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COMMENTARY

PLEIOTYPIC ACTION OF THYROID HORMONES AT THE TARGET CELL LEVEL

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Thyroid hormones influence multiple physiological functions, e.g. cell growth and differentiation, protein synthesis, basal metabolic rate. Thyroid hormone action at the cellular level proceeds by (i) the entry of the hormone into cell, (ii) the conversion of the 'prohormone' thyroxin to the active hormone metabolite, T3, inside the cell, (iii) the binding of T3 to a specific receptor protein and (iv) the possible modulation of the hormonal signal at a postreceptor level. The identification of hormone specific binding sites is essential for our understanding of hormone action at the target cell level. 'Receptors' are defined as macromolecules which are able to recognize and to bind selectively to a ligand, thus generating a signal, that in turn initiates a chain of events leading to the biological response. The interaction between receptor and ligand is characterized by (i) rapid and usually reversible binding, (ii) limited binding capacity, (iii) high affinity and (iv) specificity.

For thyroid hormones, low capacity and high affinity binding sites have been demonstrated in the nucleus, the mitochondria, the plasma cell membrane, and probably for the cytosol [for a review c.f. 1–6]. Accordingly, the percentage of total cellular T3 recovered 15 min after injection of the hormone was estimated to be 12% for the nuclear, 19% for the mitochondrial and 59% for the soluble cell fraction [6, 7]. From this it is evident, that T3 binds to different cellular sites.

Thyroid hormones are generally considered as gene activating hormones. However, recent data support the physiological relevance of extranuclear events. This article sets out to review the interaction of thyroid hormones with their different specific binding proteins. From this it becomes evident, that the thyrometabolic state of the single cell is the result of the simultaneous action of T3 at the nucleus, the mitochondria as well as the plasma membrane. Thus, the diversity of T3 action at the cellular level is a consequence of the pleiotypic action of the hormone.

NUCLEUS

Specific nuclear binding sites for thyroid hormones have been described from various organs accounting for 5847 sites/nucleus in the anterior pituitary, 4515 in the liver, 3923 in the kidney, 2960 in the heart, about 2000 in the brain, while for spleen and testis only 133 or 17 sites/nucleus respectively are reported [2, 5]. Except for the 'adult' brain a correlation exists between the nuclear binding capacity and the thyromimetic effect of the hormone metabolites [2].

Thyroid hormone nuclear receptor is situated in 'active' chromatin, and appears to be an acid 'non-histone' protein associated with the chromatin, probably a DNA binding protein [2, 3, 5, 8, 9]. There is evidence that the nuclear receptor has a single polypeptide chain [10]. The binding site is associated with the DNA linking nucleosomes in structurally modified regions of the chromatin [8]. The 'T3 receptor'-protein has a mol. wt of about 50,000 D, a sedimentation coefficient of 3.5 S and a Stokes radius of 35 Å [2, 5, 8]. Scatchard analysis of the 500-fold purified rat liver material showed an apparent affinity constant of 50 pM for T3 and 1 nM for T4 [11]. There is some evidence that the receptor is not randomly distributed in euthyroid hepatic chromatin [12].

As both the L- and the D-isomer of T3 are bound to the receptor, the criterion of stereospecificity is not fulfilled. With the exception of T4, other physiologically important iodothyronines derived from T4 are unlikely to modulate the interaction of T3 with its receptor, as their nuclear binding is less compared to T3:1 (T3 reference value); 4.4 (triac: triiodothyroacetic acid); 6.6 (T4); 56 (3,5′-diiodothyronine); 245 (3,5-diiodothyronine), 264 (rT3) and 60,000 (3′,5-diiodothyronine) [13].

Thyroid hormone receptor binding is stimulated by histones or histone like proteins [2, 5, 11]. It is interesting to note, that the acetylation of histones in GH1 cell nuclei results in a decrease in the number of nuclear associated thyroid hormone receptors, thus chromatin associated factors may influence the specificity of thyroid hormone action [14]. As proposed by some authors [5] a 'holo'-receptor exists, consisting of a 'core subunit', which has a thyroid hormone binding site and a 'regulatory binding subunit', which may be a histone protein. The interaction of the 'core' receptor with the regulatory subunit initiates an alteration in the ligand binding properties of the 'core' receptor resulting in the generation of the 'holo' receptor. The association of T3 with the 'holo' receptor induces a structural change in chromatin that influences the transcription of specific genes.

