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Investigations of 3-iodothyronamine as a novel regulator of thyroid endocrinology

by

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DISSERTATION

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Investigations of 3-iodothyronamine as a novel regulator of thyroid endocrinology

Alexandra G. Ianculescu

Abstract

3-iodothyronamine (T_1AM) is an endogenous thyroid hormone metabolite with distinct, acute biological effects that are largely opposite those of thyroid hormone. Administration of T_1AM to rodents results in rapid and profound reduction in body temperature, heart rate, and metabolism. Since its discovery only five years ago, T_1AM is emerging as a potentially key signaling molecule involved in thyroid hormone endocrinology. The structural similarities between T_1AM and monoamine neurotransmitters as well as its parent compound, thyroid hormone, suggest an intriguing role for T_1AM as both a neuromodulator and a hormone-like molecule that complements or regulates thyroid hormone action.

The known molecular targets of T_1AM include both plasma membrane and intracellular proteins, suggesting that intracellular transport of T_1AM may be an important component of its action, although no uptake mechanism has yet been described. Using various human cell lines, we show that, indeed, cellular uptake of T_1AM occurs in multiple cell types and that this process involves specific, saturable, and inhibitable transport mechanisms. These mechanisms are sodium- and chloride-independent, pH-dependent, thyronamine-specific, and do not involve the likely candidate transporters of other monoamines, organic cations, or thyroid hormones. A large-scale RNAi screen targeting the entire SLC superfamily of transporter genes reveals that the transport of T_1AM into

cells involves multiple transporters and we identify eight transporters that may contribute to the uptake of T_1AM in HeLa cells. Moreover, we demonstrate that T_1AM is taken up into the nucleus of HepG2 cells, suggesting that T_1AM might play a role in transcriptional regulation via nuclear receptors, similar to the mechanism of action of its thyroid hormone precursor.

We also investigate the effect of T_1AM on cellular entry of thyroid hormones, which is a prerequisite for their subsequent metabolism and action at nuclear thyroid hormone receptors. Transport inhibition studies reveal that T_1AM displays differential inhibition of T_3 and T_4 cellular uptake by the specific thyroid hormone transporter MCT8 as well as by the multispecific organic anion transporting polypeptides (OATPs) 1A2 and 1C1, but does not affect thyroid hormone transport by OATP1B3. Given that OATP1A2, OATP1C1, and MCT8 are all present in the brain, T_1AM may play an important role in modulating thyroid hormone delivery and activity in specific target regions in the central nervous system.

Finally, we identify α_2-Macroglobulin (α_2M) as a potential serum binding protein for T_1AM. Serum proteins are involved in the binding, transport, and extracellular storage of a wide variety of endogenous compounds, including thyroid hormones. Examination of the mode of T_1AM binding to α_2M reveals that T_1AM does not covalently bind the protein, in contrast to the reported interactions of other monoamines with α_2M. T_1AM also does not appear to bind α_2M at the same sites as other monoamines, suggesting a distinct mechanism of binding. Moreover, T_1AM binding does not result in the

conversion of the native form of α_2M to the activated form, a conformational change that does occur upon binding of α_2M to proteases and that is necessary for its clearance from the body. α_2M is known for various functions in the body, including its unique role as a pan-protease inhibitor, as well as its potential significance in immune defense and modulation of neurotransmitter metabolism. In addition to the possible role of α_2M as a carrier protein for T_1AM, the discovery of α_2M interaction with T_1AM opens another interesting area of investigation into this thyroid hormone derivative and its mechanisms of action in the body.

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CHAPTER 1

Introduction

THYROID HORMONE AND THYRONAMINES

Thyroid hormone is a classic endocrine hormone that regulates gene expression by binding to nuclear receptors, controlling multiple physiological processes including metabolism, growth, development, and central nervous system function (1). Thyroid hormone is biosynthesized from tyrosine and is initially produced in the tetraiodinated form thyroxine, or T_4, which is then deiodinated to the triiodothyronine T_3, which is the active form of the hormone (Figure 1), having greater affinity for the thyroid hormone nuclear receptors (2, 3). By binding to and activating thyroid hormone receptors that regulate transcription of T_3-responsive genes, T_3 increases basal metabolism, heart rate and contractility, blood flow to peripheral tissues, cholesterol metabolism, lipolysis, and body temperature (4). Although thyroid hormone has been studied for decades and is well-known in the basic science and medical community, the recent discovery of a class of thyroid hormone metabolites known as thyronamines (5) is greatly expanding the potential physiological roles and regulation of thyroid endocrinology.

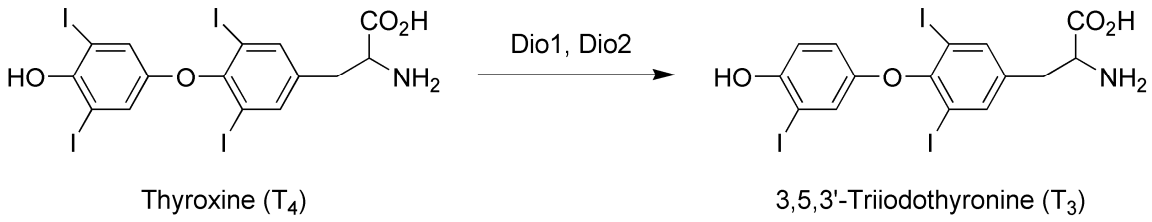

Figure 1. Thyroid hormone activation. The prohormone thyroxine (T_4) is enzymatically converted to the active form T_3 by cellular deiodinases (Dio1 and Dio2).

Thyronamines are decarboxylated derivatives of T_4 and iodothyronines (Figure 2). One of these thyronamines, 3-iodothyronamine (T_1AM), is a biogenic amine that is found in vertebrate tissues as well as in the circulatory system, and has physiological effects

opposite those of thyroid hormone (Figure 3). Intraperitoneal injection of T_1AM into mice results in profound hypothermia and bradycardia within minutes, a time scale too rapid to be explained by a transcriptional mechanism (5). In addition, T_1AM administration rapidly induces hyperglycemia in mice (6) and triggers a shift in fuel usage toward lipids and away from carbohydrates in both mice and Siberian hamsters (7). Interestingly, while thyroid hormone exerts most of its actions over a period of hours to days, certain rapidly occurring effects of thyroid hormone have been reported but remain poorly understood at the molecular level (8, 9). One intriguing possibility is that these effects are actually due to the rapid, non-transcriptional effects of T_1AM and may be a novel mechanism for regulation of thyroid hormone function in response to constantly changing physiological conditions.

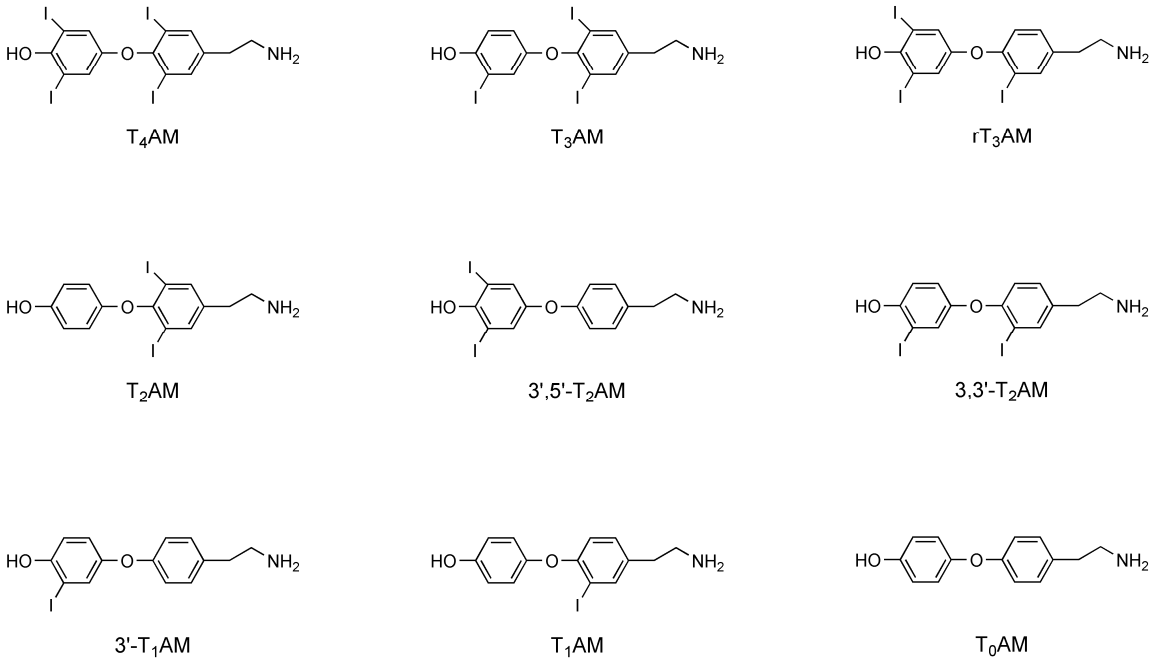

Figure 2. Chemical structures of the iodothyronamines. The entire panel of thyronamine compounds consists of the nine possible iodination states of the thyronamine scaffold.

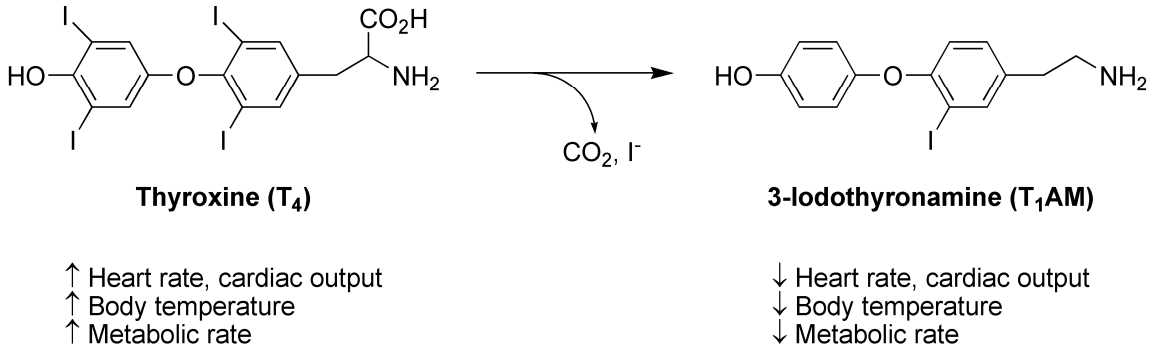

Figure 3. Conversion of thyroxine to T_1AM. Through enzymatic decarboxylation and deiodination, T_4 can theoretically be metabolized to T_1AM, which has physiological effects that are in general opposite those of thyroid hormone.

Discovery of Thyronamines as Endogenous Thyroid Hormone Derivatives

Several reports over the last few decades have described the synthesis and pharmacological properties of the general class of thyronamines, differing from one another in iodine content on the iodothyronamine scaffold (10-16), but only recently have thyronamines been demonstrated to be endogenous thyroid hormone derivatives with specific molecular targets. In 2004, both T_1AM and T_0AM were found to occur naturally in mouse brain, peripheral tissues, and blood, as detected by LC-MS/MS (5). To examine the *in vivo* effects of T_1AM and T_0AM, the synthesized thyronamines were administered to mice via intraperitoneal injection. Both compounds resulted in a rapid, dose-dependent reduction in body temperature, with T_1AM being more potent. Profound hypothermia to 31°C resulted within 30 minutes after injection of T_1AM, accompanied by behavioral inactivity, but normal core body temperature and behavior returned 6-8 hours after injection. Cardiac effects of T_1AM were also examined, and intraperitoneal injection caused an immediate drop in heart rate, while an *ex vivo* working heart preparation revealed a rapid reduction in both cardiac output and heart rate. These observed effects on heart rate and body temperature are opposite those of thyroid

hormone, suggesting that thyroid hormone and its thyronamine metabolite T_1AM could act together to maintain homeostasis, with T_1AM serving as a rapid, fine-tuning regulator of the more long-term physiological effects of thyroid hormone.

To investigate a potential molecular basis for these observed *in vivo* effects, thyronamines were examined for possible interactions with G protein-coupled receptors (GPCRs). Because of the structural similarities between thyronamines and other biogenic amines, it seemed plausible that these thyroid hormone metabolites could activate the biogenic amine-like GPCR TAAR1, the first reported member of a large family of trace amine-associated receptors (17, 18). Indeed, several thyronamines were shown to stimulate heterologously expressed mouse and rat TAAR1 to produce cAMP in a dose-dependent manner. T_1AM was identified as the most potent agonist of TAAR1, with an EC_{50} of 14 nM for rat TAAR1 and 112 nM for mouse TAAR1, and T_0AM was roughly one order of magnitude less potent. To examine the selectivity of T_1AM and T_0AM, these thyronamines were also assayed for activity towards the dopamine D_1 and β_2 adrenergic receptors and were not found to activate these GPCRs. In addition, T_1AM and T_0AM had no affinity for the thyroid hormone nuclear receptors TRα or TRβ and had no effect in TR-mediated reporter gene transactivation assays (5).

While the dramatic and rapid induction of hypothermia, bradycardia, and behavioral inactivity is consistent with a GPCR-mediated process, as opposed to the more slowly-occurring effects of thyroid hormone-regulated gene transcription, and the relative potencies of T_1AM and T_0AM towards TAAR1 *in vitro* mirrors their *in vivo* potencies to reduce body temperature and heart rate, the observed physiological effects would be more consistent with an inhibition of cAMP production through a G_i-mediated process,

whereas TAAR1 couples to G_s, at least *in vitro*. However, the physiological effects may be secondary to TAAR1 activation, such as activation of TAAR1 in an inhibitory cell that ultimately results in decreased production of cAMP in the final target cell. Alternatively, these effects may result from activation of one of the other TAAR subtypes that may be G_i-coupled. Perhaps a more likely possibility is that T_1AM exerts its physiological effects via interaction with multiple cellular targets, including extracellular GPCRs as well as other membrane proteins and intracellular targets. Most importantly, this key study was the first report of T_1AM as an endogenous thyroid hormone derivative, demonstrated its dramatic effects *in vivo*, and identified TAAR1 as a target receptor, prompting further studies of T_1AM and its physiological effects and mechanism of action.

Biological Roles of T_1AM and Molecular Mechanisms of Action

Neuromodulation

The notion of thyroid hormone metabolites as biologically active agents, in particular as neurotransmitters or neuromodulators, has been hypothesized since the 1970s (19). Intravenous administration of radiolabeled thyroxine and triiodothyronine in rats demonstrated that thyroid hormone is rapidly and selectively taken up in the nerve ending fractions, or synaptosomes, of the brain (20, 21). Approximately 90% of the detected radioactivity in synaptosomes was due to triiodothyronine, with 10% due to a single unidentified metabolite. Additional thyroid hormone metabolites, such as T_1AM, were not specifically identified in these studies because of the location of the radiolabeled iodine tag in the ligand used. Nevertheless, these findings suggested a neuroregulatory

role for thyroid hormones and their metabolites, and may help explain the significant effects of thyroid hormone, and perhaps thyronamines, on behavior and function of the central nervous system (22).

It was not until 2007 that a novel role for T_1AM itself as a neuromodulator was reported with the discovery that this thyronamine inhibits plasma membrane and vesicular monoamine transport (23). Because T_1AM is naturally present in the brain and is structurally similar to the monoamine neurotransmitters (Figure 4), it was hypothesized that T_1AM might interact with monoamine transporters. Synaptosomal monoamine transport was first studied and T_1AM was found to significantly inhibit dopamine, norepinephrine, and serotonin transport. However, when membranes were incubated with radiolabeled T_1AM, no significant uptake was observed, indicating that T_1AM itself is not a substrate. The human dopamine (DAT), norepinephrine (NET), and serotonin (SERT) transporters were then individually expressed in HeLa cells to examine the specific inhibition of monoamine transport by T_1AM. Both DAT and NET were sensitive to inhibition by T_1AM, as measured by reduced uptake of their preferred substrates. DAT and NET were also inhibited by several of the other thyronamines, and NET displayed increased transport in the presence of T_2AM, T_3AM, and rT_3AM, the significance of which is unknown. SERT, however, was not inhibited by T_1AM. Neither DAT, NET, nor SERT displayed any uptake activity of T_1AM itself. Using both purified synaptic vesicles from synaptosomal preparations as well as endosomal membranes from rat vesicular monoamine transporter 2 (VMAT2)-transfected HEK cells, T_1AM was found to inhibit the uptake of serotonin, the preferred substrate of VMAT2. In fact, all thyronamines with the exception of T_4AM inhibited VMAT2-mediated transport. Unlike

the more substrate-specific plasma membrane monoamine transporters DAT, NET, and SERT, VMAT2 transports a variety of monoamine substrates and is the only known vesicular transporter for monoamines in the central nervous system; however, no T_1AM transport by VMAT2 was detected, making T_1AM the only known endogenous phenethylamine that inhibits VMAT2 but is not recognized as a substrate.

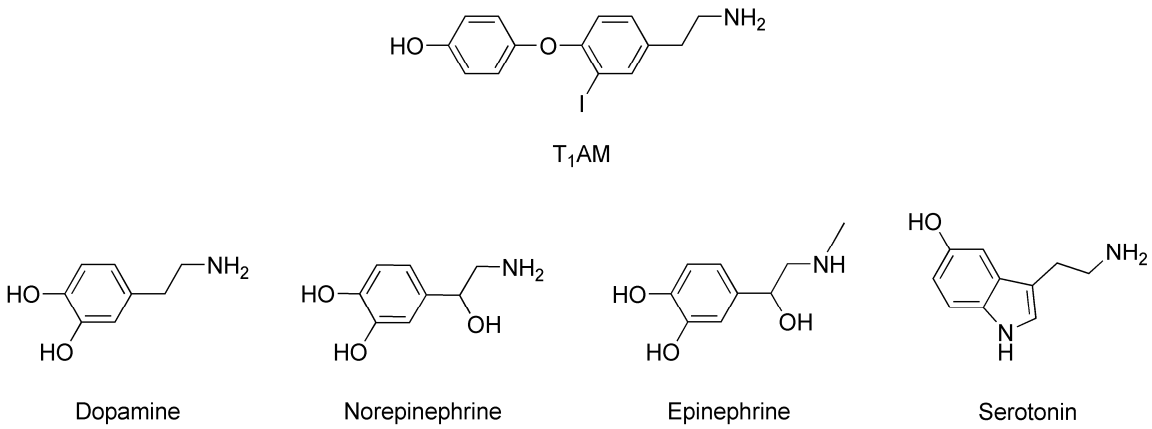

Figure 4. Structural comparison of T_1AM and other biogenic amines. The arylethylamine moiety of T_1AM is common to several classical neurotransmitters such as dopamine, norepinephrine, epinephrine, and serotonin.

Aminergic agents that regulate monoamine neurotransmission have been associated with thermoregulatory and cardiac effects, and it is plausible that T_1AM perturbation of monoamine transport may contribute to the observed *in vivo* effects of hypothermia and reduction in cardiac performance. Alternatively, T_1AM effects on monoamine transport and activity through TAAR1 may synergistically lead to the reported effects. However, the observed potencies of T_1AM inhibition of DAT, NET, and VMAT2 were all in the low micromolar IC_{50} range, while the physiological concentrations of T_1AM in the brain are estimated to be in the low nanomolar range,

similar to endogenous levels of thyroid hormone (5). Whether active localization mechanisms can elevate local concentrations of T_1AM in specific brain regions necessary to significantly affect catecholamine transport remains to be determined. If local concentrations can be elevated physiologically or pharmacologically, this study would suggest a novel neuromodulatory mechanism of monoamine levels in the brain; the data also reveals that the thyronamine scaffold and the specific iodination states can selectively modulate the different monoamine transporters, since the structure-activity relationships were different for each transporter tested, with certain thyronamines increasing monoamine transport activity, while others inhibited monoamine transport. Additional questions remaining to be answered include how thyronamines are generated and localized to synapses, as well as determining whether they have their own transport or reuptake pathways.

Cardiac effects

As is the case for its effects on body temperature, T_1AM exhibits cardiac effects that are also opposite those associated with thyroid hormone. Dramatic reduction in cardiac output and heart rate was first reported for T_1AM in 2004, and a more detailed study of the cardiac effects of T_1AM in 2007 proposed the existence of a novel aminergic system modulating cardiac function (24). T_1AM was shown to produce a rapid, reversible, dose-dependent decrease in cardiac output, aortic pressure, coronary flow, and heart rate in the isolated working rat heart. These hemodynamic effects were significantly enhanced in the presence of a tyrosine kinase inhibitor and attenuated in the presence of a tyrosine phosphatase inhibitor, suggesting that TAAR-dependent changes

in the phosphorylation of critical tyrosine residues may be important for the negative inotropic and chronotropic effects of T_1AM. Aside from TAAR1, which is a known target of T_1AM, other TAAR subtypes found to be expressed in rat heart were TAAR2, TAAR3, TAAR4, and TAAR8a. Specific, saturable binding of T_1AM was observed, with a dissociation constant in the low micromolar range, but no TAAR subtype-selective antagonist is currently available to distinguish among the potential TAAR binding sites. No change in tissue cAMP levels was detected in the study, but activation of TAAR1 in a native environment may be coupled to another second messenger pathway, or alternatively, other TAAR subtypes not coupled to cAMP could be responsible for T_1AM-mediated cardiac effects. Quantitative analysis of endogenous thyronamine content of cardiac tissues revealed average T_1AM levels of 68 pmol/g, or roughly 70 nM, in rat hearts, similar to epinephrine, dopamine, and adenosine content (25, 26), and 20- and 2-fold higher than T_3 and T_4 content, respectively (27). Interestingly, very few endogenous negative inotropic agents have been identified, making the identification of T_1AM as a novel endogenous compound with negative inotropic and chronotropic effects in the heart a finding of particular significance.

Hyperglycemia

Along with hypothermia and reduced cardiac function, T_1AM rapidly induces hyperglycemia in mice, and a recent study has investigated the potential mechanisms responsible for this hyperglycemic effect (6). A known GPCR target of T_1AM, TAAR1, is G_s coupled, but this study focused on the function of G_i family members and the receptors that regulate them. Using a genetically engineered mouse line, pertussis toxin

(PTX), which uncouples G_i family members from upstream GPCRs, was expressed in a cell type-specific Cre recombinase-dependent manner, specifically in pancreatic β islet cells. PTX expression in β cells resulted in hyperinsulinemic mice with high levels of glucose-stimulated insulin release. The improved glucose tolerance and resistance to diet-induced diabetes in these mice suggested the importance of β cell G_i and GPCR signaling in the regulation of glucose metabolism. Interestingly, while intraperitoneal administration of 50 mg/kg T_1AM increased blood glucose levels to about 250% of basal levels and decreased blood insulin levels to approximately 40% of basal levels at 2 hours following T_1AM injection in wild-type and Cre-negative mice, T_1AM had no effect on glucose and insulin levels in Cre-dependent PTX-expressing mice. These results suggest that T_1AM activates a G_i-coupled receptor on β cells to inhibit insulin secretion, although TAAR1, which is present in β cells, is coupled to G_s. The authors thus speculated that T_1AM activates G_i and inhibits insulin secretion via a GPCR in β cells other than TAAR1. Because of the similar chemical features between T_1AM and catecholamines, which are ligands for adrenergic receptors, and since the G_i-coupled α_{2A} adrenergic receptor is highly expressed in pancreatic islets, binding experiments were performed and demonstrated that indeed T_1AM is capable of binding to the α_{2A} receptor with high affinity. Moreover, coadministration of the α_{2A} receptor antagonist yohimbine with T_1AM inhibited T_1AM's hyperglycemic effects, and T_1AM administration failed to cause hyperglycemia in α_{2A} receptor knockout mice, suggesting that T_1AM's inhibitory effect on insulin release is mediated in a G_i-dependent manner by the α_{2A} receptor in β cells.

Further investigation of the role of TAAR1 in β cell response to T_1AM utilized the MIN6 insulinoma cell line, in which relative expression levels of the α_{2A} receptor and

TAAR1 are reversed. T_1AM exposure to MIN6 cells resulted in increased rather than decreased insulin secretion, which was augmented by treatment with PTX or yohimbine, suggesting that T_1AM can stimulate insulin secretion by the G_s-coupled TAAR1 and inhibit it by the G_i-coupled α_{2A} receptor (6). Thus, the TAAR1 effect appears to dominate in MIN6 cells, in which TAAR1 expression is high relative to that of the α_{2A} receptor, but in β cells *in vivo*, in which the α_{2A} receptor is more highly expressed than TAAR1, the α_{2A} receptor effect dominates. Indeed, α_{2A} receptor knockout mice became hypoglycemic after T_1AM administration, suggesting that the TAAR1 activation effect on insulin secretion became detectable only in the absence of α_{2A} receptor function. Thus, T_1AM was found to activate two GPCRs, TAAR1 and the α_{2A} receptor, to activate predominantly G_s or G_i signaling pathways depending on the relative expression levels of these receptors. However, the affinity of T_1AM for the α_{2A} receptor was in the micromolar range, an affinity at least as great as that of the endogenous adrenergic receptor agonist epinephrine, but endogenous T_1AM concentrations in the circulation are in the nanomolar range, raising the question of whether T_1AM could ever reach a level sufficient to activate the α_{2A} receptor *in vivo*. However, the decarboxylation and deiodination enzymes believed to generate T_1AM from thyroid hormones are expressed in pancreatic islets (28), and it is conceivable that local synthesis, storage, and release could indeed concentrate local T_1AM levels sufficiently to affect insulin release.

Effects on metabolism and food intake

Additional studies of T_1AM have focused on effects on metabolic rate and fuel utilization. The metabolic response to T_1AM was examined in the Siberian hamster, a

hibernating rodent species, and in mice. The administration of 50 mg/kg T_1AM led to a rapid decrease in both metabolic rate as measured by oxygen consumption and in core body temperature (7). Both hamsters and mice also showed a reduction in respiratory quotient from normal values of ~0.90 to ~0.70 for a period of several hours, an indication of a shift in metabolic pathways from primarily carbohydrate to lipid fueling in response to T_1AM. Consistent with these observations, T_1AM treatment caused ketonuria and a significant loss of body fat. The observed reduction in body temperature was slightly delayed relative to the decrease in metabolic rate, although the degree of metabolic reduction and extent of hypothermia were highly correlated, suggesting that the hypothermic response can be considered a result of the depressed metabolic rate. Moreover, the reduction in respiratory quotient, representing diminished carbohydrate metabolism, persisted for several hours after the recovery of metabolic rate and normal body temperature, indicating that some time is required to readjust the metabolic machinery from the exclusive use of lipids back to glucose utilization as the main source of fuel. This study concluded that T_1AM is an endogenous hormone that depresses metabolism by a rapid interruption of carbohydrate fueling accompanied by a compensatory rise in lipid utilization.

