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Impact of Gene Cloning, Disruption and Over-Expression of Iodothyronine Deiodinases on Thyroid Hormone Homeostasis

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RESUMO

Impacto da Clonagem, Ruptura e Super-Expressão do Gene das Desiodinases da Iodotironina Sobre a Homeostase dos Hormônios Tiroidianos. A tiroxina (T4) é o principal produto de secreção da tiróide, um pró-hormônio que deve ser obrigatoriamente ativado a T3 para que possa iniciar a ação do hormônio tiroideano. Esta reação de desiodação ocorre no anel fenólico da molécula de T4 e é catalisada por duas enzimas contendo selenocisteína, i.e. D1 e D2. Em contrapartida a esta via ativadora, tanto T4 quanto T3 são irreversivelmente inativados por desiodação do anel tirosil, uma reação catalisada pela D3, a terceira enzima constituinte do grupo das selenodesiodases. Devido à sua pronunciada plasticidade fisiológica, a D2 é considerada a principal desiodase produtora de T3 em seres humanos. Recentemente, as observações feitas no camundongo com deficiência da D1, C3H, foram expandidas com a criação dos camundongos D2 *knockout* ou com super-expressão cardíaca do gene da D2. Os resultados obtidos indicam que as selenoenzimas constituem um sistema fisiológico que contribui com a homeostase do hormônio tiroideano durante adaptação a mudanças no suprimento alimentar de iodo, exposição ao frio, em pacientes com disfunção tiroideana e talvez durante jejum e doença sistêmica. **(Arq Bras Endocrinol Metab 2002;46/4:402-411)**

Descritores: Tiróide; Tiroxina; Triiodotironina; Desiodases; Selenodesiodases; Genes D1/D2

ABSTRACT

Thyroxine (T4) is the main product of thyroid secretion, a pro-hormone that must be activated by deiodination to T3 in order to initiate thyroid hormone action. This deiodination reaction occurs in the phenolic-ring (outer-ring deiodination, ORD) of the T4 molecule and is catalyzed by two selenocysteine-containing deiodinases, i.e. D1 and D2. As a counter point to the activation pathway, both T4 and T3 can be irreversibly inactivated by deiodination of the thyrosyl-ring (inner-ring deiodination, IRD), a reaction catalyzed by D3, the third member of the selenodeiodinase group. Due to its substantial physiological plasticity, D2 is considered the critical T3-producing deiodinase in humans. Recently, the observations made in the D1-deficient C3H mouse mice were expanded by the development of mice with generalized targeted disruption or cardiac-specific over-expression of the D2 gene. The results obtained indicate that the selenodeiodinases constitute a physiological system contributing with the thyroid hormone homeostasis during adaptation to changes in iodine supply, cold exposure, in patients with thyroid dysfunction and perhaps during starvation and illness. **(Arq Bras Endocrinol Metab 2002;46/4:402-411)**

Keywords: Thyroid; Thyroxine; Triiodothyronine; Deiodinases; Selenodeiodinases; D1/D2 genes

THE CAPACITY TO METABOLIZE and incorporate iodine into a variety of organic compounds occurs widely throughout the animal and vegetal kingdoms. However, thyroid tissue is confined to and present in all vertebrates. Its role is to synthesize and secrete biologically active polyiodinated iodothyronine molecules that modulate gene expression in virtually every vertebrate tissue through ligand-dependent transcription factors, the thyroid hormone receptors (TR). It is intriguing how iodine was selected as a key inorganic element in thyroid hormone composition. Iodine is a trace element that can be found in the seawater at about the same concentration as in human serum, 50-60ug/l, a fact that is reminiscent of the origin of life at sea 3.5 billions of years ago. Once vertebrates moved to terrestrial environment 360 millions of years ago, the once steady supply of iodine became irregular, depending on the proximity to the ocean and geological nature of the soil. Even though iodine was present during the primordial development of the earth, over geological periods of time large amounts of this element were leached from the surface soil by snow and rain, and carried by wind and rivers into the sea. Therefore, the scarceness of iodine availability became an obstacle to vertebrates and the constancy of their *milieu intérieur* in the terrestrial habitat. This environmental challenge served as an evolutionary force that drove the natural selection process towards adaptation to the irregular iodine ingestion. This worked so well that in modern terrestrial vertebrates a multiplicity of physiological iodine-preserving compensatory mechanisms has evolved to mitigate the consequences of irregular iodine ingestion on thyroid hormone synthesis. In fact, these mechanisms can be so efficient that a recent survey indicates that nearly 2.3 billion people live in geographical areas with low iodine soil content.