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In the euthyroid state about 50% of the nuclear binding sites in the liver are occupied by T3, this is reduced to 30% in experimental hypo- and increased to nearly 100% in hyperthyroidism [15]. Changes in the number and in the affinity of nuclear receptors have been shown for various physiological and pathological conditions: development, fasting, different hormonal states, hepatectomy and experimental tumor implantation [2, 3, 5, 15]. As fasting and diabetes are characterized by reduced hepatic nuclear T3 binding sites, the physiological meaning of such a 'down regulation' phenomenon is questioned by the finding, that T3 induced hepatic phosphoenolpyruvate carboxykinase to a similar extent in fed, fasted as well as diabetic rats [16]. In addition hepatic α-glycerophosphate dehydrogenase stimulation by T3 is preserved in each of these states, while malic enzyme response is modulated by the nutritional state of the animal [2, 17]. From these findings, one might preliminarily assume that the cellular response of thyroid hormones is controlled more by saturation of the available receptor sites than by their absolute number [15].

In addition, a disproportion between receptor occupancy and the response of hepatic malic enzyme and α-glycerophosphate dehydrogenase has been interpreted as indicating that there has been an amplification of the signal [2]. In contrast, a linear relationship is observed between nuclear binding and growth hormone production in a pituitary tumor cell line [5]. Triac and DT4 are also strongly bound to the nuclear receptor, but show only low biological activity, documenting that binding by itself does not determine thyromimetic activity [15].

Although the solubilized receptors prepared from different organs appeared to be similar, as judged by the chromatographic analysis and their behavior when tested with various analogues of thyroid hormones, hyperthyroidism increased the synthesis of hepatic phosphoenolpyruvate kinase, whereas it decreased the synthesis of the kidney enzyme despite a similar nuclear occupancy in both organs [16, 18, 19]. This finding suggests the regulatory significance of postreceptor events for the expression of the hormonal signal.

Reduced binding affinities for T3-nuclear receptors have been proposed for a clinical state, characterized by euthyroidism, despite increased serum T4 and T3 levels and non-suppressed TSH in the absence of a pituitary adenoma or TSI [20]. Since in this state the hormone has been shown to be normal stereochemically, to undergo the normal pathways of degradation, and adequately to penetrate peripheral tissues, the defect must reside at the receptor or postreceptor level [20].

Correlating with the nuclear binding of T3, the activity of deoxyribonucleic acid-dependent ribonucleic acid polymerase is increased followed by altered chromatin template activity at a later period. Consequently the amount of poly (A)-containing nuclear RNA is enhanced, thus generally representing an increase in all major species of mRNA [21, 22]. In addition to the generalized increase in cellular RNA content, T3 stimulates a number of specific mRNA species; this has recently been documented by two dimensional gel electrophoresis data, showing a thyroid hormone dependent modulation of 8.2% of the 231 expressed mRNA species visualized [22]. The activity was stimulated in 11 and inhibited in 7 mRNA translational products [22]. Thus, thyroid hormone affects the expression of a unique and limited set of genes in the cell. These data indicate that the various physiological responses to thyroid hormones may be governed by a small set of gene products that act as key points in the regulatory pathway of responsive cells [23]. In regard to the number of genes affected, only as few as 100–1000 per cell seem to be involved, considering there are some 11,000 different mRNA sequences per cell [21].

At present thyroid hormone induced nuclear events are best characterized for the synthesis of growth hormone and prolactin by cultured rat pituitary cells [5, 24, 25] and the synthesis of $\alpha 2_u$-globulin, malic enzyme and different nucleoproteins in rat liver [2, 4, 26–29]. Recent data, using a two-dimensional gel analysis of rat liver nuclear proteins revealed that 102 of 500 protein subunits distinguished disappeared after thyroidectomy, but 13 proteins reappeared 6 hr after thyroid hormone administration, whereas after 24 hr 67 additional proteins could be demonstrated [29]. These changes in protein concentrations are the greatest in number reported as an effect of thyroid hormone [29]. As a consequence of thyroid hormone-induced changes in nucleoproteins the structure of chromatin is found to be altered; thus, it exposes more DNA to the attack of micrococcal nuclease [30]. However, the observed data could also be due to a thyroid hormone-increased solubility of released nucleosomes [30].

With respect to the number of genes affected, it is evident (as discussed above) that the domains of genes regulated by thyroid hormones are probably unique and differ from, though overlapping with the domains controlled by other hormones. This would result in synergistic and antagonistic actions between T3 and other hormones such as insulin, glucocorticoids or androgens [5, 23]. A good example of this is the hepatic synthesis of $\alpha 2_u$-globulin mRNA by thyroid hormones, glucocorticoids, growth hormones and androgens and a decrease after estrogen application. cDNA–RNA hybridization studies have recently confirmed these results [2, 3, 5]. In addition to these *in vivo* findings, T3 (1.5×10^{-6} M) directly induces the synthesis of $\alpha 2_u$-globulin in primary cultured rat hepatocytes, and acts synergistically with growth hormones and dexamethasone [26].

