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Age-related changes in renal and hepatic cellular mechanisms associated with variations in rat serum thyroid hormone levels

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Silvestri E, Lombardi A, de Lange P, Schiavo L, Lanni A, Goglia F, Visser TJ, Moreno M. Age-related changes in renal and hepatic cellular mechanisms associated with variations in rat serum thyroid hormone levels. Am J Physiol Endocrinol Metab 294: E1160-E1168, 2008. First published April 22, 2008; doi:10.1152/ajpendo.00044.2008.—Aging is associated with changes in thyroid gland physiology. Age-related changes in the contribution of peripheral tissues to thyroid hormone serum levels have yet to be systematically assessed. Here, we investigated age-related alterations in the contributions of the liver and kidney to thyroid hormone homeostasis using 6-, 12-, and 24-mo-old male Wistar rats. A significant and progressive decline in plasma thyroxine occurred with age, but triiodothyronine (T₃) was decreased only at 24 mo. This was associated with an unchanged protein level of the thyroid hormone transporter monocarboxylate transporter 8 (MCT8) in the kidney and with a decreased MCT8 level in the liver at 24 mo. Hepatic type I deiodinase (D1) protein level and activity declined progressively with age. Renal D1 levels were decreased at both 12 and 24 mo but D1 activity was decreased only at 24 mo. In the liver, no changes occurred in thyroid hormone receptor (TR) TR α_1 , whereas a progressive increase in TR β_1 occurred at both mRNA and total protein levels. In the kidney, both $TR\alpha_1$ and $TR\beta_1$ mRNA and total protein levels were unchanged between 6 and 12 mo but increased at 24 mo. Interestingly, nuclear TR β 1 levels were decreased in both liver and kidney at 12 and 24 mo, whereas nuclear TR α_1 levels were unchanged. Collectively, our data show differential age-related changes among hepatic and renal MCT8 and D1 and TR expressions, and they suggest that renal D1 activity is maintained with age to compensate for the decrease in hepatic T_3 production.

aging; deiodinase; liver; kidney

THYROID PHYSIOLOGY AND THYROID PARAMETERS are markedly influenced by genetic and by environmental factors (such as food and iodine intake), all able to modulate thyroid hormone bioactivity (18). During aging, thyroid function decreases progressively, and it is well recognized that the symptoms of aging can easily be confused with those of hypothyroidism (40). Complex age-related changes occur in the anatomy of the thyroid gland and in the physiology of the hypothalamopituitary-thyroid axis, and these could contribute to the progressive age-related decline in hormone metabolic activity. Moreover, the sensitivity of target cells to triiodothyronine (T₃) is reduced with aging (32, 35). A tissue's response to thyroid hormones (THs) is directly dependent on I) the amount of THs available, 2) the cellular uptake and efflux of THs, 3) the conversion of thyroxine (T_4) to T_3 and their degradation by different deiodinases, 4) the number of TRs available to interact with T_3 (50), and 5) post-receptor events.

Since TH homeostasis changes during an organism's life span, it is perhaps not surprising that the above-mentioned factors vary with age. Indeed, aging is associated with a decrease in serum TH levels due to a progressive decline in the functional capacity of the thyroid gland to synthesize T₄ and T₃ (see Ref. 18 and references therein). The plasma concentrations of thyroid hormones appear to be key determinants of biological activity (19). The proportions of nuclear receptor-bound T_3 originating from plasma T₃ or from local deiodination of T₄ vary between tissues but it is clear that both T₄ and T₃ must first cross the plasma membrane of target cells to exert their effects (19, 39), and total tissue uptake of T_3 is reduced in the liver and heart of aged rats (31, 33). Recently, it has been reported that cellular entry of thyroid hormones is mediated by plasma membrane transporters, with the rat monocarboxylate transporter 8 (MCT8) being identified as an active and specific TH transporter (49). However, no information is yet available on its variation during aging.

Since a large amount of serum T_3 is generated by the deiodination of T_4 in extrathyroidal tissues (24), TH homeostasis is regulated not only by hormone secretion by the thyroid gland and their cellular uptake but also by enzymatic deiodination in extrathyroidal tissues (4). To date, three iodothyronine deiodinases (D1, D2, and D3) are known, and these have different catalytic profiles, tissue distributions, and physiological functions. They contribute to TH homeostasis either by producing T_3 from T_4 (in the case of D1and D2) or by catalyzing the inactivation of both T_4 and T_3 (D1 and D3) (3, 4). The actions of the deiodinase are integrated to promote the maintenance of serum T_3 concentrations involving both transcriptional and post-translational modifications (see Ref. 4 and references therein).

