImagerMaster Total Lab v2.01 program were purchased from Amersham (Buckinghamshire, UK).

3. Results

3.1 Technical verifications

3.1.1 Semiquantitative RT-PCR

To quantify the levels of OAT and cyclophilin A mRNA by RT-PCR, we determined the appropriate number of amplification cycles to remain in the exponential phase of the amplification process and avoid saturation (Table 1). For each mRNA studied a single RT-PCR product was obtained. The amplified RT-PCR products were of the expected size (Table 1). The level of the mRNA of a housekeeping gene (cyclophilin A) did not vary significantly in our experimental conditions (data not shown).

3.1.2 OAT activity assay

The renal cortex of male and female mice was dissected and prepared to quantify OAT activity. To determine the conditions for assay of OAT activity, the incubation time was tested every 5 min up to 30 min and the protein concentration varied from 0 to 400 µg per sample. OAT activity increased linearly with the concentration of soluble proteins up to 400 µg in male mouse cortex and 300 µg in that of the female when the incubation time was fixed at 30 min (Fig.2 left, male: r^2=0.99809 and female: r^2=0.99583, $P < 0.01$). OAT activity increased linearly in proportion with the incubation time up to 30 min when the concentration of soluble proteins was fixed at 140 µg in both male and female mice (Fig. 2 right, male: r^2=0.99352 and female: r^2=0.9972, $P < 0.01$). Constantly, OAT activity was more than three-fold higher in female than in male mouse kidney. The sex-differential OAT activity found in the present results is in good agreement with published data (Levillain et al., 2007; Ventura et al. 2009).

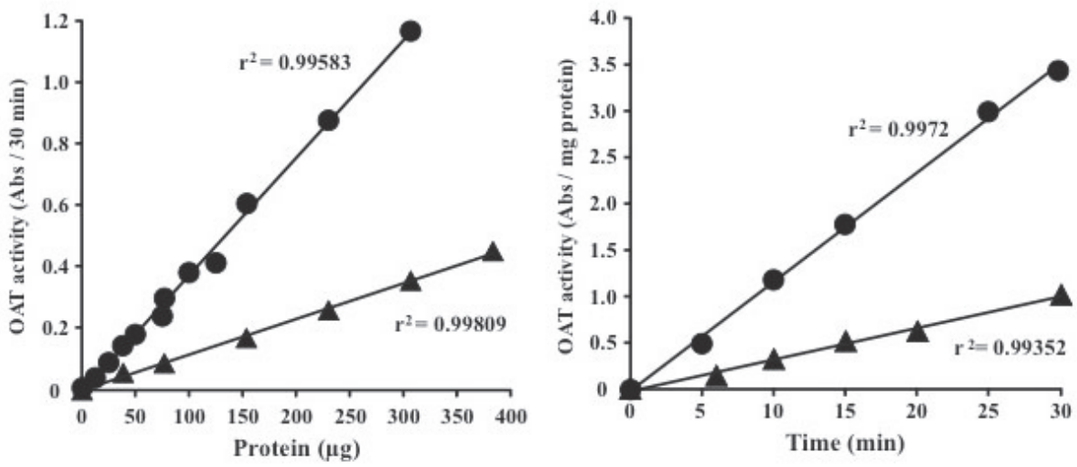

Fig. 2. Technical verifications to determine the conditions for OAT assay.

Closed circles: female mouse kidney and closed triangles: male mouse kidney. In all cases, the coefficient of correlation was statistically significant ($P < 0.01$ or less).

3.2 Influence of orchidectomy on the expression of OAT gene

A complete analysis of the expression of OAT gene in the mouse kidney was undertaken by analyzing the levels of mRNA and protein, and measuring OAT activity. The level of OAT

