The Absence of Transthyretin does not Impair Regulation of Lipid and Glucose Metabolism

Author F. Marques[1], J. C. Sousa[1], P. Oliveira[1,2], H. C. F. Oliveira[3], J. A. Palha[1]

Affiliation [1] Life and Health Sciences Research Institute (ICVS), School of Health Sciences, University of Minho, Braga, Portugal
[2] Department of Production & Systems Engineering, University of Minho, Braga, Portugal
[3] Departamento de Fisiologia e Biofísica, Universidade Estadual de Campinas, UNICAMP, Campinas, Brazil

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Abstract

Increased levels of neuropeptide Y have been reported in transthyretin-null mice. This effect might be related to transthyretin ligands (retinol and thyroxine) since, through binding to nuclear receptors, they modulate the expression of genes that control cellular metabolism. The retinoic X receptors form obligatory heterodimers with peroxisome proliferator-activated receptors and liver X receptors – potent regulators of fat, glucose and cholesterol homeostasis. We used transthyretin-null mice to investigate whether the absence of transthyretin influences metabolism. Transthyretin-null mice do not differ from controls in body weight and white adipose tissue morphology, nor in basal or fast-induced circulating levels of glucose, lipids, and leptin. Glucose tolerance tests show that transthyretin-null mice have normal capacity to remove and metabolize energy substrates. Expression of genes encoding lipid transporters and nuclear receptors are also similar in transthyretin-null and control mice. Therefore, the absence of transthyretin does not seem to influence the regulation of lipid and glucose metabolism.

Introduction

Transthyretin (TTR) is the principal carrier of thyroid hormones and retinol (vitamin A) in rodent's serum. The association to retinol is accomplished by the formation of a complex with retinol binding protein (RBP). TTR is mainly synthesized by the liver and the choroid plexus, tissues from which TTR is secreted into the blood and the cerebrospinal fluid (CSF), respectively [1]. Thyroid hormones and retinoids play pivotal roles in development and cellular homeostasis through their ability to directly regulate the transcription of target genes involved in the control of cell proliferation, differentiation, and survival [2]. Several studies have implicated retinoids and thyroid hormones in the control of adiposity and energy expenditure, namely in the differentiation of adipocytes and in the activation of uncoupling proteins, enzymes involved in the regulation of energy expenditure and fatty acid metabolism [3–8].

Mice lacking TTR (TTR-null) do not present classical features of altered thyroid hormone homeostasis [9–12] and are not vitamin A deficient [11]. Even though liver and adipose tissue RBP mRNA levels are normal, TTR-null mice present increased hepatic levels of RBP suggesting that TTR is involved in RBP secretion from hepatocytes [11]. Impairing liver secretion and/or altering kidney filtration of unbound RBP results in extremely low circulating levels of RBP and retinol in TTR-null mice. However, despite these low RBP and retinol serum levels, liver, kidney, spleen, and eye levels of retinol and retinyl esters are normal in these animals. Comparison with RBP-null mice, which have decreased tissue levels of retinyl esters and evidence of visual impairment [13], suggest that the residual RBP-bound retinol found in TTR-null mice might be responsible for their normal tissue retinoid levels. Alternatively, transport of retinyl esters by lipoproteins in TTR-null may play a relatively more substantial role for delivering retinol to tissues than in wild-type mice [14]. Circulating levels of all-*trans*-retinoic acid are 2.3-fold increased in the TTR-null mice and it is possible that these also contribute to the normal retinoid tissue pools [11]. Interestingly, previous studies indicated a slight decrease in the levels of cellular retinol-binding protein type I (CRBP-I) [11], a protein whose expression is regulated by retinoic acid availability [3, 15].

These observations, together with the report of increased neuropeptide Y (a major orexigenic

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Correspondence
J. A. Palha
Life and Health Sciences
Research Institute (ICVS)
School of Health Sciences
University of Minho
Campus de Gualtar
4710-057 Braga
Portugal
Tel.: +351/253/60 48 17
Fax: +351/253/60 48 09
japalha@ecsaude.uminho.pt

molecule) in the brain of TTR-null mice [16], prompted us to investigate whether major metabolic pathways involved in energy homeostasis are altered in TTR-null mice. Our attention focused particularly on the regulation of lipid and glucose metabolism by nuclear receptors and on the fuel removal from circulation and mobilization from tissue stores in the fed and fasted states. In the present study we show that, despite the reported increased levels of NPY in the brain of TTR-null mice [16], the absence of TTR does not seem to, directly or indirectly, influence mouse lipid or glucose metabolism.