D1 is abundantly expressed in liver and kidney, where it is induced by T_3 and suppressed by hypothyroidism in a form of autoregulation that involves adaptations to changes in thyroid status (4). In rats, it has been observed that the T_3 production rate from T_4 increases from 25% at the age of 7 mo to 60% at 11 mo, suggesting an increased T_4 deiodination during this period (21). Moreover, in vitro data reveal a marked decrease

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in D1 activity in liver and thyroid in 26-mo-old rats (10), thus indicating an impaired T_4 -to- T_3 conversion in peripheral tissues with age. However, the precise mechanism(s) underlying the changes in TH homeostasis in aging rats has not yet been elucidated. Liver and kidney, as well as being involved in the transport, storage, metabolism, and excretion of this hormone, are profoundly dependent on it. In particular, T_3 modulates ~8% of the hepatic genes (12) as well as renal growth, hemodynamics, and salt and water metabolism (7), and it thereby affects a variety of hepatic and renal metabolic processes. However, no systematic data are yet available on how the main peripheral tissues, such as liver and kidney, contribute to changes in TH homeostasis during aging, particularly with regard to differences between these tissues in the underlying mechanisms.

The aim of the present work was to investigate the molecular events regulating T_3 metabolism and bioavailability in rat liver and kidney by studying (using 6-, 12-, and 24-mo-old male rats) the in vivo age-related changes in the expressions of MCT8, TR α_1 , and TR β_1 and the expression and activity of D1. By so doing, we hoped to gain insight into the age-related changes in renal and hepatic contributions to rat thyroid hormone homeostasis.

METHODS

Experimental animals. All animal experimentation was conducted in accord with accepted standards of humane animal care, the policy of Naples University and was approved by the Dipartimento della Sanità Pubblica Veterinaria, la Nutrizione e la sicurezza degli Alimenti, Ministero della Salute, Rome, Italy (protocol 176/2005a).

Animals. Three groups of male Wistar rats (Charles River, Lecco, Italy) (each composed of 5 animals) aged 6 (adult), 12 (old), or 24 (senescent) mo were used throughout. After 7 days of acclimatization, they were housed one per cage in a temperature-controlled room at 28°C (their thermoneutral ambient temperature) with a 12:12-h lightdark cycle. Each animal was maintained for 20-30 days prior to being euthanized, a period during which food intake and body weight were daily recorded. A commercial mash (Charles River, Lecco, Italy; total metabolizable energy: carbohydrates 60.4%, proteins 29%, fat 10.6% [J/J]; 15.88 kJ gross energy/g) and water were available ad libitum. Because a diurnal variation of TH-related genes has been reported (51), the experiments were all performed using the same time schedule starting at 0900, thus avoiding differences due to circadian variations or nutrient alterations. Rats were anesthetized with an intraperitoneal injection of chloral hydrate (40 mg/100 g body wt) and killed by decapitation. Trunk blood was collected and serum isolated to allow analysis of hormone levels.

Then, liver and kidneys were excised, weighed, and either immediately processed for preparation of microsomes and nuclei or instantly frozen in liquid nitrogen and stored at -80° C for later processing. No gross pathologies were evident. Moreover, an extensive histopathological examination revealed no evidence of hepatic or nephrotic lesions in 12- and 24-mo-old rats and not even at 27 mo (46). Serum total (T)T₃, free (F)T₃, TT₄, and FT₄ levels were determined using materials and protocols supplied by Byk-Sangtec Diagnostica (Dietzenbach, Germany). Thyroid-stimulating hormone (TSH) serum levels were measured using materials and protocols supplied by Amersham Bioscience, with rat TSH as standard [Biotrak rat TSH (rTSH) ¹²⁵I assay system].

Resting metabolic rate was measured using an Oxymax indirect calorimeter (Columbus Instruments, Columbus, OH) at 28°C, as described (28). Body temperature was measured using a rectal probe (307-Digital Thermometer, P & M Industrial Refrigeration, Taiwan).

RNA extraction and RT-PCR assays. Total RNA was isolated from liver and kidney using TRIzol Reagent (Invitrogen). The protocol supplied by the manufacturer was slightly adapted to ensure effective inactivation of endogenous RNAse activity during the subsequent phenol extractions, and the quality of the RNA samples was checked on a denaturing agarose gel. Both the RT reactions and the PCR amplifications were carried out as described in Ref. 9. Random hexamers were from Invitrogen. Superscript RT, RNAse inhibitor, deoxynucleotide triphosphates (dNTPs), and SuperTaq polymerase were all from HT Biotechnology (Cambridge, UK). The oligonucleotide primers used (listed in Table 1) were from Sigma Genosys (Cambridge, UK). As an internal control, the same cDNAs were amplified using β-actin oligonucleotide primers. Parallel amplifications (20, 25, and 30 cycles) of a given cDNA were used to determine the optimum number of cycles. For each gene under study, a readily detectable signal within the linear range was observed after 30 cycles. The quantities of the PCR products were determined in separate preparations from five rats. One-half of the PCR reaction products were separated on a 2% agarose gel containing EtBr, and the corresponding signals were quantified by means of a Bio-Rad Molecular Imager FX using the supplied software (Bio-Rad Laboratories, Hercules, CA). The expression signals of the various genes were normalized to the nonregulated β -actin signal. Indeed, β -actin is known as being little sensitive to nutritional and hormonal conditions, and the β-actin mRNA was previously used as the endogenous standard for semiquantitative analysis in aging studies (35).

