BEATRIZ CAMILLE GRI GALIS FREITAS

A SINALIZAÇÃO PARÁCRINA DA TRIODOTIRONINA DERIVADA DAS CÉLULAS GLIAIS ATIVA A EXPRESSÃO DE GENES NEURONIAIS NO CÉREBRO DE ROEDORES E EM CÉLULAS HUMANAS

Orientador: Prof. Dr. Antonio Carlos Bianco
Co-orientador: Prof. Dr Rui Monteiro de Barros Maciel

São Paulo
2010
A SINALIZAÇÃO PARÁCRINA DA TRIIODOTIRONINA DERIVADA DAS CÉLULAS GLIAIS ATIVA A EXPRESSÃO DE GENES NEURONAIOS NO CÉREBRO DE ROEDORES E EM CÉLULAS HUMANAS

Tese de Doutorado apresentada ao Programa de Pós-Graduação em Endocrinologia da Universidade Federal de São Paulo para a obtenção do título de Doutor em Ciências.

**Orientador:** Prof. Dr. Antonio Carlos Bianco

**Co-orientador:** Prof. Dr Rui Monteiro de Barros Maciel

São Paulo

2010
Freitas, Beatriz Camille Grigalis


Tese de Doutorado, Programa de Pós-Graduação em Endocrinologia, Disciplina de Endocrinologia, Departamento de Medicina, Escola Paulista de Medicina, Universidade Federal de São Paulo

Orientador: Prof. Dr. Antonio Carlos Bianco
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Descritores: Hormônio tiroidiano - Desiodase - Cérebro - Astrócito e Neurônio
Autor(a): Beatriz Camille Grigalis Freitas

Título: A sinalização parácrina da triiodotironina derivada das células gliais ativa a expressão de genes neuronais no cérebro de roedores e em células humanas

Programa de Pós-Graduação em Endocrinologia, Universidade Federal de São Paulo

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3. Prof. Dr. João Roberto Maciel Martins

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ÍNDICE

LISTA DE ABREVIATURAS E SIGLAS .................................................................................................. 5
AGRADECIMENTOS .......................................................................................................................... 9
APRESENTAÇÃO ................................................................................................................................ 12
RESUMO .............................................................................................................................................. 14
ABSTRACT ............................................................................................................................................ 15
INTRODUÇÃO À FISIOLOGIA DAS DESIODASES DAS IODOTIRONINAS ................................................ 16
INTRODUÇÃO AO TEMA DA PESQUISA PRINCIPAL ......................................................................... 22
OBJETIVOS ........................................................................................................................................... 25
MATERIAIS E MÉTODOS ..................................................................................................................... 26
RESULTADOS ....................................................................................................................................... 31
DISCUSSÃO .......................................................................................................................................... 37
CONCLUSÕES ....................................................................................................................................... 41
FIGURAS ............................................................................................................................................... 42
REFERÊNCIAS BIBLIOGRÁFICAS ........................................................................................................ 51
MANUSCrito 1: PUBLICADO NA REVISTA JOURNAL OF CLINICAL INVESTIGATION (JCI) .. 59
MANUSCrito 2: PUBLICADO NA REVISTA THYROID .............................................................................. 71
REFERÊNCIAS BIBLIOGRÁFICAS DA INTRODUÇÃO ........................................................................... 80
PROJETOS REALIZADOS DURANTE A PÓS-GRADUAÇÃO ................................................................. 85
<table>
<thead>
<tr>
<th>Termo</th>
<th>Nome em Inglês</th>
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<tr>
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<td>SHH</td>
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<td>WSB-1</td>
<td>WD repeat and SOCS box-containing 1</td>
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Dedico esta tese aos meus pais e avós pelo apoio incondicional e o enorme exemplo de vida que me proporcionam, sem eles nada disso seria possível. Obrigada por tudo.

Ao mestre, prof. Rui, com carinho...
“Was mich nicht umbringt, macht mich stärker”
Friedrich Nietzsche, Götzen-Dämmerung (1888)
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Gostaria de começar essa minha seção dedicada aos agradecimentos com a citação das sábias palavras de José Saramago, que dizia: “Se podes olhar, vê. Se podes ver, repara”. Acredito que essa frase reflete o princípio mais básico, porém o mais importante da ciência: o da observação. Quando digo isso, não me refiro apenas à simples observação visual, mas à observação que vai muito além dos sentidos, que leva à reflexão, ao questionamento, às críticas, à avaliação. A curiosidade e a motivação pessoal que, enfim, conduzem a experimentação científica. Esse sempre foi o sentimento que a ciência despertou em mim. Mesmo sendo nova na carreira científica, dando aqui meu primeiro grande passo com a obtenção de um título, sei que a observação é pessoal, ou seja, cada um tem seu ponto de vista. Somente a ditadura representa e segue apenas um ponto de vista. A ciência vive do convívio, da discussão, e principalmente da participação alheia na construção do conhecimento. Desta maneira fica impossível aqui agradecer a todos, que com seus diferentes pontos de vista, ajudaram na construção da minha base científica. Deixo registrado, portanto, meus sinceros agradecimentos a todos que de uma maneira geral formaram a cientista que sou.

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Ao meu grande companheiro de vida, Vinícius, sobretudo pela compreensão das dificuldades deste meu doutorado, mostrando que tudo que é verdadeiro prevalece independente da distância, tempo e dificuldades.

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À Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), pela bolsa de Doutorado Direto (2005/55826-4) e pelo financiamento da pesquisa realizada (2005/55825-8).