Distinct from many hormones that have a highly focused functional role, the thyroid hormone exhibits an extraordinary broad range of biological effects, including cell differentiation, growth and energy expenditure. Its developmental effects are so dramatic that in human neonates the deficiency of thyroid hormone results in cretinism, a syndrome characterized by marked retardation in development of the skeleton and central nervous system. On the other hand, a thyroid hormone excess can place a heavy burden on the respiratory and circulatory systems, particularly the heart, draining the physiological reserves that normally exist to perform physical activities and meet environmental challenges, possibly resulting in catastrophic consequences.

The backbone of the thyroid hormone is an amino acid tyrosine conjugated to an alanine side chain, thus forming a biphenyl ether structure. Depending on the content and distribution of iodine in each phenyl structure a series of iodothyronines are formed, of which thyroxine (T4), T3 and reverse T3 (rT3) are the most biologically relevant. Four iodine atoms comprise ~70% of T4 molecular weight. It is tempting to speculate that the purpose of the biphenyl ether structure is to position the large iodine atoms in the correct spatial position in order to fit with high degree of specificity and affinity in the binding pocket of the TR. Thyroxine is the main product of thyroid secretion, a pro-hormone that must be activated by deiodination to T3 in order to initiate thyroid action. This deiodination reaction occurs in the phenolic-ring (outer-ring deiodination, ORD) of the T4 molecule and is catalyzed by two selenocysteine (Sec)-containing deiodinases, i.e. D1 and D2. As a counter point to the activation pathway, both T4 and T3 can be irreversibly inactivated by deiodination of the thyrosyl-ring (inner-ring deiodination, IRD), a reaction catalyzed by D3, the third member of the selenodeiodinase group.

It is challenging to elaborate about the nature and function of the deiodinase group and the need for an endocrine system constituted by a pro-hormone and an active metabolite. One major component of the evolutionary force that shaped the development of such complex mechanism was the irregular terrestrial supply of iodine and the necessity to adapt to it. T3 is the short-lived biologically active molecule ($t_{1/2}$ ~1 day) responsible for thyroid action. If T3 were the main product of thyroid secretion the availability of the biologically active thyroid hormone would be fluctuating according to the iodine ingestion. Rather, the thyroid secretes large amounts of T4, a long-lived molecule ($t_{1/2}$ ~7 days) that binds to circulating proteins and constitutes a large extrathyroidal circulating reserve pool, approximately 1umol in the adult human. Only a steady 40nmol of T4 are activated to T3 daily while the large pool of T4 and the deiodinase system accommodate changes in iodine availability by efficiently reacting to modifications of the serum T4 in order to maintain the constancy of the daily T3 production.

Thyroid hormone status is determined by the net occupation of the TRs, which depends on the intracellular free T3 concentration and the intrinsic TR affinity for this hormone. Most of the T3 is located intracellularly but at equilibrium, the small pool of serum T3 is a reliable indicator of thyroid status for

many but not *all* tissues. The set point that directs a large part of the mechanisms involved in thyroid homeostasis is the maintenance of a steady extrathyroidal T3 pool. This is accomplished by constantly adjusting both T3 production and inactivation via different deiodinase pathways according to environmental and metabolic cues. If these homeostatic mechanisms fail then changes in net occupation of the TRs will occur, altering the thyroid status of the tissues and serum T3 concentration.