mRNA, protein, and enzyme activity did not differ between control and sham-operated male mice. Surgery did not affect the expression of OAT gene in the mouse kidney (Fig. 3). In contrast, orchidectomy provoked a sharp increase in OAT mRNA, protein, and enzyme activity. In details, in castrated mice killed 11 day after the surgery, the level of OAT mRNA, OAT protein, and OAT activity were increased by 3.95-fold (Kruskal-Wallis, $P < 0.035$ followed by Mann Whitney, $P < 0.0495$), 2.13-fold (Kruskal-Wallis, $P < 0.0004$ followed by Mann Whitney, $P < 0.0039$), and 2.27–fold (Kruskal-Wallis, $P < 0.018$ followed by Mann Whitney, $P < 0.05$), respectively as compared to the sham-operated mice (Fig. 3). When orchidectomized mice were killed 18 days after the operation, OAT gene remained still highly expressed, nevertheless, its expression had a tendancy to be higher than at day 11. The level of OAT mRNA was 4.44-fold higher than that of the sham-operated mice (Mann Whitney, $P < 0.0495$), but did not differ from that of the mice killed on day 11. Similarly, the mean value of OAT protein was higher on day 18 than on day 11, but it did not reach the statistical level of significance ($P < 0.0547$). In contrast, OAT activity was significantly higher on day 18 than day 11 as compared to the sham-operated mice (Mann Whitney, $P < 0.0495$). The level of OAT mRNA was quantitated either by RT-PCR (Fig. 3) or by q-PCR (Fig. 4). The two methods gave similar results and patterns (Figs. 3 and 4). Different house-keeping genes were used to prove that the effect of castration were independent of the gene of reference. Orchidectomy provoked a 4.9- and 4.7-fold increase in the level of OAT mRNA in 11d- and 18d-castrated mice as compared to the sham-operated male mice. (Fig. 4; Kruskal-Wallis, $P < 0.0001$ followed by Mann Whitney, $P < 0.0027$ or less in both cases).

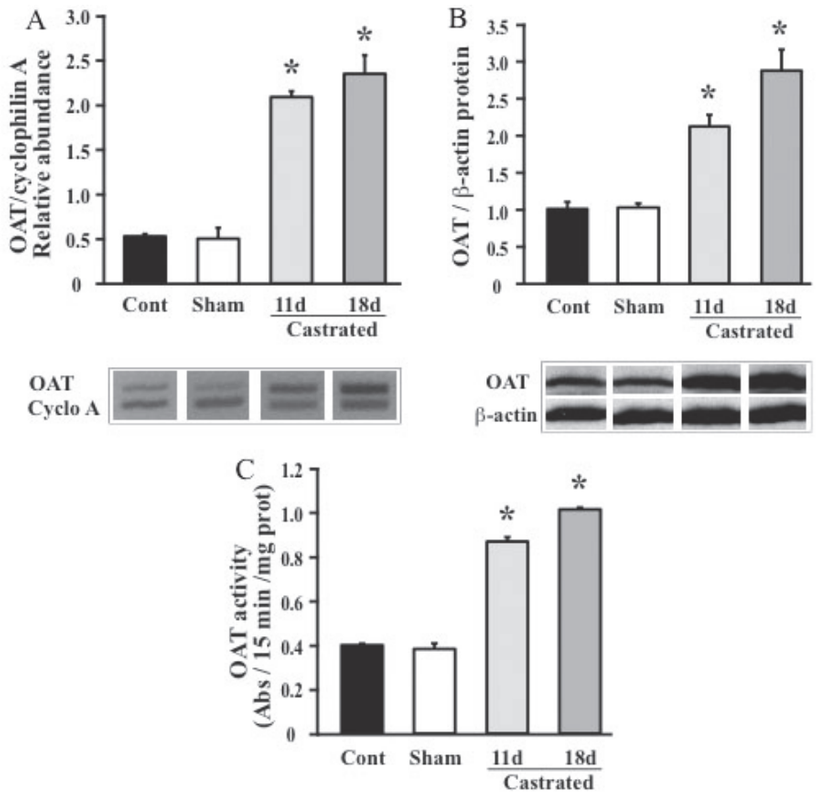

Fig. 3. Effect of orchidectomy on the expression of ornithine aminotransferase gene in the mouse kidney.