Thyroid hormone action at a pretranslational level has been investigated for hepatic malic enzymes: following acute administration of T3 to normal rats or addition of T3 to rat hepatocyte culture, the synthesis as well as the level of malic enzyme mRNA increases with a lag period of 2–3 hr [27, 31]; consequently malic enzyme activity has become a commonly employed marker of tissue effects of thyroid hormone for the rat liver [2]. Since malic enzyme is also influenced by diet, T3 then acts as a multiplier of a glucose induced signal increasing malic enzyme mRNA activity [2, 17, 27, 31, 32]. In addition, the absolute increment of malic enzyme induced by T3 declines with age [28], thus T3 is one part of a

multifactorial control system regulating malic enzyme synthesis.

With respect to the time course, there is some evidence for the liver cell that one of the early cellular actions of T3 might be on rapidly turning over mRNA-sequences, whose translational products may secondarily exert effects on the induction of other mRNA species coding for different proteins [33]. Thus, it is hypothesized that T3 exerts its primary effect on a specific, rapidly turning over mRNA sequence, coding for a protein which will stimulate in turn RNA-polymerase and other mRNA sequences e.g. coding for malic enzyme, which increase hours after T3 administration [33, 34]. The regulatory function of the labile active peptide is supported by the failure to increase RNA-polymerase activity in cycloheximide pretreated animals [33, 34]. Although our understanding of T3 mediated nuclear RNA-synthesis is still inadequate, the evidence for the existence of the putative peptide mediator of RNA-synthesis exemplifies the integrated response of cellular metabolism to thyroid hormone action [34].

The role of T3 as a specific mitogen has been extensively described: thyroid hormone stimulates DNA-synthesis, DNA-replication and the mitotic index of the cell [35]. As shown for pituitary tumor cells, T3 effected a 3,9-fold increase in the growth rate of the cell due to decrease in the G_1, G_2 and M stages of the cell cycle [36]. It is of interest that the appearance of nuclear T3 binding sites is well coordinated with DNA-synthesis in the cell cycle, consequently newly divided cells have a full complement of nuclear T3 receptor sites [37].

The potency of T3 in gene activation is also illustrated by the recent finding, that X-ray induced neoplastic transformation of mouse fibroblasts is abolished by incubation of the cells in a thyroid hormone free medium [38]. Thus, thyroid hormone may play a crucial role in neoplastic transformation [38]. In addition, the growth rate of different Morris hepatoma cell lines is inhibited by thyroid hormone deficiency. Consequently the survival of rats bearing hepatoma is increased after induction of hypothyroidism [39]. Moreover, there are data suggesting that thyroid hormones play a role in the growth of liver cell derived cancers similar to that influencing normal hepatocyte proliferation [40].

Although the limited capacity, high affinity and good correlation to the biological response strongly suggest the nuclear binding protein to be a 'true' receptor, this will be finally proven by the demonstration that the purified receptor initiates a hormone action in an *in vitro* cell free system.

Relatively little information is available regarding the influence of thyroid hormones on the translational machinery. A T3-induced increase in peptide chain assembly and ribosomal protein phosphorylation has been described [3–5, 41], whereas others have observed no alteration in the number, distribution and half-life of ribosomes in thyroidectomized rat liver [41]. As for hepatic $\alpha 2_u$-globulin specific mRNA-utilization is increased by thyroxin supporting the view of a T3-action at a step distal to the formation of mature mRNA [42]. Data for muscle and liver demonstrate, that T3 cannot stimulate the translational phase of protein synthesis in diabetic rats suggesting that minimal insulin concentrations are a prerequisite for the effect of T3 [43]

MITOCHONDRIA

Specific binding sites with high affinity and low capacity for thyroid hormones have been described for mitochondria isolated from different organs e.g. liver, kidney, intestine, lung, heart and skeletal muscle and adipose tissue [3, 44–47]. Mitochondrial binding sites have not been demonstrated in brain, spleen and testis, which are also known to show no increase in oxygen consumption after thyroid hormone application. Partial purification reveals a 150,000 D lipoprotein in the inner mitochondrial membrane with a binding constant of 0.75×10^{-11} M for T3; there are about 2000 binding sites per liver cell. The receptor binds stereospecifically the L-isomer of T3; LT4 and 3,5,3′-triiodoacetic acid are also bound [3, 46]. Recent studies using rat kidney mitochondrial membranes revealed four main T3 binding activities in outer and two T3 binding sites in the inner mitochondrial membrane [47]. Other authors could demonstrate unspecific binding sites situated on the outer mitochondrial membrane [48]. Mitochondrial adsorption of the hormone depends on the distribution of the proteins between the cytosol and the organelle; some drugs, e.g. chlorpromazine and phenobarbital appear to influence the adsorption [48]. Nevertheless it should be noted, that others have failed to demonstrate a specific mitochondrial binding protein for T3 [49].