D1 assay. D1 activity was measured in the liver and kidney microsomal fractions by analysis of the production of radioiodide from $[3',5'-^{125}I]rT_3$. Microsomes were isolated as described (48). Protein concentration was determined by the Bradford method, using BSA as standard. Aliquots of microsomes were snap-frozen on dry ice and then stored at -80° C.

Two micrograms of microsomal protein was incubated for 30 min at 37°C with 0.1 μ M rT₃ and ~100,000 cpm [3',5'-¹²⁵I]rT₃ in 200 μ l of 100 mM phosphate buffer (pH 7.2) with 2 mM EDTA and 10 mM DTT. After incubation, the reaction was stopped by the addition of 100 μ l of 5% BSA at 0°C. Protein-bound iodothyronines were precipitated by the addition of 500 μ l of 10% (wt/vol) trichloroacetic acid. After incubation of the mixture at 0°C for 10 min, it was centrifuged at 3,500 g for 15 min, and the radioactivity in the supernatant was subsequently determined using a β -counter. Enzy-

Table 1. Oligonucleotide primers used for RT-PCR experiments

Gene	Sense Oligonucleotide	Antisense Oligonucleotide
SMP30	5'-TCAAAGACTGTCTGCCGATG-3'	5'-TATCTCCCAGCAGGATCCAC-3'
D1	5'-CCTCCACAGCTGACTTCCTC-3'	5'-TAGAGCCTCTCAGGCAGAGC-3'
D3	5'-GCCTCTACGTCATCCAGAGC-3'	5'-GCCCACCAATTCAGTCACTT-3'
MCT8	5'-TGGTTACTTCGTCCCCTACG-3'	5'-CCAGGGATGGAGTCACTGAT-3'
$THR\alpha_1$	5'-AGAACCTCCAGCCCTTGGAC-3'	5'-AGCCAAGCTGTCCTTTGCAC-3'
THRβ	5'-GTTCAAGAGGAGCCACACTG-3'	5'-CAGGCTTCGGACATTCCTAC-3'
β-Actin	5'-TTGTAACCAACTGGGACGAT-3'	5'-TAATGTCACGCACGATTTCC-3'

SMP30, senescence marker protein-30; D1 and D3, type I and type III deiodinase; MCT8, monocarboxylate transporter 8; THR, thyroid hormone receptor.

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matic deiodination was corrected for nonenzymatic ¹²⁵I production (as determined in blank incubations without enzymes) and multiplied by 2 to account for random labeling and the deiodination of the 3' and 5' positions of $[3',5'-^{125}I]rT_3$ (48).

Western blotting analysis. For Western blotting analysis of MCT8, TR α_1 , and TR β_1 , liver and kidney were homogenized in lysis buffer containing 20 mM Tris·HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 2.5 mM Na₂H₂P₂O₇, 1 mM b-CH₃H₇O₆PNa₂, 1 mM Na₃VO₄, 1 mM PMSF, 1 mg/ml leupeptin, and 1% Triton X-100 (all from Sigma-Aldrich, St. Louis, MO) using an ultraturrax and centrifuged at 14,000 rpm for 10 min at 4°C (Beckman Optima TLX; Beckman Coulter, Milan, Italy).

For Western blotting analysis of nuclear TR α_1 and TR β_1 , liver and kidney nuclei were isolated in a medium consisting of 220 mM mannitol, 70 mM sucrose, 20 mM Tris·HCl, 1 mM EDTA, 5 mM EGTA, and 5 mM MgCl₂, pH 7.4, supplemented with the following protease inhibitors: 1 mM benzamidine, 4 µg/ml aprotinin, 1 µg/ml pepstatin, 2 µg/ml leupeptin, 5 µg/ml betastatin, 50 µg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone, and 0.1 mM phenylmethylsulfonyl fluoride (all from Sigma-Aldrich). Liver and kidney homogenates were centrifuged at 500 g_{av} for 10 min, and the resulting pellet containing the nuclei was taken up in lysis buffer and debris removed by centrifugation at 14,000 rpm for 10 min at 4°C (Beckman Optima TLX). The protein concentrations of the nuclear extracts were determined using Bio-Rad's DC method (Bio-Rad Laboratories).