Obrigada a Deus e a todos aqui citados.
Realizei meu Doutorado junto ao Programa de Pós-Graduação em Endocrinologia da Universidade Federal de São Paulo (UNIFESP), sob orientação do Professor Antonio Carlos Bianco e co-orientação do Professor Rui Monteiro de Barros Maciel.

De acordo com a orientação de meus preceptores e considerando-se seus interesses comuns em fisiologia e fisiopatologia tiroidianas, estive envolvida em diversos projetos de pesquisa durante meu programa de doutorado, intercalando períodos de trabalho nos laboratórios por eles dirigidos na Thyroid Section, Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Womens’ Hospital-Harvard Medical School, em Boston (Dr. Bianco) e no Laboratório de Endocrinologia Molecular, Disciplina de Endocrinologia, Departamento de Medicina, Escola Paulista de Medicina, UNIFESP em São Paulo (Dr. Maciel), com auxílio de uma bolsa de Doutorado Direto da FAPESP (2005/55826-4).

Entre estes projetos de pesquisa, destaca-se o estudo do papel dos hormônios tiroidianos no cérebro, objeto desta Tese. Este trabalho iniciou-se com estudos sobre a degradação da desiodase do tipo 2 (D2), enzima que catalisa a maior parte da transformação de T₄ para T₃ em vertebrados e considerada a desiodase de maior importância fisiológica. A D2 é uma enzima dotada de grande plasticidade funcional e degradada pelo sistema ubiquitina-proteassomo. Nesta degradação proteassômica, a D2 é unida à ubiquitina pela ação conjunta de uma enzima do tipo 1 (E1) ativadora da ubiquitina, de uma enzima do tipo 2 (E2) carregadora de ubiquitina e de uma enzima do tipo 3 (E3) ligase, que finaliza o processo, dirigindo a proteína-alvo para sua degradação. Entre as proteínas relacionadas com este sistema de degradação proteassômica específicas para a D2 destacam-se a WSB-1, que é uma E3 ligase, que diminui a meia-vida funcional da D2 e a VDU-1, uma desubiquitinadora da D2, que devolve a sua atividade enzimática.

A princípio, o projeto principal de minha Tese visava a análise funcional e estrutural, assim como a variação genética dessas proteínas envolvidas com a D2 para uma melhor compreensão da homeostase dos hormônios tiroidianos (HT). Porém, após alguns experimentos focados na detecção da atividade da D2 em diferentes tipos celulares, evidenciamos que um tipo celular originado a partir de um glioma (H4) apresentava atividade intrínseca desta proteína. A partir disso, montamos um sistema...
baseado na co-cultura destes gliomas com neurônios (possuidores apenas da desidrase do tipo 3, D3), com o objetivo de melhor compreender a homeostase dos HT no cérebro, objetivo que passou, então, a ser o projeto principal desta Tese. Os resultados deste projeto foram recentemente publicados no *The Journal of Clinical Investigation* 2010; 120: 2206-2217. Parte deste trabalho foi complementada depois de minha volta ao Brasil, ocorrida em 2008, com contribuições do novo laboratório do Dr. Bianco na Division of Endocrinology, Diabetes and Metabolism, University of Miami Miller School of Medicine, Miami, Florida, EUA, assim como com contribuições da Dra. Ann Marie Zavacki, Thyroid Section, Division of Endocrinology, Diabetes and Hypertension, Department of Medicine, Brigham and Womens’ Hospital-Harvard Medical School, Boston, Massachusetts, EUA, do Dr. Ronald M. Lechan, Tupper Research Institute, Department of Medicine, Tufts University Medical Center, Boston, Massachusetts, EUA e do grupo do Dr. Balázs Gereben, Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine, Hungarian Academy of Sciences, em Budapeste, Hungria.

Além deste trabalho, participei ativamente de uma série de outros projetos de pesquisa em curso nos laboratórios dos Professores Bianco e Maciel, mencionados ao final desta Tese, para que a Banca Examinadora possa ter uma visão mais abrangente das atividades por mim realizadas durante o Programa de Doutorado.
RESUMO

O hipotiroidismo nos humanos caracteriza-se por distúrbios neurológicos graves, frequentemente irreversíveis, que ressaltam o papel crítico dos hormônios tiroidianos (HT) no cérebro. Apesar disto, pouco se sabe sobre as vias de sinalização que controlam a ação dos HT no cérebro. O que é conhecido é que a tiroxina ($T_4$), um pró-hormônio, é convertida no hormônio metabolicamente ativo, a triodotironina ($T_3$), pela desiodase do tipo 2 (D2) nos astrócitos, embora os receptores dos HT e a desiodase do tipo 3 (D3), que inativa o $T_3$, sejam encontrados nos neurônios adjacentes. Neste trabalho, modelamos a ação dos HT no cérebro empregando um sistema de co-cultura in vitro de células de glioma humano H4, que expressam D2 e células de neuroblastoma humanos SK-N-AS, que expressam D3. Constatamos que a atividade da D2 da célula glial causou o aumento na produção de $T_3$, que agiu de maneira parácrina induzindo genes responsivos a $T_3$, tais como o gene ecto-nucleotídeo pirofosfatase/fosfodiesterase 2 ($ENPP2$) nos neurônios desta co-cultura. A atividade de D3 nos neurônios modulou estes efeitos. Adicionalmente, esta sinalização parácrina foi regulada por outras vias, tais como hipóxia, sinalização pela via de “hedgehog” ($Shh$) e inflamação induzida por LPS, como se demonstrou tanto no sistema de co-cultura in vitro, quanto nos modelos de ratos in vivo de isquemia do cérebro e modelos de inflamação em camundongos. Este estudo, portanto, apresenta o que acreditamos tratar-se da primeira evidência direta para a demonstração de uma alça parácrina ligando a atividade D2 na glia aos receptores dos HT em neurônios, identificando desta forma as desiodases como etapas potenciais de controle da regulação de sinalização dos HT no cérebro durante a saúde e doença.