However, there is some evidence for a putative 'mitochondrial pathway'. Thyroid hormones stimulate O_2-consumption in several organs but not in spleen, testis, and adult brain [1, 3, 7].

Turnover, number, size and inner surface area of mitochondria are increased in 'hyperthyroid' tissues [3, 50]. T3 directly stimulates O_2-consumption and the synthesis of ATP, as has been shown *in vitro* for isolated mitochondria, hepatocytes and perfused liver [3, 7, 51]. This effect is dose dependent and significant for a hormone concentration of 10^{-11} M [52], representing the physiological 'free' hormone concentration in rat serum. Thyroid hormone-increased respiration occurs within minutes and is not influenced by potent inhibitors of protein synthesis [3]. Thyroid hormone-induced increase in mitochondrial oxidative capacity might be explained by an elevation of the protonic electrochemical potential difference across the inner mitochondrial membrane, favouring oxidative phosphorylation [53]. In addition the exchange of various metabolites and ions is stimulated by thyroid hormones, e.g. ADP, malate, Ca^{2+} [51, 54–61]. Other effects of thyroid hormones on the mitochondrial membrane, affecting the lipid protein ratios, the proportion of different phospholipid classes, the relative contents of unsaturated fatty acid moieties and of cholesterol, together resulting in differences in membrane fluidity and function, may explain in part the alterations in metabolic processes observed in hypo- and hyperthyroidism [62, 63]. Although the mitochondrial receptor is still a matter of controversy [2, 3, 4, 5, 7],

there is more than circumstantial evidence that the mitochondria are a target of early thyroid hormone action.

PLASMA MEMBRANE

Two classes of saturable binding sites for T4 and LT3 have been described for liver and kidney plasma membrane preparations, having apparent equilibrium dissociation constants (Kd) for LT4 of about 0.6 and 23 nM and for LT3 of about 3–9 nM and 230 nM in rat liver [64, 65]. The binding site stereospecifically interacts with the L-isomer of the hormone, thus supporting the existence of a distinct thyroid hormone plasma membrane receptor. It is interesting that the Kd and the capacity for both sites are not affected by different thyroid states; obviously thyroid hormones themselves do not regulate their own plasma membrane binding sites [65].

Recent data have brought some insight with respect to the physiological relevance of such specific, high affinity binding sites in plasma membrane preparations, (i) for the cellular response to T3 and/or (ii) a receptor mediated uptake and internalization of the hormone. (i) Thyroid hormones affect the activity of various transport processes, e.g. the uptake of sugars, amino acids and Ca^{2+}. T3 stimulates glucose uptake in thymocytes by an interaction with insulin and epinephrine; this effect is significant for a hormone concentration of 5×10^{-12} M; in this system T3 affects both the transport and phosphorylation of glucose [66]. The effect depends on the availability of Ca^{2+} and is possibly mediated by cyclic AMP [67]. These biochemical responses could be correlated to the binding of T3 to specific sites in the plasma membrane of rat thymocytes [68]. (ii) As to the mechanism by which T4 and T3 traverse the liver cell membrane, free diffusion is proposed by some authors [69], but there is evidence that T3 entry into the cell is mediated by a specific transport system within the plasma membrane [70]; it is possible that plasma membrane binding is involved in the transport of the hormone into the interior of the target cell. Visualizing the internalization phenomenon by the use of Rhodamin-T3 (Rho-T3) in cultured fibroblasts, it has been shown that about 70% of the Rho-T3 binding can be blocked by unlabeled T3, while only 20% of (^{125}J)-T3 uptake is blocked by Rho-T3 [71]; assuming similar binding affinities for the T3 and Rho-T3 metabolites, these data may suggest that binding and accumulation of T3 in endocytotic vesicles are discrete phenomena [71].