The values are expressed as means ± SE. A: The level of OAT mRNA were analyzed by semi-quantitative RT-PCR. The amplified cDNAs were separated by agarose-gel electrophoresis. Band intensities were quantified and reported relative to the cyclophilin A band (n=3 mice per group). B: Immunoblot assessing the abundance of OAT and β-actin proteins in mouse kidneys. Immunoblots were loaded with samples of 100 µg soluble proteins (n=6 mice per group). β-actin was used as control of protein loading and transfer. C: OAT activity was measured in mouse kidneys (n= 4, 3, 3, 3 mice per group, respectively). To simplify the Figure, a representative RT-PCR or immunoblot that corresponds to one mouse from each group was shown. Abbreviations: control (Cont), sham-operated (Sham), and 11- and 18-day orchidectomized mice (11d and 18d, respectively), cyclophilin A (Cyclo A), ornithine aminotransferase (OAT), and absorbance (Abs). Differences between groups were statistically analyzed by Kruskal-Wallis test and followed by Mann-Whitney test. *, $P < 0.05$ or less.

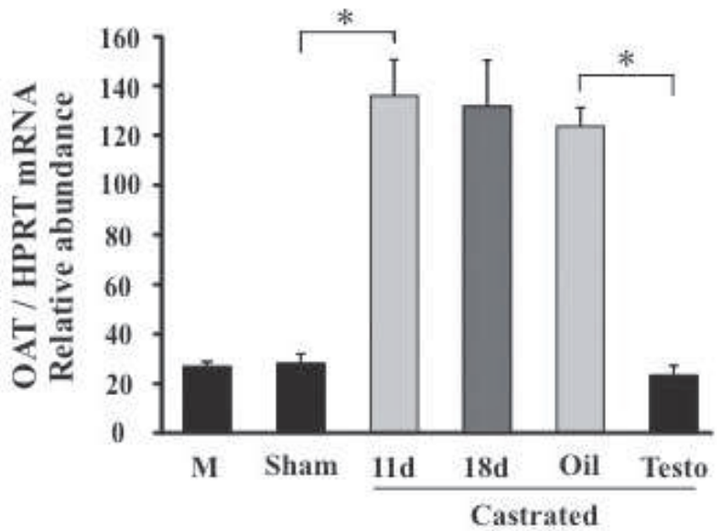

Fig. 4. Relative levels of ornithine aminotransferase mRNA in mouse kidneys.

The values are expressed as means ± SE. The relative mRNA levels of OAT were determined by qPCR in the kidneys of male (M), sham-operated (Sham), 11- and 18-day orchidectomized mice (11d and 18d, respectively), castrated mice treated with oil as solvant (Oil), and castrated mice supplemented with testosterone dissolved in oil (Testo). Abbreviations: ornithine aminotransferase (OAT), and hypoxanthine guanine phosphoribosyl transferase (HPRT). Differences between groups were statistically analyzed by Kruskal-Wallis test ($P < 0.0001$) and followed by Mann-Whitney test. *, $P < 0.0039$ or less. Number of mice used: 11, 6, 9, 7, 6, and 6, respectively.

3.3 Influence of testosterone on the expression of OAT gene

Given that orchidectomy provoked a sharp increase in the expression of OAT gene in the mouse kidney, we hypothesized that androgens control and down-regulate the expression of this gene. To verify our hypothesis, castrated male mice were subjected to testosterone-treatment. An additional group of castrated mice was subjected to sesame oil-treatment inasmuch as oil was used as a solvant to dissolve testosterone. The expression of OAT gene was analyzed at the transcriptional and traductional levels and OAT activity was measured. In