Thirty micrograms of the appropriate protein sample was electrophoresed on 10% SDS polyacrylamide gels, and specific primary antibodies against MCT8 (rabbit polyclonal antisera against human MCT8 COOH-terminal amino acid residues 527-539) (15), TR α_1 (T-17, Sc-10819; Santa Cruz Biotechnologies) and TR β_1 (N-19, Sc-10822; Santa Cruz Biotechnologies) were used. For Western blotting analysis of D1 and D3, liver and kidney microsomes lysates were electrophoresed on 13% SDS polyacrylamide gels. The polyclonal antibodies against rat D1 and D3 were generously supplied by Dr. Jack L. Leonard (University of Massachusetts Medical Center, Worcester, MA). Lysates of HEK 293 cells transfected with rat D1 cDNA or human D2 cDNA in pcDNA3 expression plasmid were used as positive and negative controls, respectively, for the D1 immunoreactive band (26, 27). As for D3, rat brain microsomes were used as positive control (results not shown).

Protein levels were finally determined using peroxidase-linked secondary antibodies by a chemoluminescence protein detection method, using a commercially available kit (NEN, Life Science Products, Boston, MA). In any case, membranes were stained with Ponceau S (Sigma) to confirm similar loading and transfers in each line. As a second approach to verify equal loading between the lines, gels were loaded in duplicate with one gel stained with Coomassie blue. Furthermore, in the case of proteins detected in total tissue lysates, membranes were stripped of the initial antibody and reprobed with β -actin. Western immunoblot bands were quantified by means of a Bio-Rad calibrated densitometer (GS-800) using the supplied software (Bio-Rad Laboratories).

Statistical analysis. Data were evaluated by ANOVA followed by the Newman-Keuls test, differences being considered significant at P < 0.05.

RESULTS

Age-related changes in serum concentrations of thyroid hormones and TSH. There was a progressive fall in mean serum T T₄ and F T₄ concentrations with age. TT₄ serum concentrations decreased markedly with age, being reduced by 31 and 53% at 12 and 24 mo, respectively, vs. the level in 6-mo-old rats (Table 2). Similarly, serum FT₄ concentrations decreased by 48 and 79% at 12 and 24 mo, respectively, vs. the level in 6-mo-old rats (Table 2). On the other hand, TT₃ and

Table 2. Metabolic and hormonal parameters of 6-, 12-, and 24-mo-old rats

	Experimental Groups		
	6 mo	12 mo	24 mo
Body weight, g	550±35a	575±28a	605±25a
Liver weight, g	16.2±1.7a	16.9±2.2a	15.0±2.1a
Kidney weight, g	1.15±0.12a	1.20±0.15a	$1.25 \pm 0.22a$
Food intake, g/day	16.2±2.3a	$15.8 \pm 2.0a$	16.1±2.2a
RMR, liters $O_2 \cdot kg^{-0.67} \cdot min^{-1}$	1.54±0.12a	$1.52 \pm 0.14a$	1.14±0.06b
Body temperature, °C	$37.5 \pm 0.05a$	37.7±0.07a	$36.8 \pm 0.08b$
tT_4 , $\mu g/dl$	$6.03 \pm 0.59a$	$4.20 \pm 0.75b$	$2.85 \pm 0.80c$
tT ₃ , ng/dl	96.4±11.8a	95.6±10.4a	$28.2 \pm 10.7b$
fT ₄ , ng/dl	1.30±0.11a	$0.68 \pm 0.13b$	$0.28 \pm 0.13c$
fT ₃ , pg/ml	2.60±0.21a	2.40±0.19a	$1.30 \pm 0.11b$
TSH, ng/ml	$0.63 \pm 0.03a$	$0.62 \pm 0.02a$	$0.60 \pm 0.02a$

Data are means \pm SE; n = 5 rats. RMR, resting metabolic rate; T₄, thyroxine; T₃, triiodothyronine; f or t, free or total. Nos. labeled with dissimilar letters a, b, and c are significantly different (P < 0.05).

 FT_3 serum levels did not change between 6 and 12 mo, but at 24 mo they decreased by 71 and 50%, respectively (Table 2). Despite the fall in serum TH concentrations, mean basal serum TSH concentrations were similar at 6, 12, and 24 mo of age (Table 2).

To assess whether differences in caloric intake could have affected TH parameters, food intake was monitored in the 3 groups of animals. As reported in Table 2, food intake and body weight did not change despite a decrease in resting metabolic rate and core temperature by 25 and 3%, respectively, at 24 mo. These data indicate no relationship between food intake and TH levels when 6-, 12-, and 24-mo-old rats are compared.

Age-related changes in senescence marker protein-30 (SMP30) mRNA expression in liver and kidney. To assess the progressive senescence of liver and kidney in the three experimental groups of rats, we measured the mRNA levels for SMP30, a protein with various functions mainly expressed in hepatocytes and proximal tubular cells, its expression decreasing with age in a sex-independent manner (16, 22). Fig. 1 shows RT-PCR-based measurements of SMP30 mRNA levels in liver and kidney in 6-, 12-, and 24-mo-old rats. In liver, SMP30 mRNA showed a progressive decrease with age (by 53% at 12 mo and 85% at 24 mo vs. 6 mo; Fig. 1A). In kidney, the SMP30 mRNA level did not vary significantly between 6 and 12 mo, but it decreased by \sim 48% at 24 mo vs. 6 mo (Fig. 1B).