Palavras-chave: Hormônio tiroidiano, desiodase, cérebro, glia e neurônio
Hypothyroidism in humans is characterized by severe neurological consequences that are often irreversible, highlighting the critical role of thyroid hormone (TH) in the brain. Despite this, not much is known about the signaling pathways that control TH action in the brain. What is known is that the prohormone thyroxine (T₄) is converted to the active hormone triiodothyronine (T₃) by type 2 deiodinase (D2) and that this occurs in astrocytes, while TH receptors and type 3 deiodinase (D3), which inactivates T₃, are found in adjacent neurons. Here, we modeled TH action in the brain using an in vitro coculture system of D2-expressing H4 human glioma cells and D3-expressing SK-N-AS human neuroblastoma cells. We found that glial cell D2 activity resulted in increased T₃ production, which acted in a paracrine fashion to induce T₃-responsive genes, including ectonucleotide pyrophosphatase/phosphodiesterase 2 (ENPP2), in the cocultured neurons. D3 activity in the neurons modulated these effects. Furthermore, this paracrine pathway was regulated by signals such as hypoxia, hedgehog signaling, and LPS-induced inflammation, as evidenced both in the in vitro coculture system and in in vivo rat models of brain ischemia and mouse models of inflammation. This study therefore presents what we believe to be the first direct evidence for a paracrine loop linking glial D2 activity to TH receptors in neurons, thereby identifying deiodinases as potential control points for the regulation of TH signaling in the brain during health and disease.

**Keywords:** Thyroid hormone, deiodinase, brain, glia and neuron.
A ativação da tiroxina (T₄) para triiodotironina (T₃) e a inativação do T₄ e T₃ são realizadas por meio de enzimas específicas chamadas desiodases. Estas enzimas estão presentes em todos os vertebrados, sugerindo que a desiodação do hormônio tiroideano seja um componente intrínseco da homeostase da glândula tiroide. O T₄ é considerado um pró-hormônio de meia-vida (t₁/₂) longa, que para iniciar seus efeitos biológicos precisa ser ativado num hormônio biologicamente ativo, o T₃. O T₃ regula a expressão gênica em virtualmente todos os tecidos dos vertebrados por meio de fatores de transcrição dependentes de ligantes específicos, os receptores dos hormônios tiroïdianos (TR) (Bianco e cols., 2002; Larsen e cols., 2008). Diferentemente de muitos hormônios que têm um papel funcional específico, os hormônios tiroïdianos (HT), T₄ e T₃, exibem uma faixa ampla de efeitos biológicos, incluindo a diferenciação e o crescimento das células e o gasto energético (Whitehead, 2001).

Figura A – Ativação e inativação da tiroxina pelas desiodases. A seta verde representa a ativação pela D2 e D1 e as setas vermelhas indicam as possíveis vias de inativação realizada pela três desiodases. Esta “inativação” refere-se a incapacidade de ligação com o receptor do hormônio tiroideano no DNA, ou seja, apenas considerando a ação genômica do hormônio tiroideano.
A desiodação de T₄ em T₃ ocorre no anel fenólico (externo) da molécula do T₄ e é catalisada por duas desiodases, D1 e D2 (Figura A). Em contrapartida a este caminho de ativação, tanto o T₄ como o T₃ podem ser inativados irreversivelmente por desiodação do anel tirosil (interno), reação catalisada preferencialmente pela D3, o terceiro membro do grupo das desiodases. Em humanos, a maior parte do T₃ é derivada da desiodação de T₄ catalisada por D2 (60-70%). Estas três desiodases contêm similaridade estrutural (com aproximadamente 50% de identidade entre si) e são proteínas de membrana com peso molecular semelhante, de 29 a 33 kDa (Bianco, 2004).

Além disso, as desiodases apresentam alta homologia em seu centro catalítico, caracterizado principalmente pela presença de uma selenocisteína em seu sítio ativo (Croteau e cols., 1995; Buettner e cols., 2000). No entanto, mesmo sendo similares, estas enzimas possuem características distintas e específicas relacionadas à sua função, distribuição tecidual, substrato (Tabela 1), meia vida, sensibilidade às drogas e cinética enzimática, conforme será discutido mais adiante (Bianco e Larsen, 2005).

### Tabela 1
Distribuição tecidual e substrato preferencial das três desiodases. Tabela modificada de Larsen e cols., 2008.

<table>
<thead>
<tr>
<th>Desiodase</th>
<th>Distribuição Tecidual</th>
<th>Substrato Pref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1</td>
<td>Fígado, rim, tireóide, hipófise</td>
<td>rT₃, T₄</td>
</tr>
<tr>
<td>D2</td>
<td>SNC, hipófise, tecido adiposo marrom, placenta, tireóide, músculo esquelético e coração – células da glia</td>
<td>T₄; rT₃</td>
</tr>
<tr>
<td>D3</td>
<td>Placenta, SNC, hemangiomas, fígado, músculo esquelético – neurônios</td>
<td>T₃; T₄</td>
</tr>
</tbody>
</table>

