DIFFERENTIAL EXPRESSION OF ORNITHINE DECARBOXYLASE AND *HA-RAS* CELLULAR ONCOGENE DURING DEVELOPMENT OF THE FEMALE RAT

M. G. Baik[1] and C. S. Park[2]

Labortory of Growth/Lactation
Department of Animal and Range Science
North Dakota State University, Fargo, ND 58105, USA

Summary

Experiments were performed to determine age-associated changes in ornithine decarboxylase (ODC) gene and *Ha-ras* cellular oncogene expression in tissues of female rats. In the kidney, ODC mRNA levels did not show age-associated changes, while ODC enzyme activities were decreased with advancing age from 3 to 10 months. These results suggest that post-transcriptional mechanism (s) are involved in the age-dependent decrease in renal ODC enzyme activity. In addition, we found no correlation between testosterone-induced renal ODC expression and DNA methylation pattern. *Ha-ras* mRNA levels in brain decreased as animals aged from 3 to 6 months, while renal *Ha-ras* mRNA levels were not influenced by age. Results demonstrate the age-dependent expression of *Ha ras* in a tissue-specific manner.

(**Key Words**: Ornithine Decarboxylase, *Ha-ras*, DNA Methylation, Rats)

Introduction

Polyamines (putrescine, spermidine, and spermine) are ubiquitous cellular components. At the molecular level, polyamines stabilize DNA against thermal denaturation and radiation damage. Ornithine decarboxylase (ODC, EC4.1.1.17) catalyzes the conversion of ornithine to the diamine, putrescine, a pivotal step in the biosynthesis of the polyamines, spermidine and spermine. There are reports on age-dependent changes in ODC expression; liver and mammary epithelium ODC activities decrease with advancing age from 7 weeks to 15 weeks (Hawrylewicz et al., 1989).

Among several regulatory mechanisms of gene expression, methylation of cytosine residues in the DNA has been implicated in transcriptional control in several eukaryotic genes (Razin and Riggs, 1980). A tissue-specific correlation between hypomethylation and gene activity has been established for a number of eukaryotic genes, including rat prolactin (Durrin et al., 1984) and rat growth hormone (Gaido and Strobl, 1989). In addition, estrogen-induced demethylation of chicken vitellogenin gene was correlated with gene activation (Burch and Evans, 1986).

Many studies have confirmed that a variety of cellular oncogenes are present in normal cells, and that many of these genes are expressed in different cell types in a tissue-specific fashion (Adamson, 1987). *Ha-ras* cellular oncogene belongs to the *ras* gene family. The protein product of *Ha-ras* p21 is a membrane-associated GTP binding protein, which exhibits GTPase activity (Sweet et al., 1984). The similarities between p21 and the adenylate cyclase regulatory proteins suggest that they are related in cellular function (Santos and Nebreda, 1989).

In the present study, we investigated age-dependent expression of ODC and *Ha-ras* oncogene in various tissues of female rats. In addition, we investigated the methylation pattern of the testosterone-induced rat ODC gene.

Materials and Methods

Animals and diet

Female Sprague-Dawley rats, purchased from Harlan Sprague Dawley (Indianapolis, IN), were housed singly in wire mesh bottom cages in a barrier facility. The air pressure within the barrier was maintained at = 5 mm Hg above the outside environment. The temperature was maintained at 22-23℃ with relative humidity ranging between

[1]Department of Genetic Engineering, Chonnam National University, Kwangju 500-757, Korea.

[2]Address reprint requests to Dr. C. S. Park, Laboratory of Growth/Lactation, Department of Animal and Range Sciences, North Dakota State University, Fargo, ND 58105, USA.

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50% and 60%. A 12 h light/dark cycle was used. Water was freely available through an automatic watering system. Rats were allowed *ad libitum* access to a modified AIN-76 diet with the ratio of approximately 1:1 of corn starch:sucrose as the carbohydrate sources (table 1).

TABLE 1. COMPOSITION OF THE MODIFIED AIN 76 DIET

Ingredients*	g/kg diet
Casein	200
DL-methionine	3
Cornstarch	320
Sucrose	330
Fiber	50
Corn oil	50
AIN mineral mix	35
AIN vitamin mix	10
Choline bitartrate	2
Chemical analysis	
Crude protein	168
Gross energy**, MJ/kg diet	19

* All ingredients were purchased from ICN Biomedicals (Costa Mesa, CA).

** Gross energy was determined by bomb calorimetry.

Tissue samples

Animals were euthanized by CO_2 at 3, 6, and 10 months of age. Brain, kidney, mammary, and liver tissues were removed and immediately frozen in liquid nitrogen and stored at −70℃ until analysis.

Testosterone implantation

At 18 wk of age, three female rats received 35 mg, 7-d release testosterone implants s.c., (Innovative Research of American, Toledo, OH), and three animals received vehicle (placebo) implants s.c. At 19 wk, all animals were killed, and kidneys were collected and frozen in liquid nitrogen and stored at −70℃ until analysis.