Age-related changes in MCT8 expression levels in liver and kidney. In liver, the MCT8 mRNA level did not change between 6 and 12 mo, but it increased by \sim 52% at 24 mo vs. 6 mo (Fig. 2A), whereas MCT8 protein expression significantly decreased (by \sim 25%) at 24 mo vs. both 6 and 12 mo (Fig. 2B). In kidney, the MCT8 mRNA level showed significant decreases with age (by \sim 38% at 12 mo and by \sim 54% at 24 mo vs. 6 mo; Fig. 3A), but at the protein level no significant differences were observed among the groups (Fig. 3B).

Age-related changes in D1 expression and activity levels in liver and kidney. Liver D1 mRNA levels showed a progressive decrease with age (by \sim 50% at 12 mo and by \sim 85% at 24 mo vs. 6 mo; Fig. 4A). D1 protein followed the mRNA levels fairly closely (it decreased by \sim 45% at 12 mo and by \sim 64% at 24 mo; Fig. 4B). Roughly in line with the changes in protein level,



Fig. 1. Hepatic (*A*) and renal (*B*) senescence marker protein-30 (SMP30) mRNA expression levels in 6-, 12-, and 24-mo-old male rats. Representative RT-PCR analysis of SMP30 and β -actin (measured as the internal standard) mRNAs. For RT-PCR analysis, each lane contained PCR product derived from the appropriate cDNA, for which 1 μ g total RNA was used. Histograms show quantification of the signals (expressed relative to the value obtained for 6-mo-old rats), which are presented separately for each time point (as indicated below the bar). Each time point represents the mean \pm SD of samples obtained from 5 rats. In each panel, bars labeled with dissimilar letters are significantly different (P < 0.05).

liver D1 activity showed significant decreases of $\sim 25\%$ at 12 mo and $\sim 30\%$ at 24 mo vs. 6 mo (Fig. 4*C*).

In kidney, D1 mRNA levels at 12 and 24 mo were \sim 58 and 54% lower, respectively, than at 6 mo (Fig. 5A). As it did in liver, D1 protein followed the mRNA levels in kidney, showing an \sim 35% decrease at 12 mo and an \sim 46% decrease at 24 mo (vs. 6 mo; Fig. 5B). Surprisingly, renal D1 activity did not change between 6 and 12 mo, although it was decreased by \sim 33% at 24 mo (vs. 6 mo; Fig. 5*C*). Since it has been reported that D3 can be reexpressed under conditions of nonthyroidal illness (5, 36), we measured D3 mRNA and protein levels both in liver and kidney from all groups. The results obtained indicated that aging does not increase D3 mRNA expression either in liver or in kidney; rather it tended to reduce the already low expression levels measurable at 6 mo of age (data not shown). Moreover, Western blot analysis revealed that the corresponding D3 protein was not detectable (compared with the brain levels) either in liver or in kidney, clearly suggesting that D1 is the only isoform playing a role in the local deiodination of thyroid hormone in these tissues.

Age-related changes in $TR\alpha_1$ and $TR\beta_1$ expression levels in liver and kidney. In liver, although $TR\alpha_1$ mRNA and protein expressions did not change with age (data not shown), $TR\beta_1$ mRNA showed a progressive increase (by 45% at 12 mo and by ~150% at 24 vs. 6 mo; Fig. 6A). Likewise, $TR\beta_1$ protein increased progressively with age (by ~27% at 12 mo and by ~90% at 24 mo vs. 6 mo; Fig. 6B). At the nuclear level, a decrease of $\sim 35\%$ was observed for TR β_1 protein at both 12 and 24 mo vs. 6 mo (Fig. 6C).

In kidney, the mRNAs for neither TR α_1 nor TR β_1 changed between 6 and 12 mo, although they were increased by ~45 and 80%, respectively, at 24 mo (Fig. 7, *A* and *D*). In total tissue lysates, neither TR α_1 protein nor TR β_1 protein changed between 6 and 12 mo, although both were increased by ~50% at 24 mo vs. 6 mo (Fig. 7, *B* and *E*). At the nuclear level, no changes were observed among the groups for TR α_1 , whereas a 30% decrease was observed for TR β_1 at both 12 and 24 mo vs. 6 mo (Fig. 7, *C* and *F*).