A separate study investigating the effects of T_1AM on food intake in rodents performed administration of T_1AM by both intraperitoneal and intracerebroventricular injection, as well as by direct injection into the arcuate nucleus of the brain (29). The hypothalamus plays an essential role in the regulation of energy homeostasis; in particular, several hypothalamic nuclei such as the paraventricular nucleus and arcurate nucleus are believed to be important in regulation of food intake and energy balance.

Because T_1AM is present in the brain, and TAAR1 is expressed in hypothalamic nuclei including the arcuate nucleus, T_1AM was hypothesized to play a role in regulating energy homeostasis. Notably, the amounts of T_1AM administered in these studies were markedly lower than the 50 mg/kg amounts used in previous studies. At 1.42 μg/kg, T_1AM delivered by intraperitoneal injection significantly increased food intake but did not affect metabolic rate or locomotor activity, in contrast to the earlier studies involving higher doses of T_1AM administration. In addition, administration of 0.043-0.43 μg/kg T_1AM intraventricularly or directly into the arcuate nucleus of male rats also significantly increased food intake, leading the authors to conclude that T_1AM is an orexigenic compound that may act through the arcuate nucleus to increase food intake in rodents.

Generation and Metabolism

Although their biosynthesis is not known with certainty, thyronamines presumably arise from the enzymatic decarboxylation and deiodination of thyroid hormones. Three deiodinases (Dio1, Dio2, and Dio3 isozymes) catalyze the conversion of iodothyronines and various iodothyronine metabolites to control the levels of thyroid hormones. For example, Dio1 and Dio2 mediate the conversion of the prohormone T_4 to the more active T_3, and Dio3 catalyzes the conversion of T_4 to the inactive rT_3 (30). Thyronamines are also substrates of the three deiodinases, supporting a role for these enzymes in thyronamine biosynthesis from thyroid hormone precursors. The isozyme selectivity of iodothyronamine deiodination was recently investigated and, interestingly, differs from that of iodothyronines (31). In contrast to the deiodination reactions of T_4 by Dio1 and Dio2, T_4AM is not a substrate of these isozymes and is instead a substrate of

Dio3, which catalyzes only deiodinations of the inner, tyrosyl ring, to yield rT_3AM. Sequential deiodination reactions of rT_3AM by Dio1 and Dio2 could then produce T_1AM, providing a specific biosynthetic pathway for endogenous T_1AM production from the parent iodothyronamines T_4AM or rT_3AM, which would result from decarboxylation of the corresponding iodothyronines T_4 or rT_3, presumably catalyzed by aromatic amino acid decarboxylase, an enzyme with relatively broad substrate specificity (32).

Aside from deiodinases, thyronamines are also substrates for sulfotransferases (SULTs), which are phase II drug-metabolizing enzymes that catalyze the sulfation of many endogenous compounds including monoamine neurotransmitters and thyroid hormones (33). Metabolism of thyronamines via sulfation could be a possible mechanism for their deactivation and termination of thyronamine action. Studies of thyronamines as substrates for human liver sulfotransferases revealed that T_1AM led to the highest SULT activity (33). Of the eight SULTs examined in the study, SULT1A2, SULT1A3, and SULT1E1 exhibited the highest activity towards T_1AM. Human brain and cardiac tissues, known targets of T_1AM action in the mouse and rat, were also tested with T_1AM as the substrate and were both found to be capable of T_1AM sulfation. The potential significance of SULT action on T_1AM activity is the attenuation and consequent regulation of T_1AM-induced effects such as hypometabolism, hypothermia, and decreased cardiac output.

Intracellular Transport

In addition to sulfation as an important clearance mechanism for regulating free circulating levels of T_1AM, a cellular uptake mechanism could also serve to terminate the

signaling of T_1AM at its extracellular receptors, *i.e.* TAAR1 and α_{2A} adrenergic receptor, analogous to the function of reuptake transporters of the monoamine neurotransmitters. In this dissertation research, we identify a specific and saturable intracellular transport mechanism for T_1AM (34). Given that the known molecular targets of T_1AM include both plasma membrane (TAAR1, α_{2A} adrenergic receptor, DAT, and NET) and intracellular proteins (VMAT2), we hypothesized that intracellular transport of T_1AM may be an important component of its action. Indeed, using various cell lines, cellular uptake of T_1AM was observed in multiple cell types. By conducting inhibition experiments of radiolabeled T_1AM uptake with increasing concentrations of unlabeled T_1AM, an IC_{50} of ~7.7 μM was measured in HeLa cells, a good approximation of the K_m for T_1AM transport. This value is similar in magnitude to the K_m values of the endogenous substrates of monoamine transporters (35, 36), organic cation transporters (37), and thyroid hormone transporters (38). T_1AM transport was sodium- and chloride-independent, pH-dependent, and thyronamine-specific, and interestingly, did not involve likely candidate transporters of other monoamines, organic cations, or thyroid hormones.

A large-scale RNAi screen was subsequently conducted targeting the entire solute carrier (SLC) superfamily of 403 transporter genes and revealed that T_1AM transport into cells involved multiple transporters. Eight transporters that may contribute to the uptake of T_1AM were identified in the RNAi knockdown screen, including several organic anion transporters, amino acid transporters, a monocarboxylate transporter, a nucleoside transporter, and a copper transporter, but overexpression studies of the individual candidate transporters did not definitively identify any of these transporters as capable of stimulating T_1AM uptake in a heterologous expression system. The presence of high

background uptake of T_1AM in the cell lines studied suggests the existence of ubiquitous, endogenous transport mechanisms and may have made it difficult to detect enhancement of uptake over basal levels. Alternatively, T_1AM uptake may be mediated by a non-SLC transporter, or a distinct mechanism such as receptor-mediated endocytosis (39) may be responsible for T_1AM transport. Nevertheless, in this dissertation we demonstrate for the first time a specific transport mechanism for T_1AM into the cell, a process important for the termination of T_1AM action at extracellular receptors as well as a necessary means of providing access of T_1AM to intracellular targets or to perform intracellular functions currently unknown.

Therapeutic Applications

Although much remains to be studied regarding the physiological roles of T_1AM and the underlying molecular mechanisms of action, the dramatic observed *in vivo* effects of T_1AM administration could be exploited for therapeutic purposes. For instance, the hyperglycemic and hypothermic effects could have potential exciting applications in medical therapy.

As previously mentioned, T_1AM has been shown to inhibit insulin secretion and induce hyperglycemia in mice via G_i signaling through the α_{2A} receptor. T_1AM activation of the G_s-coupled TAAR1, on the other hand, stimulates insulin secretion and results in hypoglycemia when the α_{2A} receptor is blocked or absent. However, the normal physiological relevance is uncertain, since the affinity of T_1AM for the α_{2A} receptor is significantly higher than the estimated endogenous circulating levels of T_1AM, although local synthesis and concentration of T_1AM in pancreatic islets could conceivably raise

T_1AM levels considerably. Nevertheless, the pharmacological effects of T_1AM at TAAR1 and the α_{2A} receptor could be explored to regulate insulin secretion for therapeutic purposes in diabetes and other disorders of glucose metabolism, insulin sensitivity, and pancreatic β cell dysfunction.

A possible mechanism already discussed by which T_1AM could cause hypothermia is through the alteration of monoamine transport, although inhibition of DAT, NET, and VMAT2 once again occurs at T_1AM concentrations higher than those present in the circulation. Specific mechanisms of action and questions of endogenous thyronamine levels notwithstanding, one of the potential therapeutic applications of T_1AM as a rapid inducer of transient hypothermia was recently reported as being neuroprotective against stroke injury (40). Both T_1AM and T_0AM caused hypothermia in mice within 30 minutes of intraperitoneal administration, which was maintained for 6 hours but lost by 10 hours after injection. The hypothermic response occurred with no apparent long-term adverse effects and, interestingly, with no evidence of shivering and piloerection, suggesting that the induced hypothermia was not opposed by the natural homeothermic response to cold temperatures. Both T_1AM and T_0AM treatment resulted in significantly smaller brain infarcts when given one hour after stroke induction in a mouse model of focal ischemia, with T_1AM showing an average 35% reduction in infarct volume and T_0AM giving a 32% reduction. Neither thyronamine showed any neuroprotective effect when the induction of hypothermia was prevented. Furthermore, mice preconditioned with T_1AM, having received a single 50 mg/kg dose two days before stroke induction, also displayed a similar 34% reduction in infarct volume, making T_1AM the first cryogen shown to provide prophylactic neuroprotection in situations of

anticipated ischemic injury, for instance surgical procedures in high-risk patients. Both pre- and post-ischemia treatments required the induction of hypothermia, and T_1AM and T_0AM treatment *in vitro* did not confer neuroprotection against ischemia. Although several synthetic compounds have been studied for their therapeutic hypothermia-inducing effects, T_1AM and T_0AM, as endogenous substances, may be better tolerated with fewer side effects.

Conclusions

Since the discovery only a few years ago of T_1AM as an endogenous thyroid hormone derivative with dramatic *in vivo* actions, a number of studies have focused on understanding its mechanism of action. The full spectrum of its effects is unlike that of any other known drug or endogenous biologically active compound. Extracellular signaling at GPCRs such as TAAR1 and the α_{2A} adrenergic receptor, modulation of neurotransmitter packaging and recycling pathways, metabolic processing, and cellular transport mechanisms of T_1AM are all important findings that provide clues regarding the biological role of this thyroid hormone derivative. A summary of the identified molecular targets and effects of T_1AM is depicted in Figure 5. Ongoing work includes studying the uptake of T_1AM into the nucleus and its potential regulation of gene transcription, similar to its thyroid hormone precursor, as well as T_1AM modulation of thyroid hormone action via inhibition of thyroid hormone transport at the cell membrane. Binding of T_1AM to serum proteins for purposes of regulating free concentrations and for transport throughout the body is yet another important area of investigation. Further studies into the molecular mechanisms of T_1AM action will be invaluable in not only

understanding the basis of its physiological effects, but also in expanding our knowledge of thyroid endocrinology and the potential elucidation of certain endocrine or neurological pathologies.

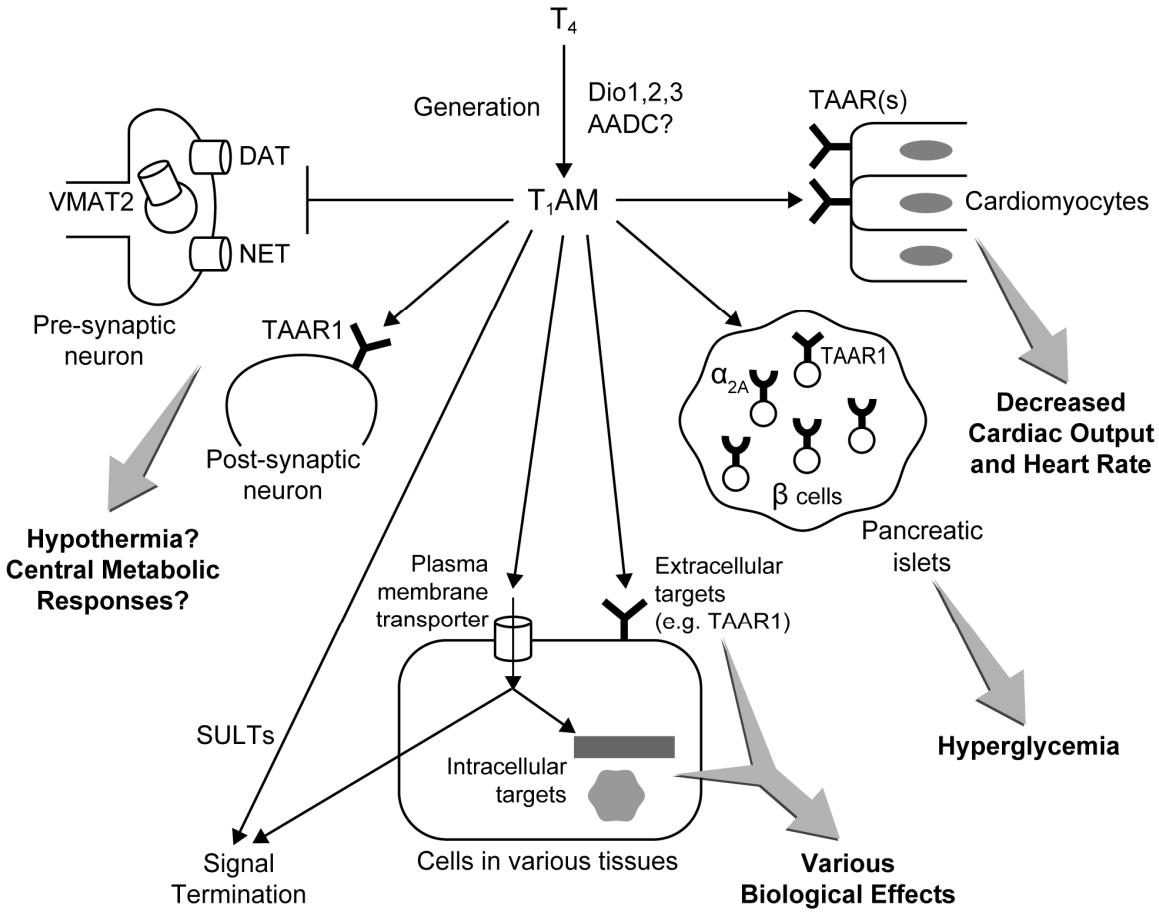

Figure 5. Proposed model of T_1AM action. T_1AM is generated by enzymatic deiodination and decarboxylation of T_4. Inhibition of monoamine transport as well as activation of TAAR1 or other receptors present in the brain may be in part responsible for the hypothermic effects and other central metabolic responses, such as changes in fuel utilization, food intake, and behavioral activity. Activation of one or more TAAR subtypes present in the heart contributes to the negative inotropic and chronotropic effects of T_1AM. Hyperglycemic effects are mediated by activation of the α_{2A} adrenergic receptor in pancreatic β islet cells. Specific plasma membrane transport machinery facilitates the entry of T_1AM into multiple cell types throughout the body, possibly to allow access to intracellular targets currently unknown. Uptake into the cell, as well as metabolism by SULT enzymes, likely contribute to the termination of T_1AM signaling at extracellular targets.

THE SOLUTE CARRIER TRANSPORTERS

Membrane transporters are essential for all cells, controlling the uptake and efflux of compounds across the plasma membrane and intracellular organelles. The solute carrier (SLC) gene series of transporters regulate transport of sugars, amino acids, nucleotides, organic and inorganic ions, and drugs (41). Transporters can be classified as either passive or active transporters; passive, or facilitated, transporters allow the passage of solutes down their electrochemical gradients, while active transporters use energy-coupling mechanisms, for instance ATP hydrolysis, to create ion or solute gradients across membranes. SLC transporters include passive transporters, coupled transporters and exchangers, mitochondrial, and vesicular transporters. Other, non-SLC transport proteins include water and ion channels, ATP-binding cassette (ABC) transporters, and ion pumps. As of 2003, the SLC gene series of transporters consisted of 43 families and 298 transporter genes, although new members are continually being identified and the SLC transporter list currently consists of 46 families and 403 transporter genes. The Human Genome Organization (HUGO) database provides the latest updates for the SLC transporter superfamily (41).

Thyroid Hormone Transporters

Thyroid hormone exerts its actions on virtually all tissues. Action and metabolism of thyroid hormones are intracellular events and thus require their uptake across the plasma membrane. Although thyroid hormones, given their lipophilic nature, were originally believed to enter target cell membranes by simple diffusion, it is now known that specific plasma membrane transporters are responsible for their uptake.

Some of these transporters have recently been identified at the molecular level, and experimental studies have established their physiological relevance and uncovered links between thyroid hormone transporter dysfunction and human disease (42).

Although several transporter families are capable of transporting thyroid hormones, including certain members of the SLC7 family of cationic amino acid transporters (43), the SLCO family of organic anion transporting polypeptides or OATPs (44), and the SLC16 family of monocarboxylate transporters or MCTs (45), only OATP1C1, MCT8, and MCT10 show a high degree of specificity towards thyroid hormones (46). Because thyroid hormone is formed within the thyroid follicle by the coupling of iodinated tyrosine residues on thyroglobulin and thus retains an amino acid moiety within the iodothyronine structure, it seems logical that thyroid hormones would be substrates of amino acid transporters. Indeed, both System L (leucine-preferring) transporters that transport large, neutral amino acids, and System T (tryptophan-preferring) transporters that are specific for aromatic amino acids accept thyroid hormones as substrates. MCT10 (SLC16A10) is one such T-type amino acid transporter that demonstrates uptake of both T_3 and T_4 and has a wide tissue distribution, including the intestine, kidney, liver, and placenta. No polymorphism or mutation in this transporter has thus far been correlated with any human disease, however (46).

Of the multispecific family of OATPs, which accept anionic, neutral, and even cationic compounds, several have been shown to transport thyroid hormones, but OATP1C1 (SLCO1C1) is the family member showing the highest specificity and affinity towards iodothyronines, T_4 and rT_3 in particular. OATP1C1 is highly expressed in brain capillaries, suggesting an important role in T_4 transport across the blood-brain barrier.

However, as in the case of MCT10, no mutations in OATP1C1 have been associated with thyroid hormone resistance syndromes, so the *in vivo* function of this transporter remains an important area of investigation (47). Although OATP1C1 has been clearly shown to mediate transport of T_4 and rT_3 and increase the access of these substrates to the intracellular active sites of the deiodinases, a recent study did not observe any effect of genetic variation on the function of this important thyroid hormone transporter (48).

In contrast, mutations in the transporter MCT8 (SLC16A2) have been clearly associated with X-linked psychomotor retardation and elevated T_3 levels (49, 50). MCT8 is also the most specific and active thyroid hormone transporter identified to date; it transports both T_3 and T_4, but does not transport sulfated iodothyronines, the amino acids phenylalanine, tyrosine, tryptophan, and leucine, or the monocarboxylates lactate and pyruvate (51). Moreover, cells cotransfected with MCT8 and one of the deiodinases exhibited a significant increase in thyroid hormone metabolism, suggesting that MCT8 increases the availability of thyroid hormone for intracellular metabolism by the different deiodinases (46). MCT8 shows a broad tissue distribution, in particular the liver and heart. Importantly, MCT8 is also found in various regions of the brain, being localized in neurons of the paraventricular, supraoptic, and infundibular nucleui of the hypothalamus and in glial cells of the ependymal lining of the third ventricle (52). MCT8 is also expressed in the folliculostellate cells of the pituitary (53). It is assumed that these sites are involved in the negative feedback control of the hypothalamus-pituitary-thyroid axis by thyroid hormone (46). The physiological importance of MCT8 for normal brain development has been demonstrated by X-linked psychomotor retardation syndromes (the MCT8 gene is located on the X chromosome) and abnormally high serum T_3 levels

resulting from mutations in MCT8. The current model for local control of T_3 availability in the brain proposes that an intracellular deiodinase, specifically Dio2, in astrocytes is responsible for the conversion of T_4 to the active T_3, which is then taken up via MCT8 into neurons, which themselves do not express Dio2 required for thyroid hormone activation. Neurons express a different deiodinase, Dio3, which converts T_3 to the inactive T_2, thereby terminating T_3-dependent transcription of various genes required for normal neuronal function and brain development (46). OATP1C1 is the transporter responsible for T_4 delivery across the blood-brain barrier, but the transporters involved in T_4 uptake and T_3 efflux in astrocytes are not currently known.

Aside from mutations in thyroid hormone transporters, the availability of thyroid hormones to their target cells and tissues can be modulated by inhibitors of thyroid hormone transporters. Indeed, the transcriptionally inactive rT_3, previously believed to be just an inactive byproduct of thyroid hormone metabolism, has been shown to be a potent competitive inhibitor of thyroid hormone uptake by several different transporter types (43). Thus, thyroid hormone metabolites that do not bind the nuclear thyroid hormone receptors can nevertheless be important regulators of thyroid hormone bioavailability and subsequent action.

SERUM BINDING PROTEINS

Many proteins found in plasma bind to various ligands, such as ions, vitamins, and hormones. Human serum albumin is the most abundant protein found in the plasma, comprising about half of the blood serum protein, and binds different classes of ligands at multiple sites. Albumin is responsible for the binding and transport of thyroid and other

hormones, fatty acids, bilirubin, a variety of essential and toxic metal ions, and many drugs. The abundance of albumin in plasma makes it an important factor in the pharmacokinetics of many drugs, affecting their efficacy and rate of delivery (54, 55).

In addition to albumin, many other serum binding proteins are important for the transport throughout the body and bioavailability of various compounds. Almost all thyroxine produced and secreted by the thyroid gland, for instance, is protein-bound, principally to thyroxine-binding globulin (TBG), as well as to transthyretin and albumin (56, 57). The unbound or free fraction of thyroid hormone is responsible for biological activity, although only 0.04% of T_4 and 0.4% of T_3 are found free in the serum (58). TBG has the highest affinity for T_3 and T_4 and carries most (~70%) of the circulating thyroid hormones. Transthyretin binds about 10% of circulating T_4, and has a much lower affinity for T_3. Because the dissociation of thyroid hormones from transthyretin is rapid, it is a source of readily available T_4. Due to its high abundance in serum, albumin carries about 15% of circulating T_3 and T_4, and the rapid dissociation rates of thyroid hormones from albumin also make this carrier a major source of free hormone (58). Although the transport proteins are not essential for thyroid hormone activity, they do form a storage pool of readily available free hormone, allow the delivery of thyroid hormones to all tissues because the small fraction of free hormone is continually replenished as the hormones are absorbed by tissues, and they protect tissues from massive hormone release. On the other hand, serum binding proteins dedicated to the transport of biogenic amines such as dopamine, norepinephrine, and tyramine throughout the body have not, to our knowledge, been described.

α_2-Macroglobulin (α_2M) is another important plasma protein, with a wide variety of activities in the body. It is primarily known for its unique property as a pan-protease inhibitor, capable of binding and inhibiting a wide variety of proteases regardless of their specificity or catalytic mechanism. The structure of α_2M is a 725 kDa homotetramer composed of a non-covalently linked pair of disulfide-linked dimers. The molecule contains a "bait" region that reacts with proteases, trapping and thus inhibiting them, in the process resulting in a conformational change of α_2M from the native, "slow" form to the activated, "fast" form, so named for their differences in electrophoretic mobility (59). In addition to its importance in the control of extracellular proteolytic activity, α_2M is also a potential regulator of neuronal development and function. While the bait region of the molecule is susceptible to cleavage by proteases, nucleophilic attack at thioester bonds of α_2M by monoamines results in monoamine-activated α_2M that selectively binds various neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4 (60). Serotonin-activated α_2M also depresses dopaminergic and cholinergic neurotransmitters in the central nervous system, suggesting a potential regulatory role in neurotransmitter metabolism (61).

The goal of this research has been to elucidate some of the functions of the recently discovered endogenous thyroid hormone derivative, T_1AM. The major focus was the identification and characterization of a specific plasma membrane transport mechanism of T_1AM into cells (Chapters 2 and 3), motivated by the known physiological importance of membrane transporters of other biogenic amines and thyroid hormone for the proper functioning of neurological and endocrine systems. In addition, because of its

physiological effects that appear to be opposite those of thyroid hormone, T_1AM was investigated as an inhibitor of thyroid hormone transport into cells (Chapter 4). Finally, because many biological compounds including thyroid hormone bind to various serum proteins important for their storage and transport throughout the body, the existence of a serum binding protein for T_1AM was investigated (Chapter 5). The findings presented here provide important insight into the potential roles of T_1AM, and further studies in these and other aspects of T_1AM action will undoubtedly yield invaluable knowledge of this thyroid hormone derivative and its physiological implications in what appears to be a novel and rapidly expanding field of thyroid endocrinology.

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CHAPTER 2

Identification and characterization of

3-iodothyronamine (T_1AM) intracellular transport

Abstract

3-iodothyronamine (T_1AM) is a naturally-occurring thyroid hormone metabolite with distinct biological effects that are opposite those of thyroid hormone. The known molecular targets of T_1AM include both plasma membrane and intracellular proteins, suggesting that intracellular transport of T_1AM may be an important component of its action, although no uptake mechanism has yet been described. Using various human cell lines, we show that, indeed, cellular uptake of T_1AM occurs in multiple cell types and that this process involves specific, saturable, and inhibitable transport mechanisms. These mechanisms are sodium- and chloride-independent, pH-dependent, thyronamine-specific, and do not involve the likely candidate transporters of other monoamines, organic cations, or thyroid hormones. Moreover, we demonstrate that T_1AM is taken up into the nucleus of HepG2 cells, suggesting that T_1AM might play a role in transcriptional regulation via nuclear receptors, similar to the mechanism of action of its thyroid hormone precursor.

Introduction

Thyronamines are a recently discovered class of compounds arising from the decarboxylation of thyroid hormone (1), a classic endocrine hormone that acts by regulating transcription of target genes involved in many important physiological actions, including regulation of growth, development, and metabolic functions (2). One of these thyronamines, 3-iodothyronamine (T_1AM), is a biogenic amine that is found in vertebrate tissues as well as in the circulatory system. Intraperitoneal injection of T_1AM into mice results in profound hypothermia and bradycardia within minutes, a time scale too rapid to

be explained by a transcriptional mechanism (1). In addition, T_1AM administration rapidly induces hyperglycemia in mice (3) and rapidly triggers a shift in fuel usage toward lipids and away from carbohydrates in both mice and Siberian hamsters (4). Interestingly, while thyroid hormone exerts most of its actions over a period of hours to days, certain rapidly occurring effects of thyroid hormone have been reported but remain unexplained (5, 6). The rapid, non-transcriptional effects of T_1AM may be a novel mechanism for regulation of thyroid hormone function in response to constantly changing physiological conditions. Insight into the mechanism of action of this thyroid hormone metabolite would thus greatly contribute to our current understanding of thyroid endocrinology.

Like other trace amines, T_1AM is a potent agonist of the rat and mouse trace amine associated receptors 1 (TAAR1), members of the G protein-coupled receptor (GPCR) family (1). T_1AM may also have a neuromodulatory role as an inhibitor of the dopamine and norepinephrine transporters responsible for the reuptake of these classical neurotransmitters, as well as the vesicular monoamine transporter VMAT2, an intracellular transporter which packages monoamines into synaptic vesicles (7). While TAAR1 signaling mechanisms and modulation of monoamine transport may help explain some of the pharmacological effects of thyronamines *in vivo*, a greater understanding of the actions of T_1AM is needed.

Other structurally related compounds, including the biogenic amine neurotransmitters dopamine, serotonin, and norepinephrine, are translocated across plasma membranes by various transporters (8, 9). As such, we hypothesized that there might likewise exist plasma membrane transport mechanisms for the uptake of T_1AM.