this new study, our results confirmed that castration of male mice provoked an overexpression of OAT gene (Fig. 5). The levels of OAT mRNA, protein, and enzyme activity were increased and strongly resemble those presented in Figure 3. However, the level of OAT mRNA was increased only by two-fold in kidneys of castrated mice of the second study, (Fig. 5) as compared to four-fold for the first study (Fig. 3). In kidneys of castrated mice treated with a single injection of sesame oil, the level of OAT mRNA, protein, and OAT activity did not differ from those of the castrated mice (Fig. 5). In contrast, a single injection of a physiological dose of testosterone decreased by 2-fold the level of OAT mRNA (Kruskal-Wallis, $P < 0.036$ followed by Mann Whitney, $P < 0.0495$), OAT protein by 1.58-fold (Kruskal-Wallis, $P < 0.032$ followed by Mann Whitney, $P < 0.05$), and OAT activity by 1.62-fold (Kruskal-Wallis, $P < 0.0045$ followed by Mann Whitney, $P < 0.0209$) (Fig. 5). The level of OAT mRNA was also quantitated either by RT-PCR (Fig. 5) or by q-PCR (Fig. 4). Different house-keeping genes were used to prove that the effect of castration were independent of the gene of reference. Again, the same pattern was found with the two methods (Figs. 4 and 5). Oil-treatment had no effect whereas testosterone-treatment provoked a 5.3-fold decrease in the level of OAT mRNA as compared to oil-treated mice. (Fig. 5; Kruskal-Wallis, $P < 0.0001$ followed by Mann Whitney, $P < 0.0039$). The plasma levels of corticosterone had a tendancy to increase after orchidectomy and decrease in testosterone-treated mice. Unfortunately, the difference did not reach statistical significance (Kruskal-Wallis, $P < 0.2435$). The plasma level of corticosterone were inversely correlated with those of testosterone (Fig. 5 and Levillain et al. 2005).

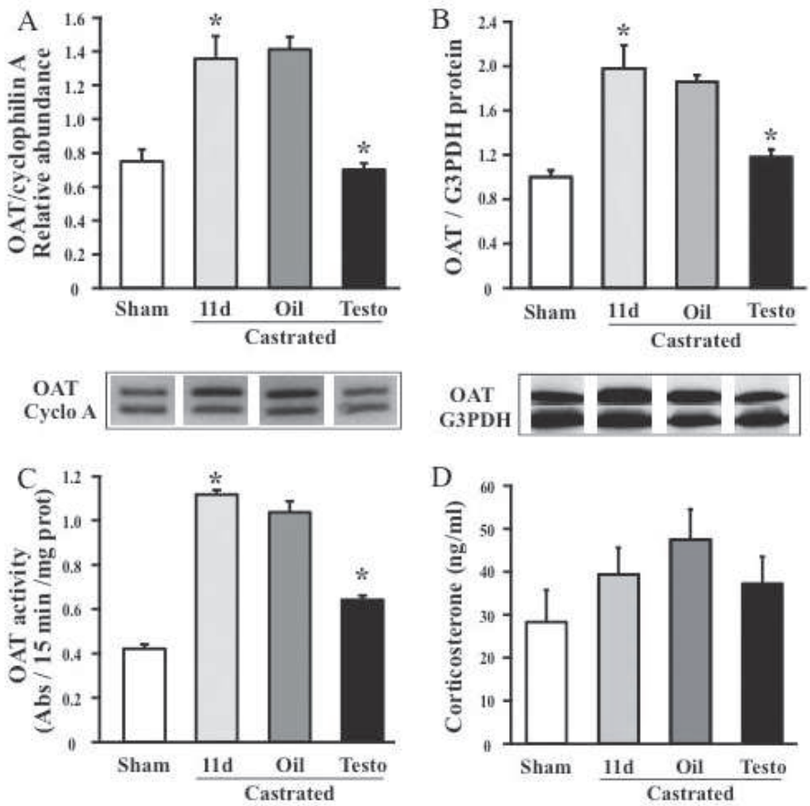

Fig. 5. Effect of testosterone treatment on the expression of ornithine aminotransferase gene in the mouse kidney.

The values are expressed as means ± SE. A: The level of OAT mRNA were analyzed by semi-quantitative RT-PCR. The amplified cDNAs were separated by agarose-gel electrophoresis. Band intensities were quantified and reported relative to the cyclophilin A band (n=3 mice per group). B: Immunoblot assessing the abundance of OAT and G3PDH proteins in mouse kidneys. Immunoblots were loaded with samples of 100 µg soluble proteins (n=3 mice per group). G3PDH was used as as control of protein loading and transfer. C: OAT activity was measured in mouse kidneys (n=4 mice per group). D: Plasma levels of corticosterone (n = 9, 10, 10, and 9 mice, respectively). To simplify the Figure, a representative RT-PCR or immunoblot that corresponds to one mouse from each group was shown. Abbreviations: sham-operated (Sham), and 11-day orchidectomized mice (11d), cyclophilin A (Cyclo A), ornithine aminotransferase (OAT), G3PDH (glyceraldehyde-3-phosphate dehydrogenase), Testo (oil + testosterone), and absorbance (Abs). Differences between groups were statistically analyzed by Kruskal-Wallis test and followed by Mann-Whitney test. *, $P < 0.05$ or less.