In iodine sufficient humans most T3 is derived from D2-catalyzed T4 deiodination (~60-70%) (1,2). However, D2 is not so widely distributed in rodents and a different physiological role of D1 and D2 is observed in the rat. In this animal model the thyroid contributes ~40% of the T3 production, at least double of what the human thyroid does, and each of the T3-producing deiodinases, D1 and D2, provide equal contributions to the residual ~60% (3-5). However, while D1-produced T3 equilibrates rapidly with the extrathyroidal T3 pool, D2-produced T3 has a longer residence-time in the tissue where it is produced, slowly equilibrating with the rest of the T3 pool. While we assume that part of this variation can be explained by different subcellular localization and catalytic activity between D1 and D2, this phenomenon promotes a disequilibrium within the T3 pool because in the D2-containing tissues there are subcompartments (e.g. nucleus) that have higher T3 concentration than do non-D2 containing tissues. In fact, brain, pituitary gland and brown adipose tissue (BAT) all have higher net saturation of the nuclear TRs, reaching levels that would cause thyrotoxicosis in other tissues.

Due to its substantial physiological plasticity D2, not D1, is considered the critical homeostatic T3-producing deiodinase during adaptation to changes in iodine supply, during cold exposure or in patients with thyroid dysfunctions. Three important D2 properties account for its plasticity: low Km for T4, which is within the physiological range of serum free T4 (1000-fold lower Km(T4) *vs.* D1), short half-life (~45min *vs.* 8h for D1), and cAMP-responsiveness. The presence of a cAMP-responsive element in Dio2 constitutes the basis for the rapid neural stimulation of D2 in a number of tissues, including brown adipose tissue (BAT) and skeletal muscle. This links D2 expression with the sympathetic nervous system (SNS) and the hypothalamus, widening the spectrum of environmental and endogenous stimuli that can potentially influence D2 level and adaptive T3 production (6 for review).

D2's intrinsic instability is due to its susceptibility to ubiquitination and selective proteasomal degradation (7-9). However, substrate-induced proteasomal proteolysis is the critical D2 property that confers it such adaptive role in T3 homeostasis. During periods of low iodine intake T4 production falls, prolonging D2's half-life and enhancing the T4 to T3 conversion rate. The falling serum T4 signals the D2-containing pituitary thyrotrophs to increase TSH secretion and accelerate intra-thyroidal T4 to T3 conversion, increasing the relative T3 output in the thyroidal secretion. The inactivating IRD of T4 and T3 via D3 complete this scenario fine-tuning the T3 concentration. T3 stimulates D3 at a transcriptional level. Therefore, if iodine intake becomes so low that exhausts the capacity of the thyroid gland and D2 to maintain the T3 pool within normal range, serum T3 falls and so does D3 activity, prolonging the half-lives of both T4 and T3. This constitutes a second line of defense whose purpose is to delay the onset of iodine-deficient hypothyroidism.

Studies in two amphibian species crystallize the relevance of the deiodinases in regulating metamorphosis by adjusting intracellular T3 concentration in a pre-programmed timely fashion. The D3 expression pattern correlates with protection against thyroid hormone-induced changes, while the expression of D2 correlates with susceptibility to metamorphic change. For example, during metamorphosis the eyes shift from a lateral position in tadpoles to a more rostral and dorsal location in frogs so that they may have overlapping visual fields. Retinal cells follow this shift with an asymmetrical growth, a phenomenon that is stimulated by thyroid hormone (10-12). To develop asymmetrically, however, a subset of dorsal cells must grow at a slower rate. This is achieved by promoting transient local hypothyroidism mediated by the expression of D3 (13).

Deiodinases Genes and mRNAs

All three thyroid hormone deiodinases are selenoproteins and contain a Sec insertion sequence (SECIS) element. Each active center contains a single Sec, which is critical for the deiodination reaction. Replacement of Sec with Cys, which essentially replaces Se with sulfur (S), reduces the affinity for substrates and the catalytic activity (K_{cat}). However, replacement with other amino acids, e.g. Leu or Ala, results in full inactivation of all three deiodinases. This relates to the mechanism of deiodination and supports the notion that a Se-I (or S-I) intermediate compound is formed during the deiodination reaction (6 for review).