This transport mechanism could serve to terminate the signal of T_1AM at its extracellular receptors or provide a means of recycling the compound, analogous to the critical function of reuptake transporters of the monoamine neurotransmitters.

Thyroid hormone itself is transported across the cell membrane by a variety of transporters, the dysfunction of which results in certain disease states (10-13). Despite our knowledge of T_1AM's action at the TAAR1 GPCR and its neuromodulatory activities, the mechanism of physiological action of T_1AM remains largely unknown. Although T_1AM does not bind the nuclear thyroid hormone receptors (1), it may nevertheless have other important roles inside the cell yet to be discovered. An understanding of the cellular transport of T_1AM would provide insight into its mechanisms of action and possible role in regulation of thyroid hormone activity.

With the goal of expanding our knowledge of the molecular mechanisms underlying T_1AM action, the aim of this study was to determine the mechanisms by which T_1AM enters cells. In particular, we were interested in characterizing the processes involved in the intracellular uptake of T_1AM and determining whether logical candidate transporters of related compounds could be responsible for T_1AM transport.

Materials and methods

T_1AM Transport Assay

Cell lines were grown in the appropriate recommended ATCC complete growth medium for the particular cell line at 37°C with 5% CO_2 and 95% humidity. In preparation for uptake assays, cells were seeded into 24-well tissue culture plates and uptake experiments were performed the following day. Cells were washed and

preincubated with prewarmed KRTH (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM Tris, 10 mM HEPES, pH 7.4) for 15 min at 37°C. Uptake was initiated by the addition of a tracer amount of ^{125}I-T_1AM, synthesized as described previously (14), with or without various concentrations of unlabeled compounds diluted in KRTH. Uptake was terminated after 20 min at 37°C, the cells were washed twice with cold KRTH and solubilized in 1% SDS, and the accumulated radioactivity was determined by scintillation counting. For uptake assays performed in sodium- or chloride-free buffer, KRTH was modified by replacing sodium with choline or chloride with gluconate, respectively. For uptake assays performed at varying pH, HCl or NaOH was added to unmodified KRTH to achieve the desired pH. The Qproteome Cell Compartment Kit (Qiagen) was used according to manufacturer's protocols to fractionate cells after standard uptake conditions to determine the subcellular localization of ^{125}I-T_1AM. In separate studies, after the standard assay for ^{125}I-T_1AM uptake, cells were permeabilized for 5 min at r.t. in 12.5 mM HEPES-KOH, pH 7.4, 50 mM PIPES-KOH, pH 6.9, 1 mM $MgSO_4$, and 4 mM EGTA-KOH containing 40 μM digitonin, washed twice with cold PBS, solubilized in 1% SDS, and the accumulated radioactivity remaining in the permeabilized cells was determined by scintillation counting. Transport activity for each condition was measured in triplicate on at least three separate occasions. Data given for relative uptake of T_1AM show representative uptake for a single experiment done in triplicate.

Generation of Stable Cell Lines

Stable cell lines were used for all of the experiments testing the function of

individual transporters. FlpIn HEK 293 cells (Invitrogen) were maintained in DMEM of high glucose supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (UCSF Cell Culture Facility). Stable cell lines were created by introducing a construct containing the complete CDS of the particular transporter gene cloned into the pcDNA5/FRT vector or the pcDNA5/FRT vector alone, according to the manual of the FlpIn system. The MCT8 cDNA was generously supplied by the laboratory of Theo Visser from Erasmus University Medical Center, Rotterdam, The Netherlands, and used to construct the MCT8 HEK stable cell line. Stably transfected HEK FlpIn cells were selected after 48 h by the addition of 75 µg/ml hygromycin. The PMAT MDCK stable cell line and corresponding pcDNA3 empty vector cell line were received from the laboratory of Joanne Wang from the University of Washington, Seattle, Washington (15). For transport assays performed with control substrates, cells were incubated with the appropriate radiolabeled substrate for the particular transporter tested under transport assay conditions identical to those for T_1AM described above.

Nuclear Uptake Assay

HepG2 cells were grown in Eagle's MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (UCSF Cell Culture Facility). In preparation for uptake assays, cells were seeded into 6-well tissue culture plates and uptake experiments were performed the following day. Cells were washed and preincubated with prewarmed Eagle's MEM growth media lacking serum and antibiotics for 15 min at 37°C. Uptake was initiated by the addition of a tracer amount of ^{125}I-T_1AM or ^{125}I-T_3 (Perkin Elmer) diluted in Eagle's MEM. After incubation for various time

points at 37°C, the cells were washed twice with cold PBS, scraped in 1 ml PBS, and transferred to eppendorff tubes. NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) were used to isolate nuclear and cytoplasmic cellular fractions according to manufacturer's protocols. The accumulated radioactivity in each fraction was determined by scintillation counting. Transport activity was measured in triplicate on at least three separate occasions. Data given for nuclear uptake of T_1AM and T_3 show representative uptake for a single experiment done in triplicate.

Statistical Analyses

Statistical analyses were performed with the GraphPad Prism version 4.00 software, with values expressed as means ± SD.

Results

Intracellular uptake of T_1AM in multiple cell lines involves facilitated transport mechanisms

To characterize T_1AM uptake in cultured cells, we examined several diverse cell lines for specific uptake of T_1AM by incubating the cells with ^{125}I-T_1AM (14) either alone or in the presence of excess unlabeled T_1AM. The variety of cell lines screened included rodent cell lines L6 (rat skeletal muscle) and BC3H1 (mouse brain tumor), insect Sf9 cells (pupal ovarian tissue), and the human cell lines CAKI-1 (kidney), U2OS (bone), Hep G2 (liver), HISM (smooth intestine), HeLa (cervix), HEK 293 (kidney), and 293T (kidney) cells. For all cell lines screened, we observed significantly reduced uptake of ^{125}I-T_1AM in the presence of 50 μM unlabeled T_1AM, suggesting the existence of

specific transport mechanisms of T_1AM *in vitro*. Relative uptake of ^{125}I-T_1AM was similar among all cell lines tested, and in most cell lines, the addition of 50 μM unlabeled T_1AM resulted in approximately a three-fold reduction in uptake (Figure 1A). By varying the concentration of unlabeled T_1AM during the uptake experiments, we observed a dose-dependent inhibition of radiolabeled T_1AM uptake with an IC_{50} of ~7.7 μM in HeLa cells (Figure 1B). Collectively, the data indicate that T_1AM uptake occurs in multiple cell types and involves facilitated transport mechanisms. Observation of T_1AM uptake in cultured cell lines derived from a variety of tissue sources suggests that T_1AM may have actions throughout the body and is consistent with its endogenous presence in several different vertebrate tissues.

Other thyronamines compete with T_1AM for uptake

We next determined the effect of other thyronamines on T_1AM uptake. The complete panel of thyronamines has been chemically synthesized (1) and consists of the nine possible iodination states, including the non-iodinated T_0AM. At least two of the thyronamines, T_1AM and T_0AM, are present endogenously (7). Because of the close structural similarity among all the thyronamines, which differ only by the number and position of iodine molecules, it seemed likely that several may be transported by the same mechanism as T_1AM. Since radiolabeled versions of the other thyronamines were not available, we indirectly tested for their uptake by conducting competition experiments with T_1AM.

With the exception of T_4AM, we observed a dose-dependent decrease in T_1AM uptake for all thyronamines, suggesting competition with T_1AM uptake. While the extent

of competition was similar among the remaining thyronamines, rT_3AM, T_0AM, and 3',5'-T_2AM appeared to be slightly less potent (Figure 1C). Although 3,3'-T_2AM reduced the uptake of ^{125}I-T_1AM to a greater extent at 50 μM than T_1AM itself, the difference was not statistically significant and unlabeled T_1AM was among the most potent of the thyronamines at competing with uptake of radiolabeled T_1AM.

Sodium- and chloride-independent, pH-dependent uptake of T_1AM

To further characterize T_1AM transport, uptake experiments were performed in buffer lacking sodium or chloride to determine whether T_1AM uptake was dependent on these ions. Similar levels of uptake were observed for the different buffer compositions, revealing that transport is sodium- and chloride-independent (Figure 1D). These results suggest that T_1AM transport does not involve a sodium and chloride cotransport mechanism.

The pH of the uptake buffer was also varied to investigate the effect on T_1AM transport. The fold increase in uptake over background (*i.e.* in the presence of excess unlabeled T_1AM) remained constant; however, after subtracting the background uptake, specific ^{125}I-T_1AM uptake increased with increasing pH (Figure 1E), suggesting that T_1AM uptake is driven by an outwardly-directed proton gradient (or inwardly-directed hydroxide gradient). The observed increase in total and background T_1AM uptake at higher pH probably reflects enhanced passive diffusion, as there would be an increased fraction of the deprotonated form of the monoamine, which is positively charged at physiological pH of 7.4.

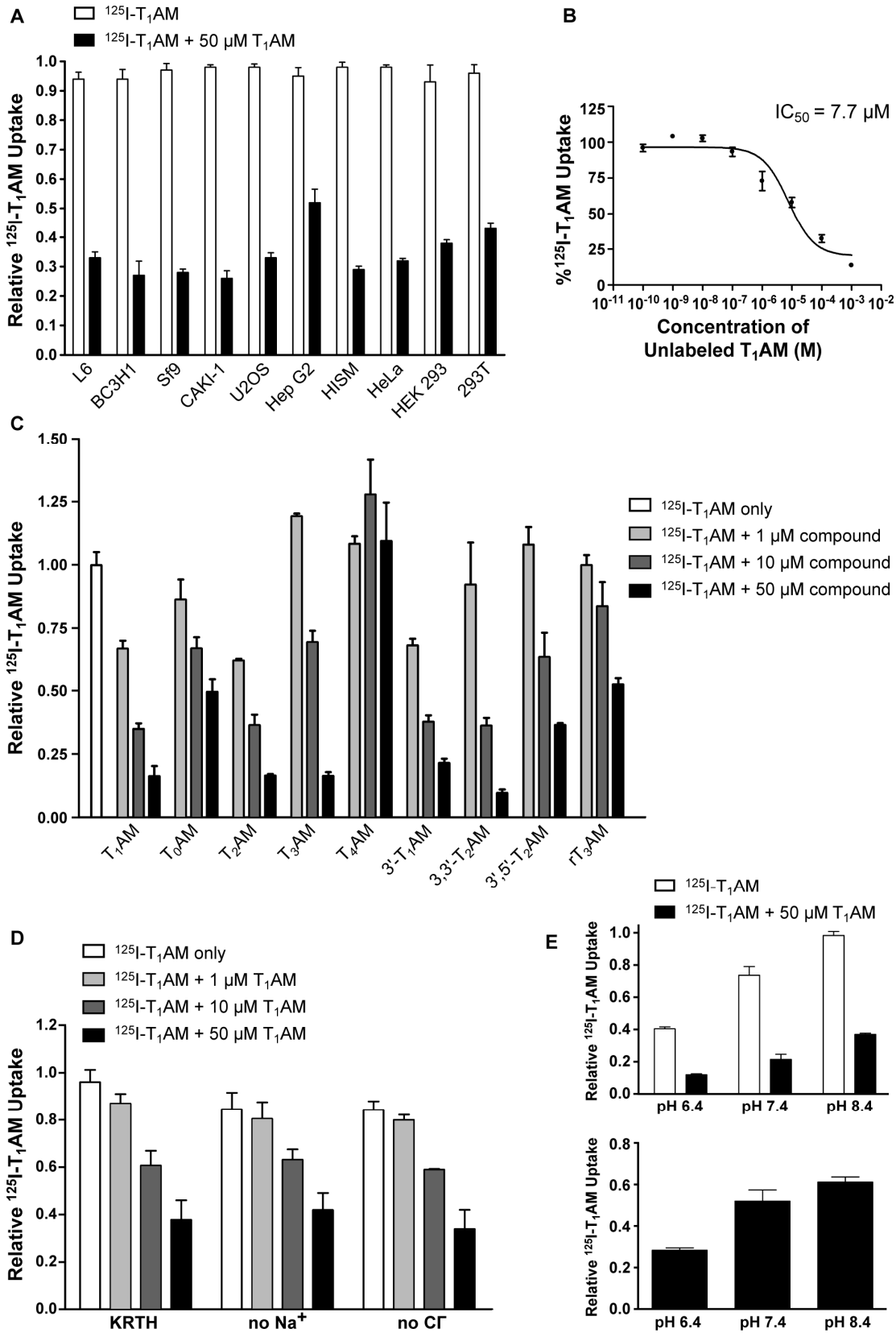

Figure 1. Identification and properties of T_1AM transport. *Legend next page.*

Figure 1. Identification and properties of T_1AM transport. A) Specific uptake of ^{125}I-T_1AM can be demonstrated in a variety of cell lines. B) Dose-dependent inhibition of ^{125}I-T_1AM with unlabeled T_1AM in HeLa cells. This response suggests a specific mechanism of T_1AM uptake with an IC_{50} of ~7.7 μM. C) Effect of thyronamines on T_1AM uptake. Uptake is inhibited to varying degrees and in a dose-dependent manner by all of the other thyronamines, with the exception of T_4AM. D) Effect of sodium and chloride on T_1AM uptake. Experiments performed in uptake buffer with and without sodium and chloride show that uptake is independent of these ions. For sodium-free buffer, sodium was replaced by choline, and for chloride-free buffer, chloride was replaced by gluconate. E) Effect of pH on T_1AM uptake. Top: Experiments performed in uptake buffer at different pH show that, while total uptake increases with increasing pH, the fold increase in uptake over background remains the same. Bottom: Subtraction of background uptake shows an increase in ^{125}I-T_1AM uptake with increasing pH, suggesting that T_1AM uptake may be driven by an outwardly-directed proton gradient. The increase in total and background T_1AM uptake at higher pH may be a result of an increased fraction of the deprotonated form of the monoamine, which can more readily diffuse across the plasma membrane. In each of the graphs, relative ^{125}I-T_1AM uptake values are normalized either to the maximum uptake signal (DPM radioactivity counts) obtained with ^{125}I-T_1AM incubation alone (panels A, C, D) or to the overall maximum uptake signal obtained for all the conditions depicted in the graph (panels B and E).

Subcellular localization of T_1AM

To determine the cellular compartments containing T_1AM after cellular uptake, cells were fractionated into cytosolic, total membrane (plasma membrane as well as all organelle membranes except the nuclear membrane), nuclear, and cytoskeletal components following incubation with ^{125}I-T_1AM. Almost 40% of the radiolabel was identified in the cytosolic fraction and approximately 30% was associated with membranes, with undetectable levels in the nucleus and cytoskeleton (Figure 2A). Compared to the total radioactivity present in unfractionated cells, some amount of ^{125}I-T_1AM was lost in the extraction procedures. Nevertheless, these results show that T_1AM is indeed being transported into the cells, although a significant portion is membrane-

associated, perhaps bound to a plasma membrane transporter, membrane receptor, or even a vesicular transporter or receptor.

In addition, cells were treated with digitonin following T_1AM uptake experiments to determine the amount of radioactivity remaining after cell permeabilization. Digitonin-permeabilized cells lost over 75% of the radiolabel (Figure 2B), again another confirmation that T_1AM is being transported into the cell rather than merely binding to the cell membrane, for example.

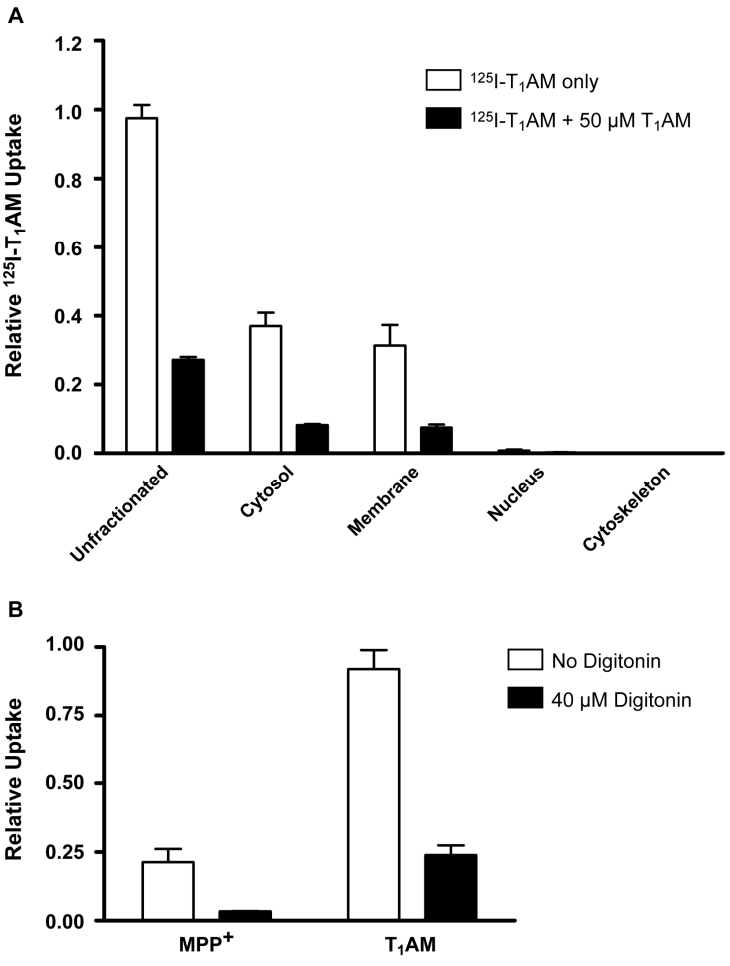

Figure 2. Cellular localization of T_1AM. A) Cellular fractionation of cells after incubation with radiolabeled T_1AM identifies T_1AM in both cytosol and membrane compartments, with none present in the nucleus or cytoskeleton. Relative ^{125}I-T_1AM uptake values are normalized to the uptake signal (DPM radioactivity counts) present in unfractionated cells. B) Digitonin treatment following incubation with radiolabeled T_1AM reveals that the majority of ^{125}I-T_1AM is lost after cells are permeabilized, confirming that ^{125}I-T_1AM is internalized during the incubation period. 1-methyl-4-phenylpyridinium (MPP^+) is used as a control, since cells express some background uptake of MPP^+ due to endogenous transporters. Relative uptake levels of ^{3}H-MPP^+ and ^{125}I-T_1AM are normalized to the maximum uptake signal (DPM radioactivity counts) obtained with ^{125}I-T_1AM incubation.

Specificity of T_1AM uptake

As an initial attempt at identifying membrane transporters that are involved in T_1AM uptake, we performed competition experiments using prototypical substrates of several major classes of transporters. These unlabeled compounds were incubated with ^{125}I-T_1AM to examine the specificity of the T_1AM uptake mechanism. The monoamine neurotransmitters dopamine, serotonin, and norepinephrine are transported by both high-affinity transporters of the SLC6 family (9) as well as low-affinity, high-capacity transporters such as the plasma membrane monoamine transporter (PMAT) (15) and transporters of the SLC22 family (16). Thyroid hormone is also transported by a variety of transporters, including organic anion transporters, amino acid transporters, and the more thyroid hormone-specific MCT8 (13). The biogenic amines dopamine, serotonin, and norepinephrine, as well as the thyroid hormone T_3, were added during ^{125}I-T_1AM incubation, and no competition for uptake was observed (Figure 3A). These observations suggest that T_1AM is not taken up into the cell via the same mechanism as these other compounds.

Moreover, several known substrates and inhibitors of the class of organic cation transporters (OCTs), organic anion transporters (OATs), and the equilibrative nucleoside transporters (ENTs), which include PMAT (ENT4), were also added at concentrations significantly greater than their observed K_m or K_i values (15, 16) and did not inhibit the uptake of T_1AM in a dose-dependent manner (Figure 3B), suggesting that T_1AM uptake does not occur through these transporters.

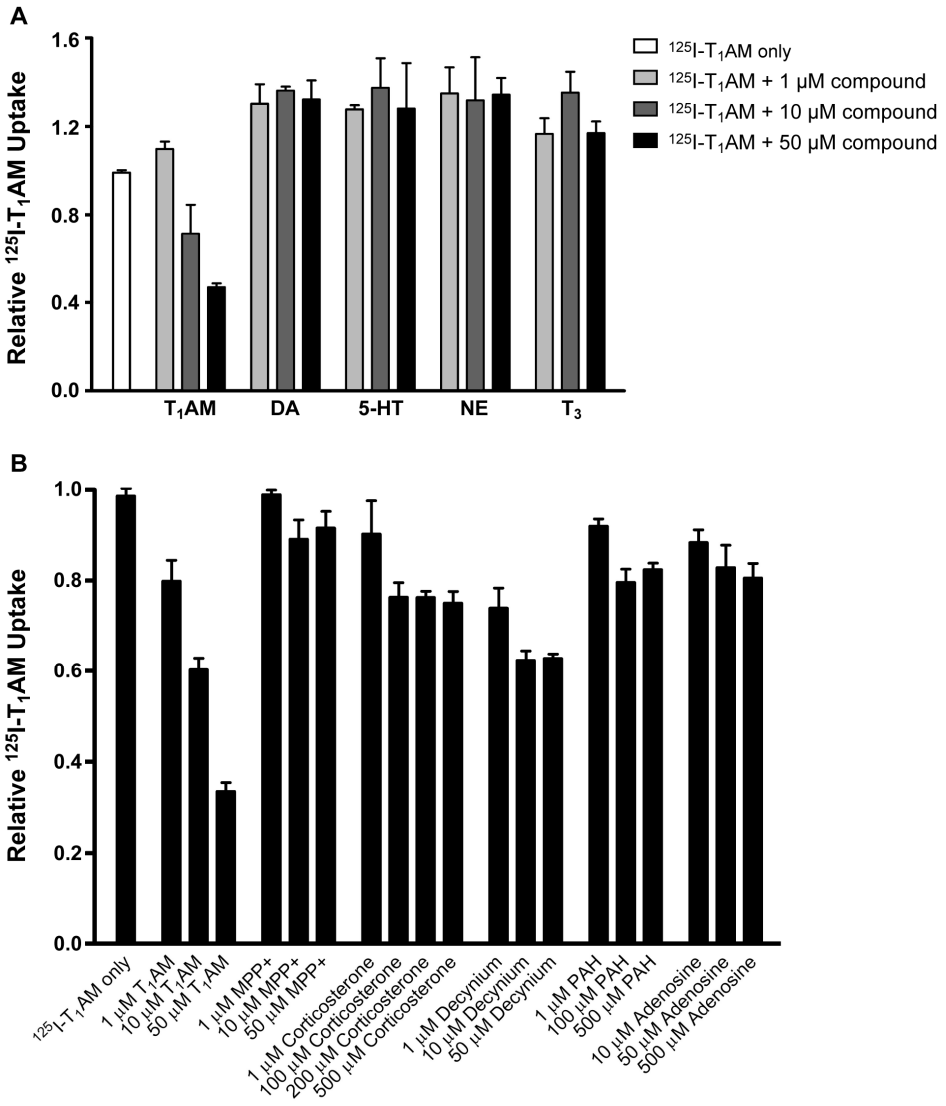

Figure 3. Specificity of T_1AM transport. A) Effect of other biogenic amines and thyroid hormone on T_1AM uptake. T_1AM uptake is not inhibited by the presence of excess unlabeled dopamine (DA), serotonin (5-HT), norepinephrine (NE), or thyroid hormone (T_3), showing that uptake is specific to T_1AM. B) Effect of other compounds on T_1AM uptake. T_1AM uptake is not significantly or dose-dependently inhibited by other substrates and inhibitors of the OCT/OAT/ENT transporter families, suggesting that T_1AM uptake does not occur via these transporters. 1-methyl-4-phenylpyridinium (MPP^+) is a prototypical organic cation and substrate of the OCT family of transporters as well as PMAT, and para-aminohippurate (PAH) is a prototypical organic anion and substrate of the OAT family of transporters. The nucleoside adenosine is a substrate of the ENT family of transporters. Corticosterone is an OCT inhibitor and decynium is an OCT and PMAT inhibitor. In both graphs, relative ^{125}I-T_1AM uptake values are normalized to the maximum uptake signal (DPM radioactivity counts) obtained with ^{125}I-T_1AM incubation alone.

Testing of potential candidate transporters for uptake of T_1AM

Our next step in identifying T_1AM transporters was a rational candidate-based approach. Despite the observed lack of competition of T_1AM uptake by prototypical substrates of certain transporter families, it is still conceivable that T_1AM may be a substrate of one of these transporters, but that the existence of multiple T_1AM transporters could mask the effects of these substrates. Our pharmacological characterization of T_1AM uptake revealed sodium- and chloride-independence, which is a property shared by the SLC22 family of transporters (16), as well as by PMAT (SLC29A4) (15) and the thyroid hormone transporter MCT8 (SLC16A2) (17). Additionally, as an organic cation, T_1AM is a logical candidate substrate of the polyspecific organic ion transporters of the SLC22 family. Being a monoamine, T_1AM could likewise be transported by PMAT, and because of its close structural similarity to thyroid hormone, might be a substrate of MCT8. Thus, we directly tested several of these transporters for uptake of T_1AM using stable cell lines overexpressing the transporters. While the stable cell lines exhibited increased uptake of their respective control substrates, none of these tested candidate transporters displayed increased uptake of T_1AM relative to empty vector stably transfected cells (Figure 4). Together with the competition experiments performed, these direct transport studies strongly suggest that some of the most likely organic ion and monoamine transporters are not responsible for T_1AM uptake and instead a unique mechanism is involved. Identification of T_1AM transporters thus requires a more general approach, as the transporters may be previously characterized transporters of unrelated compounds or orphan transporters.