DISCUSSION

Aging in rats is a multifaceted process in which several physiological changes occur at both the tissue and the whole animal level. Several studies have demonstrated a progressive, age-related impairment in energy expenditure due to a reduction in basal metabolic rate (47), physical activity (44), and hormonal alterations (45). Studies on circulating TH levels in aged rats have consistently shown a reduced T_4 serum concentration (11, 20, 30, 37), although the serum T_3 concentration remains more controversial (13, 17, 30, 37, 38). In the present study, 6-, 12-, and 24-mo-old rats showed no differences in body weight and food intake, although a reduction in resting metabolic rate was observed at 24 mo. Serum concentrations of TT_4 , FT_4 , TT_3 , and FT_3 changed significantly during aging, in accord with other studies (11, 20, 38). These changes lead to a



Fig. 2. Hepatic (MCT8) expression levels in 6-, 12-, and 24-mo-old male rats. A: representative RT-PCR analysis of MCT8 and β -actin (measured as the internal standard) mRNAs. For details regarding RT-PCR, see legend to Fig. 1. B: representative Western blotting analysis of MCT8 protein levels. Each lane contained 30 µg protein of total tissue lysate from a single rat. β -Actin was used to control for loading differences. Histograms show quantification of the signals (expressed relative to the value obtained for 6-mo-old rats), which are presented separately for each time point (as indicated below the bar). Each time point represents the mean \pm SD of samples obtained from 5 rats. In each panel, bars labeled with dissimilar letters are significantly different (P < 0.05).



Fig. 3. Renal MCT8 expression levels in 6-, 12-, and 24-mo-old male rats. A: representative RT-PCR analysis of MCT8 and β -actin (measured as the internal standard) mRNAs. For details regarding RT-PCR, see legend to Fig. 1. B: representative Western blotting analysis of MCT8 protein levels. Each lane contained 30 µg protein of total tissue lysate from a single rat. β -Actin was used to control for loading differences. Histograms show quantification of the signals (expressed relative to the value obtained for 6-mo-old rats), which are presented separately for each time-point (as indicated below the bar). Each time point represents the mean \pm SD of samples obtained from 5 rats. In each panel, bars labeled with dissimilar letters are significantly different (P < 0.05).

higher FT_3/FT_4 ratio in 24-mo-old rats that could be related to the much more marked decrease in FT_4 levels (79% vs. 6-mo-old rats), possibly due to a higher expression of TBG, as reported by Savu et al. (42). Normally, decreased TH serum levels should stimulate TSH secretion and result in increased TSH serum levels. In accord with other studies (6, 23), we did not observe a feedback response in this study, as serum TSH levels remained unchanged in aging rats, which would suggest central hypothyroidism in aging rats, rendering them unable to respond efficiently to the decreased negative modulation by thyroid hormones. It could be argued that in 12-mo-old rats the lack of response of TSH to decreased T₄ levels could be due to the unaltered T₃ serum levels; this would not fit with the unchanged serum TSH in 24-mo-old rats, which had significantly decreased serum T₄ and T₃ levels. However, in these animals an augmented conversion of T₄ to T₃ within the pituitary itself (4) as well as sex-related hormonal differences cannot be excluded (29).

Liver and kidney, which are target tissues for TH and important peripheral sites in TH homeostasis, may contribute to the progression of age-related TH modifications. They are each subject to senescence processes during aging, [albeit in a differential manner as indicated by the different pattern of age-related changes in the mRNA levels for SMP30, an important aging marker (16, 22)]. A decrease in SMP30 was already evident in liver at 12 mo but was apparent in kidney only at 24 mo. On this basis, liver appears to be more prone to senescence, and a tissue-specific response or contribution to the age-related decrease in plasma TH levels can be hypothesized one compares liver and kidney.

In any physiological context, for both tissues to be able to carry out their metabolic activities, T_3 and T_4 need to be transported into the cells, and their transport across the cell membrane is a limiting factor for their metabolism and action (14). Reduced T_3 uptake in liver, heart, abdominal muscle, soleus muscle, and brain has been found in aged rats in vivo by tissue sampling after a single T_3 injection (31, 33). Here, we provide evidence that in liver and kidney MCT8 is differentially modified by aging. Indeed, MCT8 mRNA levels were increased in the liver of 24-mo-old rats but decreased in the kidney of 12- and 24-mo-old rats, which in itself suggests a tissue-specific regulation of MCT8 transcription. However, at



Fig. 4. Hepatic levels of type I deiodinase (D1) mRNA expression (A) and protein (B) and protein activity (C) in 6-, 12-, and 24-mo-old male rats. A: representative RT-PCR analysis of D1 and β-actin (measured as the internal standard) mRNAs. For details regarding RT-PCR, see legend to Fig. 1. B: representative Western blotting analysis of D1 protein levels in liver microsomes (right) and in lysates of HEK 293 cells transfected with rat D1 cDNA (D1) or rat D2 cDNA (D2) in pcDNA3 expression plasmid (left). Each lane contained 30 µg microsomal protein from a single rat. A representative selected band of Ponceau S of the same membrane is depicted below to visualize loading and blotting efficiency. Histograms show quantification of the signals (expressed relative to the value obtained for 6-mo-old rats), which are presented separately for each time point (as indicated below the bar). Each time point represents the mean \pm SD of samples obtained from 5 rats. C: liver D1 activity expressed as $pmol \cdot min^{-1} \cdot mg$ protein⁻¹ is the mean \pm SD of samples obtained from 5 rats for each group. In each panel, bars labeled with dissimilar letters are significantly different (P < 0.05).