Dio1 gene: The elucidation of the *Dio1* gene structure was derived from studies comparing a polymorphism in the *Dio1* gene between the C57BL\6J and C3H\HeJ mouse strains (14,15). The human gene is found on chromosome 1 p32-p33, in a region syntenic with mouse chromosome 4, the location of *mDio1* (16). The mouse and human *Dio1* genes consist of 4 exons. The transcription start site is ~25 nucleotides upstream of the initiator methionine. The UGA Sec codon is in exon 2 and the UAG STOP codon and the SECIS element are in the 953 nucleotide fourth exon. The coding sequences of the mouse and rat D1 proteins are virtually identical. Both contain a Sec residue at position 126 (15). The complete cDNA sequences have been determined for rat, human, mouse, dog, chicken and tilapia D1 proteins (15,17-21). The mRNA sizes are about 2-2.1kb and all contain a UGA codon in the region encoding the active center, which is highly conserved among species.

Dio2 gene: The *Dio2* gene is present as a single copy located on the long arm of the 14th human chromosome in position 14q24.3 (22,23). It is about 15kb in size and the coding region is divided into two exons by an ~7.4kb intron. The exon/intron junction is located in the coding region in codon 75 and is found at an identical position in the human and mouse *Dio2* genes (22,24,25). For the human gene, there are three transcriptional start sites (TSS), 707, 31 and 24nt 5' to the initiator ATG. As shown, the longest 5'-UTR of the hD2 mRNA contains an ~300bp intron that can be alternatively spliced (25). The human, mouse and rat *Dio2* 5'-FR have been isolated and functionally characterized. All contain a functional cAMP responsive element (CRE) but only h*Dio2* has Thyroid Transcription Factor-1 (TTF-1) binding sites (25-27). The cloning of the complete D2 cDNA has been difficult. An RT-PCR based method using oligonucleotides designed for conserved D1 and D3 regions provided the first D2 fragment from *Rana catesbeiana* (28). The fragment was extended by 5' and 3' rapid amplification of cDNA ends (RACE) and the identity of the mRNA confirmed by expression in oocytes. A partial rat cDNA containing the coding region and portions of the 5'- and 3'-UTR was subsequently isolated leading to the identification of the coding region of the human cDNA in GenBank (29). Both the rat and the human proteins were expressed *in vitro* and showed classical D2 kinetics (29,30). However, neither of these clones included a SECIS element. The D2 coding region has also been cloned from a teleost fish, *Fundulus heteroclitus* (31). A ~5.1kb rat D2 fragment has also been reported, but this also lacks a SECIS element (32). Human, mouse and chicken D2 cDNAs containing intact 3'-UTR (5-7.5kb) were successfully identified using GenBank searches and library screening combined with 3' RACE PCR. These D2 cDNAs encode functional D2 proteins in *Xenopus laevis* oocytes and/or by transient expression (24,33,34).

The rat and human D2 mRNA are ~7.5kb and the chicken ~6kb (29,30,33,34). The ~7.5kb band of the rat D2 mRNA appears as a doublet in different brain regions but not in BAT. The difference in size between the 2 bands is ~500-1000bp (29). The same phenomenon is present in human thyroid, brain and heart (25,35). A detailed analysis involving nuclease mapping, 5' RACE, primer extension and Northern blots indicates that the hD2 mRNAs can exist as 4 different transcripts in thyroid, brain and possibly in other tissues (25). The longest transcript is ~7.5kb, it starts from the most 5' TSS, 708nt 5'+ to the ATG, and is the only transcript found in placenta. A shorter, minor ~7.2kb D2 species uses the same TSS but the ~300bp intron is spliced out. Two shorter transcripts of ~6.8kb differing by only 7nt utilize 3' TSSs located close to the translation initiation site. It is not known whether the rat and mouse genes utilize the same two major TSSs but this is likely to be so for D2 in rat brain (29). The chicken, mouse, rat and human D2 enzymes contain two Sec codons. The first is in the active center of the enzyme while the second is located close to the COOH terminus. In fish and frog D2, only the Sec codon in the active center is present (24,28-31,34). The D2 SECIS elements are FORM 2 structures located close to the 3' end of D2 mRNA and separated from the UGA codon in the active center by ~5kb of A/U rich sequences (24,33,34). This is the longest separation between a Sec encoding UGA and SECIS element in any eukaryotic selenoprotein mRNA reported to date.