A desiodase do tipo 1, D1 (Figura B) foi a primeira a ser clonada em 1991 por Berry e colaboradores e caracteriza-se por ser inibida pelo composto propiltiouracil (PTU) (Oppenheimer e cols., 1972). A D1 efetua também a inativação do hormônio pela desiodação do anel interno do T₃, gerando assim o T₂, porém com menos afinidade por esse substrato quando comparado com a D3 baseado em sua Constante de Michaelis e Menten (Km) (Fekkes e cols., 1982; Bianco e cols., 2002). Seu Km para T₄ é de aproximadamente 1μM e para rT₃ de 0.5 μM, com t½ de 8 horas (Bianco, 2004). A expressão de D1 em humanos ocorre em maior quantidade no fígado, mas também está presente nos rins e na tireóide (Larsen e cols., 2008). Embora já tenha sido identificada a expressão de D1 na hipófise e no ovário de tecidos de vertebrados, os resultados são
contraditórios (Connor e cols., 2005). Mesmo presente nestes órgãos, a importância da D1 na homeostase dos HT é questionada, pois sua expressão varia entre linhagens diferentes de camundongos (Bianco e Larsen, 2005; Connor e cols., 2005; St. Germain e cols., 2005; Zhou e cols., 2009); ademais, a D1 é a única desiodase que não participa de nenhuma etapa do desenvolvimento dos anfíbios, vertebrado onde os HT são essenciais para seu desenvolvimento completo (Galton, 1992).

**Figura B** • Atividade celular da D1. A tiroxina e o T₃ proveniente do meio extra-celular entra na célula através de um transportador específico (representado pela cor marrom) podendo ser MCT8 ou OATP14, entre outros, variando conforme o tipo celular. A posição da D1 na membrana permite a ativação da tiroxina em T₃ como também sua inativação para rT₃ e T₂ a partir do T₄ e T₃, respectivamente.

A desiodase do tipo 2, D2 (Figura C), é uma proteína de 32 kDa residente do retículo endoplasmático (RE) com uma porção NH₂ terminal no lúmen e apenas um domínio transmembrana contido nos seus primeiros 40 aminoácidos. Sua porção catalítica encontra-se no citoplasma da célula, com um átomo de selênio em seu sitio ativo com distribuição tipicamente perinuclear (Maciel e cols., 1979; Baqui e cols., 2000; Curcio e cols., 2001). Foi a última das desiodases a ser clonada, primeiramente em *Rana catesbeiana* (Davey e cols., 1995), em seguida em ratos e humanos (Croteau e cols., 1996; Salvatore e cols., 1996), e camundongos (Davey e cols., 1999), entre outros vertebrados (Valverde e cols., 1997; Gereben e cols., 1999).
Devido à sua plasticidade fisiológica, a D2 é a maior produtora de T₃ em situações críticas, como ausência de iodo (Obregon e cols., 2005) ou exposição ao frio Silva e (Larsen, 1983; Christoffolete e cols., 2004). Três importantes propriedades da D2 são responsáveis por esta plasticidade: t₁/₂ curto (~45 minutos vs. 8 horas para a D1); Km baixo para T₄, dentro da faixa fisiológica de T₄ livre no soro (1000 vezes mais baixo que a D1) e resposta ao estímulo com AMP cíclico (cAMP). A presença de um elemento responsive ao cAMP no gene da D2 (DIO2) constitui a base para a estimulação neural rápida de D2 em alguns tecidos, incluindo-se o tecido adiposo marrom (BAT) e o músculo esquelético. Este fato liga a expressão da D2 com o sistema nervoso simpático (SNS) e com o hipotálamo, o que amplia o espectro de estímulos ambientais e endógenos que podem potencialmente influenciar o nível de D2 e a produção adaptativa de T₃ (Bianco e Larsen, 2005).

**Figura C** • Atividade celular da D2. Após ser transportado do meio extra-celular por transportadores específicos, a tiroxina pode ser direcionada para o retículo endoplasmático e o T₃ pode seguir diretamente ao núcleo ativando seus receptores específicos. A D2 está localizada no retículo endoplasmático onde pode ativar o T₄ em T₃ e este é direcionado para o núcleo, onde ativa genes responsivos à T₃.

A instabilidade intrínseca da D2 é proporcionada principalmente por sua susceptibilidade à ubiquitinação e à degradação proteassômica seletiva (Gereben e cols., 2000). Entretanto, esta proteólise proteossomal induzida por substrato é a propriedade crítica da D2, o que confere à mesma um papel adaptativo na homeostase de T₃
(Steinsapir e cols., 2000). Por exemplo, durante períodos de ingestão baixa de iodo, a produção de T₄ cai, prolongando o t₁/₂ da D₂ e intensificando a taxa de conversão de T₄ para T₃. A conseqüente queda do T₄ sérico diminuiu a retroalimentação negativa sobre os tirotrofos estimulando a secreção de TSH e acelerando simultaneamente a conversão de T₄ para T₃, principalmente nos tanicitos (células da glia), células que contem D₂. Além disso, a desiodação inativadora de T₄ e T₃, por meio da D₃, completa esse cenário, ajustando a concentração final de T₃. O T₃ estimula a D₃ em nível transcricional; portanto, se a ingestão de iodo tornar-se tão baixa que esgote a capacidade da glândula tireoide de produzir hormônio e falte D₂ para manter o reservatório de T₃ dentro da faixa normal, o T₃ sérico cai e assim diminui também a atividade da D₃, prolongando o t₁/₂ tanto do T₄, quanto do T₃. Este mecanismo constitui uma segunda linha de defesa, cujo propósito é retardar o início do hipotiroidismo por deficiência de iodo (Gereben e cols. 2008a).

Além desses processos descritos para o controle da atividade da D₂, há outros mecanismos colaboradores para a homeostase dos HT, pois o mRNA da D₂ pode sofrer “splicing” alternativo e sua ativação ser causada por diversos outros fatores transcricionais, como NKX2-5, GATA-4 e TTF-1. A existência de tantas vias buscando o controle da atividade da D₂ confirmam a sua importância no controle da disponibilidade dos HT em suas células alvo (Gereben e Salvatore, 2005).