Fig. 5. Renal levels of D1 mRNA expression (A) and protein (B) and protein activity (C) in 6-, 12-, and 24-mo-old male rats. A: representative RT-PCR analysis of D1 and \beta-actin (measured as the internal standard) mRNAs. For details regarding RT-PCR, see legend to Fig. 1. B: representative Western blotting analysis of D1 protein levels in liver microsomes (right) and in lysates of HEK 293 cells transfected with rat D1 cDNA (D1) or rat D2 cDNA (D2) in pcDNA3 expression plasmid (left). Each lane contained 30 µg microsomal protein from a single rat. A representative selected band of Ponceau S of the same membrane is depicted below to visualize loading and blotting efficiency. Histograms show quantification of the signals (expressed relative to the value obtained for 6-mo-old rats), which are presented separately for each time point (as indicated below the bar). Each time point represents the mean \pm SD of samples obtained from 5 rats. C: renal D1 activity expressed as pmol·min⁻¹·mg protein⁻¹ is the mean \pm SD of samples obtained from 5 rats for each group. Bars labeled with dissimilar letters are significantly different (P < 0.05).

the protein level no changes were observed in kidney, whereas a significant decrease occurred in liver at 24 mo. These data may help to explain previous results showing a reduced hepatic transcellular transport of T_3 during aging (8) and may furnish a putative molecular mechanism. Our data also suggest that, in liver, MCT8 protein may be prone to degradation with age, following a generalized senescence of the hepatic tissue (indicated by the progressive decrease in the expression level of SMP30).

At the cellular level, T_4 and T_3 are deiodinated by D1 in both liver and kidney. In vitro data from other groups have shown a marked decrease in hepatic D1 activity in 26-mo-old rats (10). In vivo, however, T_3 production rate from T_4 has been reported in 11-mo-old rats compared with 7-mo-old rats (21), indicating that the mechanisms underlying the age-related changes in the peripheral conversion of T_4 into T_3 remain poorly understood.

At the transcriptional level, D1 is itself induced by T_3 and suppressed by hypothyroidism in a form of autoregulation that occurs in response to changes in thyroid status (2, 25). Thus, if a signal leads to a decrease in D1, the result will be a decrease in T_3 production that leads to a further decrease in D1 expression, initiating a negative vicious circle that can, however, be broken by the opposite regulation of D2 expression in other tissues. Moreover, it is known that D1 as well as TR expression are under diurnal rhythm control in relation to the animal's biological clock and feeding behavior (52). Here, by performing experiments at beginning of the light period and within a restricted time schedule, we have shown that, in both liver and kidney, D1 mRNA expressions and protein levels decrease progressively with age, with the liver D1 expression level showing the greater sensitivity to aging. This is in line with the



Fig. 6. Hepatic thyroid hormone receptor $(TR)\beta_1$ expression levels in 6-, 12-, and 24-mo-old male rats. *A*: representative RT-PCR analysis of $TR\beta_1$ mRNA (β -actin was measured as internal standard). For details, see legend to Fig. 1. Representative Western blotting analysis of $TR\beta_1$ protein levels in total tissue lysates (*B*) and in liver nuclei (*C*). Each lane contained 30 µg protein from a single rat. A representative selected band of Ponceau S of the same membrane is depicted below to visualize loading and blotting efficiency. Histograms show quantification of the signals (expressed relative to the value obtained for 6-mo-old rats), which are presented separately for each time point (as indicated below the bar). Each time point represents the mean \pm SD of samples obtained from 5 rats. Bars labeled with dissimilar letters are significantly different (P < 0.05).

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Fig. 7. Renal $TR\alpha_1$ and $TR\beta_1$ expression levels in 6-, 12-, and 24-mo-old male rats. Representative RT-PCR analysis of TR α_1 (A) and TR β_1 (D) mRNAs (β -actin was measured as internal standard). For details, see legend to Fig. 1. Representative Western blotting analysis of TRa1 (B) and $TR\beta_1$ (E) protein levels in total tissue lysates. Each lane contained 30 µg microsomal protein from a single rat. A representative Western blotting analysis of $TR\alpha_1$ (C) and $TR\beta_1$ (F) protein levels in kidney nuclei. Each lane contained 30 µg microsomal protein from a single rat. A representative selected band of Ponceau S of the same membrane is depicted below each Western to visualize loading and blotting efficiency. Histograms show quantification of the signals (expressed relative to the value obtained for 6-mo-old rats), which are presented separately for each time point (as indicated below the bar). Each time point represents the mean ± SD of samples obtained from 5 rats. Bars labeled with dissimilar letters are significantly different (P < 0.05).