Material and Methods

Animals

All experimental procedures were carried out in accordance with European Union rules and the National Institute of Health guidelines for the care and handling of laboratory animals.

Five month-old 129SV wild-type and TTR-null mice [12] were maintained under a 12-hour light/dark cycle at 22.5°C and 55% humidity and fed with regular rodent chow and tap water *ad libitum*. All experiments were performed 2–3 times each, including at least 5 animals per group.

Glucose and lipid measurements

TTR-null and control mice were sacrificed at the end of the dark cycle (fed state) or after a 24-hour fast that started at the beginning of the light cycle (fasted state). Serum was obtained by decapitation and stored at −20°C until used. Glucose, cholesterol, and triglycerides were measured by enzymatic-colorimetric methods (Spinreact, Santa Colomba, Spain). Leptin was measured using an ELISA kit (Linco Research, St. Charles, MO).

Glucose tolerance test

Food was removed 4 hours before the beginning of the experiment. Glucose (2 g/Kg of body weight) (Merck, Darmstadt, Germany) was administered by intraperitoneal injection, blood was obtained by orbital sinus puncture, and glucose levels were measured at 0, 20, 60, 120, and 180 minutes.

Gene expression

Total RNA was isolated from tissues using TRIzol (Invitrogen Inc.,. Carlsbad, CA). Total RNA was reverse-transcribed into first strand cDNA using the superscript first-strand synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instructions. Semi-quantitative multiplex PCR reactions were performed as previously described [17]. Briefly, each PCR cycle was composed of the following steps: 94°C for 30 seconds, annealing temperature for 45 seconds, and 72°C for 60 seconds. A sequential series of PCR reactions using each primer pair was performed initially to determine the number of cycles in which the amplification resides within the exponential phase of the amplification curve both for the gene under study and for the reference gene β-actin.

The oligonucleotide primers were synthesized using the Primer3 software [18] on the basis of the following respective GenBank sequences: AF085745 (LXR); NM013454 (ABCA1); NM011144 (PPARα); NM011146 (PPARγ), NM009024 (RARα), NM011243 (RARβ), NM011305 (RXRα), NM011306 (RXRγ), and X03672 (β-actin). All primer sequences are available upon request.

Aliquots of the PCR products were separated by 2% agarose gel electrophoresis and stained with ethidium bromide. Gels were visualized with AlphaImager 2200 (AlphaInnotech, San Leandro, CA) and analyzed densitometrically with the corresponding AlphaEase software. The expression level of the reference gene β-actin was used as internal standard, to which other PCR amplification products were normalized.

Adipocyte histology

White adipose tissue was removed from the abdominal deposits, fixed in aqueous 10% formaldehyde, and stained with hematoxilin eosin as previously described [19]. The number of adipocytes was calculated using StereoInvestigator software (MicroBrightField, Williston, VT) and a camera (DXC390; Sony, Tokyo, Japan) attached to a motorized microscope (Axioplan 2; Zeiss, Oberkochen, Germany). Average cell numbers per area (N_A) were estimated using an equally spaced grid of 2.4 cm^2. The grid was distributed at determined inter-distance of 400 m starting at a random position. Average areas were calculated in at least 100 adipocytes per animal.

Statistical analysis

Values are reported as mean ± SE. Statistical significance was determined using Student's *t*-test, except for the glucose tolerance test in which repeated measures ANOVA was used. Differences were considered significant at $p < 0.05$. The glucose tolerance test curves were obtained considering the mean values of the observations for a given time.

Results

Body weight and serum glucose and lipid measurements

Body weight at 5 months of age does not differ between the two groups of mice [control: 34.4 ± 4.5 g (n = 37) and TTR-null: 32.6 ± 4.4 g (n = 36); $p > 0.05$].