A desiodase do tipo 3 (D₃) é a principal responsável pela inativação dos HT, promovendo a remoção de um átomo de iodo do anel interno das iodotironinas, convertendo o T₄ em rT₃ e o T₃ em T₂, moléculas consideradas biologicamente inativas. Esta função inativadora da D₃ garante a homeostase tecidual, além de prevenir a exposição de tecidos sensíveis ao excesso de HT. Nos camundongos deficientes de D₃ (D₃KO) foi possível observar o papel crítico desta enzima na maturação e função do eixo tiroidiano (Hernandez e cols., 2006), enquanto que sua expressão aumentada em tumores vasculares causa hipotiroidismo grave (Huang e cols., 2000; Huang e cols., 2002). Estes animais D₃KO também demonstraram deficiência na fertilidade e no crescimento, acompanhados de maior taxa de mortalidade perinatal (St. Germain e cols., 2005; Hernandez e cols., 2006).

A D₃ tem aproximadamente 32 kDa e catalisa a desiodação do anel interno tirosil da tiroxina com um Km de 10⁻⁹. Esta enzima não é sensível à inibição por PTU (Salvatore e cols., 1995), forma dímeros como as outras desiodases (Curcio-Morelli e cols., 2003)

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com \( t_{1/2} \) longo (~12 horas). Ainda não se descreveu nenhuma via de regulação pós transcriucional para D3 (Gereben e cols., 2008b).

O status tiroidiano tem grande influência sobre os níveis de atividade e expressão de D3 diante de diferentes situações fisiológicas, principalmente no sistema nervoso central. A atividade da D3 é aumentada no hipertiroidismo e diminuída no hipotiroïdismo. O aumento da expressão do mRNA da D3 na transição do estado de eutiroidismo para de hipertiroïdismo chega a ser de 4 a 50 vezes (Escobar-Morreale e cols., 1997; Tu e cols., 1999).

**Figura D**  • Atividade celular da D3. As moléculas de tiroxina e \( T_3 \) podem entrar na célula por transportadores específicos e serem redirecionadas para a membrana plasmática para sua inativação pela D3. Sua posição na membrana plasmática permite a rápida inativação do \( T_4 \) em \( rT_3 \) e \( T_3 \) em \( T_2 \) (Baqui e cols., 2003).
Um dos aspectos mais devastadores do hipotiroidismo neonatal é a deterioração neurológica grave e o retardo mental, frequentemente associados com fácies anormal, surdo-mudez e espasticidade motora (1, 2). Estas características são, de modo geral, irreversíveis e vários países criaram, há décadas atrás, os programas de detecção precoce de hipotiroidismo congênito, com o objetivo de identificar e tratar os recém-nascidos com esta condição. O cérebro adulto também é sensível aos hormônios tiroidianos (HT). Assim, pacientes com hipotiroidismo são frequentemente letárgicos, têm coordenação motora prejudicada, deterioração da memória e uma tendência para depressão e perturbações do humor. Por sua vez, o hipertiroidismo está associado a irritabilidade, ansiedade e oscilações do humor (3).

Embora estas síndromes ilustrem o papel crítico desempenhado pelos HT no cérebro, pouco se sabe sobre as vias de sinalização que controlam a ação dos HT neste órgão. Em contraste com a maioria dos tecidos, a maior parte dos HT no cérebro é produzido localmente pela ativação de T₄ para T₃ por meio da ação da D₂ (4). Isto tem sido estudado diretamente por técnicas de marcação dupla e recentemente confirmado com a observação de que camundongos onde o gene DIO2 foi nocauteado (do inglês knockout, ko) exibem quase a metade de T₃ em seu cérebros quando comparados com camundongos normais (5). Assim, é amplamente aceito que a expressão de D₂ em determinadas áreas do cérebro aumenta a sinalização dos HT, mecanismo que tem sido associado a funções cerebrais importantes, tais como desenvolvimento coclear, o “feedback” de hormônio liberador de tirotrofina/hormônio estimulador da tireoide (TRH/TSH) e a procriação sazonal em pássaros (6, 7). Ao mesmo tempo, a via inativadora dos HT, por meio da D₃, também está ativa em áreas específicas do cérebro, amortecendo a ação dos HT. Não surpreendentemente, as sinalizações da D₂ e D₃ são sincronizadas inversamente do ponto de vista espacial e temporal. Assim, assume-se que o balanço destas duas vias, isto é, D₂ versus D₃ e, menos significantemente, a contribuição do nível de T₃ plasmático, determinem processos críticos do cérebro, tais como mielinização, migração neuronal, diferenciação das células da glia e neurogênese (1, 8, 9).
Os neurônios expressam os receptores do hormônio tiroidiano (TRs) e são presumivelmente o principal alvo do T₃ no cérebro, mas a D2 é expressa em astrócitos, não em neurônios. Isto suscita uma questão anatômica: o T₃ gerado em astrócitos alcança os TRs nos neurônios (2, 10, 11)? Adicionalmente, a expressão de D3 nos neurônios limita esta via parácrina? Tal mecanismo parácrino tornou-se mais plausível com a descoberta do transporte ativo dos HT para os neurônios por meio de transportadores, tais como o transportador do ácido monocarboxílico do tipo 8 (MCT8, do inglês monocarboxylate TH transporter-8): mutações no MCT8 explicam a base molecular para a síndrome de Allan-Herndon-Dudley (AHDS), uma doença rara ligada ao cromossomo X, caracterizada por anormalidades neurológicas que incluem retardo global no desenvolvimento, hipotonia central, nistagmo rotatório, audição prejudicada e espasticidade (12, 13). Uma outra linha de evidência apoiando o conceito de transporte dos HT de maneira parácrina no cérebro é derivada a partir de estudos do metabolismo dos HT no hipotálamo, onde a D2 é expressa nos tanicitos, células giais especializadas localizadas nas paredes inferior e infralateral do terceiro ventrículo no hipotálamo mediobasal (HMB) (14, 15). Tem sido sugerido que a geração de T₃ via D2 em tanicitos poderia afetar a expressão génica em neurônios do tipo TRH, localizados no núcleo paraventricular (NPV), assim explicando porque o T₄ é crítico no “feedback” negativo de TRH (16). Ao mesmo tempo, a regulação positiva de D2 em tanicitos foi demonstrada num modelo em roedores para jejum e na doença crítica não-tiroidiana, sugerindo que um aumento relativo local na ação dos HT media o hipotiroidismo central, frequentemente observado nestas circunstâncias (17-19). Na codorna japonesa, a expressão de D2 no HMB é induzida por luz. A administração de T₃ nos ventrículos cerebrais simula a resposta fotoperiódica, enquanto que o ácido iopanóico (inibidor de D2) impede o crescimento gonadal, indicando que a expressão de D2 induzida por luz no HMB pode estar envolvida na resposta fotoperiódica de gônadas neste tipo de animal (7).