3.4 Influence of testosterone on the expression of eIF4-E gene

The eukaryotic initiation factor eIF4-E has been reported to be rate-limiting for OAT translation (Fagan et al., 1991). We analyzed whether the level of eIF4-E mRNA was altered by orchidectomy and testosterone replacement. Our data show that, in the mouse kidney, the level of eIF4-E mRNA had a tendancy to be decreased by orchidectomy and enhanced by testosterone replacement. Nevertheless, the difference did not reach statistical significance (Fig. 6, Kruskal-Wallis, $P < 0.1574$).

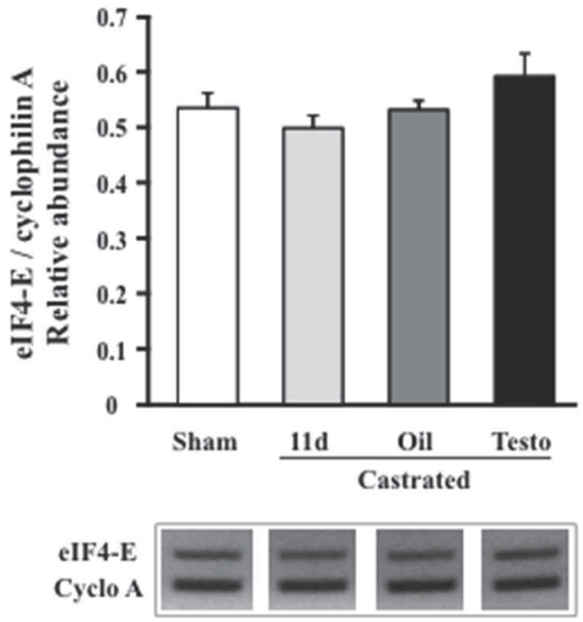

Fig. 6. Effect of testosterone treatment on the expression of eIF4-E gene in the mouse kidney.

The values are expressed as means ± SE. The levels of eIF4-E mRNA were analyzed by semi-quantitative RT-PCR. The amplified cDNAs were separated by agarose-gel electrophoresis. Band intensities were quantified and reported relative to the cyclophilin A band (n=3 mice

per group). To simplify the Figure, a representative RT-PCR that corresponds to one mouse from each group was shown. Abbreviations: sham-operated (Sham), and 11-day orchidectomized mice (11d), cyclophilin A (Cyclo A), eukaryotic initiation factor eIF4-E (eIF4-E), and oil + testosterone (Testo). Differences between groups were statistically analyzed by Kruskal-Wallis test, $P = 0.1574$.

3.5 Time course changes in the expression of OAT gene

This experiment was performed to determine the delay requested for testosterone to down-regulate the expression of OAT gene in the mouse kidney. The time-course effect of testosterone on the expression of OAT gene was analyzed at the transcriptional and post-translational levels 8, 24, 28, and 32 hrs following hormone administration. Eight hours following testosterone treatment, the level of OAT mRNA was reduced by 8%, but did not reach statistical significance (Fig. 7A). In contrast, 24, 28, and 32 hrs after testosterone replacement, the renal levels of OAT mRNA were decreased by 45%, 40%, and 36%, respectively (Kruskal-Wallis $P < 0.0028$ followed by Mann Whitney, $P < 0.0209$ for each case). The pattern of OAT activity along this time-course study strongly resembled that of OAT mRNA except that the decrease in OAT activity was about 2-fold lower than that of OAT mRNA (Fig. 7B). Indeed, 24, 28, and 32 hrs after testosterone administration, OAT activities were decreased by 20%, 21%, and 23%, respectively (Kruskal-Wallis $P < 0.0071$ followed by Mann Whitney, $P < 0.0209$ for each case). The efficiency of the hormonal treatment was checked by measuring testosteronemia. Our results show that testosteronemia was very high 8 hrs after a single injection of the hormone and regularly decreased over the experimental period. Thirty-two hours after the treatment, testosteronemia was about 2-fold higher than the physiological concentration of testosterone reported for control male mice (Levillain et al., 2007). Corticosteronemia were quantified in the same mice to determine whether testosteronemia modified the plasma level of corticosterone. A high concentration of corticosterone was detected 8 hrs after testosterone treatment (Fig. 7C). One day later (24 and 28 hrs), the plasma level of corticosterone were decreased by 3-fold whereas at 32 hrs, it increased again by 2-fold (Fig. 7D). Unfortunately, given that it was impossible to have access to the animal room during the night, no mice could be sampled during the dark period.