No differences are observed between TTR-null and control in fed state serum glucose, triglycerides, cholesterol, and leptin levels. In addition, both groups of animals respond as well to 24-hour fast: while glucose and triglyceride levels decrease about 30 and 55%, respectively, cholesterol levels remain constant in the fasted state (**Fig. 1**). Leptin levels are similar in the fed state (wild type: 5.33 ± 1.0 ng/ml and TTR-null: 6.82 ± 1.2 ng/ml; $p > 0.05$) and decrease to below sensitivity levels upon fasting.

Glucose tolerance test

In order to understand whether the lack of TTR could influence glucose homeostasis, we tested the response of TTR-null mice to a standard glucose load. Both TTR-null and control mice present the same ability to remove glucose from the circulation. Following an initial rise that peaks 20 minutes after the administration of glucose, glucose levels start do decrease towards basal values (**Fig. 2**).

RT-PCR analysis

Since nuclear receptors are master regulators of energy homeostasis, we next investigated the expression levels of genes encoding recognized modulators of glucose and lipid metabolism. The absence of TTR does not influence the expression of genes encoding ABCA1, LXRα, PPARα, PPARγ, RARα, RARβ, RXRα, and RXRγ both in liver and/or in WAT (**Fig. 3**). Therefore, major regulators of metabolism are not influenced by the low circulat-

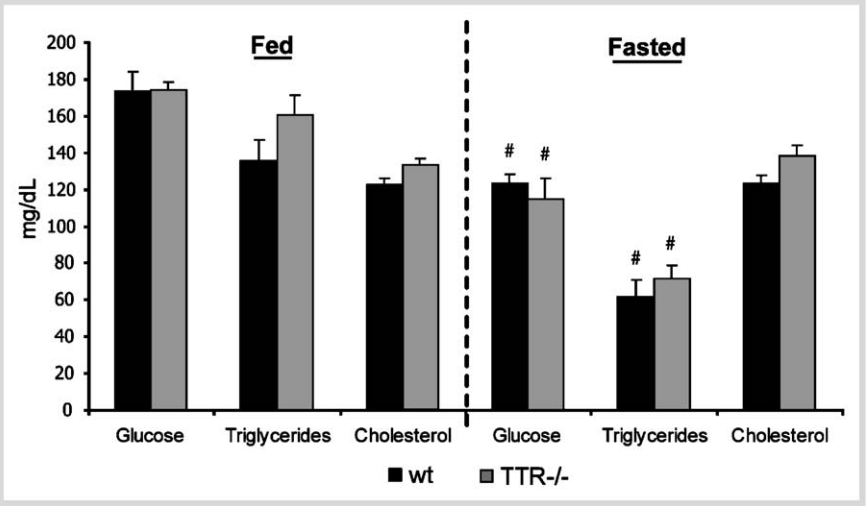

Fig. 1 Glucose and lipid serum levels in the fed and fasted states. 24-Hour fasting induced identical metabolic adaptation in TTR-null (TTR–/–) and wild-type (wt) mice, as seen by the reduction in circulating triglycerides and glucose serum levels when compared to the normal fed state. $^{\#}p<0.05$ when compared to the fed state.

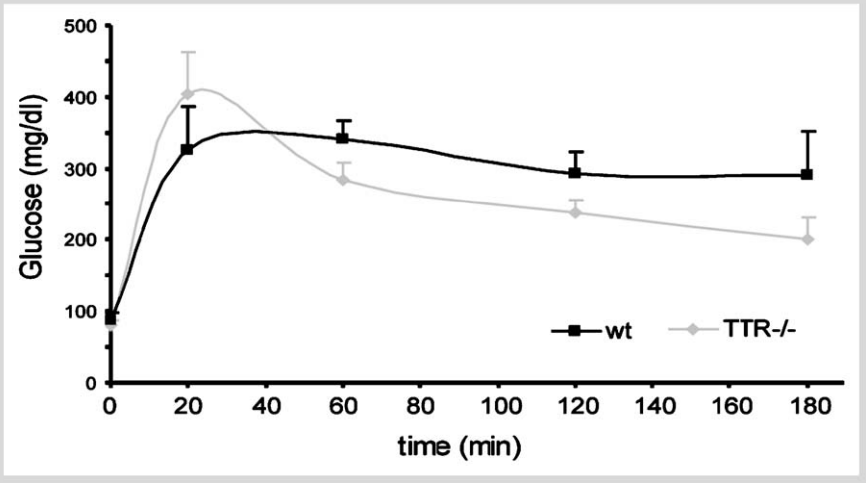

Fig. 2 Glucose tolerance curves. Glucose serum levels did not differ between wild-type (wt) and TTR-null (TTR–/–) mice at several time points after intraperitoneal injection of 2 g glucose/kg body weight.

ing levels of retinol and thyroxine that result from the absence of TTR.