Se esta conexão glial-D2/neurônio-TR existir de fato, este mecanismo poderia ter a vantagem de permitir uma regulação muito mais sofisticada da ação dos HT no cérebro, com o controle por meio da desiodação glial ou neuronal. Um número de vias de sinalização, que foram recentemente demonstradas como relevantes para as desiodases, podem também serem operantes no cérebro, como por exemplo, a ativação
de HIF-1α na D3 em tecido hipóxico (20), a inativação de D2 mediada pela família da proteína “hedgehog” (Shh) e a ativação de D3 (20-22).

A evidência direta de uma ação transcricional de T3 mediada por desiodases em neurônios ainda não é disponível. Neste trabalho nós modelamos esta via in vitro por cocultura de células de glioma H4 que expressam D2 com células neuronais SK-N-AS que expressam D3. Usando este sistema nós constatamos que o T3 gerado por células da glia (por meio da atividade de D2), foi capaz de atuar de modo parácrino, induzindo a expressão de genes responsivos a T3 em neurônios da co-cultura, a despeito da presença de atividade de D3. Adicionalmente, nós constatamos que o sistema é regulado por vias como hipóxia, proteínas da via de sinalização do “hedgehog” e inflamação induzida por LPS. Estudos in vivo usando isquemia e LPS adicionalmente validaram a relevância destes achados. Em nosso conhecimento, estes dados representam a primeira evidência direta para uma alça parácrina ligando D2 em células gliais a TRs em neurônios, identificando as desiodases como etapas de controle para a regulação da sinalização dos HT no cérebro na saúde e na doença.
OBJETIVOS

- Identificar tipos celulares que imitem o padrão de expressão das desiodases *in vivo* encontradas no cérebro, entre vários tipos de glioblastoma e neuroblastoma disponíveis;

- Elaborar e construir um sistema de co-cultura *in vitro* com as células previamente identificadas com presença ou ausência da tiroxina;

- Submeter o modelo elaborado de co-cultura à situações de estresse fisiológico como hipóxia e aos estímulos de moléculas da via de “Hedgehog”;

- Analisar o impacto da inflamação celular induzida por LPS no modelo de co-cultura estabelecido.
MATERIAIS E MÉTODOS

Reagentes. T₄, T₃, forskolin, ciclopamina, soro fetal bovino (SFB), ampicilina, gentamicina e selenito sódico foram comprados da Sigma-Aldrich; rT₃ da Calbiochem; DMEM da Gibco. O sistema de Transwell® foi adquirido da Corning Scientific. Todo o material de transcriptase reversa, DNAse, RNase out, DNTp mix, EDTA, SuperScript II, DAPI, anticorpos, α-tubulina (camundongo), α-mouse IgG de cabra conjugado com Alexa Flúor 594 e Trizol foram adquiridos da Invitrogen. As células da linhagem celular SK-N-AS foram doadas por S. Huang (Children’s Hospital, Boston, Massachusetts, USA), as células H4 por M. LaVoie (Brigham and Women’s Hospital, Boston, Massachusetts, USA) e as células HEK-TLR4-MD2-Elam por M. Genest (Eisai Research Institute, Andover, Massachusetts, USA).

Animais. Camundongos adultos machos C57BL/6 ou D2-KO e ratos Sprague-Dawley foram usados conforme detalhado nas seções descrevendo a infecção por LPS e os modelos de hipóxia animal. Os animais foram mantidos num ambiente em condições padronizadas (iluminação entre 06:00-18:00 horas, temperatura 22 ± 1ºC, ração padrão e água disponível ad libitum). Os animais foram mantidos e testados de acordo com os protocolos aprovados pelo Animal Care and Use Committees das Universidades de Miami e Tufts, de acordo com os modelos do NIH. Os protocolos animais seguiram o European Communities Council Directive de 24 de Novembro de 1986 (86/609/EEC) e, quando apropriado, foram revisados e aprovados pelo Animal Welfare Committee do Institute of Experimental Medicine da Hungarian Academy of Sciences.