The D2 5'UTRs are greater than 600 nucleotides and contain 3-5 short open reading frames. These full-length 5'UTRs reduce the D2 translation efficiency approximately 5-fold. The inhibition by human D2 5'UTR is localized to a region containing the first short open reading frame encoding a tripeptide-MKG. This inhibition is abolished by mutating the AUG start codon and weakened by modification of the essential purine of the Kozak consensus. Deletion of the 3.7-kb 3'UTR of the chicken D2 mRNA increases D2 activity approximately 3.8-fold due to an increase in D2 mRNA half-life. These mechanisms, together with the short half-life of the protein (see below), ensure limited expression of this key regulator of T4 activation (36).

Dio3 gene: The D3 gene has been localized to human chromosome 14q32 and to mouse chromosome 12F1 (37). Genomic cloning revealed a unique feature of the *Dio3* gene when compared to other deiodinases: no introns are present in the mouse or human genomic gene. In this regard, D3 can be included among those rare genes in the eukaryotic kingdom (6% of total) that have no introns in their genomic structure (37). In 1994, the cDNA for *Xenopus Laevis* D3 was cloned using a PCR–based gene expression study (38). Subsequently, the corresponding cDNAs of many species (rat, human, chicken, tilapia) were isolated. The human D3 mRNA is 2066 nucleotides long and contains 220bp of 5'-UTR, an 834bp open reading frame and a 3'-UTR of 1012bp (39). The deduced amino acid sequence predicts a protein of 278 residues, with a molecular mass of 31.5kDa. Hydropathy analysis reveals a hydrophobic NH_2-terminal portion consistent with a transmembrane domain analogous to D1. All D3 cDNAs identified to date include a Sec-encoding TGA codon, as well as a SECIS element in the 3'-UTR. There is a high degree of identity between the human and other species, particularly in the putative active center, where the Sec is located. The conservation of this enzyme from *Xenopus laevis* tadpoles to humans implies that its role in regulating thyroid hormone inactivation during embryological development is essential. Although the 2.3-kb band is the major mRNA band in most tissues, at least four differently sized mRNAs from the rat CNS hybridize with the D3 cDNA, and dramatic changes in the relative intensity of these occur depending on thyroid status (40). Because the structure of the rat D3 gene has yet to be clarified, it is not known whether the differences in transcript sizes are due to alternative splicing, differences in polyadenylation, and/or the use of different poly (A) adenylation signals.

Deiodinases Molecules

The predictions from topology software and experimental data (41) indicate that all three deiodinases have a single transmembrane domain that lies within the first 30-40 NH_2-terminal residues.

The D1 cDNA encodes a protein of about 27kDa that is highly similar in size (26-30kDa) and sequence with a few informative exceptions (42). There is physicochemical evidence that rD1 is a homodimer of 50-60kDa although it is not yet certain that this is required for its catalytic activity (43-45). The D1 monomer is a Type 1 integral membrane protein oriented with a 12 amino acid NH_2-terminal extension in the endoplasmic reticulum (ER) lumen and a single transmembrane domain exiting the ER at about position 36 (41). The hydrophobic nature of the NH_2-terminus suggests that this portion of the molecule is an uncleaved signal recognition sequence and incorporates both signal and STOP-transfer functions. The transmembrane domains of other proteins such as 17α-hydroxylase (P450-17) or D3 cannot substitute for the NH_2-terminus of D1 even though these permit synthesis of a membrane protein. This orientation is in agreement with earlier studies showing that trypsinization of kidney microsomes caused both loss of enzyme activity and N-bromoacetyl (BrAc) T3 labeling (46-48). Studies of the *in vitro* translated Sec126Cys mutant of rat D1 show that, while the NH_2-terminal and transmembrane portions of the enzyme are not catalytically active, their sequence is critical since even minimal exchanges of amino acids in the transmembrane domain reduce the efficiency of its transient expression. These mutations do not interfere with the activity of the protein, *per se*, nor do they affect the catalytic function of the protein that is successfully synthesized (41).