Adipocyte histology

Total number of adipocytes from visceral depot present in the 30 grids is similar in both groups of animals (43 ± 5 for wild-type and 45 ± 4 for TTR-null; $p>0.05$). Similarly, no differences are found for the estimated adipocyte volumes (0.11 ± 0.01 mm^2 for wild-type and 0.10 ± 0.01 mm^2; $p>0.05$).

Discussion

The regulation of energy homeostasis is a complex system in which several orexigenic and anorexigenic signals interplay for a final balance, so detailed analysis of the various influencing pathways might be warranted. By measuring the expression levels of key modulators of short- and long-term energy homeostasis, and serum parameters of energy substrate mobilization, we show here that TTR does not influence major pathways of energy homeostasis metabolism, and that retinoid and thyroid hormone-dependent metabolic pathways seem normally regulated in organs such as the liver and WAT.

In the normal fed state, basal levels of glucose, triglycerides and cholesterol did not differ between TTR-null and control mice. When acutely exposed to ingestion of glucose, both TTR-null and control mice showed identical ability to metabolize it from the circulation. In addition, TTR-null mice responded as the wild-type when a challenge of 24-hour fasting was imposed. To meet energy demands, fasting induces increased mobilization of triglyceride stores and gluconeogenesis [20, 21]. In both groups of mice, fasting induced augmented usage of triglycerides resulting in decreased triglyceride and glucose serum levels.

We decided to evaluate whether the normal ability of TTR-null mice to metabolize triglycerides and glucose could result from adaptations in molecules that centrally regulate energy homeostasis. We chose to study mainly the expression levels of ligand-activated nuclear receptors described to regulate the expression of key genes of the lipid and glucose metabolism. Delivery of lipids to tissues requires release of free fatty acids from triglycerides hydrolysis, a process indirectly regulated by PPARα and PPARγ [22] and influenced by retinoic acid [23]. The expression levels of these nuclear receptors, as well as liver and WAT RXRs and RARs, are similar in the TTR-null and control mice. These observations are in accordance with the basal triglyceride circulating levels and with the ability to normally remove triglycerides from the circulation. Another downstream

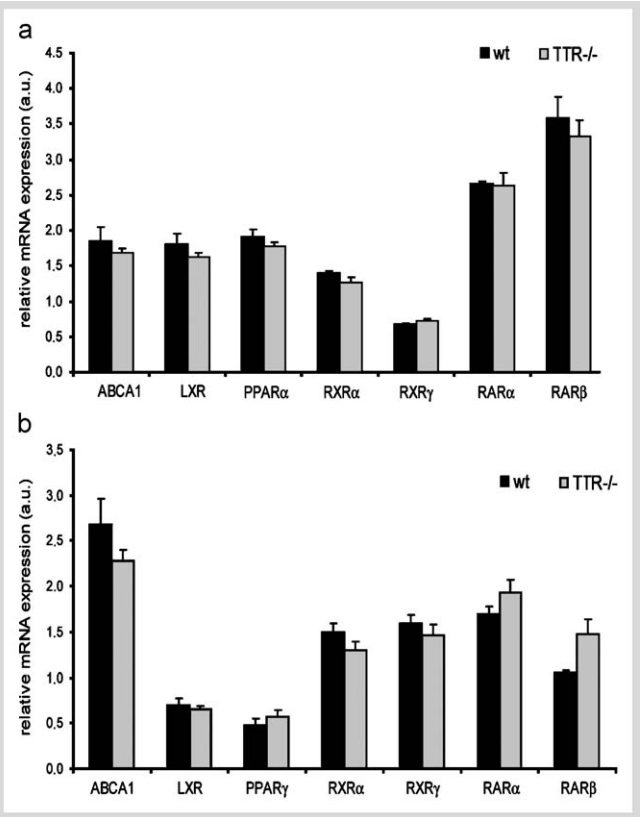

Fig. 3 mRNA levels of lipids and glucose metabolism modulators. Liver (**a**) and WAT (**b**) expression levels of nuclear receptors and transporters involved in lipid and carbohydrate metabolism are not altered by the absence of TTR. Wt: wild-type, TTR-null: TTR–/–.