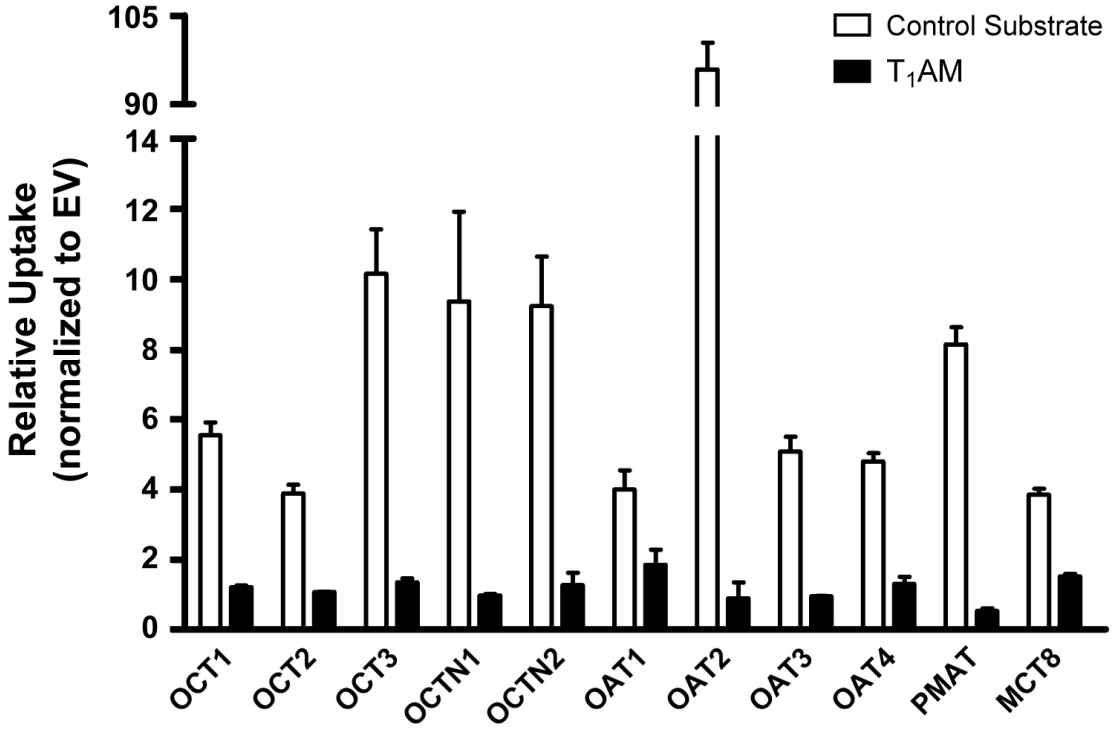

Figure 4. Uptake of control substrates and T_1AM in cells expressing various candidate transporters. A selection of logical candidate transporters were tested for uptake of T_1AM in FlpIn HEK 293 cells (or MDCK for PMAT) stably expressing the transporter. The control substrates tested were MPP^+ (OCT1, OCT2, OCT3, PMAT), TEA (OCTN1), carnitine (OCTN2), PAH (OAT1), acyclovir (OAT2), estrone sulfate (OAT3, OAT4), and T_3 (MCT8). Relative to empty vector-transfected cells, an increased uptake of at least four-fold was observed for the control substrates, but no significant increase in uptake was found for T_1AM. For each substrate, uptake values are normalized to the average uptake signal measured for that particular substrate in the empty vector-transfected cells.

Nuclear uptake of T_1AM occurs in HepG2 cells

To determine whether intracellular T_1AM is taken up into the nucleus, we isolated nuclear and cytoplasmic fractions of HepG2 cells after incubation with ^{125}I-T_1AM at various time points from 15 min to 4 hr. T_1AM indeed accumulates in the nucleus and uptake saturates with time. For comparison, T_3 nuclear uptake experiments were also performed at the same time. A greater percentage of T_1AM (~7.4%) appears to

accumulate in the nucleus when compared with T_3 (~3.6%), and saturation of T_1AM nuclear uptake occurs by 2 hr vs. 45 min for T_3 (Figure 5). The nuclear uptake observed for T_3 is comparable to that previously reported (18). Our data suggests that T_1AM is accumulated in nuclei in a similar fashion as T_3, although amount of accumulation and saturation time differ.

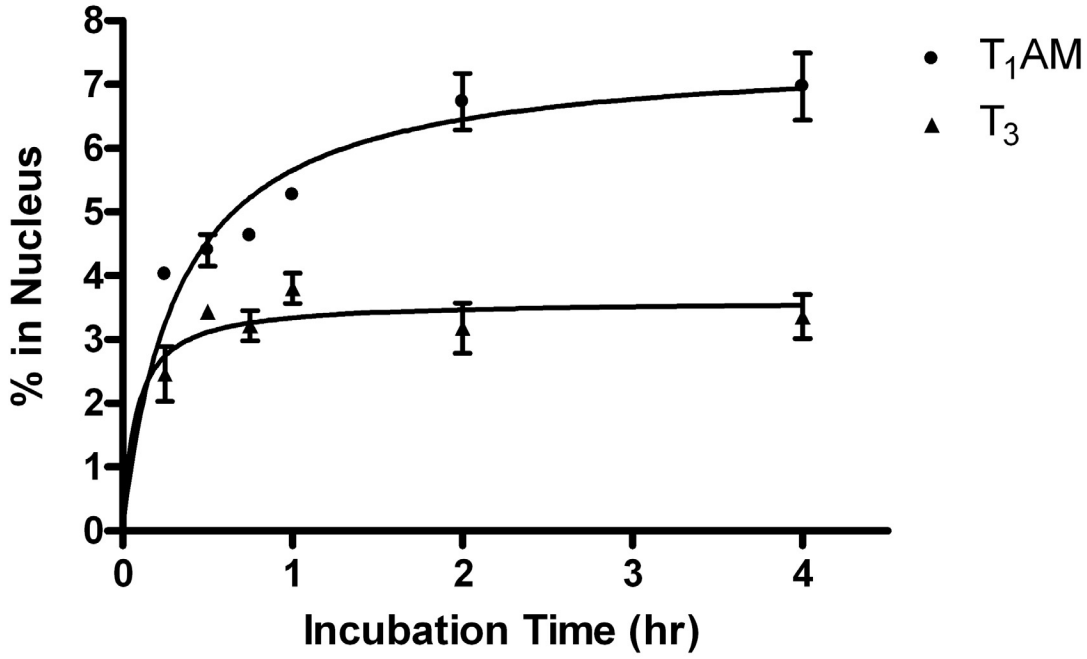

Figure 5. Nuclear uptake of T_1AM in HepG2 cells. Cellular fractionation of cells after incubation with radiolabeled T_1AM and T_3 reveals accumulation of T_1AM into nuclei in a manner similar to that of T_3, with an even greater percentage of intracellular T_1AM found in the nucleus. Nuclear uptake of T_1AM and T_3 is expressed as the percentage of radioactivity measured in the nucleus out of the total radioactivity measured (cytoplasmic plus nuclear fractions).

Discussion

T_1AM is a recently discovered endogenous metabolite of thyroid hormone with dramatic physiological actions when administered *in vivo*. In the current study, specific

cellular uptake of T_1AM was observed in a variety of cultured cell lines, suggesting a ubiquitous transport mechanism consistent with the widespread tissue accumulation of T_1AM and its wide range of actions, including hypothermia, bradycardia, hyperglycemia, and general behavioral inactivity. Although multiple transporters throughout the body likely contribute to intracellular accumulation of T_1AM, the uptake mechanism is relatively specific. Because thyronamines are the only molecules found to compete with T_1AM for uptake, it appears that the cellular uptake mechanism of T_1AM is specific for certain thyronamines and is distinct from that of the classical monoamine neurotransmitters, thyroid hormone, and other organic ions. Nearly all of the other thyronamines inhibited uptake of T_1AM, but varied somewhat in potency. The specific iodination states of the thyronamines, therefore, are likely to be important to some degree for uptake. Interestingly, the only thyronamine not found to compete with T_1AM for uptake, T_4AM, was also the only other member of the thyronamine compounds that did not display inhibitory activity against the vesicular monoamine transporter VMAT2 (7).

Many endogenous compounds and xenobiotics have multiple transporters responsible for their uptake into cells. For example, although thyroid hormones had been originally thought to enter target cells by passive diffusion, several transport mechanisms are now known to be responsible for their uptake. A broad range of transporter types mediate intracellular entry of thyroid hormones, including monocarboxylate transporters, amino acid transporters, and classic multispecific organic anion/cation transporters such as several OATP family members (19-21). The transport of thyroid hormones into their target tissues by saturable mechanisms is critical for proper physiological control of both their action and metabolism. Thus, to gain an understanding of the physiological

function and regulation of T_1AM, it is necessary to study its transport mechanisms into cells. The discovery of a specific transport mechanism for T_1AM presented in this study provides important additional insight into the role of this relatively new class of signaling molecules, although further elucidation of the particular transporters involved is required.

Although TAAR1 is an extracellular receptor known to be a target of T_1AM, and while T_1AM does not bind nuclear thyroid hormone receptors (1), observation of nuclear uptake of T_1AM, to an even greater degree than that of T_3, which exerts its actions by binding to nuclear thyroid hormone receptors to regulate transcription of target genes, suggests an intriguing possibility that T_1AM might also play a role in transcriptional regulation by binding its own nuclear receptors. Nuclear uptake of T_1AM also increases the significance and importance of plasma membrane transport into the cell as a necessary means of delivering T_1AM to its nuclear targets. In addition, although rapid effects of T_1AM are observed *in vivo*, presumably resulting from non-transcriptional effects, nuclear uptake of T_1AM expands its potential mechanisms of action and physiological roles.

In conclusion, we have demonstrated that there exists specific intracellular transport of T_1AM, an important endogenous metabolite of thyroid hormone with physiological actions opposite those of its precursor and with previously demonstrated functions as a neuromodulator. Identification of the particular transporters responsible for T_1AM uptake would provide additional insight into its mechanism of action and specific biological roles, but these transporters currently remain unknown. Transporters involved in uptake of structurally related compounds or with substrate profiles that would appear to include molecules like T_1AM are, interestingly, not involved in cellular uptake

of T_1AM. On the other hand, given the apparent narrow specificity of the T_1AM uptake mechanisms for thyronamines, it is likely that either uncharacterized, orphan transporters or transporters involved in uptake of unrelated compounds may be involved in T_1AM uptake. Because our rational candidate-based approach did not identify a T_1AM transporter, a more general, large-scale may be more successful in identifying T_1AM transporters. Also, the observation of nuclear uptake of T_1AM is particularly significant because it suggests that T_1AM may have actions similar to its thyroid hormone precursor in regulation of transcription. An additional motivation for identifying transporters of T_1AM is the potential elucidation of certain endocrine or neurological pathologies. Just as thyroid hormone transporter dysfunction has clearly been linked to particular disorders, such as MCT8 mutations leading to X-linked psychomotor retardation, improper functioning of T_1AM transporters could also lead to disease syndromes. Further studies into the recently discovered transport mechanism of T_1AM described here for the first time would be invaluable in our understanding of not only T_1AM action but also its potential implications for thyroid hormone regulation and possible involvement in thyroid-related pathologies.

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CHAPTER 3

Development of a large-scale RNAi screening method to identify T_1AM transporters

Abstract

Like its thyroid hormone precursor and other structurally related biogenic amines, T_1AM is transported across cell membranes by facilitated transport mechanisms. Notably, these transport mechanisms are specific for thyronamines and do not involve the likely candidate transporters of other monoamines, organic cations, or thyroid hormones. T_1AM transport has been observed in a variety of cell lines, suggesting a ubiquitous transport mechanism consistent with the widespread tissue accumulation of T_1AM and its wide range of physiological actions, and it is likely that multiple transporters may be involved in the cellular uptake of T_1AM. Because a rational candidate-based approach was unable to identify any T_1AM transporters, a more general approach towards transporter identification is required, as the transporters may be previously characterized transporters of unrelated compounds or orphan transporters. Here we describe the development of a novel transporter siRNA screening method to identify transporters involved in T_1AM uptake. A large-scale RNAi screen targeting the entire SLC superfamily of transporter genes reveals that the transport of T_1AM into cells involves multiple transporters and we identify eight transporters, including several organic ion and amino acid transporters, a monocarboxylate transporter, a nucleotide transporter, and a copper transporter, that may contribute to the uptake of T_1AM in HeLa cells. This type of transporter siRNA screening approach can be used in general to identify the constellation of transporters that participate in the intracellular disposition of compounds, in particular for systems in which there is high endogenous uptake that may pose challenges for an expression cloning method.

Introduction

Previously we have demonstrated that cellular uptake of T_1AM occurs in multiple cell types and that this process involves specific, saturable, and inhibitable transport mechanisms that are sodium- and chloride-independent, pH-dependent, and thyronamine-specific (1). Because plasma membrane transporters are crucial for all cells, controlling uptake and efflux compounds such as sugars, amino acids, nucleotides, hormones, organic and inorganic ions and drugs, the identification of T_1AM plasma membrane transporters would be invaluable to understanding the mechanism of action and physiological functions of this thyroid hormone derivative. For example, several monoamine transporters possess reuptake transporters that are critical for terminating their signaling at extracellular receptors and for recycling the compounds (2, 3). In addition, a variety of transporters are responsible for the cellular uptake of thyroid hormones and their consequent intracellular metabolism and action (4, 5). Moreover, mutations in the specific thyroid hormone transporter MCT8 have been linked to severe psychomotor retardation syndromes (6-8), highlighting the importance of proper functioning of plasma membrane transporters for the normal physiological actions of their substrates.

Because of the structural similarity between T_1AM and other monoamines and thyroid hormones, as well as the broad substrate specificity of certain classes of organic ion transporters, a rational candidate-based approach was previously used to test various characterized transporters for T_1AM uptake function, but none of these displayed T_1AM transport activity (1). Identification of T_1AM transporters thus requires a more general approach. Since high levels of endogenous T_1AM uptake activity occur in a wide variety

of cell lines, an expression cloning approach is likely to be difficult in terms of the ability to detect increased uptake levels over background levels. Thus, we considered that a knockdown rather than overexpression approach may be more successful in identifying transporters responsible for T_1AM uptake.

The solute carrier (SLC) superfamily of transporters consists of 46 families and 403 transporter genes and includes both plasma membrane transporters that function as passive transporters, ion-coupled transporters, and exchangers, as well as intracellular transporters such as vesicular and mitochondrial transporters. The remaining non-SLC transporter-related genes include ion channels, aquaporins, and ATP-driven efflux pumps (9). Since we were interested in transporters involved in the cellular uptake of T_1AM across the plasma membrane, and wished to use an unbiased, general method for identifying specific T_1AM transporters, we considered all SLC transporters, rather than just the previously tested SLC transporter candidates, for T_1AM uptake.

Here we describe the development of a novel, high-throughput RNAi screening approach as a relatively rapid way of testing a large number of transporters for function in T_1AM transport. Specifically, we used a library consisting of siRNAs targeting all of the currently known SLC transporters to knock down each of the transporters and identify those which displayed reduced T_1AM uptake function as a consequence of decreased transporter expression.

Materials and methods

siRNA Transfection and Screening

A custom siRNA library consisting of three unique targets each against 403 transporters, for a total of 1209 siRNAs, was obtained from Ambion. Positive control GAPDH siRNA and negative control siRNA were also from Ambion. HeLa cells were transfected with individual siRNAs using NeoFX (Ambion) using the standard method of reverse transfection in 96-well tissue culture plates according to the manufacturer's protocols. 48 h after transfection, cells were washed and preincubated with prewarmed KRTH (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM Tris, 10 mM HEPES, pH 7.4) for 15 min at 37°C and T_1AM uptake assays were conducted as previously described (1). T_1AM uptake for transporter siRNA-transfected cells was compared relative to that of negative control siRNA-transfected cells.

For each transfection, a set of cells were also transfected with GAPDH siRNA and GAPDH gene knockdown was verified by qRT-PCR as a positive control for successful transfection. Transporter gene knockdown of the final eight transporter candidates was also ultimately verified by qRT-PCR. Gene expression levels were calculated using the comparative threshold cycle (Ct) method. 48 h after siRNA transfection, HeLa cells were harvested using the Cells-to-Signal protocol (Ambion) and gene-specific primers and probes were obtained as TaqMan assays (Applied Biosystems). The Ct values resulting from amplification were normalized to either PGK1 when assessing GAPDH knockdown or GAPDH when assessing transporter knockdown to give ΔCt values. The target ΔCt values were then normalized with the ΔCt values of the calibrator samples, which consisted of cells transfected with negative control siRNA, to

give ΔΔCt values. The formula $2^{-\Delta\Delta Ct}$ was used to obtain the normalized gene expression levels.

Transport Experiments Performed for Transporters Identified by siRNA Screening

The eight transporters eventually identified as potential transporters of T_1AM were individually cloned and the cDNA containing the complete CDS of each transporter was introducted into the pcDNA5/FRT vector. Stable HEK FlpIn cells were then generated as previously described (1). For some of the transporters with high expression levels in HEK cells, MDCK or CHO FlpIn stable cell lines were also constructed. Two variants of SLCO3A1 were tested using CHO FlpIn stable cell lines kindly provided by the laboratory of Bruno Stieger from the University Hospital Zurich, Switzerland (10). Transport assays for radiolabeled control substrates and T_1AM were conducted for each stable cell line under experimental conditions identical to those used during siRNA screening, with the exception that assays were performed in a 24-well rather than 96-well plate format.

Statistical Analyses

Statistical analyses were performed with the GraphPad Prism version 4.00 software, with values expressed as means ± SD.

Results

Development of an RNAi screening method to identify T_1AM transporters

Using a library of siRNAs against 403 membrane transporters consisting of all of the SLC series of transporters characterized at this time, including pseudogenes and orphan transporters associated with this superfamily, the goal was to identify transporters that, when knocked down, resulted in decreased uptake of T_1AM. Table 1 lists the 46 families of transporters targeted by the siRNA library. The siRNAs were designed and constructed by Ambion and received as a custom transporter siRNA library. Three different siRNAs per target, for a total of 1209 unique siRNAs, were individually transfected into HeLa cells and T_1AM uptake was measured 48 hours after transfection under the standard uptake conditions used previously. Figure 1 shows the experimental workflow of the RNAi screen. Transporters identified as positive hits were those for which at least two of the three siRNA probes against that particular transporter target resulted in a 30% or greater reduction in T_1AM uptake when compared to cells transfected with a negative control siRNA targeting no specific part of the genome. A 30% reduction in T_1AM uptake corresponds to a reduction level that is 1.5 standard deviations greater than the mean reduction in uptake observed for all 1209 siRNAs. A positive control of siRNA against GAPDH was used to confirm successful transfection and gene knockdown. For each set of transfection experiments, the positive GAPDH siRNA control transfection resulted in at least 70% knockdown of GAPDH mRNA as determined by qRT-PCR.

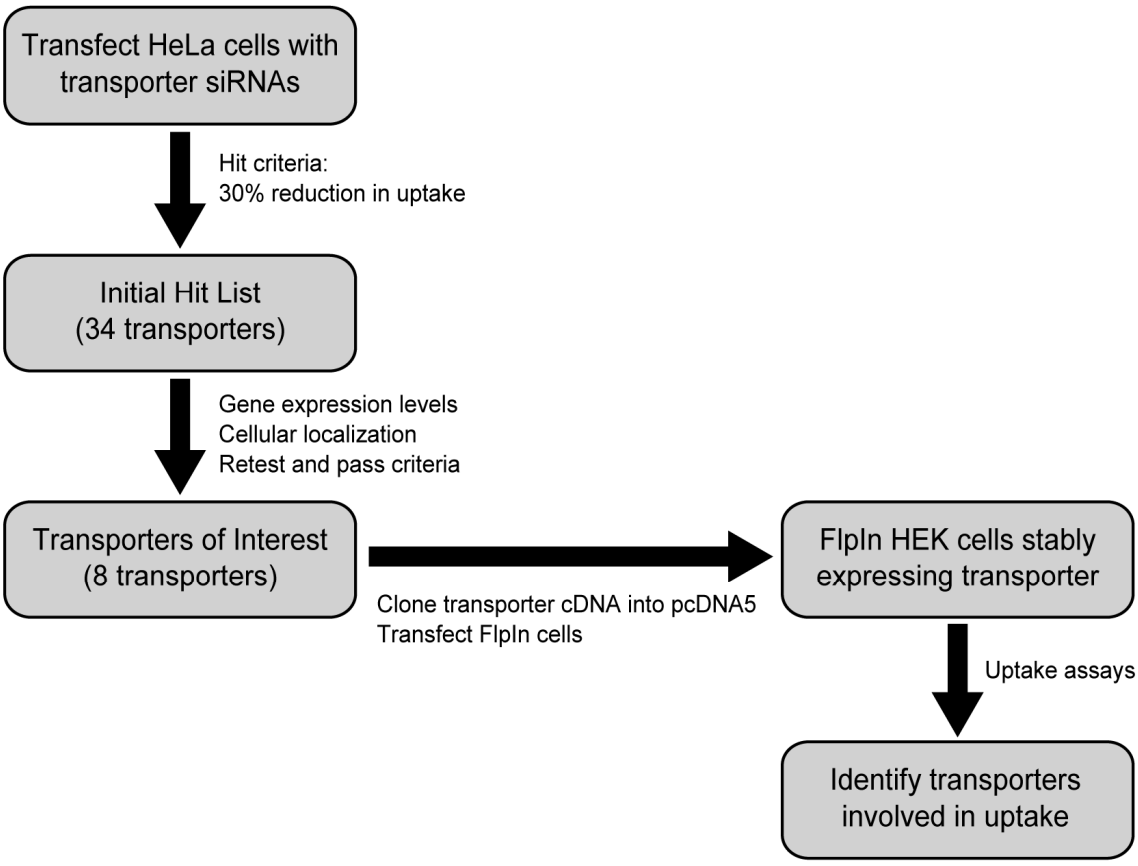


Figure 1. RNAi screen design. The experimental scheme for high-throughput screening of the transporter siRNA library to identify transporters involved in uptake of T_1AM is shown. The siRNA library used for the screen consisted of 403 total transporter targets, including all SLC series transporters, with three distinct siRNAs per target. A total of 1209 unique siRNAs were individually transfected into HeLa cells using NeoFX transfection agent and the transfected cells were assayed for uptake of T_1AM 48 hours after transfection. A positive hit was defined as a target for which at least two of the three siRNAs produced a 30% or greater reduction in T_1AM uptake, when compared to cells transfected with negative control siRNA; these hits comprised the preliminary transporter candidates. After analysis of endogenous gene expression levels and cellular localization (plasma membrane vs. vesicular/mitochondrial membrane), the transporter siRNAs were retested and the candidate transporters were reduced to a subset of eight transporters that were each cloned and transfected into FlpIn HEK cells for direct measurement of T_1AM uptake.

Table 1. The 46 families of 403 transporters in the HUGO Solute Carrier Series.

SLC1: The high affinity glutamate and neutral amino acid transporters	SLC24: The $Na^+/(Ca^{2+}\text{-}K^+)$ exchangers
SLC2: The facilitative GLUT transporters	SLC25: The mitochondrial carriers
SLC3: The heavy subunits of the heteromeric amino acid transporters	SLC26: The multifunctional anion exchangers
SLC4: The bicarbonate transporters	SLC27: The fatty acid transport proteins
SLC5: The sodium glucose cotransporters	SLC28: The Na^+-coupled nucleoside transporters
SLC6: The Na^+- and Cl^--dependent neurotransmitter transporters	SLC29: The facilitative nucleoside transporters
SLC7: The cationic amino acid transporter/glycoprotein-associated	SLC30: The zinc efflux proteins
SLC8: The Na^+/Ca^{2+} exchangers	SLC31: The copper transporters
SLC9: The Na^+/H^+ exchangers	SLC32: The vesicular inhibitory amino acid transporter
SLC10: The sodium bile salt cotransporters	SLC33: The Acetyl-CoA transporter
SLC11: The proton coupled metal ion transporters	SLC34: The type II Na^+-phosphate cotransporters
SLC12: The electroneutral cation-Cl^- cotransporters	SLC35: The nucleoside-sugar transporters
SLC13: The human Na^+-sulfate/carboxylate cotransporters	SLC36: The proton-coupled amino acid transporters
SLC14: The urea transporters	SLC37: The sugar-phosphate/phosphate exchangers
SLC15: The proton oligopeptide cotransporters	SLC38: The System A & N, Na^+-coupled neutral amino acid transporters
SLC16: The monocarboxylate transporters	SLC39: The metal ion transporters
SLC17: The vesicular glutamate transporters	SLC40: The basolateral iron transporter
SLC18: The vesicular amine transporters	SLC41: The MgtE-like magnesium transporters
SLC19: The folate/thiamine transporters	SLC42: The Rh ammonium transporters
SLC20: The type III Na^+-phosphate cotransporters	SLC43: The Na^+-independent, system L-like amino acid transporters
SLC21/SLCO: The organic anion transportins	SLC44: The choline-like transporters
SLC22: The organic cation/anion/zwitterion transporters	SLC45: The putative sugar transporters
SLC23: The Na^+-dependent ascorbic acid transporters	SLC46: The heme transporters

Preliminary transporter candidates resulting from siRNA screening

SLC6A18	Neurotransmitter transporter	SLCO6A1	Organic anion transporter
SLC7A1	**Cationic amino acid transporter**	SLC26A11	Anion exchanger
SLC7A2	Cationic amino acid transporter	SLC27A1	Fatty acid transporter
SLC7A14	Cationic amino acid transporter	SLC28A3	Concentrative Na^+-nucleoside transporter
SLC9A4	Sodium/Hydrogen exchanger	**SLC29A2**	**Equilibrative nucleoside transporter**
SLC9A5	Sodium/Hydrogen exchanger	SLC30A8	Zinc transporter
SLC9A6	Sodium/Hydrogen exchanger	SLC30A10	Zinc transporter
SLC9A7	Sodium/Hydrogen exchanger	**SLC31A1**	**Copper transporter**
SLC9A8	Sodium/Hydrogen exchanger	SLC35C2	Ovarian cancer overexpressed 1
SLC9A9	Sodium/Hydrogen exchanger	SLC35D2	UDP-N-acetylglucosamine transporter
SLC16A7	**Monocarboxylate transporter**	SLC37A1	Glycerol-3-phosphate transporter
SLC17A5	**Anion/Sugar transporter**	SLC42A2	Rhesus blood group, B glycoprotein
SLCO1A2	Organic anion transporter	SLC42A3	Rhesus blood group, C glycoprotein
SLCO3A1	**Organic anion transporter**	**SLC43A3**	**System-L like amino acid transporter**
SLCO4A1	**Organic anion transporter**	SLC45A2	Putative sugar transporter
SLCO4C1	Organic anion transporter	SLC45A4	Putative sugar transporter
SLCO5A1	Organic anion transporter	SLC46A1	Heme transporter

Preliminary candidates consisted of 34 transporters out of the 403 included in the siRNA library, giving an 8% hit rate. Only twelve of these transporters (SLC7A1, SLC16A7, SLC17A5, SLCO3A1, SLCO4A1, SLC29A2, SLC31A1, SLC43A3, SLC9A6, SLC9A9, SLC35C2, and SLC35D2) are expressed in HeLa cells, and four of these (SLC9A6, SLC9A9, SLC35C2, and SLC35D2) are only expressed intracellularly rather than at the plasma membrane. The final remaining eight transporter candidates are shown in bold.