4. Discussion

OAT plays a pivotal role in the intermediary metabolism because this enzyme is at the crossroad of several pathways. OAT controls the production of glutamate and ornithine, competes with the polyamine pathway, and may be a source of carbon for the renal gluconeogenesis. These pathways are essential for many physiological roles including growth and energy supply. Several reports clearly documented the hormonal regulation of OAT gene in the rat liver and kidney. In this species, the renal expression of OAT gene is up-regulated by estrogens and triiodothyronine (Herzfeld & Knox, 1968; Lyons & Pitot, 1977; Mueckler & Pitot, 1983; Mueckler et al., 1984). During the post-natal development of the rat kidney, the sexual dimorphism of the expression of OAT gene increased in parallel with the endogenous synthesis of estrogens (Herzfeld & Knox, 1968). In contrast, testosterone affected OAT activity neither in liver nor in kidneys of male and female rats (Herzfeld & Knox, 1968). The authors concluded that estrogen naturally control and upregulate the expression of OAT

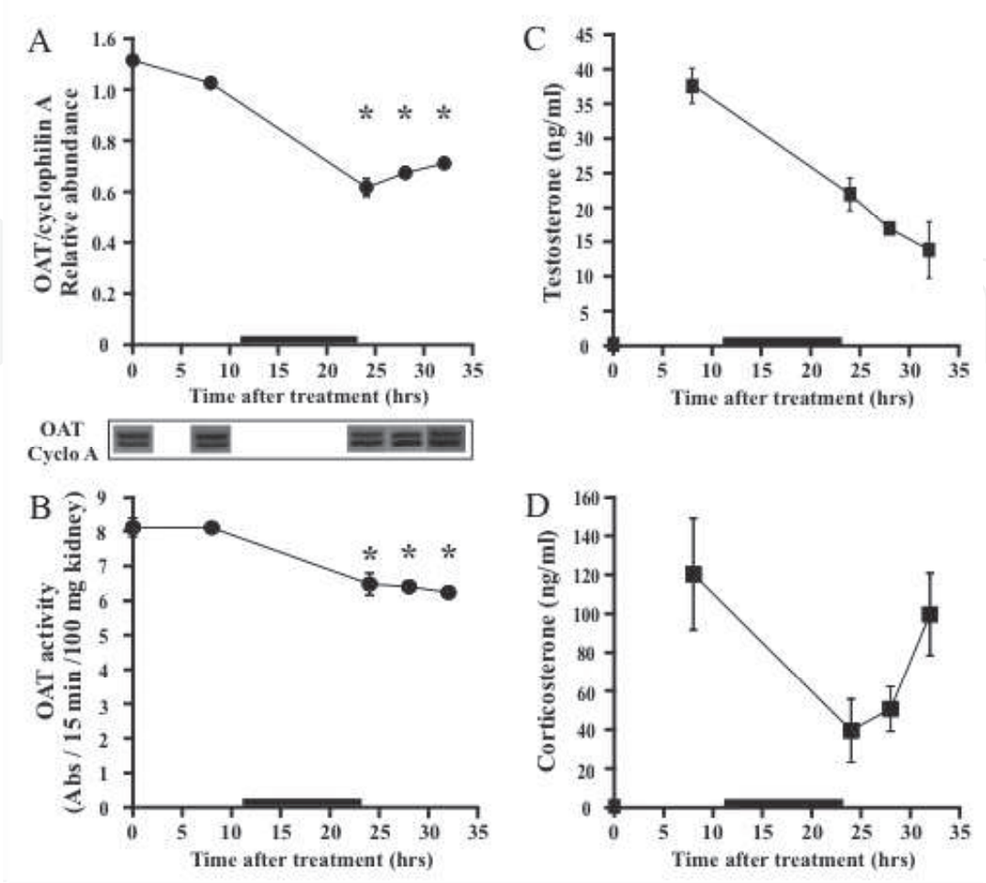

Fig. 7. Time course effect of testosterone on the expression of ornithine aminotransferase gene in the mouse kidney. A single dose of testosterone was injected to castrated-male mice. Mice were killed 8, 24, 28, and 32 hrs after the hormonal-treatment. Untreated castrated mice were used as controls. The values are expressed as means ± SE, (n=4 mice per group). A: The level of OAT mRNA were analyzed by semi-quantitative RT-PCR. The amplified cDNAs were separated by agarose-gel electrophoresis. Band intensities were quantified and reported relative to the cyclophilin A band. To simplify the Figure, a representative RT-PCR that corresponds to one mouse from each group was shown. B: OAT activity was measured in mouse kidneys. C: Plasma levels of corticosterone. D: Plasma levels of testosterone. The black bar represents the dark period (night). Differences between groups were statistically analyzed by Kruskal-Wallis test and followed by Mann-Whitney test. *, $P < 0.0209$ in all cases.

gene in the female rat kidney. As reported for the rats, the mouse kidney also exhibits a sexual dimorphism in the expression of OAT gene. Although the hormonal regulation of this gene has been poorly studied in both liver and kidney of male and female mice, it has been clearly shown that the endogenous production of testosterone was responsible for a decrease in the renal expression of OAT gene (Levillain et al., 2007). Interestingly, in contrast to the rats, ovariectomy altered neither the level of OAT protein nor OAT activity in the murine kidney (Levillain et al., 2007). Estrogens do not control the renal expression of OAT in the mouse kidney. Consequently, testosterone naturally controls and down-regulates the expression of