The subcellular location of mature D1 is likely to be the plasma membrane. This has been specifically demonstrated in the LLCPK1 proximal binding tubule cell line by BrAcT3 labeling and enzyme markers and in pig thyroid cells by immunohistochemical studies using primary D1 antiserum (47,49,50). More recently a basolateral plasma membrane location was confirmed in cells constitutively expressing a green fluorescent protein (GFP)-tagged D1 (45). However, early studies of the rat liver were conflicting, some evidence suggesting that D1 in hepatocytes co-localized with ER proteins such as protein-disulfide isomerase (PDI) and NADPH cytochrome-C reductase and glucose 6-phosphatase with other results supporting a plasma membrane localization (48,51). A preliminary report using transiently expressed GFP-D1 and D2 noted an ER location for both enzymes (52). The reasons for the discrepancies with results from our laboratory are under investigation (53). Depending on the detergent used the molecular weight of the solubilized wild type enzymes is about 50-60kDa suggesting it may be a homodimer (54).

More recently, using either NH_2-terminal or COOH-terminal FLAG epitope-tagged transiently expressed rat D1, confocal laser microscopy of transiently expressed D1 in human embryonic kidney cell line (HEK293) or the neuroblastoma cell line (NB2A) shows it is located at the plasma membrane. It does not co-localize with the ER resident protein BiP (53). Furthermore, when either COOH- or NH_2-terminal

FLAG tagged D1 is transiently expressed in HEK cells which are then subjected to limited permeabilization of the plasma membrane with digitonin, the FLAG tag is visualized at the plasma membrane, even though BiP cannot be visualized. This observation confirms the earlier assignment of D1 to plasma membrane of kidney and thyroid cells. Whether hepatocytes process D1 differently remains to be determined.

Thus, D1 topological studies predict that the catalytic site of D1 is cytosolic. Its plasma membrane location could be viewed teleologically as offering ready access of circulating reverse T3 and T4 to the enzyme as well facilitating the entry of the T3 produced from T4 into the plasma compartment. The localization of D1 in the plasma membrane is in striking contrast to the ER localization of D2 in the same cell types (53) (see below). This differential subcellular localization of D1 and D2 may explain why there is such a minimal contribution of the T3 generated via D1 to intranuclear T3 and such a large contribution of D2-generated T3 to this compartment (55,56).

The deduced hD2 protein is ~31kDa and contains a hydrophobic NH_2 terminus (29,30,34). The catalytic center is ~100% conserved between the frog, chicken, rat and human enzymes. The similarity of the chicken and human D2 proteins between the NH_2-terminus and the first Sec and between the first and second Sec residues is 88% and 90%, respectively (34). It is not known how often the COOH-terminal UGA codon is translated as Sec. The full-length cDNA is transiently expressed as a ^{75}Se-labelled doublet but there are no kinetic differences between these two D2 proteins (57).

Early attempts were made to determine the subcellular localization if D2 in rat cerebral cortex using cell fractionation and activity measurements. D2 activity was associated with membrane fractions but possible differences in the localization of PTU-sensitive and insensitive 5' deiodinase activities could not be resolved (58). The availability of the D2 cDNA allowed more specific studies of the intracellular distribution of the transiently expressed human D2 labeled with the FLAG epitope tag. The FLAG-tagged D2 is catalytically active. D2 is an integral membrane protein and protease protection assays reveal that the NH_2-terminus remains in the ER lumen while the COOH-terminal portion is in the cytosol (53). Immunofluorescent confocal microscopy of FLAG-D2 transfected HEK-293 or neuroblastoma cells shows that transiently expressed D2 co-localizes with BIP, an ER resident marker protein while FLAG-D1 is present on the plasma membrane region. This indicates that intrinsic protein sorting signals determine the subcellular localization of D2 and D1. While these studies were performed with transiently expressed proteins and need to be confirmed with primary antibodies, these different subcellular localizations can explain the ready access of T3 generated from T4 by D2 but not D1 to the nuclear compartment, a phenomenon noted in the earliest kinetic studies of this subject (55,59,60).