Modelo de infecção por LPS. Os experimentos foram conduzidos em camundongos C57BL/6 WT ou D2-KO (linhagem originada do C57BL/6, Jackson Laboratories) pesando entre 20 a 30g. Os animais D2KO foram caracterizados anteriormente (46). O tratamento com LPS foi realizado de acordo com estudo anterior (18), usando 250 µg de LPS bacteriano (O127:B8; Sigma-Aldrich) dissolvido em solução salina por 100 g de massa corpórea numa injeção intraperitonial de LPS bacteriano (O127:B8; Sigma-Aldrich) dissolvido em solução salina. Doze ou 24 hrs após o tratamento, os camundongos foram sacrificados com uma dose elevada de pentobarbital por perfusão na aorta ascendente com 4% paraformaldeído (PFA) em 0.1M solução de PBS-salina. Os cérebros foram fixados por 4 horas no mesmo fixador, crio-protegido em solução de 30% sacarose e,
em seguida, processados para hibridização in situ para TRH como descrito anteriormente (47).

**Hipóxia cerebral causada por oclusão da artéria cerebral média cirurgicamente.** A isquemia cerebral em ratos foi induzida (n=10) por oclusão da artéria cerebral média (OACM) durante 60 minutos sob anestésico Nembutal (60 mg/kg i. p.), conforme descrita anteriormente (48, 49). Animais falsamente operados (“sham”) (n=10) foram submetidos ao mesmo procedimento cirúrgico, mas sem inclusão de filamentos na artéria carótida. O déficit intra-isquêmico neurológico foi confirmado por detecção dos padrões de marcha anormais caracterizados por movimentos circulares ou movimentos para esquerda nos animais com sucesso no procedimento de OACM. Animais que não demonstraram déficit foram excluídos do estudo. Após 60 minutos de isquemia, os animais eram perfundidos com solução salina de 0.1 M PBS com 4% PFA.

**Cultura de células e o sistema Transwell®.** SK-N-AS e H4 foram propagadas em DMEM contendo 10% SFB com 15 μg de gentamicina e 50 μg de ampicilina por litro de meio. As células foram incubadas durante a noite com meio contendo 10% de soro previamente tratado com carvão ativado na noite anterior ao tratamento com T₄ ou T₃. O meio de todas as células foi suplementado com solução 10⁻⁷ M de selenito sódico. As células foram incubadas sob condições de normóxia ou hipóxia em câmaras fechadas de hipóxia (Billups-Rothenberg Inc.). Para o sistema da Transwell, foram plaqueadas 1,5 x 10⁶ células de H4 dentro do inserto, e 7,5 x 10⁵ células de SK-N-AS foram plaqueadas em placas de seis poços comuns (Figura 5). As células foram plaqueadas em placas separadas para evitar a mistura dos tipos celulares e ao final do dia foram colocadas na mesma placa quando o meio foi trocado para DMEM com 10% SFB tratado previamente com carvão ativado. As células HEK-TLR4-MD2-Elam expressam permanentemente o complexo TLR4-MD2. Essas células foram usadas para estabelecer a concentração e o tempo necessários para a resposta celular ao LPS (29). As culturas destas células foram mantidas em DMEM com 10% SFB suplementado com 150 μg/mL Zeocina, 300 μg/mL G418, e 50 μg/mL higromicina. As células foram tratadas em triplicata por duas horas ou cinco horas com 1 μg/mL de LPS (026:B6; Sigma-Aldrich); ou com PBS como controle. Os astrócitos corticais primários de camundongos foram isolados conforme descritos (50, 51) e mantidos em meio MEM suplementados com 10% de soro fetal de novilho de 3 a 4 semanas com apenas uma passagem. Conforme indicado, um antisoro de alta afinidade anti-T₃ policional de coelho foi adicionado ao meio com a concentração de 1:5000 para
aprisionar o T₃ liberado; quando apropriado, o mesmo volume usado era feito com soro de coelho normal adicionado nos grupos controles. Para os estudos de imunofluorescência, as células SK-N-AS e H4 foram plaqueadas com baixa confluência em uma placa de 35-mm de vidro (MatTek Corp.), e após 24 horas, fixadas com 10% formalina em PBS por 30 minutos; em seguida, as células foram lavadas com tampão de glicina (100 mM glicina, pH 7.4), permeabilizadas com 0.1% Triton X-100 diluído em PBS por 10 minutos e incubadas durante a noite com anticorpo primário α-tubulina (Sigma-Aldrich). O anticorpo secundário usado foi um α-camundongo IgG conjugado com Alexa Flúor 594 (cabra; Molecular Probes, Invitrogen). As amostras foram incubadas por 24 horas e visualizadas com um microscópio confocal Zeiss LSM META 510 (Carl Zeiss).

**Cromatografia das iodotironinas por UPLC.** As culturas celulares foram incubadas com aproximadamente 250000 cpm de ¹²¹I-T₄/mL ou ¹²³I-T₃/mL, totalizando 2 mL por poço. A concentração de iodotironina livre total foi de 20 pM. Nos tempos indicados (24 horas), 100 μL de meio foi analisado, misturado com 100 μL de 100% METOH, e adicionado no sistema AcQuity UPLC (Waters). As frações foram automaticamente processadas em um Flow Scintillation Analyzer Radiomatic 610TR (PerkinElmer) para radiometria.

**RT-PCR.** O RNA total foi extraído pelo método do Trizol. A reação de transcriptase reversa foi realizada usando 3.0 μg de RNA total com o sistema de síntese da SuperSript First-Strand. A análise quantitativa do PCR em tempo real foi feita usando o kit de PCR com IQ SYBR GREEN (Bio-Rad) usando primers específicos desenhados pelo software Primer3 (v. 0.4.0) conforme ilustrado na tabela 2. A expressão de ciclofilina A foi usada como um gene de controle interno de expressão (*housekeeping gene*).
Tabela 2 • Seqüências dos primers utilizados. S representa a sequência senso e AS antisenso. A letra h indica a origem humana do gene.