regulator of lipid metabolism is the ABCA1 transporter. Expression of ABCA1 transporter is regulated by the cellular content of cholesterol through oxysterol-dependent regulation of LXR [24,25]. PPARα and PPARγ have been reported to induce LXR transcription thereby indirectly inducing ABCA1 transcription. The observation that ABCA1 expression levels are normal not only agrees with the normal mRNA levels of its direct and indirect modulators, LXR and PPAR (α and γ), respectively, but also with the normal serum levels of cholesterol we found in the TTR-null mice.

PPARγ is mainly expressed in adipose tissue [26] where it has been shown to be essential for adipocyte differentiation [27] and, in mature adipocytes, to promote lipid storage [28]. Retinoids, themselves, are able to influence adipocyte differentiation [8], and regulate intracellular fatty acid metabolism. Unaltered ability to store and mobilize lipids in the absence of TTR is further corroborated by the normal histology of WAT: TTR-null mice present no differences in adipocyte cell number or size.

PPARγ is also implicated in carbohydrate homeostasis. By modulating adipocytokine production, activation of PPARγ improves insulin sensitivity [28,29]. We found normal WAT expression levels of PPARγ, which probably explains why TTR-null mice responded equally well as the wild-type mice when submitted to a glucose tolerance test. Given their capacity to normally remove and metabolize glucose, it is reasonable to suggest that they also have the same sensitivity to insulin as the control mice.

It has been recently reported that TTR-null mice have increased CSF and brain levels of NPY [16], a potent orexigenic molecule that stimulates food intake. This observation could suggest increased body weight and obesity [30,31]. However, as we show here, TTR-null mice display identical body weights as the wild-type mice, and the white adipose tissue histology does not reflect increased storage of lipids, as referred above. Interestingly, we find that the TTR-null serum levels of leptin, a major modulator in the long-term regulation of food intake and body weight homeostasis that downregulates the expression of NPY [32,33], does not differ from the controls. This is in agreement with their normal body weight and white adipose tissue mass, since leptin release into circulation, mainly by the adipose tissue, correlates with the body fat mass [34].

Another important issue in the context of energy substrate mobilization is the ability of TTR-null mice to maintain a normal energy homeostasis under prolonged stressful conditions. We have recently shown that, when exposed to cold, TTR-null mice are as able as control animals to maintain body weight, by increasing food intake and inducing brown adipose tissue expression of uncoupling protein 1 and deiodinase type II [35]. Uncoupling proteins are responsible for the production of heat necessary for facultative and adaptative thermogenesis. A decrease in body temperature is usually associated with upregulation of uncoupling proteins, mainly in brown adipose tissue [36,37]. Wild-type and TTR-null mice have the same expression of brown adipose tissue UCP1 in basal conditions, and are equally able to up-regulate its expression by exposure to cold. This also supports the normal availability of thyroid hormone and retinoic acid status of the TTR-null mice since both [8], together with norepinephrine [38], modulate the expression of UCP1 [39–41] and we found no influence of the absence of TTR, whether at room temperature or in the cold. This is in accordance with the normal body temperature reported in young adult TTR-null mice [16].

All together, these data clearly indicate that TTR does not influence major pathways of cellular energy metabolism, and that retinoid and thyroid hormone-dependent metabolic pathways seem to be normally regulated in organs such as the liver and the adipose tissue. Whether the brain is receiving a normal supply of retinoic acid is unknown. Interestingly, retinoic acid, one of the several players in the NYP signaling system [42], has been shown to negatively regulate the expression of the NPY gene [43]. Despite the complexity of the system, in which other molecules might display counter-acting effects, one can speculate that in specific regions of the brain retinoic acid availability is impaired in the TTR-null mice. Deficiency of an inhibitor of NPY expression would result in the increased brain NPY levels reported in the TTR-null mice [16]. Therefore, these altered NPY levels reported might have implications in the central nervous system not related to energy expenditure, but rather to behavior. In agreement, NPY is reported to influence anxiety- and depressive like behaviors [44–47], and TTR-null mice present decreased anxiety- and depression-like behaviors [35].

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