OAT gene in the male mouse kidney. Furthermore, orchidectomy completely abolished the sexual dimorphism of the renal expression of OAT gene (Levillain et al., 2007). The present findings confirm these statements. Moreover, our results show that testosterone negatively regulated the expression of OAT gene at the transcriptional level. Indeed, the levels of OAT mRNA, protein, and enzyme activity were markedly increased following castration. The castrated male mice were killed either 11 days or 18 days after the surgery to completely deplete the endogenous pool of testosterone. Plasma testosterone levels showed that all mice were testosterone-free as soon as 11 days after castration (Levillain et al., 2005).

To further explore the mechanismes involved in the regulation of OAT gene by testosterone in the mouse kidney, experiments were conducted to test the consequence of testosterone replacement on the expression of OAT gene at the transcriptional, translational, and post-translational levels. The dose of testosterone injected was calculated to reach a physiological plasma level of testosterone. The efficiency of testosterone to lower the level of OAT mRNA in the mouse kidney was confirmed. These results demonstrate that testosterone regulates OAT gene expression at the transcriptional level. The regulation of OAT gene at the transcriptional level has been also reported in the rat tissues (Mueckler & Pitot, 1983; Mueckler et al., 1983, 1984). The levels of OAT protein and OAT activity were also diminished by a single injection of testosterone, however, this decrease was less pronounced than that of OAT mRNA. This difference may be explained by the long half-life of OAT protein. The half-life of OAT protein is estimated at about 48 hrs (Ip et al., 1974). The time-course study of the effect of testosterone on the expression of OAT gene in castrated male mice also supports this timeline with the level of OAT mRNA decreasing more rapidly than OAT activity. This experiment also revealed a lag-time of 8 hrs or more from testosterone administration before detecting significant changes in the level of OAT mRNA. Similar results were observed with injection of testosterone to female mice (paper in preparation). This delay from administration to mRNA expression suggests that several genomic and/or non-genomic events may take place in the signaling cascade of testosterone (Heinlein & Chang, 2002). One hypothesis is that a target molecule such as the eukaryotic initiation factor eIF4-E is rate-limiting for OAT translation (Fagan et al., 1991). However, in our expriments, the level of eIF4-E mRNA was altered neither by orchidectomy nor by testosterone replacement.