Using these initial criteria, 34 transporters were identified (Table 1, bottom). To eliminate potential false positive hits, gene expression levels of the transporters in HeLa cells were determined experimentally by RT-PCR (Figure 2) and verified using previously published microarray data from The Genomics Institute of the Novartis Foundation (11); those transporters not expressed in HeLa cells were discarded. Of the twelve transporters remaining, only eight are expressed at the plasma membrane as opposed to intracellular vesicles, based on Entrez Gene, an NCBI database of gene-specific and general protein information (12). The final list of transporters both expressed in HeLa cells and localized to the plasma membrane are highlighted in bold in Table 1 and included several organic ion transporters, a nucleoside transporter, an amino acid transporter, a monocarboxylate transporter, a copper transporter, and an orphan transporter belonging to the SLC43 family. The average levels of T_1AM uptake resulting from transfection with the siRNAs against these transporters is displayed in Figure 3. These eight transporters were retested at least three times, and the data shown depict average values from a representative experiment with each condition performed in triplicate. In addition, we directly measured levels of gene knockdown of these eight transporters, rather than relying on only a GAPDH siRNA positive transfection control. Knockdown efficiency of these transporters as determined by qRT-PCR varied among the different transporters but was typically at least 50-60%, rather than the 70% or greater knockdown observed for GAPDH (Figure 4), which is not unexpected given the extent of revalidation of the commercially available GAPDH siRNA, as opposed to the previously unvalidated efficacy of the transporter siRNAs.

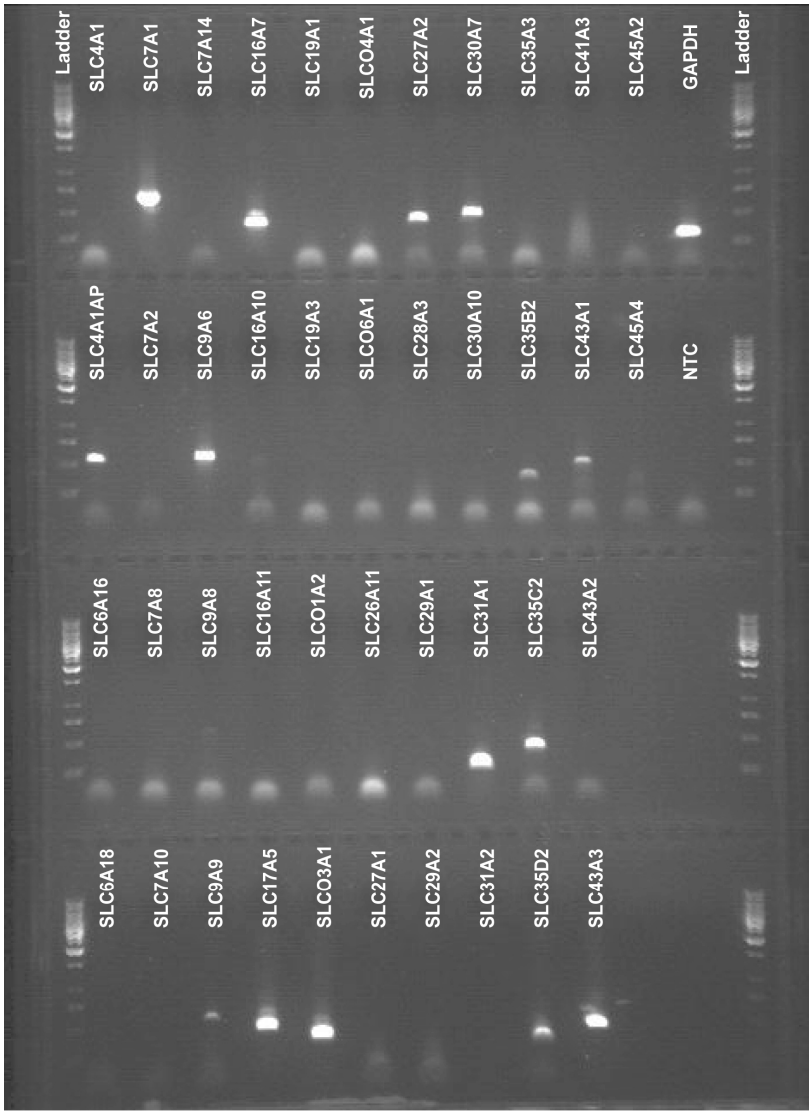


Figure 2. RT-PCR to determine endogenous expression of a selection of membrane transporters in HeLa cells. RNA was isolated from HeLa cells using the Micro-to-Midi Total RNA Purification System (Invitrogen) and cDNA was constructed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Primers targeting the various transporters were used for PCR with the cDNA template and the products were run on an agarose gel. GAPDH primers were used as a positive control, and a no template control (NTC) in which GAPDH primers without any cDNA template in the reaction is also shown. Each of the lanes is labeled with its corresponding target. The transporters shown include some transporters that were not preliminary candidates (*i.e.* SLC4A1, SLC4A1AP, SLC6A16, refer to Table 1). Expression levels were also examined using previously published microarray data from The Genomics Institute of the Novartis Foundation (Ref. 11 in the manuscript), which revealed expression of SLCO4A1 and SLC29A2 in HeLa cells, despite the negative RT-PCR results shown on the gel. In this way, twelve of the 34 preliminary transporter candidates were found to be expressed in HeLa cells: SLC7A1, SLC9A6, SLC9A9, SLC16A7, SLC17A5, SLCO3A1, SLCO4A1, SLC29A2, SLC31A1, SLC35C2, SLC35D2, and SLC43A3. Note that SLC4A1AP, SLC27A2, SLC30A7, SLC35B2, and SLC43A1, although showing HeLa cell expression on the gel, were not ultimately chosen as preliminary transporter candidates because they did not meet the criteria of 30% reduction in T_1AM uptake after siRNA transfection.

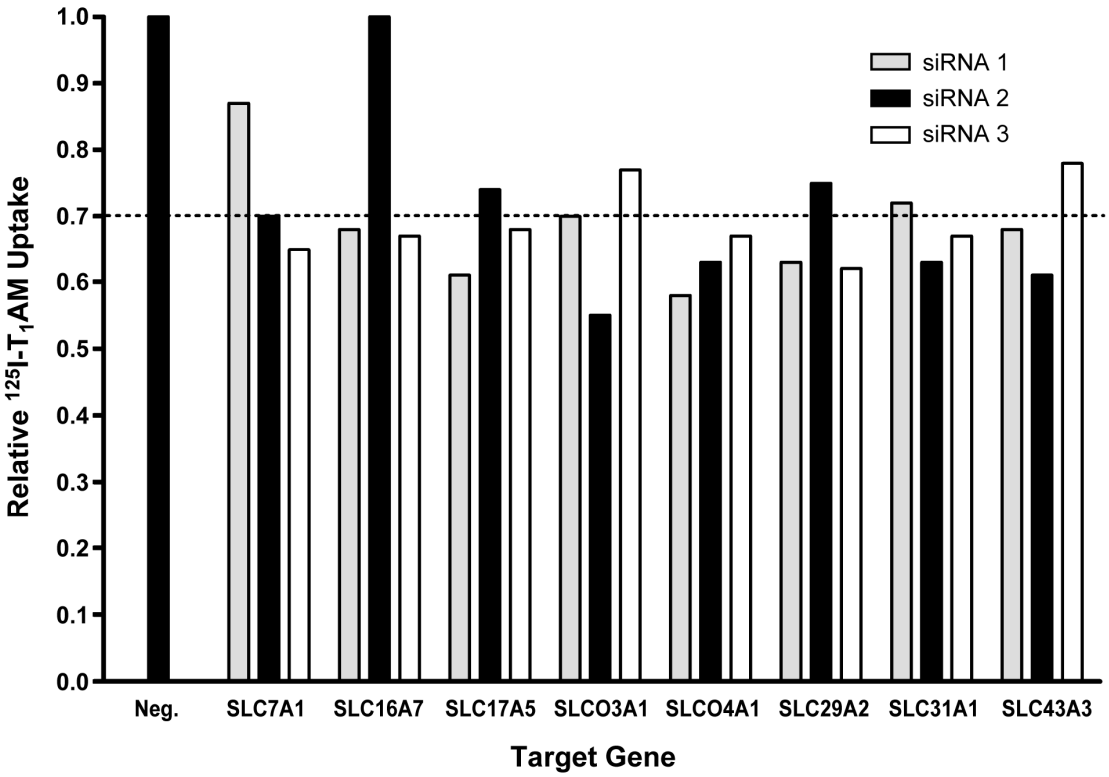


Figure 3. Candidate T_1AM transporters identified in RNAi screen. Average levels of T_1AM uptake in HeLa cells transfected with siRNAs targeting the final eight transporter candidates identified in the screen are depicted, expressed relative to uptake in cells transfected with negative control (Neg.) siRNA. Each value represents the mean of triplicate determinations with variations of 1-6%.

Functional assessment of candidate transporters resulting from RNAi screening

To examine the effect of overexpressing the transporters identified in the RNAi screen, we cloned each of the transporters and transfected them into FlpIn HEK cells. One transporter, SLC29A2, is unlikely to be a T_1AM transporter because the uptake of the control substrate, inosine, was enhanced, whereas the uptake of T_1AM was not. However, we were unable to use overexpression to confirm or refute the results of the RNAi screen for the remaining seven transporters.

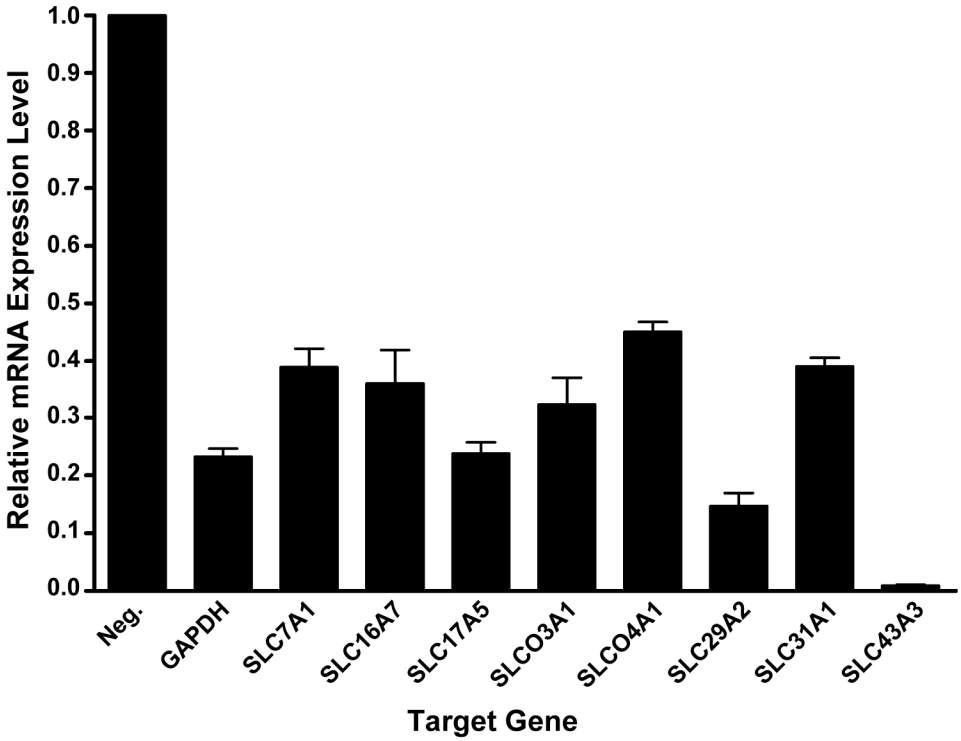


Figure 4. Expression of membrane transporters after siRNA transfection. The mRNA levels of GAPDH after positive control GAPDH siRNA transfection were determined by qRT-PCR and used to confirm successful transfection and gene knockdown for each set of transfections performed during transporter siRNA library screening. GAPDH expression was normalized to PGK1 and for each transfection experiment at least 70% knockdown of GAPDH mRNA was observed. After obtaining the final eight transporter candidates, qRT-PCR was used to determine levels of gene knockdown after siRNA transfection for each of these specific transporters and was typically at least 50-60%, although knockdown efficiency varied among the different transporters. Transporter expression levels were normalized to GAPDH in cells transfected only with transporter siRNA, and the levels obtained after transfection with each of the three siRNAs targeting the particular transporter were averaged. All expression levels are shown relative to the expression of the corresponding target gene in cells transfected with negative control (Neg.) siRNA and were calculated using the comparative threshold cycle (Ct) method.

Five of the seven transporters (SLC7A1, SLC16A7, SLC31A1, and the OATPs SLCO3A1 and SLCO4A1) are abundantly expressed in multiple cell lines including HEK cells (11); therefore their activities are difficult to measure over the background, and we

did not observe increased uptake of various prototypical substrates or of T_1AM. For instance, the cationic amino acid transporter SLC7A1 transports lysine and arginine (13) and substrates of the monocarboxylate transporter SLC16A7 include lactate and pyruvate (14). SLCO3A1 and SLCO4A1 have been found to transport thyroid hormones and prostaglandins when expressed in *X. laevis* oocytes and in CHO cells (10, 15, 16). However, we were unable to detect a significant increase in uptake of the various control substrates in HEK cells stably overexpressing these transporters, so their potential role in transport of T_1AM is unknown. In an attempt to find a lower background system, MDCK and CHO stable cell lines were constructed or obtained for these five transporters but they also did not exhibit a reproducible increase in uptake of T_1AM or any control substrate. Additionally, HEK 293T and COS-1 cells were transiently transfected with the transporter constructs in an attempt to obtain higher, although temporary, expression levels and a resulting increase in function, but these likewise displayed no significant enhancement of substrate uptake.

SLC43A3 is an orphan transporter of the sodium-independent, system L-like amino acid transporters, and interestingly is highly expressed in the thyroid (Figure 5), but the SLC43A3 HEK FlpIn stable cell line failed to show increased uptake of any compound tested, including T_1AM, T_3, T_4, tyrosine, and phenylalanine. An obvious challenge in studying an orphan transporter like SLC43A3 is the lack of a known substrate with which to test functionality of the stable cell line. We constructed a SLC43A3-GFP stable cell line in parallel and, while we did observe clear plasma membrane localization of the transporter (Figure 6), suggesting functional expression, we did not identify any substrate of this transporter.

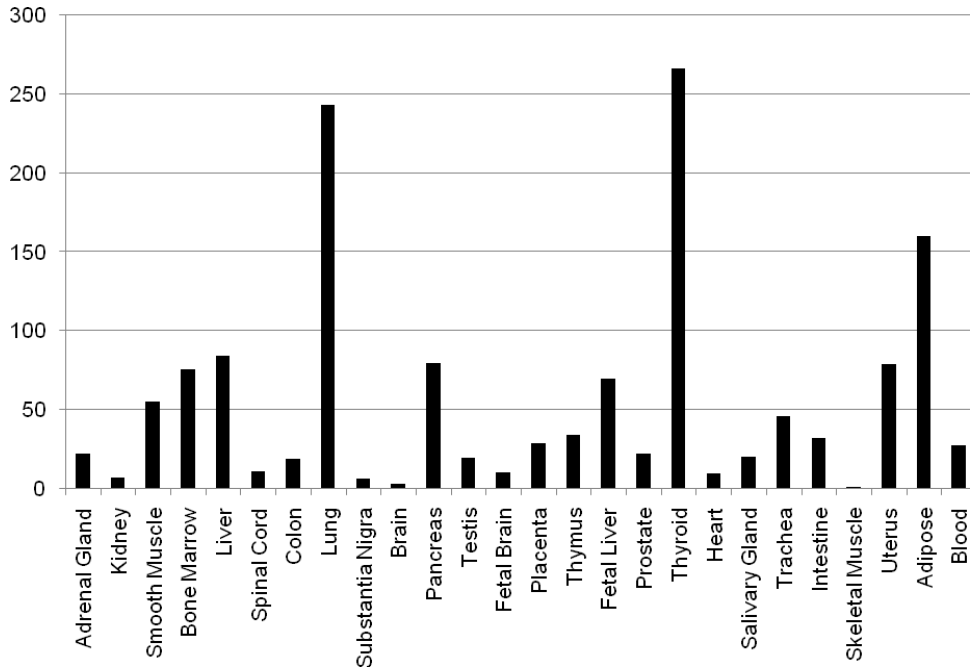
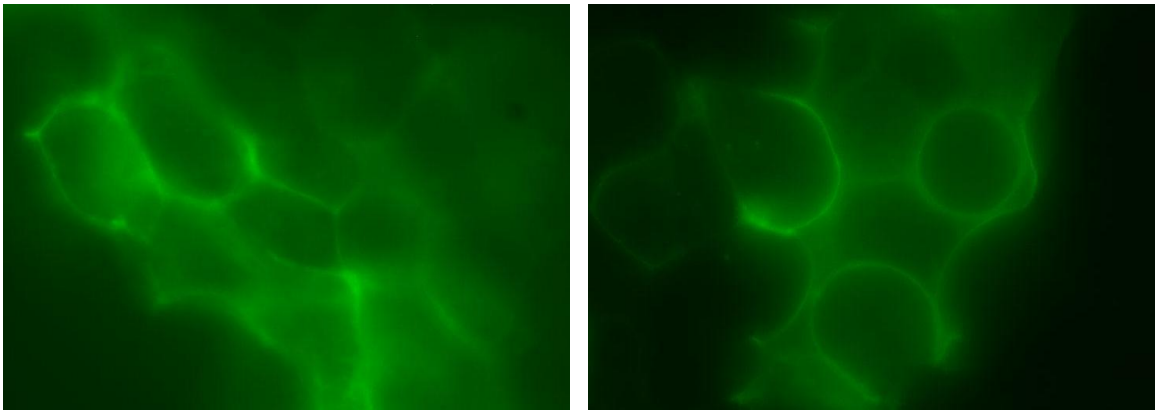


Figure 5. Tissue expression of SLC43A3. cDNA was constructed by reverse transcription from RNA of various tissues and qRT-PCR was performed using an SLC43A3-specific probe. Relative expression levels are shown normalized to skeletal muscle, the tissue displaying the lowest level of expression.



SLC43A3-GFP **SLC22A1-GFP**

Figure 6. Localization of SLC43A3-GFP and SLC22A1-GFP fusion proteins in stably expressing HEK FlpIn cells. GFP fluorescence is clearly seen at the plasma membrane, suggesting functional expression of the SLC43A3 transporter protein. SLC22A1-GFP HEK FlpIn cells, previously validated for functional uptake of the control substrate MPP^+, are also shown for comparison.

Discussion

Specific and saturable transport mechanisms for the cellular uptake of T_1AM have been recently identified and described (1). In an attempt to identify plasma membrane transporters responsible for the uptake of T_1AM, we developed a high-throughput RNAi screening method in which a library of siRNAs targeting all of the solute carrier series of membrane transporters was transfected into HeLa cells and the siRNAs producing the greatest degree of reduction of T_1AM uptake were identified. The transporters targeted by these siRNAs are likely to be involved in T_1AM uptake into cells. A total of 34 out of 403 transporters were initially identified as facilitating T_1AM uptake in HeLa cells. The 34 included several heavy metal transporters, and various inorganic and organic ion transporters. As would have been expected, none of the likely candidate transporters previously tested and ruled out as T_1AM transporters (1) displayed reduced T_1AM uptake after siRNA transfection. After examining endogenous expression levels in HeLa cells and cellular localization of the 34 transporters, we obtained a list of eight transporters that were retested and consistently displayed decreased T_1AM uptake function when knocked down.

Direct testing of transporters identified by the RNAi screen, however, was inconclusive. Even though decreased T_1AM uptake was observed when several transporters were knocked down in HeLa cells, overexpression of these transporters did not show the expected increase in T_1AM uptake. One of the challenges posed by the particular transporter candidates resulting from the RNAi screen included high background expression of the transporter, making it difficult to detect an increase in substrate uptake over an already high level. On the other hand, the lack of a control

substrate for the orphan transporter SLC43A3, which has relatively low expression in HEK cells, made it difficult to determine whether the stable cell line created was indeed functional, or if experiments were being performed under optimal uptake assay conditions particular to this transporter.

Moreover, some transporter candidates identified by the RNAi screen might in fact be the result of indirect effects. Of the 34 initial candidates, only eight transporters are expressed in HeLa cells and localized to the plasma membrane. The high rate of false positives is likely due to secondary effects, such as cellular toxicity or disruption of membrane integrity. In addition, knocking down a transporter may affect the cellular content of another substance that influences the activity of the true transporter directly responsible for ligand uptake. For instance, SLC16A7 is a lactate transporter, and although it cannot be ruled out as a T_1AM transporter (indeed, the thyroid hormone transporter MCT8 is an SLC16 family member), this transporter may affect the intracellular ion content and pH of cells and consequently alter T_1AM uptake.

Nevertheless, the RNAi screening method developed here for T_1AM is a broadly applicable approach to potentially identify all transporters involved in the uptake of any particular compound in a particular cell type or tissue. Unlike the use of expression cloning to identify a particular gene responsible for activity, such an RNAi screen is advantageous because it could in theory be used even in a system where there is high background uptake of the compound of interest, since it relies on the knockdown of the activity rather than the enhancement over background signal. This inhibitory screening method and the traditional technique of expression cloning can be mutually complementary approaches with their own benefits depending on the circumstances. For

instance, when there is a source of high activity compared to the expression system, a cDNA library is likely to provide an enriched pool of genes responsible for the activity, which is a major advantage in expression cloning. However, when all available sources exhibit similar levels of activity, it might be more difficult to construct a complete cDNA library representing each gene at a sufficiently high level for functional detection. In this case, a high-throughput siRNA library screen can be more technically feasible. First, rather than testing every gene of the genome, the set of genes examined can be specified to a certain subset of interest, in this case membrane transporters. In addition, the growing popularity of RNAi methods is resulting in an improvement in siRNA design algorithms employed in the creation of commercially available library collections. Finally, large numbers of siRNAs can be screened relatively quickly with optimized transfection conditions, followed by functional assessment.

In conclusion, using a novel RNAi screening method, we have identified eight transporters that when knocked down reproducibly result in reduced T_1AM transport in HeLa cells. It is possible that these transporters collectively participate in the regulation of intracellular levels of T_1AM. On the other hand, transport of T_1AM may also be mediated by a non-SLC transporter, as novel transporter genes are continually being discovered. Alternatively, a distinct transport mechanism altogether may be involved. For example, megalin has been identified as an endocytic receptor for the cellular uptake of steroid hormones including vitamin D, androgens, and estrogens, although megalin can act as a receptor for a wide variety of ligands (17). Potential T_1AM transport mechanisms thus include specific membrane uptake transporters and receptor-mediated endocytosis. These mechanisms could serve in regulation of T_1AM action in several

ways and are depicted in Figure 7. The action of T_1AM at extracellular targets such as TAAR1 may be terminated by its uptake into the cell, similar to the reuptake mechanisms for the monoamine neurotransmitters. Passage of T_1AM into the cell may also serve to provide access to intracellular targets, such as the vesicular monoamine transporter VMAT2, or to perform intracellular functions currently unknown. We have previously demonstrated saturable nuclear uptake of T_1AM, suggesting that T_1AM may have nuclear targets and may, like thyroid hormone, be involved in regulation of gene transcription. That nuclear uptake occurs is an additional motivation for identifying plasma membrane transporters of T_1AM. Future work includes using a lower background system, such as expression in oocytes, to further investigate whether or not the transporter candidates identified here might indeed play a role in T_1AM intracellular transport. In addition, endocytosis inhibitors could be useful in determining whether cellular uptake of T_1AM occurs by receptor-mediated endocytosis.

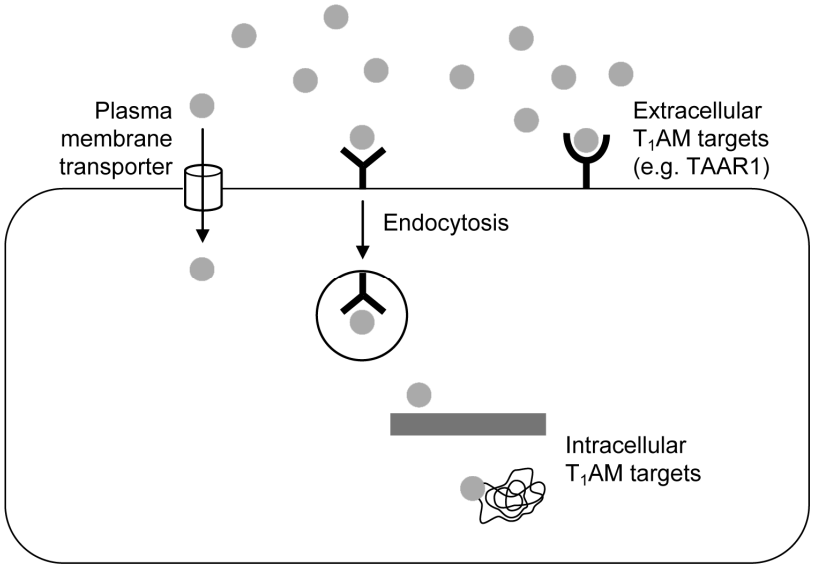


Figure 7. Potential T_1AM transport mechanisms include specific membrane uptake transporters and receptor-mediated endocytosis. The action of T_1AM (represented by gray circles) at extracellular targets such as TAAR1 may be terminated by its uptake into the cell, similar to the reuptake mechanisms for the monoamine neurotransmitters. Passage of T_1AM into the cell may also serve to provide access to intracellular targets or to perform intracellular functions currently unknown.

Acknowledgements

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CHAPTER 4

T_1AM selectively inhibits transport of thyroid hormones

Abstract

Thyroid hormone transporters are responsible for the cellular uptake of thyroid hormones, which is a prerequisite for their subsequent metabolism and action at nuclear thyroid hormone receptors. A recently discovered thyroid hormone derivative, T_1AM, has distinct biological effects that are opposite those of thyroid hormone. Here we investigate the effects of T_1AM on thyroid hormone transporters as a potential mechanism for its opposing effects on thyroid hormone action. Using COS-1 cells transfected with the multispecific organic anion transporting polypeptides (OATPs) 1A2, 1B3, and 1C1, as well as the specific thyroid hormone transporter MCT8, we show that T_1AM displays differential inhibition of T_3 and T_4 cellular uptake by these transporters. T_1AM inhibits T_3 and T_4 transport by OATP1A2 with IC_{50} values of 270 nM and 2.1 μM, respectively. T_4 transport by OATP1C1, which is thought to play a key role in thyroid hormone transport across the blood-brain barrier, is inhibited by T_1AM with an IC_{50} of 4.8 μM. T_1AM also inhibits both T_3 and T_4 uptake via MCT8, the most specific thyroid hormone transporter identified to date, with IC_{50} values of 83.1 μM and 26.1 μM, respectively. By contrast, T_1AM has no effect on thyroid hormone transport by OATP1B3. Although endogenous circulating levels of T_1AM have not yet been established, active localization mechanisms of thyroid hormone and its derivatives could result in local concentrations of T_1AM in certain regions of the brain that may exceed circulating levels and be sufficient to affect thyroid hormone transport. Given that OATP1A2, OATP1C1, and MCT8 are all present in the brain, T_1AM may play an important role in modulating thyroid hormone delivery and activity in specific target regions in the central nervous system.