In addition, the inhibition of receptor mediated endocytosis by monodansylcadaverin reduced the nuclear accumulation of T3 [72, 73], proposing that T3 mediated nuclear events depend on receptor mediated T3 uptake. From these data, the functional significance of plasma membrane binding sites for T3 is evident. Thyroid hormone action on plasma membrane may be an alternative pathway for rapid, nuclear independent effects of T3.

CYTOSOL

To date very few reports have been concerned with possible cytosolic binding proteins for thyroid hormones: these proteins are characterized by a low affinity and an about 100 times higher capacity than nuclear receptor proteins (apparent Ka about 10^{-7}–10^{-8} M [34, 74–77]. Different cytosolic proteins have been described, differing in some aspects from thyroxin binding globulin, e.g. by higher affinities for T3 than T4; binding studies reveal a dose-dependent sigmoidal relationship, favouring the hypothesis that these proteins contain more than one binding site per molecule with a cooperative effect between the different sites [74, 78]. Moreover, cytosolic binding proteins differ from nuclear receptors by their kinetic properties, sedimentation constants and electrophoretic mobility [74]. Distinct binding proteins for T4 and T3 have been demonstrated in human liver cytosol [79].

In contrast to these data a specific T3-binding protein was recently described for the cytosol fraction of human breast adipose tissue: it displayed saturability, high affinity (apparent Ka 3×10^{-9} M) and a single class of binding sites [80]. Furthermore, this protein appeared similar to thyroxin binding globulin, which could be demonstrated by a specific radioimmunoassay in the cytosol fraction [80].

However, the physiological meaning of cytosolic binding proteins for thyroid hormones remains to be established: at present there appear to be no connections between cytosolic binding of T3 and the biological actions of the hormone. Obviously thyroid hormones are extremely hydrophobic, the presence of hydrophilic carriers in cytoplasm is necessary to transport the hormone to specific intracellular receptor sites.

With respect to the cellular uptake of thyroxin, one major factor might be the intracellular (e.g. the cytosolic) binding system: the net cellular retention of thyroid hormones is a function of the tissue binding index relative to the plasma binding index [34]. As there is still discussion whether T4 and T3 traverse the cellular membrane by free diffusion or are actively transported through the cell membrane (c.f. above), the assumption that cytosolic binding proteins represent one part of the cellular transport system for thyroid hormones requires further investigation.

The physiological significance of cytosolic binding may relate to the storage and distribution of thyroid hormones between the cellular compartments. Unlike steroid hormones, cytosol binding is apparently not a prerequisite for the interaction of thyroid hormones with nuclear receptors though competition between cytosolic binding proteins and nuclear receptors for the ligand has been proposed [5]. As cytosolic binding proteins bind T4 more avidly than T3, it has been postulated that the 'core receptor' is converted to a 'holoreceptor' after binding to chromatin, thus, cytosolic binding proteins may have a function in the recognition of T3 by the nuclear receptor [5].

Cytosolic binding proteins have been demonstrated in brain cortex and cerebellum of the newborn rat declining with age from the 3rd postnatal day with respect to their number, whereas the Ka-value remained constant [81–83]. The physiological function of cytosolic binding proteins in the developing brain is still obscure but may be related to

the well known role of thyroid hormones in the functional differentiation of the developing mammalian central nervous system.

At present, it must be stated, that the functional significance of the cytoplasmic thyroid hormone binding proteins is unknown. They may even serve as a high capacity intracellular reservoir of thyroid hormones [34].

CONCLUSION

There is strong correlative evidence for the importance of the nuclear receptor. (1) Its distribution in thyroid hormone responsive tissues; (2) its correlation to thyromimetic activity; (3) the occupancy-response relationship with respect to protein synthesis, e.g. an increase in a specific mRNA in the liver, coding for malic enzyme with a concomitant increase in its enzyme synthesis, or pituitary growth hormone production. Furthermore there is good evidence for mitochondrial and plasma membrane receptors for T3 as a primary site of thyroid hormone action. This is substantiated by the recent findings that the hormone at physiological concentrations immediately increases mitochondrial O_2-consumption, ATP synthesis and the uptake of sugars and amino acids in several organs, these effects are independent of thyroid hormone induced nuclear activity.

Considering the diversity of thyroid hormone action at the cellular level, T3 evidently acts on multiple sites within the cell: the mitochondrial inner membrane as well as plasma membrane represent the target of the primary or initiating action of the hormone, whereas the nucleus is responsible for the late sustained effects of T3. The thyroid hormone induced integrated cellular response may be considered as the resultant of the 'mitochondrial-', 'plasma membrane-' and 'nuclear-pathway'. Thus, pleiotypic action of thyroid hormones implies that multiple binding sites are involved in the final expression of the hormonal effect.

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