In order to explain a direct genomic action of testosterone, an attempt was made to identify ARE on the promoter of the mouse OAT gene. We carried out an *in silico* search for homology to the consensus ARE motifs by selecting either the left half-site 5′-AGAACA-3′ or the right half-site 5′-TGTTCT-3′ motifs by search computational using MatInspector (http://www.genomatix.de/) (Beato et al., 1989; Fabre et al., 1994; Roche et al., 1992; Wang et al., 2007; Merkulov & Merkulova, 2009). Several ARE (5′-AGAACAnnnTGTTCT-3′) and glucocorticoid responsive element (GRE, 5′-GGTACAnnnTGTTCT-3′) which share the same sequence 5′-TGTTCT-3′ (Nelson et al., 1999, Verrijdt et al., 2003) were identified on the promoter of the mouse OAT gene. Interestingly, four GRE have been found in the human OAT gene (Zintz & Inana, 1990). Further experiments will be required to determine the functionality of these elements in response to hormone administration.

Recently, it has been reported that, in Swiss CD1 mice, sex hormones influence the weight of the adrenal gland and the plasma level of corticosterone (Bastida et al, 2007). Indeed, the weight of the female adrenal gland was 2.8-fold higher than that of the male (Bastida et al, 2007). Orchidectomy enhanced the weight of the male adrenal gland by 2.1-fold whereas

testosterone treatment decreased by 2.6-fold the weight of the female adrenal gland which did not differ from that of the control male adrenal gland (Bastida et al, 2007). The plasma level of corticosterone was 2.8-fold higher in the females as compared to the males (Bastida et al, 2007). These results led us to hypothesize that testosterone may control the plasma level of corticosterone which may secondarily regulate the expression of OAT gene in the mouse kidney. For this reason the plasma level of corticosterone were measured in the different groups of mice. Our results did show a trend for the plasma levels of corticosterone to be higher in orchidectomized mice than in untreated male mice and in castrated male mice treated with testosterone, however, with a low sample number and high standard error the difference did not reach statistical significance. Contrarily, when analyzing the time course effect of testosterone 8 hrs after the hormonal treatment the plasma level of corticosterone and testosterone were both very high suggesting that testosterone does not control the rate of synthesis and release of corticosterone. The presence of GRE and ARE in the promoter of the mouse OAT gene offers new insight on the hormonal regulation of this gene. Further experiments utilizing male mice subjected to both orchidectomy and adrenalectomy may afford a model to resolve this point.

Hormonal regulation of OAT gene by steroids can impact human disease model, such as prostate cancer (PCa). Androgen receptor (AR) targets have been recently identified in a model of androgen-dependent (LNCaP) and a model of castrate-resistant (C4-2B) human PCa cell lines (Jariwala et al., 2007) with OAT expressed at a higher level in the castrate resistant (C4-2B) cells. Expression of OAT was repressed by siRNA in C4-2B cells demonstrating that OAT was an AR regulated gene, although it did not respond to dihydrotestosterone (DHT). Furthermore, the expression of OAT was analyzed in primary PCa tumors and primary PCa tumors after 3 months of androgen ablation therapy. OAT appears to be negatively regulated by the AR *in vivo*.

In conclusion, in the mouse kidney, testosterone down-regulates the expression OAT gene at the transcriptional level. This finding may have interesting consequences for human diseases as reported for prostate cancer.

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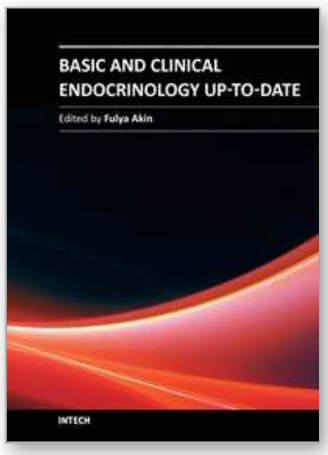

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