Introduction

Thyroid hormone has many important physiological roles and is critical for growth, development, and regulation of metabolic processes throughout life (1). Entry of thyroid hormone into the cell is required for its action and metabolism, since it acts by binding to nuclear thyroid hormone receptors to regulate transcription of target genes. The deiodinases involved in thyroxine (T_4) conversion to the more active form of the hormone, 3,3',5-triiodothyronine (T_3), are also located intracellularly. Although the lipophilic hormone was originally believed to enter cells by passive diffusion, several plasma membrane transporters responsible for thyroid hormone uptake have recently been identified (2-4).

Some of the transporters now known to be involved in the uptake of thyroid hormones include members of the polyspecific organic anion transporting polypeptide (OATP) family (5) and the monocarboxylate transporter (MCT) family, in particular the specific thyroid hormone transporter MCT8 (6). The OATPs comprise a large family of proteins present in numerous organs and tissues that are responsible for the transport of a wide variety of ligands, including anionic as well as neutral and even cationic compounds such as bile salts, bilirubin and bilirubin glucuronides, estrogen conjugates, oligopeptides, prostaglandins, lipohphilic organic cations, and thyroid hormones (4). Some examples of OATPs capable of thyroid hormone transport include OATP1A2, which is expressed in the brain, liver, and kidney and likely plays a role in the delivery of T_4 to the kidney and across the blood-brain barrier, the liver-specific OATP1B3 involved in the uptake of T_4 into hepatocytes and the release of T_3 into blood plasma, and OATP1C1, the OATP with

the highest affinity and specificity for T_4 and expressed in brain capillaries, suggesting a particular importance for transport of T_4 across the blood-brain barrier (2).

In contrast to the numerous OATPs, which transport a wide variety of compounds aside from thyroid hormone, MCT8 is the most active and specific thyroid hormone transporter identified to date (7) that has also been clearly linked to human disease. Mutations in this transporter result in a severe X-linked mental retardation syndrome with dramatic neurological deficits (6, 8, 9) due to impaired delivery of T_3 to neurons in the brain, underscoring a crucial role for MCT8 in the development of the central nervous system. Aside from expression in the brain, MCT8 is also widely distributed in other tissues and likely plays an essential role in thyroid hormone delivery for regulation of developmental and metabolic functions throughout the body.

Aside from enabling intracellular delivery of thyroid hormones, thyroid hormone transporters are also required for the metabolism of thyroid hormones. In addition to the conversion of T_4 to T_3, enzymatic deiodination of thyroid hormones to receptor-inactive iodothyronine metabolites also influences the biological activity of thyroid hormone. Further enzymatic reactions could lead to another class of compounds, known as thyronamines, which arise from the decarboxylation of thyroid hormone (10). One of these thyronamines, T_1AM, is a biogenic amine that is found in vertebrate tissues as well as in the circulatory system. T_1AM has many intriguing pharmacological actions, including effects on thermal regulation, cardiac performance, and metabolism (10, 11). For example, T_1AM administration triggers a shift in fuel usage toward lipids and away from carbohydrates in both mice and Siberian hamsters (12). The molecular target(s) of T_1AM action is not entirely clear at present.

Like other trace amines, T_1AM is a potent agonist of the rat and mouse trace amine associated receptors 1 (TAAR1), members of the G protein-coupled receptor (GPCR) family (10). T_1AM may also have a neuromodulatory role as an inhibitor of the dopamine and norepinephrine transporters responsible for the reuptake of these classical neurotransmitters, as well as the vesicular monoamine transporter VMAT2, an intracellular transporter which packages monoamines into synaptic vesicles (13). While TAAR1 signaling mechanisms and modulation of monoamine transport may help explain some of the pharmacological effects of thyronamines *in vivo*, a greater understanding of the actions of T_1AM is needed.

The importance of thyroid hormone transporters in normal thyroid hormone physiology raises the possibility that the modulation of transporter function may serve to regulate the actions of thyroid hormone. Since T_1AM has been detected in the circulation (10), and because the physiological actions of T_1AM are opposite those associated with excess levels of thyroid hormone, we hypothesized that T_1AM may in some way directly oppose the effects of thyroid hormone. Just as T_1AM has been found to inhibit monoamine transport, it is conceivable that T_1AM may also have an effect on the cellular uptake of thyroid hormones and thus play a role in regulation of thyroid hormone activity and metabolism.

With the goal of expanding our knowledge of the molecular mechanisms underlying T_1AM action, the aim of this study was to determine whether T_1AM influences the activity of thyroid hormone transporters. In particular, we were interested in determining whether T_1AM could inhibit thyroid hormone uptake by a selection of

thyroid hormone transporters and thereby limit the access of thyroid hormone to the intracellular thyroid hormone receptors.

Materials and methods

Constructs

The OATP1A2 pSG5, OATP1B3 pSG5, OATP1C1 pSG5, MCT8 pcDNA3, and CRYM psG5 plasmids were kindly provided by the laboratory of Theo Visser from Erasmus University Medical Center, Rotterdam, The Netherlands, and used for transient transfection of COS-1 cells for thyroid hormone transport studies.

Cell Transfection and Uptake Studies

COS-1 cells were maintained in DMEM of high glucose supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin (UCSF Cell Culture Facility) at 37°C with 5% CO_2 and 95% humidity. The day before transfection, cells were seeded into 24-well tissue culture plates in complete media lacking antibiotics. Cells were transfected with 200 ng of transporter construct or empty vector and 200 ng of CRYM, using FuGENE6 Transfection Reagent (Roche) according to manufacturer's protocols. 24 h after transfection, cells were washed and preincubated with prewarmed HBSS for 15 min at 37°C. For inhibition studies, uptake was initiated by the addition of a tracer amount of ^{125}I-T_3 or ^{125}I-T_4 (Perkin Elmer), with or without various concentrations of unlabeled T_1AM diluted in HBSS. For uptake studies, tracer amounts diluted in HBSS of ^{125}I-T_3, ^{125}I-T_4 (Perkin Elmer), or ^{125}I-T_1AM, synthesized as described previously (14), were each added alone to the cells. Uptake was terminated after 60 min at 37°C, the cells

were washed twice with cold HBSS and solubilized in 1% SDS, and the accumulated radioactivity was determined by scintillation counting.

Statistical Analyses

Statistical analyses were performed with the GraphPad Prism version 4.00 software, with values expressed as means ± SD. Dose-response inhibition curves were calculated using the equation Y = Bottom + (Top – Bottom)/(1 + 10^(X – Log IC_{50})).

Results

T_1AM inhibits thyroid hormone uptake by OATP1A2 and OATP1C1

To examine the effect of T_1AM on transport of thyroid hormone by several plasma membrane transporters of the OATP superfamily, COS-1 cells transiently transfected with constructs containing the entire coding region of OATP1A2, OATP1B3, and OATP1C1 were incubated with the radiolabeled substrates ^{125}I-T_3 and ^{125}I-T_4, either alone or in the presence of various concentrations of unlabeled T_1AM. For all thyroid hormone uptake studies, cells were cotransfected with μ-crystallin (CRYM), a high-affinity cytosolic thyroid hormone-binding protein that prevents the efflux of internalized iodothyronines (15, 16). T_1AM inhibited OATP1A2-mediated transport of both thyroid hormones, showing greater potency towards T_3 as revealed by a nearly ten-fold lower IC_{50} value of 270 nM as compared to an IC_{50} of 2.1 μM for T_4 transport (Figure 1). The observed high basal uptake levels of both ^{125}I-T_3 and ^{125}I-T_4 in cells transfected with empty vector and CRYM is probably due to thyroid hormone uptake by endogenous transporters present in COS-1 cells.

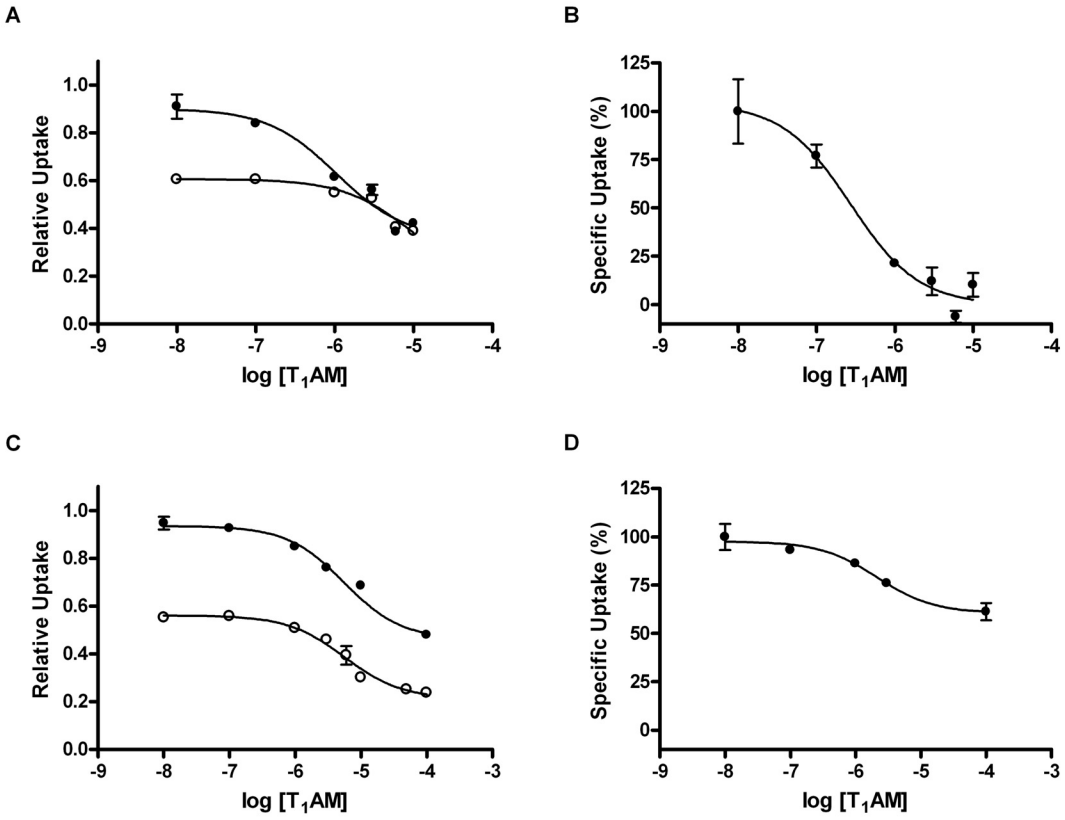

Figure 1. Thyroid hormone transport by OATP1A2. A, B) T_3 uptake by COS-1 cells transfected with OATP1A2 (solid circles) or empty vector (empty circles) and CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 60 min at 37°C with ^{125}I-labeled T_3 in the presence of various concentrations of T_1AM. Total uptake (A) and net uptake levels corrected for background uptake observed in cells transfected with empty vector (B) are shown. T_1AM inhibits T_3 uptake by OATP1A2 with an IC_{50} of 270 nM. C, D) T_4 uptake by COS-1 cells transfected with OATP1A2 (solid circles) or empty vector (empty circles) and CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 60 min at 37°C with ^{125}I-labeled T_4 in the presence of various concentrations of T_1AM. Total uptake (C) and net uptake levels corrected for background uptake observed in cells transfected with empty vector (D) are shown. T_1AM inhibits T_4 uptake by OATP1A2 with an IC_{50} of 2.1 μM.

By contrast, T_1AM did not inhibit uptake of either T_3 or T_4 by OATP1B3. OATP1B3 expression did not enhance uptake of T_3 much above the basal level, and the presence of T_1AM had a similar effect on T_3 uptake in both OATP1B3-transfected and

empty vector-transfected cells (Figure 2A, 2B). Although T_4 uptake was stimulated over basal levels to a greater degree in OATP1B3-overexpressing cells, the addition of increasing concentrations of T_1AM did not have a significant effect on uptake (Figure 2C, 2D).

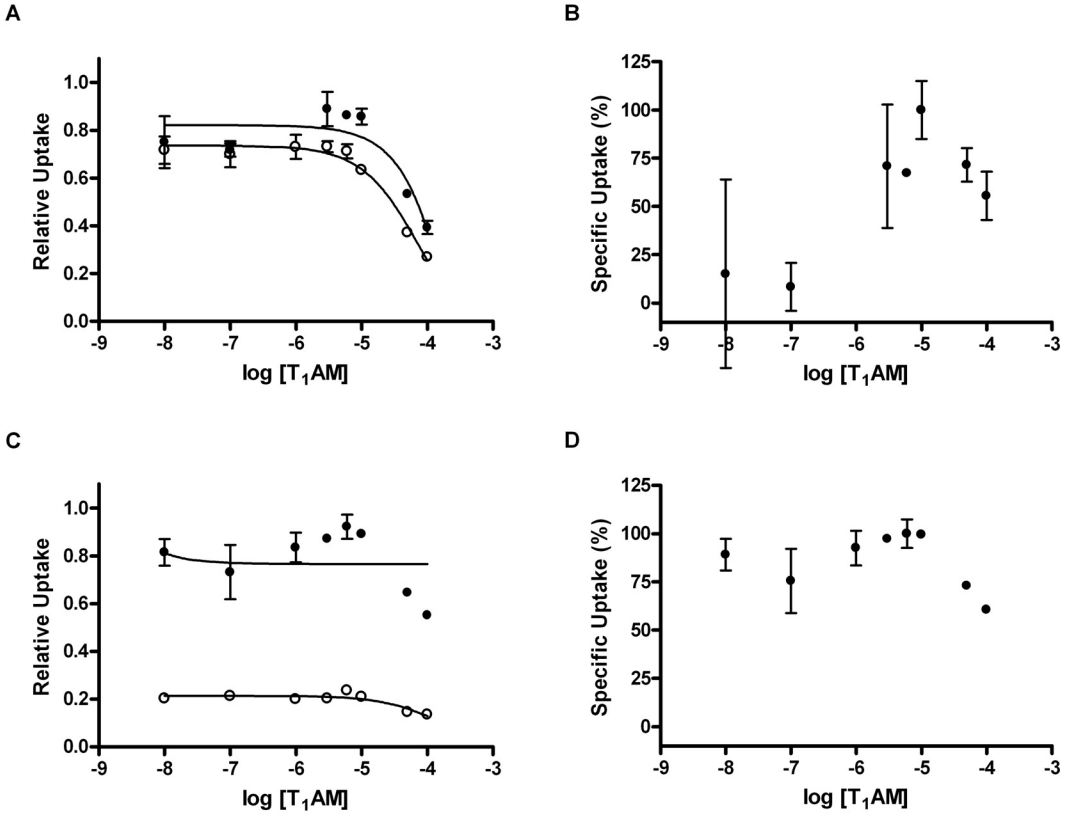

Figure 2. Thyroid hormone transport by OATP1B3. A, B) T_3 uptake by COS-1 cells transfected with OATP1B3 (solid circles) or empty vector (empty circles) and CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 60 min at 37°C with ^{125}I-labeled T_3 in the presence of various concentrations of T_1AM. Total uptake (A) and net uptake levels corrected for background uptake observed in cells transfected with empty vector (B) are shown. C, D) T_4 uptake by COS-1 cells transfected with OATP1B3 (solid circles) or empty vector (empty circles) and CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 60 min at 37°C with ^{125}I-labeled T_4 in the presence of various concentrations of T_1AM. Total uptake (C) and net uptake levels corrected for background uptake observed in cells transfected with empty vector (D) are shown. T_1AM displays no inhibition of OATP1B3-mediated uptake of either T_3 or T_4.

OATP1C1 is known to transport T_4 but not T_3 (16), and indeed we also observed increased uptake levels of T_4 in cells transfected with OATP1C1 and CRYM as compared to cells transfected with empty vector and CRYM. Moreover, T_1AM inhibited T_4 uptake in a dose-dependent manner, with an IC_{50} of 4.8 μM (Figure 3).

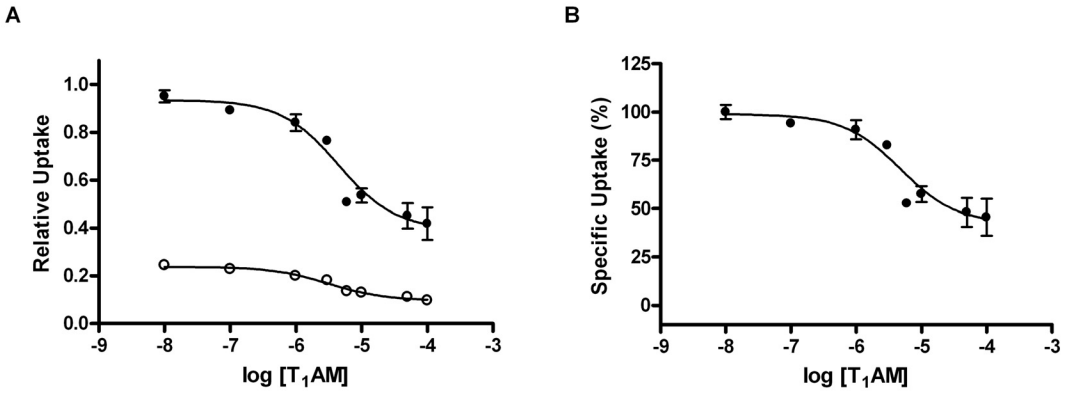

Figure 3. Thyroid hormone transport by OATP1C1. T_4 uptake by COS-1 cells transfected with OATP1C1 (solid circles) or empty vector (empty circles) and CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 60 min at 37°C with ^{125}I-labeled T_4 in the presence of various concentrations of T_1AM. Total uptake (A) and net uptake levels corrected for background uptake observed in cells transfected with empty vector (B) are shown. T_1AM inhibits T_4 uptake by OATP1C1 with an IC_{50} of 4.8 μM.

T_1AM inhibits thyroid hormone uptake by MCT8

We next determined the effect of T_1AM on thyroid hormone uptake by MCT8, the most specific thyroid hormone transporter identified to date (7). COS-1 cells were transiently transfected with a construct containing the complete coding region of MCT8 and subsequently incubated with the radiolabeled substrates ^{125}I-T_3 and ^{125}I-T_4, either alone or in the presence of various concentrations of unlabeled T_1AM. As in the transport studies involving OATPs, cells were cotransfected with CRYM. T_1AM

inhibited the uptake of both thyroid hormones but showed an approximately three-fold higher potency for T_4 transport inhibition, with an IC_{50} of 26.1 μM for T_4 as compared to an IC_{50} of 83.1 μM for T_3 uptake (Figure 4).

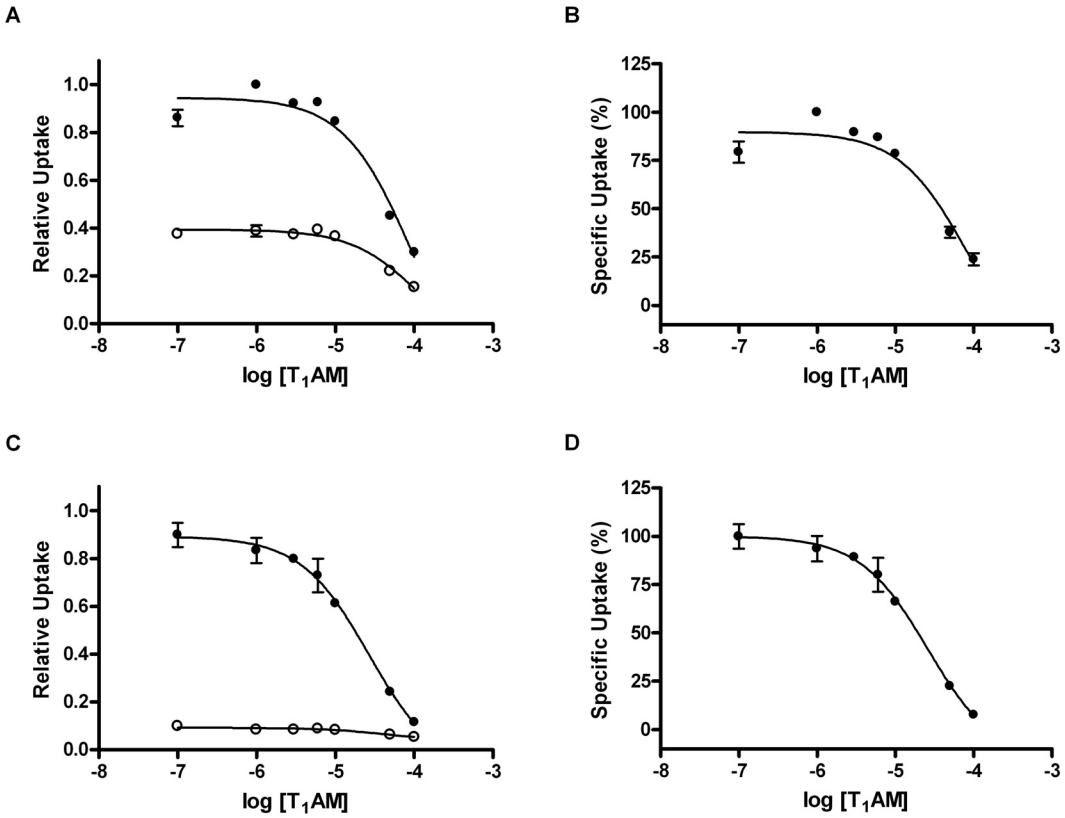

Figure 4. Thyroid hormone transport by MCT8. A, B) T_3 uptake by COS-1 cells transfected with MCT8 (solid circles) or empty vector (empty circles) and CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 60 min at 37°C with ^{125}I-labeled T_3 in the presence of various concentrations of T_1AM. Total uptake (A) and net uptake levels corrected for background uptake observed in cells transfected with empty vector (B) are shown. T_1AM inhibits T_3 uptake by MCT8 with an IC_{50} of 83.1 μM. C, D) T_4 uptake by COS-1 cells transfected with MCT8 (solid circles) or empty vector (empty circles) and CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 60 min at 37°C with ^{125}I-labeled T_4 in the presence of various concentrations of T_1AM. Total uptake (C) and net uptake levels corrected for background uptake observed in cells transfected with empty vector (D) are shown. T_1AM inhibits T_4 uptake by MCT8 with an IC_{50} of 26.1 μM.

T_1AM is not a substrate of the thyroid hormone transporters OATP1A2, OATP1B3, OATP1C1, and MCT8

To verify that T_1AM is not a substrate of the thyroid hormone transporters tested, we incubated COS-1 cells transiently transfected with each thyroid hormone transporter or empty vector and CRYM with ^{125}I-T_3, ^{125}I-T_4, and ^{125}I-T_1AM. As previously observed, T_3 uptake is not significantly enhanced in OATP1B3-expressing cells, and as has been previously reported (16), T_3 is not transported by OATP1C1 (Figure 5A). On the other hand, T_4 uptake levels are increased in cells overexpressing OATP1A2, OATP1B3, OATP1C1, and MCT8 (Figure 5B). However, T_1AM uptake levels in cells transfected with the thyroid hormone transporters are not enhanced over the basal uptake levels seen in cells transfected with empty vector (Figure 5C), revealing that T_1AM is not a substrate of these transporters.

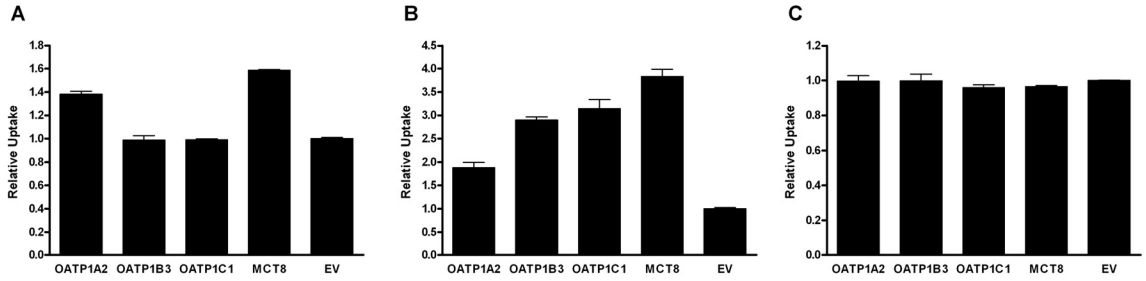

Figure 5. Uptake of thyroid hormones and T_1AM by thyroid hormone transporters. Transport of T_3 (A), T_4 (B), and T_1AM (C) in COS-1 cells transfected with either OATP1A2, OATP1B3, OATP1C1, MCT8, or empty vector and CRYM, an intracellular thyroid hormone-binding protein. Cells were incubated for 60 min at 37°C with the corresponding ^{125}I-labeled substrate. Uptake levels are expressed relative to uptake observed in empty vector-transfected cells.

Discussion

T_1AM is a recently discovered endogenous derivative of thyroid hormone with dramatic pharmacological actions when administered *in vivo*, including hypothermia, bradycardia, hyperglycemia, and general behavioral inactivity, that are in general opposite those effects associated with excess levels of thyroid hormone. Little is known about the mechanism of action of T_1AM. As a trace amine, it is a potent agonist of TAAR1 (10). T_1AM has also been found to inhibit the dopamine and norepinephrine plasma membrane transporters, as well as the vesicular monoamine transporter VMAT2 (13). Specific cellular uptake of T_1AM has also been identified in various cell lines, although the particular plasma membrane transporters involved remain unknown (17).

In the current study, we describe an additional action of T_1AM in the differential inhibition of thyroid hormone transport by several thyroid hormone transporters. A broad range of transporter types mediate intracellular entry of thyroid hormones, including classic multispecific organic anion/cation transporters such as several OATP family members and monocarboxylate transporters such as MCT8 (5, 6). The transport of thyroid hormones into their target tissues by saturable mechanisms is critical for proper physiological control of both their action and metabolism. Thus, the potential modulation of thyroid hormone transport and subsequent delivery to intracellular targets by T_1AM provides important additional insight into the role of this relatively new class of signaling molecules.