idea that the liver D1 enzyme has the greater importance in the body's response to modest fluctuations in TH levels. Our data indicate that D1 activity is differentially modulated by aging in liver and kidney: 1) in liver, the progressive decrease in D1 activity was closely related to its expression pattern, whereas 2) in kidney, although the bulk of the decline in both D1 mRNA and protein levels had occurred already at 12 mo, D1 activity showed no change between 6 and 12 mo and was decreased only at 24 mo. The data about kidney D1 activity suggest a posttranslational modulation of the enzyme, a mechanism that has already been demonstrated in $TR\alpha_1^{-/-}$ mice (1). It is unknown whether this posttranslational modulation of renal D1 is related to the observations of a substrate-induced inactivation of the enzyme reported previously (43). Our results indicate that in 12-mo-old rats there may be a hepatic decrease in systemic T₃ production but a maintained T₃ production rate in kidney. The presence of low D1 mRNA levels in both liver and kidney in aging rats (at 12 and 24 mo) points toward a reduced gene transcriptional efficiency and suggests an alteration in the kidney's D1 mRNA levels that is independent of the local production of T₃.

Previous studies have indicated an age-related reduction in the general sensitivity to the actions of THs (8, 34). Actually, it is known that TRs undergo important ontogenic changes in the rat from fetal to young adult life (41), but little information is available on the physiology of TRs during aging. Here, we report that aging influences TR isoform expressions in a tissue-specific way. Indeed, I in liver, TR α_1 levels did not change with age, whereas TR β_1 levels increased progressively, and 2) in kidney, both isoforms were unaltered at 12 mo but increased at 24 mo (vs. 6 mo). These results seem in themselves to suggest that both liver and kidney compensate for the reduced T₃ levels (i.e., low T₃ plasma levels and low D1 activity) at 24 mo by increasing $TR\beta_1$ levels, with the liver response being activated earlier. However, our data on D1 mRNA expression do not support this idea. Indeed, at both 12 and 24 mo, liver and kidney nuclei accumulated less $TR\beta_1$ (vs. 6 mo) despite the presence of higher expression levels, thus indicating an age-related modulation of the nuclear translocation efficiency of $TR\beta_1$. The presence of decreased nuclear TR β_1 levels in both kidney and liver in aged rats may explain the downregulation of D1 mRNA expression (and the lower TH level) and suggest an altered biological responsiveness of the tissues to TH (35). Recently, it has been reported that in liver TRs set both the basal and the T₃-induced D1 expression, whereas in kidney there is less dependence on TRs, the basal expression of D1 being substantial even in the absence of TRs (1). Thus, other transcription factors seem likely to play a more prominent role in kidney than in liver, and further studies will be needed to identify the factors involved in the regulation of the nuclear translocation of TRs in aged tissues. Our data did not support an involvement of D3 in liver and kidney TH deiodination, as D3 mRNA levels were very low at 6 mo (compared with the brain levels) and even lower in aging liver and kidney (data not shown), and the corresponding protein was not detectable in any of the experimental groups explored.

As a whole, our data demonstrate in aging rats *1*) a significant decrease in serum concentrations of TT_4 , FT_4 , TT_3 , and FT_3 , whereas serum TSH levels remained unchanged; *2*) an unchanged MCT8 protein level in the kidney and a decreased MCT8 level in the liver; *3*) a decreased D1 protein level and activity in liver and a decreased renal D1 protein level at both 12 and 24 mo, with D1 activity being decreased only at 24 mo; *4*) unchanged liver $TR\alpha_1$ but a progressively increased $TR\beta_1$ mRNA level and increased kidney $TR\alpha_1$ and $TR\beta_1$ mRNA levels; and *5*) decreased nuclear $TR\beta_1$ but unchanged $TR\alpha_1$ protein levels in both liver and kidneys.

From a physiological point of view, despite the fact that aging is a complex process that cannot be explained only by decreased TH signaling, our study is the first to provide an interorgan overview of the aging-related changes in the renal and hepatic contributions to TH homeostasis. These tissues appear to respond and/or contribute in a differential way to the aging-induced changes in serum T₄ and T₃ levels. An agedependent downregulation of D1 mRNA expression in liver and kidney is apparent when either serum T_3 or nuclear TR β expression decreases or in consequence of a combination of both. In kidney, an additional relative increase in D1 protein activity vs. D1 mRNA may play a role in limiting the detrimental effects of conditions altering TH levels. However, we cannot exclude that changes in D1 activity in the pituitary and thyroid gland (4), changes in D2 or D3 activity (4), as well as sex-related hormonal differences (29), may further contribute to the net change in THs in aging rats.

GRANTS

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