ODC enzyme activity

Activities of ODC enzyme were determined as previously described (Baik et al., 1992). Briefly, enzyme activity was determined by measuring the release of $^{14}CO_2$ from L-[1-^{14}C] ornithine in a mixture consisting of the assay buffer, radio-labeled and unlabeled ornithine, and the tissue extract to which the enzyme cofactor, pyridoxal phosphate, was added. One unit of ODC activity corresponds to one mole of $^{14}CO_2$ released per second.

RNA blotting

Total RNA was extracted from tissues with guanidine/cesium chloride as previously described (Sambrook et al., 1989). The RNA was blotted onto nylon membranes (Zetaprobe blotting membrane, Bio Rad, Richmond, CA). Yeast RNA was used as a negative control. Dot-blotted membranes were hybridized with α^{32}P-dCTP labeled *Ha-ras* cDNA (American Type Culture Collection, Rockville, MD) and ornithine decarboxylase (kind gift from Dr. Perry Blackshear, Duke Medical School, Durham, NC) probes as described (Baik, et al. 1992). After hybridization and washing of the nylon membranes as recommended by the supplier, the membranes were exposed to XAR-5 X-ray film (Eastman Kodak, Rochester, NY) with an intensifying screen at −70℃ for 2 d. Relative intensity was determined by scanning densitometry of individual dots on autoradiograms and calculated from the midpoints of linear regression equations ($r^2 > .90$) (Park et al., 1988).

Southern blotting

Genomic DNA from kidney of testosterone-implanted animals was extracted from a crude nuclear fraction with a proteinase K and RNase A digestion (Sambrook et al., 1989). Hpa II or Msp I digestion of genomic DNA was performed according to the assay conditions specified by supplier. Digested genomic DNA fragments were separated by electrophoresis on a 0.7% agarose gel and transferred to a nylon membrane. The membrane was hybridized with a α^{32}P-dCTP labeled ODC probe as described above. Hybridization signals were visualized by autoradiography.

Statistics

The statistical difference between various ages was determined by ANOVA using the general linear models routine of SAS/STAT™ (SAS Institute Inc., 1988). All pairwise comparisons of means were made by least significant difference tests using appropriate error terms when differences among ages were obtained.

Results

ODC mRNA levels in mammary tissue, kidney, brain, and uterus were evaluated by RNA blotting at various ages. No age-associated differences were detected (table 2). ODC mRNA levels, however, showed tissue-specific differences. The highest ODC mRNA levels were detected in the kidney, with intermediate differences in brain, mammary tissue, and uterus. Blackshear et al. (1989) have reported similar findings.

We also determined ODC enzyme activities in mammary tissue and kidney at 3, 6, and 10 months of age (table 3). Renal ODC enzyme activities decreased as animals aged from 3 to 10 months, while ODC activities in mammary tissues were not influenced by age.

Southern analysis was performed to determined whether testosterone implants affect DNA methylation patterns of the ODC gene in the kidney. Hpa II can cleave unmethylated CCGG sequences but can not cleave methylated CmCGG sequences. Msp I can cleave both CCGG and CmCGG sequences. Hpa II-digested DNA showed three bands, while Msp I-digested DNA did not show clear bands (figure 1), indicating the ODC gene has some methylated CmCGG sequences. There were no differences in the methylation patterns of ODC DNA in the kidneys of placebo- and testosterone-implanted rats. Testosterone induces renal ODC mRNA expression (Baik et al., 1992; Wing, 1990; Janne et al., 1988; Berger et al., 1984); therefore, testosterone-induced ODC expression is not related to changes in DNA methylation.

Levels of *Ha-ras* oncogene RNA transcripts in mammary tissue, liver, kidney, and brain taken at 3 months of age were determined by RNA dot blotting. Brain had the highest level of *Ha-ras* mRNA, while liver had the lowest level (table 4). We also examined mRNA levels of *Ha-ras* in the brain and kidney at 6 months of age. *Ha-ras* mRNA levels in brain were lower at 6 months than those at 3 months, while renal *Ha-ras* mRNA levels were not influenced by age. These results demonstrate the age-dependent expression of *Ha-ras* with tissue-specific pattern.

TABLE 2. ORNITHINE DECARBOXYLASE mRNA IN FEMALE RAT TISSUES AT VARIOUS AGES

Tissue	Age (months)			SE[1]	P[2]
	3	6	10		
	(Specific activity[3])				
Brain	ND[4]	23.2	31.6	6.2	0.31
Kidney	57.5	63.2	60.0	5.4	0.24
Mammary	ND[4]	10.4	14.2	4.8	0.54
Uterus	30.6	33.7	26.5	7.5	0.24

[1] Standard error of the mean, n = 3.

[2] Significance level of F-test for equality of three age groups.

[3] Relative levels of ornithine decarboxylase mRNA transcript determined by dot blotting were expressed as specific activity, where specific activity = relative intensity/(μg RNA $\times$ 10^{-1}).

[4] Not determined.

TABLE 3. ORNITHINE DECARBOXYLASE ACTIVITY IN FEMALE RAT TISSUES AT VARIOUS AGES

Tissue	Age (months)			SE[1]
	3	6	10	
	(10^{13} $\times$ units[2]/mg protein)			
Mammary tissue	0.72	0.60	0.56	0.06
Kidney	1.20[a]	0.93[b]	0.71[c]	0.07

[1] Standard error of mean, n = 3.