Of the wide variety of known thyroid hormone transporters, we chose to focus on three members of the OATP superfamily as well as the more specific thyroid hormone transporter MCT8. Of the eleven different OATPs in humans, families 1A and 1B are

believed to be involved in overall body detoxification, and family 1C contains high-affinity T_4 transporters and appears to be important for thyroid hormone metabolism (5). While OATP1A2 has been shown to transport a broad range of organic anionic, neutral, and cationic compounds (18), it also transports T_3 and T_4. Because of its expression in the endothelial cells of the blood-brain barrier, and in the kidney and liver, OATP1A2 is thought to be involved in the delivery of thyroid hormones to the kidney and across the blood-brain barrier, as well as function in the removal of thyroid hormone from peripheral tissues for elimination by the liver (5). OATP1B3 also has wide substrate specificity aside from thyroid hormones, and is expressed specifically in the liver. On the other hand, OAT1C1 is perhaps the most interesting OATP because it is the most thyroid hormone-specific transporter of the OATP superfamily and is also the thyroid hormone transporter with the highest affinity towards T_4 (2). OATP1C1 is expressed only in brain and testis, and because of its preferential localization in brain capillaries may be important for T_4 transport across the blood-brain barrier. We also chose to study the effect of T_1AM on MCT8 transport function, as this transporter shows the highest specificity towards thyroid hormones and has also been linked to human disease, underscoring a particular importance of MCT8 for proper thyroid hormone physiology. With its high expression in many tissues, including liver, kidney, heart, brain, placenta, lung, and skeletal muscle (6), MCT8 is likely to be important for thyroid hormone delivery to target tissues throughout the body and particularly crucial to thyroid hormone transport into neurons, since mutations in the transporter result in severe neurological deficits (8).

The inhibition profile of T_1AM towards thyroid hormone uptake by the transporters studied is interesting in its selectivity for certain transporters and differing potencies for T_3 and T_4 (Table 1). Although T_1AM has no effect on transport by the liver-specific OATP1B3, T_1AM inhibits thyroid hormone uptake by the other three transporters to varying degrees. Interestingly, OATP1A2, OATP1C1, and MCT8 are all expressed in the brain, and at least two of these transporters are believed to play an essential role in thyroid hormone delivery to the brain. Taken together with the previous finding of T_1AM as a potential neuromodulator in its inhibition of monoamine transport (13), the possible function of T_1AM in regulation of thyroid hormone transport in the brain underscores an intriguing potential role of this thyroid hormone metabolite in development and proper functioning of the central nervous system.

Table 1. Summary of T_1AM inhibition profile.

Transporter	IC_{50} (T_3)	IC_{50} (T_4)
OATP1A2	0.27 μM	2.1 μM
OATP1B3	No inhibition	No inhibition
OATP1C1	N/A	4.8 μM
MCT8	83.1 μM	26.1 μM

Although the micromolar potencies of T_1AM inhibition of the thyroid hormone transporters are higher than their endogenous circulating levels, active trafficking and subsequent metabolism of thyroid hormone in brain synaptosomes could conceivably result in local concentrations of T_1AM in certain regions of the brain that may far exceed

circulating levels (19-21). In addition, with the exception of OATP1C1, the reported K_m values for thyroid hormone transport by the various thyroid hormone transporters are also in the micromolar range, far above their endogenous circulating levels, but have nevertheless been shown to be essential for thyroid hormone delivery into target organs and tissues (5, 6). Interestingly, T_1AM shows the highest inhibition potency towards T_3 transport by OATP1A2, with the only sub-micromolar IC_{50} measured in this study (Table 1). Although subsequent studies are required to investigate the potential significance of this finding, T_1AM might affect the OATP1A2-mediated clearance of T_3 from the body.

In conclusion, we have demonstrated that T_1AM is an inhibitor of thyroid hormone uptake by several of the identified thyroid hormone transporters. Although T_1AM has profound effects when administered *in vivo*, much remains to be discovered regarding the mechanisms by which it exerts these effects. A role in regulation of thyroid hormone delivery to target sites presents a novel mechanism of action for this endogenous thyroid hormone metabolite. Further studies of T_1AM and its effect on thyroid hormone transport and action would be invaluable in our understanding of not only T_1AM action but also its potential implications for thyroid hormone regulation and possible involvement in certain endocrine and neurological pathologies.

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CHAPTER 5

Identification of α_2-Macroglobulin (α_2M) as a serum binding protein for T_1AM

Abstract

Serum proteins are involved in the binding, transport, and extracellular storage of a wide variety of endogenous compounds, including thyroid hormones. As a thyroid hormone derivative present in the circulation, T_1AM could conceivably also be transported by one or more serum binding proteins. Using ultrafiltration and protein precipitation methods, we first determined that indeed the majority of T_1AM is associated to protein in whole rat serum. Subsequent analysis by native gel electrophoresis and autoradiography using ^{125}I-T_1AM incubated in rat serum revealed a single high-molecular-weight protein associated with T_1AM. Using affinity purification methods and mass spectrometry analysis, we identified α_2-Macroglobulin (α_2M) as a serum binding protein for T_1AM. Further analysis of the mode of T_1AM binding to α_2M revealed that T_1AM does not covalently bind the protein, in contrast to the reported interactions of other monoamines with α_2M. T_1AM also does not appear to bind α_2M at the same sites as other monoamines, suggesting a distinct mechanism of binding. Moreover, T_1AM binding does not result in the conversion of the native form of α_2M to the activated form, a conformational change that does occur upon binding of α_2M to proteases and that is necessary for its clearance from the body. α_2M is known for various functions in the body, including its unique role as a pan-protease inhibitor, as well as its potential significance in immune defense and modulation of neurotransmitter metabolism. Although the significance of T_1AM binding to α_2M is unclear, the discovery of α_2M as a serum binding protein for T_1AM opens another interesting area of investigation into this thyroid hormone derivative and its mechanisms of action in the body.

Introduction

Many endogenous compounds and drugs travel in the circulation bound to proteins in the serum. Serum binding proteins play an important role in the pharmacokinetics of many drugs, influencing their efficacy and rate of delivery (1, 2), and in regulating the biological activity of various endogenous compounds including vitamins and hormones. For example, the majority of thyroid hormone (over 99%) is bound to proteins in the serum, and only the unbound or free fraction of hormone is responsible for biological activity. The principal thyroid hormone binding proteins are thyroxine-binding globulin (TBG), transthyretin, and albumin (3-5). Although these proteins are not essential for thyroid hormone activity, they form a storage pool of readily available free hormone and protect tissues from massive hormone release.

While some serum binding proteins are relatively specific for certain ligands, others are capable of binding a wide variety of compounds. Albumin, for instance, is the most abundant protein in plasma and binds many ligands other than thyroid hormone, including other hormones, fatty acids, bilirubin, a variety of metal ions, and many xenobiotics (1). Another major plasma protein in humans is α_2-Macroglobulin (α_2M), and homologous proteins appear to be found in all other vertebrates (6). The human protein is a 725 kDa homotetramer consisting of a non-covalently associated pair of disulfide-linked dimers and is particularly interesting for its role as a molecular trap for a wide variety of protease molecules. Once bound and enclosed within the α_2M molecule, proteases are unable to act on protein substrates or to bind antibodies. α_2M contains a "bait" region that reacts with proteases, trapping and thus inhibiting them, and reacts with a great majority of proteases, regardless of their precise specificity or catalytic

mechanism. Reaction with proteases causes an irreversible change in conformation of the α_2M molecule from the native or "slow" form to the activated or "fast" form, so named for their differences in electrophoretic mobility (7). The changed conformation of α_2M leads to rapid clearance from the circulation and this protein may represent an ancient defensive system of the body, as α_2M is the only plasma inhibitor of a number of proteases used by pathogens and parasites in attacking the body. α_2M also shares an evolutionary relationship with some of the complement components (6).

Besides its role in controlling extracellular proteolytic activity, it is thought that α_2M is also involved in the regulation of neuronal development and function. Nucleophilic attack at thioester bonds in the α_2M molecule by monoamines results in monoamine-activated α_2M that selectively binds various neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4 (8). In addition, serotonin-activated α_2M has also been found to depress dopaminergic and cholinergic neurotransmitter systems in the central nervous system, suggesting a potential regulatory role in neurotransmitter metabolism (9).

Because of the importance of serum proteins in the transport of compounds throughout the body, their role in regulating the amount of free or biologically active hormones and other molecules, as well as the alteration in function of certain serum proteins upon binding their ligands, as in the case of protease-α_2M and monoamine-α_2M, we sought to investigate whether T_1AM is carried or transported in the circulation by a serum binding protein. Just as thyroid hormone itself is principally bound to several proteins in the serum, we hypothesized that there might exist one or multiple serum proteins that bind the thyroid hormone derivative T_1AM. The goal of this study,

therefore, was to first determine whether a portion of T_1AM is associated with protein in whole serum and if so, attempt to identify the T_1AM serum binding protein.

Materials and methods

Serum Protein Ultrafiltration and Fractionation

Rat serum (Sigma) was incubated with ^{125}I-T_1AM, synthesized as described previously (10), or ^{125}I-T_3 (Amersham) for 1 h at r.t. Centricon centrifugal filter devices with Ultracel YM-10 filter membranes having a 10,000 MW cutoff (Millipore) were used for serum ultrafiltration according to manufacturer's protocols, and the radioactivity present in the filtrate and retentate fractions was determined by scintillation counting. Ammonium sulfate precipitation was used separately for total serum protein fractionation. After incubation of rat serum with ^{125}I-T_1AM or ^{125}I-T_3, saturated ammonium sulfate solution (Pierce) was added to the samples and 1 h later the samples were centrifuged at 13,000 rpm for 10 min. The radioactivity present in both the supernatant and protein pellet was measured.

Polyacrylamide Gel Electrophoresis and Autoradiography

Rat serum was incubated with ^{125}I-T_1AM or ^{125}I-T_3 in the presence and absence of excess unlabeled ligand for 1 h at r.t. Similar incubations were also conducted using purified, commercially available proteins instead of whole rat serum. Pure human α_2M (Sigma) and albumin (Pierce) were incubated with either ^{125}I-T_1AM or ^{125}I-T_3 for 1 h at r.t., with or without various unlabeled compounds. For all incubations with purified

proteins, the final protein concentration was 1 mg/mL. After the incubation period, sample aliquots were examined using polyacrylamide gel electrophoresis (PAGE).

NativePAGE or NuPAGE gels (Invitrogen) were used for gel electrophoresis of samples under native or denaturing conditions, respectively, following the guidelines of the manufacturer. The gels were then exposed to film for at least 2-3 h, after which the films were developed to produce the autoradiographs used to determine localization of the ^{125}I signal.

Affinity Purification

Affinity chromatography was used to fractionate rat serum proteins in order to identify proteins bound to T_1AM. An eight-carbon linker attaching a carboxyl group to the T_1AM molecule was immobilized to a gel support using the UltraLink EDC/DADPA Immobilization Kit (Pierce) following manufacturer's protocols. Briefly, the carbodiimide EDC (1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide•HCl) cross-linker was reacted with the carboxyl-containing T_1AM derivative and coupled to the primary amines on DADPA (diaminodipropylamine) conjugated to the UltraLink Biosupport Medium, resulting in a gel support containing the immobilized T_1AM derivative that was used for affinity purification. Whole rat serum diluted in PBS was then applied to the prepared column, incubated for 1 h at r.t., and washed with 12 ml PBS. The bound protein was then eluted with 8 ml glycine buffer (100 mM, pH 3.0) and collected in 1 ml fractions. These elution fractions were then analyzed for protein content by measuring their absorbance at 280 nm and the fractions containing the majority of protein content

were analyzed by mass spectrometry (Protein Sciences Facility/Carver Biotechnology Center, University of Illinois at Urbana/Champaign).

T_1AM Uptake Assay

T_1AM uptake experiments in HeLa cells were conducted in 24-well tissue culture plates as previously described (11), using either KRTH uptake buffer (120 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5 mM Tris, 10 mM HEPES, pH 7.4) or KRTH uptake buffer containing 1 mg/mL α_2M. Cells were washed and preincubated with prewarmed uptake buffer for 15 min at 37°C. Uptake was initiated by the addition of a tracer amount of ^{125}I-T_1AM and terminated after incubation for 20 min at 37°C, the cells were washed twice with cold KRTH and solubilized in 1% SDS, and the accumulated radioactivity was determined by scintillation counting.

Statistical Analyses

Statistical analyses were performed with the GraphPad Prism version 4.00 software, with values expressed as means ± SD.

Results

Similar fractions of T_1AM and T_3 are associated with proteins in whole rat serum

Ultrafiltration of whole rat serum following incubation with ^{125}I-T_1AM revealed that over 99% of the total radioactivity was found in the retentate, that is, the sample fraction retained by the filter membrane (MW cutoff = 10,000) that contains most of the serum proteins. Virtually identical results were obtained for serum incubated with ^{125}I-

T_3, with more than 99% of the total radioactivity present in the retentate, which is also in accordance with the reported fraction of T_3 that is protein-bound in the serum (5).

Total protein precipitation using saturated ammonium sulfate also yielded similar results for T_1AM as for T_3. For both compounds, approximately 60% of the radioactivity was found in the protein pellet, with the remainder of the signal in the supernatant, which presumably consists of few if any proteins. The percentage of T_1AM as for T_3 bound by proteins in the pellet is considerably lower than the percentage retained during ultrafiltration, which might be explained by the inability to achieve a high enough salt concentration or ionic strength to precipitate all the serum proteins.

T_1AM binds to a single high-molecular-weight protein present in rat serum

Since the results of ultrafiltration and protein precipitation of rat serum appeared to strongly suggest that the majority of T_1AM, like T_3, is protein-bound in serum, we next wished to determine whether T_1AM binds to a single protein or to multiple proteins. After incubation of rat serum with ^{125}I-T_1AM or ^{125}I-T_3, serum samples were run on a native protein gel under non-denaturing conditions and the locations of the radioligand were subsequently examined by autoradiography. The ^{125}I-T_1AM signal was visualized at only one location on the gel, bound to a protein with a high molecular weight of ~720 kDa, while the ^{125}I-T_3 signal, on the other hand, was seen as a high-intensity smear on the autoradiograph apparently bound to one or more proteins of sizes ~55 kDa to ~400 kDa. Furthermore, the presence of excess unlabeled T_1AM or T_3 displaced the majority or entirety of the corresponding radiolabeled ligand (Figure 1), indicating that the binding is specific. The major serum binding proteins for T_3 are thyroxine-binding globulin (TBG),

transthyretin, and albumin, which have molecular weights of approximately 54 kDa, 55 kDa, and 65 kDa, respectively (5). Very high abundance of albumin in serum, as well as a high affinity for T_3 binding to TBG, which carries about 70% of circulating thyroid hormone, are probably partly responsible for the smeared appearance of the ^{125}I-T_3 signal. In addition, in native gel electrophoresis, proteins are not denatured and therefore are separated according to their charge-to-mass ratio rather than their mass alone, as occurs during denaturing SDS-PAGE.

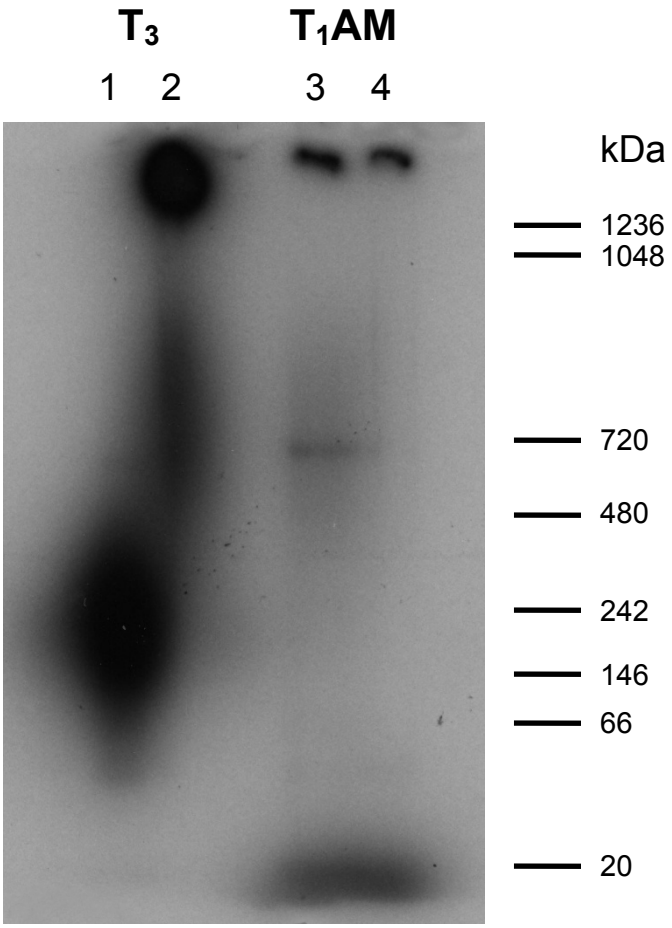

Figure 1. Identification of a rat serum protein bound to T_1AM. After incubation with ^{125}I-labeled T_3 (Lanes 1 and 2) or T_1AM (Lanes 3 and 4), rat serum proteins were separated by gel electrophoresis on a native protein gel under non-denaturing conditions. Subsequent autoradiography of the gel reveals a single, high-molecular-weight band of ~720 kDa when serum is incubated with ^{125}I-T_1AM alone (Lane 3). The high-intensity, lower-molecular-weight band seen with ^{125}I-T_3 incubation (Lane 1) is presumably albumin, TBG, and transthyretin. The top-most band in Lanes 2-4 corresponds to the bottom of the well in which sample was loaded and represents radiolabeled substrate caught in the loading well. Note that the addition of an excess amount (100 μM) of unlabeled ligand during incubation with serum reduces or eliminates the protein-bound signal (Lanes 2 and 4), revealing that the binding is specific.

Affinity purification of rat serum reveals several potential binding proteins for T_1AM

After determining that T_1AM appears to be principally bound to a single protein in rat serum, we used affinity purification to fractionate rat serum proteins in an attempt to identify this protein. By constructing a column in which a T_1AM derivative was immobilized to the gel matrix (Figure 2), we identified a fraction of proteins in rat serum that were bound to the T_1AM derivative.

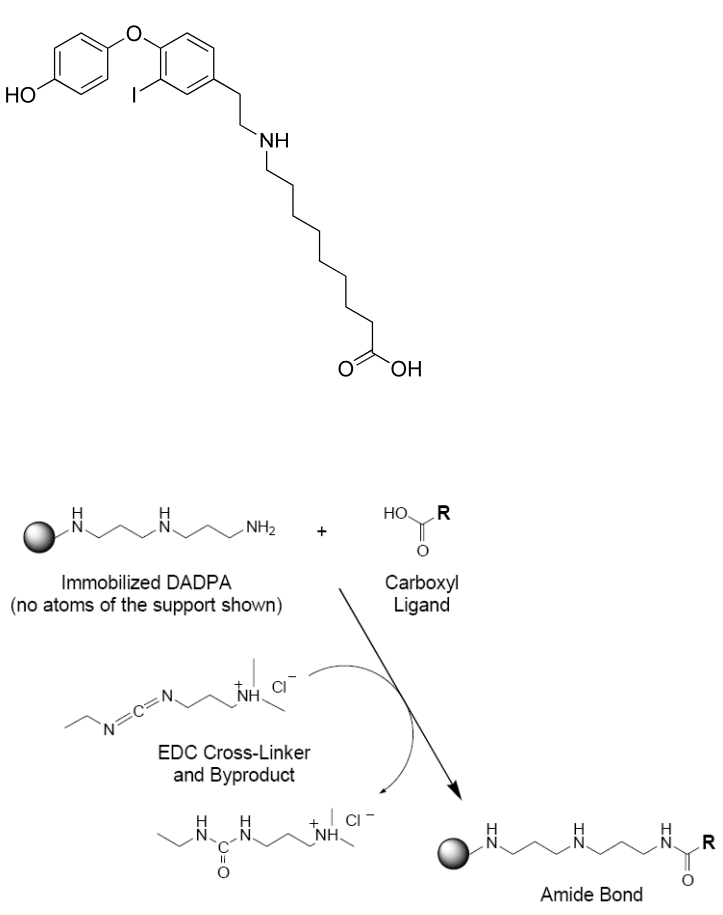

Figure 2. Structure of the T_1AM derivative that was used for affinity purification. An eight-carbon linker attaching a carboxyl group to the T_1AM molecule ("carboxyl ligand," top) was immobilized to a gel support by cross-linking with EDC and conjugation to DADPA (reaction scheme, bottom) using the UltraLink EDC/DADPA Immobilization Kit (Pierce) according to manufacturer's protocols. The application of whole rat serum to the constructed column resulted in the collection of a single elution fraction containing the majority of proteins presumably bound to T_1AM.

SDS-PAGE of the elution fraction containing the majority of protein as measured by absorbance at 280 nm revealed a major band at greater than 200 kDa, as well as several smaller, minor bands (Figure 3). The major, higher molecular weight band might

correspond to a subunit of the denatured ~720 kDa protein previously observed as the ^{125}I-T_1AM signal on the autoradiograph of the native protein gel of whole rat serum. Mass spectrometry analysis of this fraction identified α_2M as a major component, as well as albumin, keratin, and several unnamed protein products. However, the previous autoradiograph of the native protein gel run with a sample of whole rat serum after incubation with ^{125}I-T_1AM did not reveal a signal at the correct size for albumin or other smaller proteins, in contrast to the ^{125}I-T_3 signal observed on the autoradiograph.

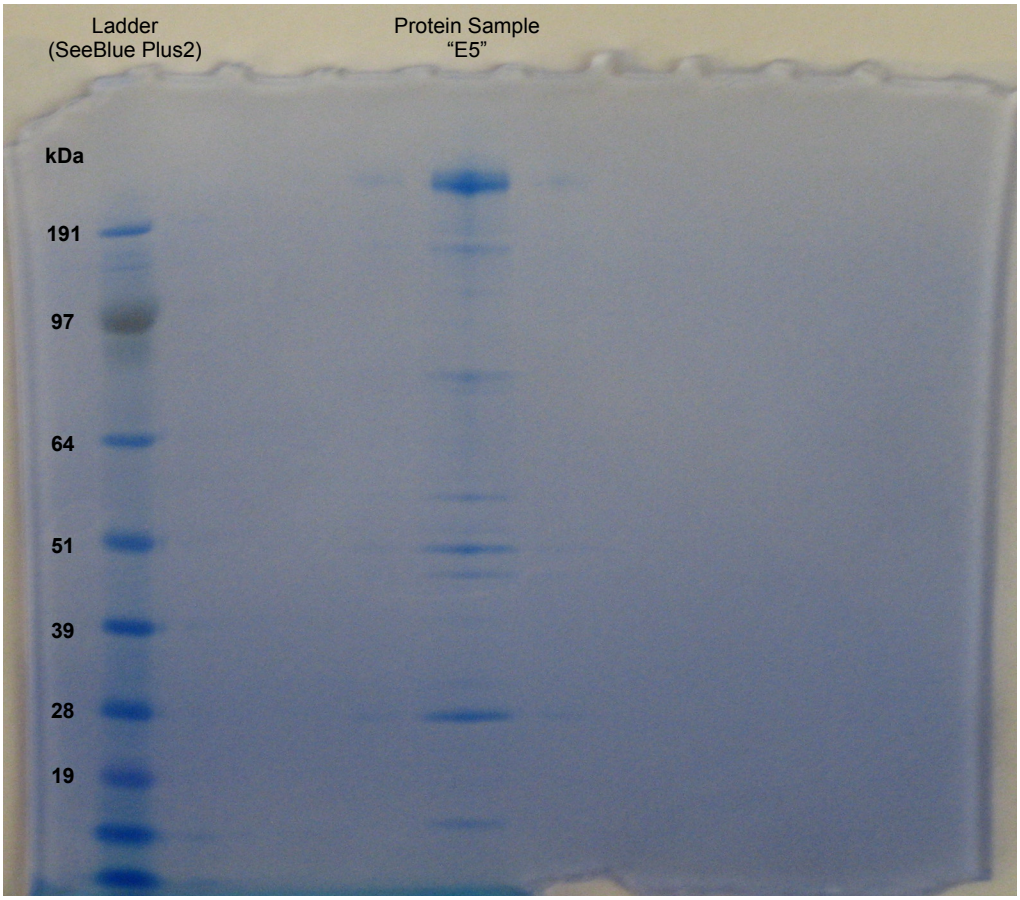

Figure 3. SDS-PAGE gel of the major protein-containing elution fraction resulting from affinity purification. After immobilization of a T_1AM derivative to a solid support, affinity purification of whole rat serum identified an elution fraction containing the majority of eluted protein collected. This fraction was examined by SDS-PAGE and revealed a major protein band over 200 kDa in size, as well as several smaller, minor protein bands.

T_1AM binds to human α_2M

Only a limited number of proteins have such a high molecular weight as that of the T_1AM binding protein identified in rat serum. Mass spectrometry analysis of the proteins in the elution fraction of interest resulting from affinity purification alerted our attention to the major protein product identified. α_2M is ~725 kDa in size and, interestingly, is also known to bind monoamines such as methylamine and serotonin (9, 12). Because of the results of mass spectrometry analysis, the close size similarity between α_2M and the T_1AM binding protein detected on our initial autoradiograph, and since T_1AM is a monoamine, α_2M appeared to be a good candidate for the T_1AM serum binding protein and could be quickly tested, since α_2M purified from human serum is commercially available. Moreover, rat and human α_2M have approximately the same size and are structurally and functionally homologous.

Consequently, we tested for T_1AM binding to α_2M by incubating the purified protein with ^{125}I-T_1AM and analyzing the sample by native PAGE followed by autoradiography. Indeed, a single, sharp band of radioligand was detected at ~720 kDa (Figure 4), the same location as the T_1AM binding protein present in whole rat serum, demonstrating that T_1AM binds to human α_2M. T_1AM also likely binds to rat α_2M, but no purification and direct testing was performed.

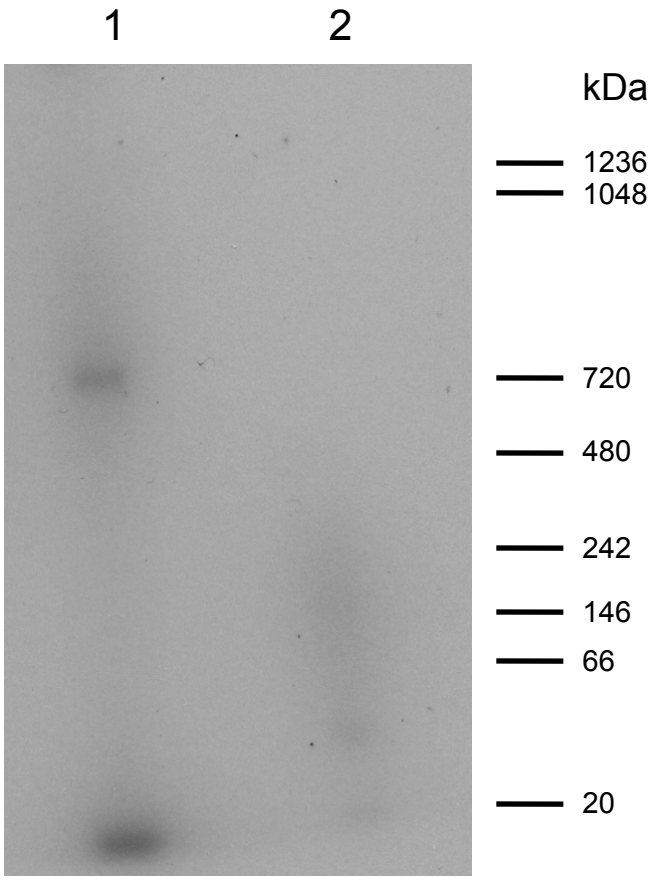

Figure 4. T_1AM binds to human α_2M. After incubation of ^{125}I-T_1AM with α_2M purified from human plasma, a single band appears on autoradiography following native PAGE (Lane 1) and is at the same position at ~720 KDa as the single band observed after ^{125}I-T_1AM incubation with whole rat serum. Rat and human α_2M proteins are structurally and functionally homologous and have similar molecular weights. ^{125}I-T_3 was also incubated with purified human albumin as a control, and a diffuse band due to alumbin-bound ^{125}I-T_3 can be visualized lower on the gel (Lane 2), again very similar to the signal seen after ^{125}I-T_3 incubation with whole rat serum.