A hydropathy analysis of hD3 revealed a hydrophobic NH_2-terminal portion (~aa 10-35) conserved in all species consistent with a transmembrane domain. The microsomal localization of D3 activity, similar to D1 and D2, indicates that D3 is also an integral membrane protein, resistant to extraction from microsomal membranes by high pH (42,61). The precise topology of D3 has not yet been assigned.

Genetic Alterations in Deiodinase Expression

Only recently the first and only human genetic variant of the Dio2 gene was reported (see below) (62), whereas no variants were ever reported for Dio1 and Dio3. What could be the reason for such lack of patients with deiodinases dysfunction? Probably, multiple redundant mechanisms involving T3 homeostasis. In order to have clinical manifestations of deiodinase dysfunction a patient should have alterations in serum T3 causing hypo- or hyperthyroidism. However, serum T3 is regulated not only by three deiodinases, D1, D2 and D3, but also by direct thyroidal secretion. One could argue that a genetic deficiency of either enzyme would be clinically silent because the other three remaining mechanisms would compensate for the missing pathway. This hypothetical symptoms-free human phenotype is confirmed in mice with severe D1 deficiency (C3H) (14,15,63,64) or targeted disruption of the Dio2 gene (Dio2$^{-/-}$) (65,66).

C3H mouse

A screening of different inbred mouse strains in their capacity to metabolize iododioxin led to the serendipitous discovery that the C3H/HeJ (C3H) mouse is D1-deficient, as both liver and kidney D1 activities and the *Dio1* mRNA are only ~10-20% that in the C57 strain (14,63). Labeling with BrAc[^{125}I]T3 or $^{75}Se_2SO_4$ confirmed the reduction in D1 protein levels. This is due to decreased transcription of the C3H Dio1 due to the presence of a series of five GCT repeats located in the promoter of this gene that impairs promoter function (15,64). Based on what was discussed about T3 homeostasis it is easy to understand how C3H animals adapt to sustain systemic euthyroidism. The loss of D1 activity results in

decreased efficiency of T4 to T3 conversion. This is mitigated by a compensatory adjustment in the hypothalamic-pituitary-thyroid axis to increase T4 secretion until the effect of D1 loss is alleviated and normal serum free T3 is achieved.

Targeted disruption of Dio2

More recently, Dr. Valerie Galton, Dartmouth Medical School, in collaboration with our group developed a mouse with targeted disruption of the *Dio2* (Dio2$^{-/-}$) (66). The animals are apparently euthyroid because they grow normally, have normal serum T3 and hepatic D1 activity, a well-known marker of thyroid status. The compensation mechanism of the Dio2$^{-/-}$ animal includes an elevation in serum T4 but, unlike the C3H animal, TSH is elevated. This suggests that the adjustment in T3 homeostasis is only partial in the Dio2$^{-/-}$ animals because it is difficult to compensate for the higher T3 concentration of tissues where D2 is normally expressed without making the whole animal hyperthyroid. Therefore, it is understandable that Dio2$^{-/-}$ animals have functional shortcomings in tissues that require T3 production by D2 for nuclear T3, such as pituitary gland (high TSH), cochlea (deafness) and BAT (impaired adaptive thermogenesis and hypothermia) (65,66). In this respect, for decades it has been known that thyroid hormone is essential for survival in the cold but the exact mechanism for this dependence has been missing. With the recognition that BAT has the SNS-responsive D2, which is activated by the hypothalamus during cold exposure (67), it became clear the contribution of this enzyme to energy expenditure and the critical adaptive role of the deiodinase system in maintaining the constancy of the *milieu intérieur*.