[2] One unit of ODC activity = one mole of $^{14}CO_2$ released per second.

[a,b,c] Values in the same row with different superscripts differ significantly ($p < 0.05$).

TABLE 4. HA-RAS mRNA IN FEMALE RAT TISSUES AT VARIOUS AGES

Tissue	Age (months)		SE[1]	P[2]
	3	6		
	(Specific activity[3])			
Brain	89.2	35.6	7.2	.04
Kidney	40.5	37.0	5.0	.76
Mammary	51.3	ND[4]	–	–
Liver	14.7	ND[4]	–	–

[1] Standard error of the mean, n = 3.

[2] Significance level of F test for equality of two age groups.

[3] Relative levels of *Ha-ras* mRNA determined by dot blotting were expressed as specific activity, where specific activity = relative intensity/(μg RNA × 10^{-3})

[4] Not determined.

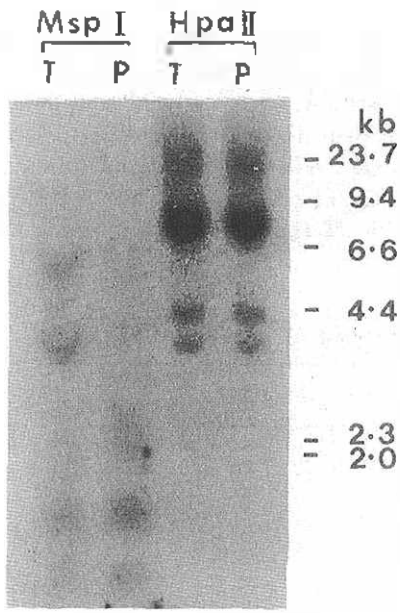

Figure 1. Southern blot analysis of renal genomic DNA from female rats. Genomic DNA (10 μg / lane) from the kidneys of either placebo (P) or testosterone (T)-implanted female rats was digested with either Msp I or Hpa II. The digested DNA fragments were electrophoresed on a 0.7% agarose gel, transferred to a nylon membrane, and hybridized with a 2.2 kb Eco RI fragment of pODC-2. Size standards are Hind III-digested lambda DNA fragments.

Discussion

ODC is regulated by three different processes: transcription, translation, and enzyme degradation. Marked induction (50- to 100-fold) of ODC activity by feeding a 50% casein diet is preceded by only a several-fold increase in ODC mRNA levels, suggesting an involvement of post-transcriptional mechanisms (Kameji, 1987). Our previous studies have also suggested that translational or post-translational mechanism (s) are involved in energy restriction-induced renal ODC activity (Baik et al., 1992). In this study, ODC enzyme activities in the kidney decreased with advancing age from 3 to 10 months with no age-associated changes in ODC mRNA. We believe that ODC gene activity is controlled through post-transcriptional mechanisms.

It has been speculated that methylation of DNA may exert a controlling influence over gene expression. In many reports concerning DNA methylation and gene activity, a correlation between hypomethylation, in particular in the 5'-flanking region of genes, and gene activity has been demonstrated (Razin and Riggs, 1980). This interrelationship, however, cannot be generalized since a number of genes have either been found to be extensively methylated though at the same time efficiently transcribed or to remain silent after chemically-induced demethylation (Razin and Szyf, 1984). Our previous study reveals rat liver DNA exhibits age related hypomethylation while hypermethylation is significant in mammary DNA with advancing age through 6 months (Baik et al., 1992). The slow turnover rate of hepatic cells may explain the decrease in DNA methylation with advancing age (Hoal-Van Helden and Van Helden, 1989).

The present study showed no correlation

between testosterone-induced ODC expression and DNA hypomethylation. Methylation in animal DNA is found exclusively in the CG dinucleotides, and it should be noted that a correlation cannot be strictly ruled out since restriction enzymes, Hpa II and Msp I, do not assay every available CG residue, but detect only CCGG sequences. Also, most methylation sites may not be of major regulatory importance to influence transcription of the genes.

Mammalian *ras* genes are expressed in all cell lineages and organs, although there are differences in expression through pre- and post-natal development, and certain adult tissues preferentially express one member of the family over another (Furth et al., 1987; Lean et al., 1987). *Ha ras* mRNA is highly expressed in an early stage of rat liver development and gradually decreases as liver develops (Baik et al., 1992; Zhang et al., 1988). In the present study, an age-dependent decrease in *Ha-ras* mRNA levels was observed in brain tissues. The *Ha-ras* mRNA was the highest in the brain compared with liver, kidney, and mammary tissue. Furth et al. (1987) reports that the highest level of *ras* proteins among any human tissues is that found in the brain. These patterns of expression are consistent with the idea that *Ha-ras* gene products may be required in *different amounts at different times in a tissue*-specific manner (Santos and Nebreda, 1989). More studies regarding the role of *Ha-ras* genes in the tissue specific differentiated function are needed to understand *Ha-ras* related organ development and possibly tumorigenesis.

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