The mode of T_1AM binding to α_2M is distinct from that of other monoamines

After establishing that α_2M appears to be the major serum binding protein for T_1AM, we next wished to characterize the mode of binding and compare it with that of other monoamines. Serotonin and other nucleophilic amines react readily with thioester bonds within α_2M and form covalently linked conjugates (12). To determine whether T_1AM likewise binds covalently to α_2M, we incubated purified α_2M with ^{125}I-T_1AM as before, but then ran the sample using SDS-PAGE under reducing and denaturing conditions. The resulting protein stain of the gel showed the denatured protein at ~190-200 kDa, which corresponds well to the sizes of the individual subunits of α_2M, and is also consistent with the major protein band previously observed in the SDS-PAGE gel of

the major protein-containing elution fraction following affinity purification, but autoradiography of the gel revealed that none of the ^{125}I-T_1AM was bound to the protein subunits but instead was found at the bottom of the gel as free, unbound ^{125}I-T_1AM (Figure 5). Had ^{125}I-T_1AM formed a covalent linkage with α_2M, radioligand would have still been detected co-localized with the protein in spite of the denaturing conditions, while a non-covalent linkage would have been disrupted by the protein reducing and denaturing conditions. Thus, T_1AM binds α_2M non-covalently, in contrast to the covalent linkage between α_2M and other monoamines.

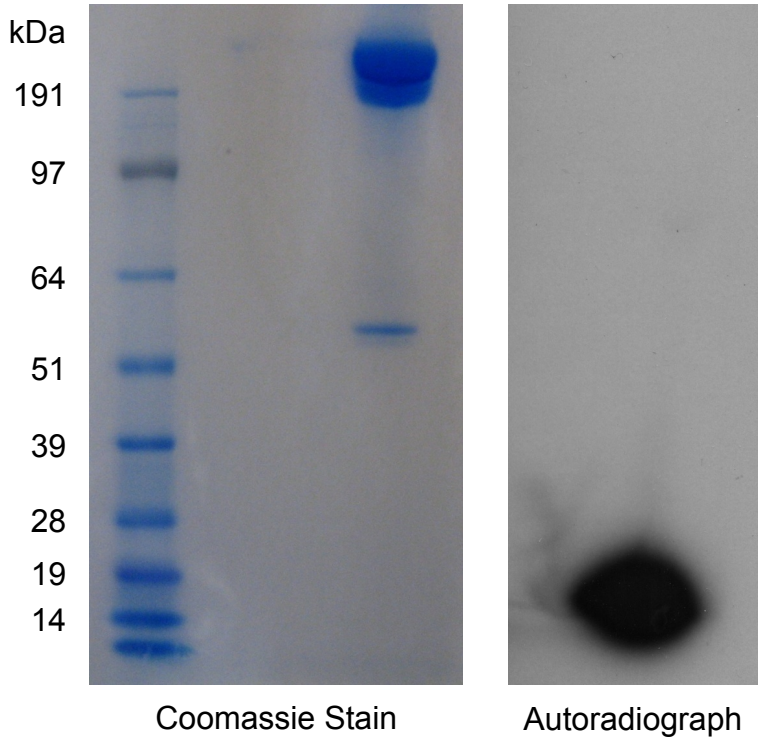

Figure 5. T_1AM does not bind covalently to α_2M. ^{125}I-T_1AM was incubated with α_2M and run on an SDS PAGE gel, under reducing and denaturing conditions. Subsequent autoradiography reveals no protein-bound ^{125}I-T_1AM, as all the signal is found as free ^{125}I-T_1AM at the bottom of the gel, suggesting that the T_1AM-α_2M linkage was non-covalent and was thus disrupted during protein denaturing. The high intensity band at ~190-200 kDa seen on the Coomassie stain of the gel represents the individual subunits of α_2M, since the homotetramer has been denatured during gel electrophoresis. The faint band at ~60 kDa probably represents a minor impurity.

To next determine whether T_1AM and serotonin might nevertheless compete for the same binding site on the α_2M molecule, but just form different types of linkages, purified α_2M was incubated with ^{125}I-T_1AM either alone or in the presence of excess unlabeled serotonin. Native gel electrophoresis followed by autoradiography showed that serotonin did not displace any of the ^{125}I-T_1AM bound to α_2M, although excess unlabeled T_1AM displaced all of the protein-bound radioligand (Figure 6). This result shows that serotonin does not inhibit or compete with T_1AM for binding to α_2M, suggesting that T_1AM binds α_2M at a site distinct from that of serotonin.

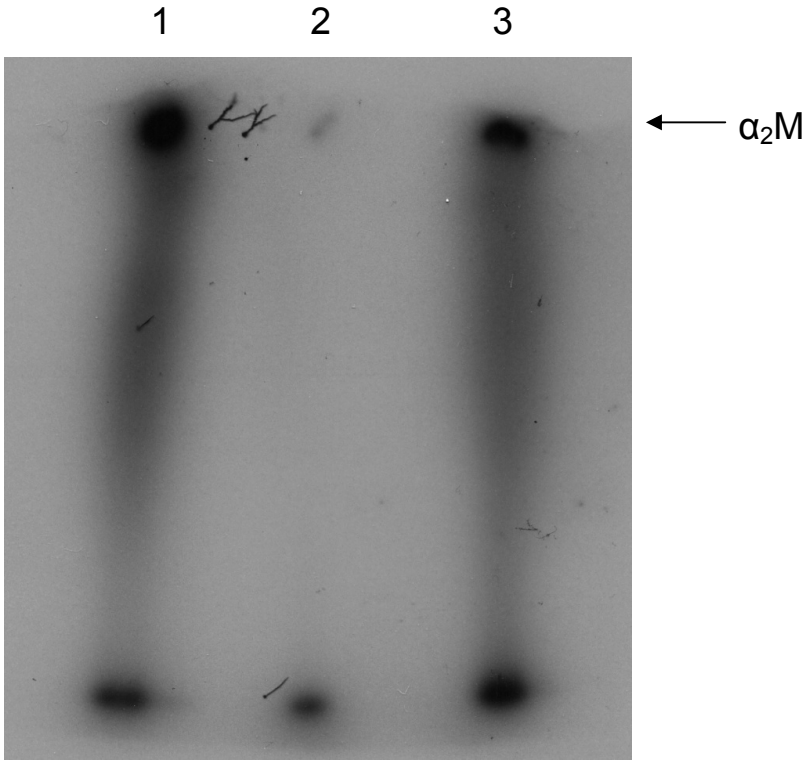

Figure 6. T_1AM binds α_2M at a site distinct from that of serotonin. Samples were run on a native PAGE gel and examined by autoradiography. The band near the top of the autoradiograph represents ^{125}I-T_1AM bound to α_2M, while the bottom band is free ^{125}I-T_1AM. When ^{125}I-T_1AM and α_2M are incubated alone (Lane 1), much of the ^{125}I-T_1AM is bound to α_2M. When an excess (100 μM) of unlabeled T_1AM is present during the incubation (Lane 2), ^{125}I-T_1AM is displaced from α_2M, indicating specific binding of ^{125}I-T_1AM to α_2M. However, when 100 μM unlabeled serotonin is present during the incubation (Lane 3), ^{125}I-T_1AM is not displaced from α_2M, indicating that T_1AM and serotonin bind α_2M at different sites.

T_1AM binding to α_2M does not induce a conformational change and is independent of the conformation of α_2M

When proteases such as trypsin react with α_2M, the protease-reacted α_2M is converted from the native, "slow" form to the "activated" or "fast" form of α_2M, so named because its electrophoretic mobility increases (7). Amine-modified α_2M resulting from monoamine binding has also been reported to undergo a conformational change to the fast form of the protein (9, 12). To investigate whether binding to T_1AM alters the conformation of native α_2M or affects the protease- or monoamine-mediated conversion, pure α_2M was incubated with T_1AM, serotonin, or trypsin, the latter two with and without T_1AM added. The protein samples were then examined by native gel electrophoresis. As expected, when incubated with trypsin, α_2M underwent a conformational change and some amount of protein was converted to the fast form, as was evident by the appearance of a second band slightly lower on the gel than the original band corresponding to the native, slow form of α_2M; the presence of T_1AM during α_2M incubation with trypsin had no effect on the location or intensity of the band corresponding to fast α_2M, indicating that T_1AM does not prevent protease-mediated α_2M activation, or conversion from slow form to fast form. However, we did not observe the expected conformational change resulting from α_2M incubation with serotonin. Finally, T_1AM did not induce a conformational change in α_2M, as all the protein remained in the slow form identical in appearance to α_2M alone (Figure 7).

All of the previous binding experiments have examined the binding of T_1AM only to the native, slow form of α_2M. To determine whether T_1AM can also bind to the fast form, α_2M was pre-incubated with trypsin, after which ^{125}I-T_1AM was added for an

additional incubation period. The resulting protein separation by native gel electrophoresis and autoradiograph revealed two slightly separated but distinct bands of ^{125}I-T_1AM signal, showing that T_1AM binds both slow and fast forms of α_2M (Figure 8).

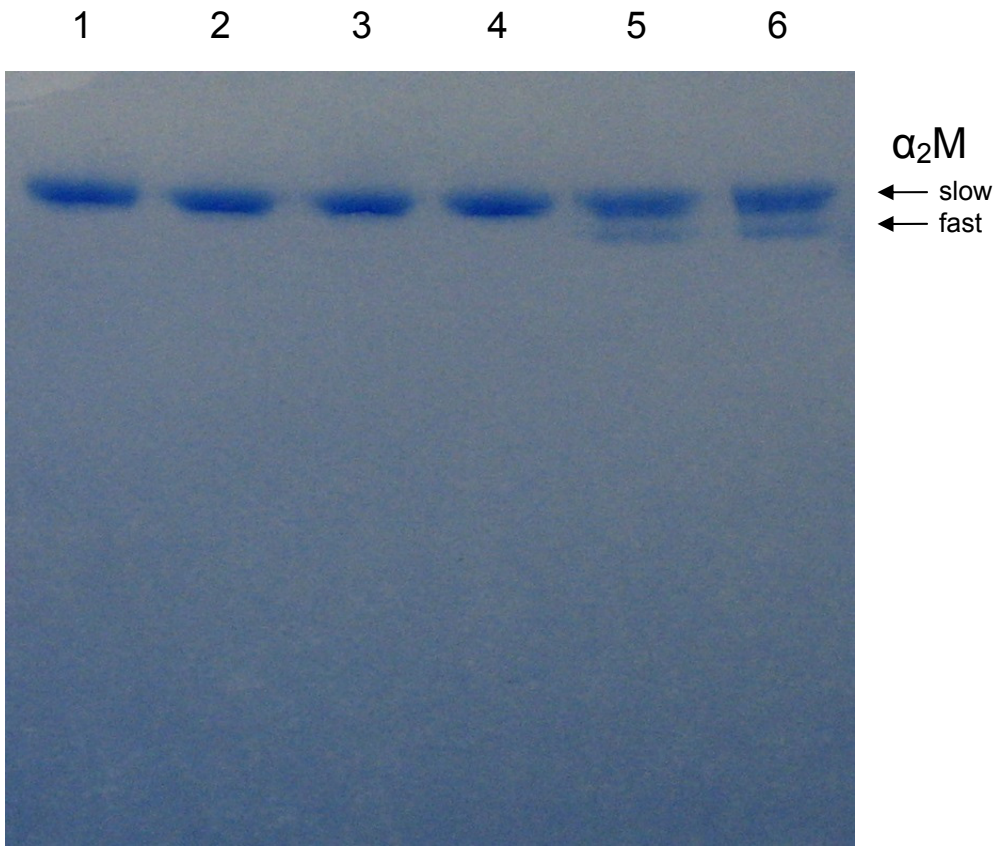

Figure 7. T_1AM binding to α_2M does not result in a conformational change in the protein, nor does T_1AM prevent the protease-mediated conversion of α_2M from slow form to fast form. Protein staining of a native PAGE gel after running sample incubations of α_2M alone (Lane 1), α_2M + T_1AM (Lane 2), α_2M + serotonin (Lane 3), α_2M + serotonin + T_1AM (Lane 4), α_2M + trypsin (Lane 5), and α_2M + trypsin + T_1AM (Lane 6) reveals that neither T_1AM nor serotonin converts α_2M from the native, slow form to the fast form (compare Lanes 2-4 with Lane 1), but trypsin does convert some amount of native α_2M to the faster-migrating band corresponding to the activated or fast form of α_2M (Lane 5). Furthermore, the presence of T_1AM during α_2M incubation with trypsin does not affect this trypsin-mediated conformational change of α_2M (Lane 6).

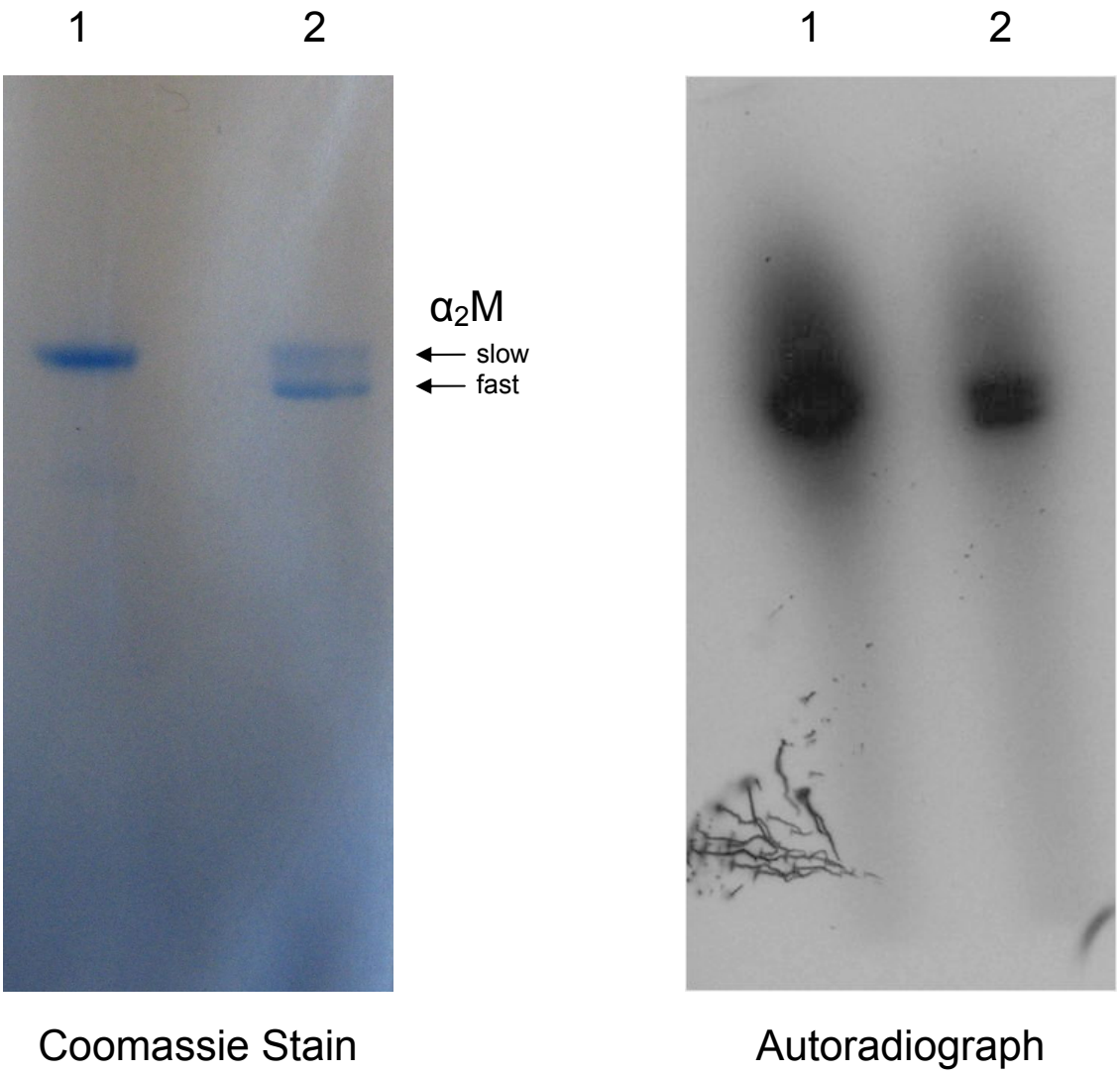

Figure 8. T_1AM binds both slow and fast forms of α_2M. From the protein stain of the native PAGE gel, the locations of native α_2M (Lane 1) and the slow and fast forms resulting from pre-incubation of α_2M with trypsin (Lane 2) can be seen. After incubation with ^{125}I-T_1AM, the autoradiograph reveals that ^{125}I-T_1AM binds to both bands corresponding to the slow and fast forms of α_2M.

Effect of α_2M on cellular uptake of T_1AM

If the majority of T_1AM in circulation is bound to the serum protein α_2M, the free T_1AM level is drastically reduced and the amount of T_1AM freely available to enter cells likewise decreases. However, albumin has been found to have a facilitatory effect on

iodothyronine uptake by MCT8-expressing oocytes (13), presumably because albumin provides a buffer of loosely bound ligand surrounding the cell and in the absence of albumin the ligand would be rapidly depleted. To determine whether α_2M has an inhibitory or facilitatory effect on T_1AM uptake, HeLa cells were incubated with ^{125}I-T_1AM diluted in standard uptake buffer with and without α_2M present. In the presence of 1 mg/mL α_2M, the uptake of T_1AM into HeLa cells is dramatically reduced by nearly four-fold (Figure 9), implying that much of the T_1AM is protein-bound and unable to access specific plasma membrane transport machinery responsible for its uptake into the cell.

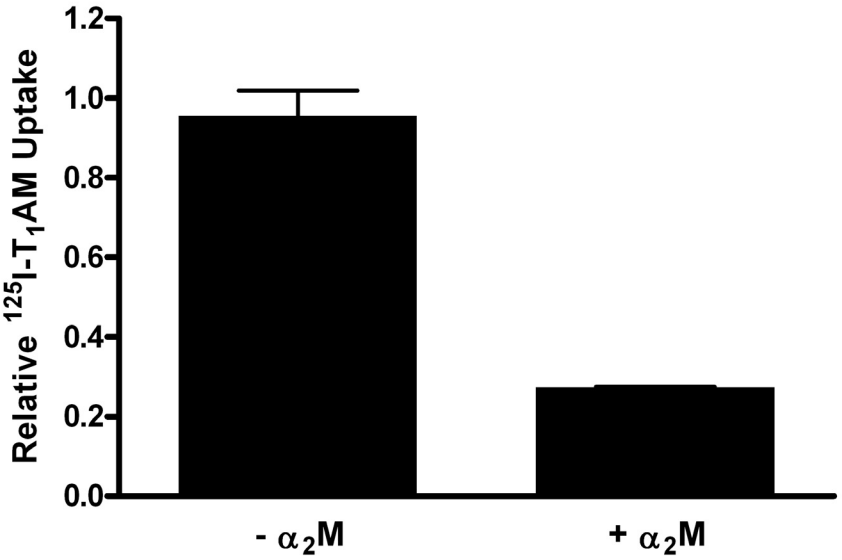

Figure 9. T_1AM cellular uptake is inhibited by α_2M. The addition of 1 mg/mL α_2M to KRTH uptake buffer during incubation of ^{125}I-T_1AM with HeLa cells under otherwise standard uptake assay conditions shows that α_2M significantly decreases the amount of T_1AM transported into cells.

Discussion

As an endogenous thyroid hormone derivative present in the circulation with profound physiological actions when administered *in vivo* (14), and with a variety of extracellular and intracellular targets in many cell types (11, 15-17), T_1AM transport throughout the body is required to reach its target tissues. Carrier proteins for thyroid hormones transport the hormones in plasma and are important for regulating the amount of free thyroid hormone readily available to tissues as well as maintaining a large extracellular storage pool of hormone. Here we report the identification of α_2M as a possible serum binding protein for T_1AM that could likewise be important for its transport in plasma and delivery to target tissues, and in regulating the fraction of free T_1AM capable of accessing its molecular targets to exert its biological actions. Our initial ultrafiltration and protein precipitation methods revealed that, like T_3, T_1AM is primarily protein-bound in serum, possibly suggesting that α_2M protects against excessive T_1AM signaling or intracellular delivery. α_2M could also be important in maintaining a large extracellular pool of T_1AM so that constant biosynthesis of the thyroid hormone metabolite is not required.

α_2M is also a particularly interesting serum binding protein because of its reported involvement in a variety of physiological activities, including regulation of protease activity, immune system function, and neuromodulation. Perhaps one of its most intriguing roles is that, when activated by monoamines, α_2M is capable of binding several neurotrophins and modulating dopaminergic and cholinergic neurotransmitter systems (8, 9). In this study, we found that T_1AM does not bind α_2M by the same mechanism or at the same binding sites as serotonin. While the serotonin binds α_2M covalently, T_1AM

was shown to dissociate from α_2M under protein denaturing conditions, suggesting a non-covalent interaction. Such an interaction, however, would be required for unloading of T_1AM upon reaching T_1AM target sites. Although the mode of T_1AM binding to α_2M is distinct from that of serotonin, it would nevertheless be of interest to investigate whether the T_1AM-α_2M complex possesses neuromodulatory activities similar to those of other monoamine-α_2M complexes. T_1AM has previously been reported as a potential neuromodulator in its inhibition of several membrane transporters of monoamine neurotransmitters (16), meriting investigation of other possible functions of T_1AM in neuromodulation.

Protease-mediated conversion of α_2M to the activated or fast form is required for its clearance from the circulation (6), and we have found that T_1AM does not cause a similar conformational change in the α_2M molecule upon binding. This finding is again consistent with the postulated function of α_2M as a carrier or transport protein for T_1AM, rather than a mechanism for T_1AM clearance from the body. That T_1AM binds both the native, slow form of the protein, as well as the protease-activated fast form, might suggest a role for T_1AM in affecting the rate of clearance of protease-α_2M complexes, but this potential function requires additional investigation.

It is also critical to determine the affinity of T_1AM binding to α_2M by measuring its dissociation rate. Several methods attempting to measure the dissociation rate constant, including filter binding and equilibrium dialysis experiments, have been unsuccessful, but future studies investigating T_1AM-α_2M binding should focus on determining the affinity of this interaction. Knowing the dissociation rate of T_1AM from

α_2M is important for understanding the role of α_2M in carrying and unloading T_1AM to various tissues.

Although α_2M was the only protein identified in this study as a T_1AM binding protein, the relatively high abundance of α_2M in serum might potentially mask the interactions of other, less abundant serum proteins capable of binding T_1AM. The use of α_2M-depleted serum might be useful in revealing additional binding proteins for T_1AM.

In conclusion, this study has identified α_2M as a serum binding protein for T_1AM. However, important aspects of its binding properties, such as the binding affinity and identification of binding sites, should be further investigated in future studies. In addition, since α_2M is known to be involved in a wide variety of physiological activities, the significance of α_2M as a binding protein for T_1AM, beyond its presumed function as a carrier protein, could provide important additional information regarding the mechanism of action of T_1AM itself.

Acknowledgements

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CHAPTER 6

Concluding remarks

Since the discovery in 2004 of T_1AM as an endogenous thyroid hormone derivative with dramatic *in vivo* actions, a number of studies have focused on understanding its mechanism of action. The full spectrum of its effects is unlike that of any other known drug or endogenous biologically active compound. Extracellular signaling at GPCRs such as TAAR1 and the α_{2A} adrenergic receptor, modulation of neurotransmitter packaging and recycling pathways, and metabolic processing are all important findings that provide clues regarding the biological role of this thyroid hormone derivative.

The goal of this research has been to augment our understanding of the functions of T_1AM. The major focus was the identification and characterization of a specific plasma membrane transport mechanism of T_1AM into cells, motivated by the known physiological importance of membrane transporters of other biogenic amines and thyroid hormone for the proper functioning of neurological and endocrine systems. Several transporter candidates were identified by a large-scale RNAi screen, and warrant further investigation. Future studies should identify the particular plasma membrane transporters or other mechanisms by which T_1AM enters cells. Nuclear uptake of T_1AM suggests a potential role in transcriptional regulation, and merits further study to identify potential nuclear receptors and T_1AM-responsive genes.

This work has also investigated T_1AM as an inhibitor of thyroid hormone transport into cells, thereby regulating its action and metabolism. The *in vivo* effects of T_1AM appear to be largely opposite those of thyroid hormone, and inhibition of the transport and intracellular activity of thyroid hormone may in part explain these effects. Further studies should focus on whether the observed inhibition has physiological

relevance, that is, whether sufficiently high concentrations of T_1AM can be reached in tissues where these thyroid hormone transporters are abundant, particularly in the brain.

Finally, because many biological compounds including thyroid hormone bind to various serum proteins important for their storage and transport throughout the body, we have identified α_2M as a serum binding protein for T_1AM. α_2M has various functions in the body, including a unique role as a pan-protease inhibitor, and has potential importance for immune defense and modulation of neurotransmitter metabolism. In addition to the possible role of α_2M as a carrier protein for T_1AM, the discovery of α_2M interaction with T_1AM opens another interesting area of investigation into this thyroid hormone derivative and its mechanisms of action in the body.

The findings reported here provide new and important additional insight into the potential roles of T_1AM, and further studies in these and other aspects of T_1AM action will undoubtedly yield invaluable knowledge of this thyroid hormone metabolite and its physiological implications in what appears to be a novel and rapidly expanding field of thyroid endocrinology. Furthermore, understanding the mechanisms of action underlying its broad spectrum of effects may reveal novel therapeutic applications of T_1AM in areas as diverse as cardiology, neurology, and modulation of metabolic processes throughout the body.

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