Association between a variant of the Dio2 Thr92Ala and insulin resistance

The scanning of the human Dio2 revealed the existence of a variant that is associated with obesity and insulin resistance. The Thr92Ala variant was found in a population of 50 obese Caucasians. Association studies in 972 nondiabetic patients, 135 of whom underwent euglycemic-hyperinsulinemic clamps, showed that subjects with the Thr92Ala variant had lower glucose disposal rate. Association analysis of the entire group showed evidence for a synergistic effect between the Thr92Ala in the Dio2 and Trp64Arg in the beta-3 adrenergic receptor (ADRB3) variants on BMI. Thr92Ala is the only description of a missense mutation of Dio2 in humans. This variant strongly associates with insulin resistance and, in subjects with the Trp64Arg ADRB3 variant, an increased BMI, suggesting an interaction between these two common gene variants (62).

Isolated myocardial D2 overexpression causes cardiac thyrotoxicosis

The heart is one of the most sensitive organs to variations in plasma thyroid hormone level. Thyroid hormone can increase myocardial inotropy and heart rate, as well as dilate peripheral arteries to reduce afterload. At molecular level, many cardiac genes respond to thyroid hormone: among them, the myosin heavy chains (MHC), the hyperpolarization-activated cyclic nucleotide-gated channel 2 (HCN2) and the sarcoplasmic reticulum calcium ATPase. The expression of D2 in human cardiac and skeletal muscle and its absence from the corresponding tissue in rodents is one of the most intriguing differences in mammalian deiodinase physiology. It raises the possibility that, in humans, this tissue can respond not only to changes in plasma T3, but also to those in T4, thus resembling the pituitary and brain with respect to sources of intracellular T3. Furthermore, the presence of an intracellular T3-producing enzyme in the human heart may preserve this organ from the systemic reduction in thyroid hormone concentrations in iodine deficiency, although the potential contribution of cardiac D2 to intracellular or peripheral T3 production is still unknown. However, based on the tissue distribution of D2, rodents are not faithful models of the human situation.

To provide a model which might better reflect the human myocardium with respect to sources of T3, a transgenic mouse model has been prepared in which human D2 is highly expressed in the heart. This was obtained inserting the D2 coding sequence downstream of the cardiac specific mouse α-MCH promoter (68). These transgenic mice expressed high myocardial D2 activity, but surprisingly, the myocardial T3 concentration was only minimally increased. The reason for this is under investigation. It is not due to rapid diffusion of T3 from the myocardium since circulating T3 and T4 concentrations are normal. It may relate to the amount of T4 uptake by the mouse myocardium. Although plasma T4 and T3, growth rate and heart weight were not affected by D2 expression, myocardial thyrotoxicity was detected in the performance of isolated hearts. Consistent with the effect observed with endogenous thyroid hormone-induced thyrotoxicosis, there was an increase of about 20% in heart rate and a 30% increase in the rate pressure product, i.e. 284 ± 12 to 350 ± 7 beats/min. This was accompanied by an increase in pacemaker channel HCN2 but not in a-MHC or SERCA II mRNA levels. The former has been shown to

be T3 responsive in rats (69). Biochemical studies and ^{31}P NMR analysis demonstrated a significant reduction in phosphocreatine and creatine in transgenic animals, which may make the cardiac tissue of these mice more susceptible to ischemic challenge since hypoxia would cause greater myocardial depletion in ATP concentrations than occurs in the wild type.

These changes were somewhat unexpected, since many of the alterations caused by short-term high dose exogenous thyroid hormone did not occur. In humans, the clinical syndrome of modest increases in serum T3 and T4 (within the normal range) accompanied by suppressed TSH creates the state of "subclinical" hyperthyroidism. This can occur spontaneously but it is intentionally induced by excess replacement with exogenous T4 in patients with thyroid cancer. Since the changes demonstrated in these mice occur with minimal increases in myocardial T3, these modest increases in circulating T4 and T3 are likely to have similar effects on the human myocardium. Supporting this concept, a recent report demonstrated an increase in heart rate in patients with normal thyroid hormone levels but suppressed TSH compared with age-matched controls (70). These results suggest that even mild chronic myocardial thyrotoxicosis can cause tachycardia and associated changes in high-energy phosphate compounds. This "humanized mouse heart" model is being evaluated to identify the consequences of mild chronic cardiac-specific thyrotoxicosis on cardiac functions to elucidate the direct effects of T3 on the heart uninfluenced by effects of thyroid hormone-induced changes in total body metabolism.

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