<table>
<thead>
<tr>
<th>Gene</th>
<th>S/AS</th>
<th>Sequência</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCyclo A</td>
<td>S</td>
<td>GGCAATGCTGACCCACAC</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>TGCCATCCCTGGACCAAAAGC</td>
</tr>
<tr>
<td>hENNP2</td>
<td>S</td>
<td>ACTCCGTGAAGGCAAGAGA</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CAAGATCCGAGATTTGTTG</td>
</tr>
<tr>
<td>hMCT8</td>
<td>S</td>
<td>AGCTTGATCCTATCCCGCGAG</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTGGGCTACTTCACCTACGG</td>
</tr>
<tr>
<td>hMCT10</td>
<td>S</td>
<td>GTGCCTCATCATGGGTCTCT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>ACGAACTAACCTGCAATGG</td>
</tr>
<tr>
<td>hOATP14</td>
<td>S</td>
<td>AGAGGCCAGGAAAGAAGG</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>TGGACCACACACCACAGACT</td>
</tr>
<tr>
<td>hRC3</td>
<td>S</td>
<td>TCAAGTTCGCCGAGAGA</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTAAAAGGGGACGGACTCAG</td>
</tr>
<tr>
<td>hTR α1</td>
<td>S</td>
<td>GGCTGCTGTCTATATGTCAA</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CGGAGGTGCTGATTTCTCAT</td>
</tr>
<tr>
<td>hTR β</td>
<td>S</td>
<td>AGCTGGAAATGGGGGTCTT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>TCAGTGTTTGCCTGAAACAG</td>
</tr>
<tr>
<td>hUCP2</td>
<td>S</td>
<td>TCTTTCCCCCACCCTCTCTT</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>AGGACGAAAGATTCTGGCTGA</td>
</tr>
</tbody>
</table>

As condições dos ciclos do PCR foram as seguintes: 2 minutos e 30 segundos a 95ºC; 50 ciclos de 30 segundos a 95ºC, 30 segundos a 60ºC e 45 segundos a 72ºC seguidos do protocolo da curva de dissociação para verificação da especificidade do amplicon gerado. A expressão gênica foi determinada pela geração de uma curva padrão conforme descrita anteriormente (52). A síntese de cDNA para RT-PCR semi-quantitativo foi realizada em duas culturas separadas de astrócitos corticais usando procedimentos padrão. No controle negativo para controle da reação de transcrição, a transcriptase foi substituída por água. O cDNA foi amplificado com Taq polimerase por 35 ciclos usando primers excluindo regiões intrónicas. Nos controles negativos a Taq foi substituída por água.

Imunohistoquímica da D3. Os cérebros foram fixados com 4% PFA durante a noite e, em seguida, infiltrados com uma solução de 20% sacarose até que eles se reduzam. Antes do corte, os cérebros foram marcados por micro-furos para identificação...
subseqüente. Cortes de 25 µm foram feitos com um micrótomo de congela-mento. Cada seis cortes de cada cérebro dos animais falsamente operados (“sham”) e do grupo dos operados (OACM) foram processados juntos na mesma cuba de acordo procedimentos imunocitoquímicos descritos anteriormente (53). As amostras foram incubadas com 1 µg/mL de anticorpo policlonal de coelho anti-D3 do soro de ratos (NBP1-05757B feito para os resíduos dos aminoácidos 250-300 da proteína de ratos; Novus Biologicals) por 36 horas a 4ºC, seguido de um anticorpo secundário anti-coelho biotinilado de burro (1:1000; *Jackson ImmunoResearch Laboratories*) por duas horas e, finalmente, tratado com complexo de avidina-biotina conjugado a peroxidase por uma hora e meia (CBA, 1:3000; Vector Laboratories). Os tecidos foram lavados com PBS entre os passos da imunioistoquímica. Os sitios imunoreativos foram visualizados com 2,5% 3,3-DAB e 0.15% de sulfato de amônia com niquel dissolvido em buffer TRIS, pH 7,6. Em alguns cortes, o produto da imuno-reação foi intensificado com prata-ouro (53). Os cortes foram em seguidas montados, desidratados, passados em xilol e cobertos com lamínulas com DPX (Fluka). O padrão de coloração obtido pelo anti-soro NBP1-05767B foi comparável ao obtido com o anti-soro (NB110-96414 Novus Biologicals) reconhecendo um epitopo diferente da proteína D3 composta pelos aminoácidos 1-100 (não mostrado) e na hibridização in situ para o sinal do mRNA da D3 em ratos (23). Quando diluições crescentes dos anticorpos foram utilizadas, o sinal da imunoistoquímica decrescia em cada sitio positivo para D3 na porção do telencéfalo. Quando o anticorpo primário (NBP1-05767B) foi omitido durante a imunoistoquímica nenhum sinal foi visualizado ou quando a solução de trabalho do anti-soro foi incubada previamente com seu peptide de bloqueio (NBP1-07PEP; Novus Biologicals).

**Ensaios de desiodases.** As análises foram feitas usando sonicados celulares preparados com solução de 0.1 M fosfato e 1 mM EDTA com pH 6.9 com 10 mM DTT e 0.25M sacarose. Os ensaios de D1, D2 e D3 foram feitos conforme descrito previamente (20).

**Análises estatísticas.** As comparações foram feitas por um teste t Student 2-tailed, e as múltiplas comparações foram feitas por ANOVA seguida do teste Student-Newman-Keuls. P<0.005 foi usado para rejeitar a hipótese nula.
RESULTADOS

Células H4 e SK-N-AS imitam o padrão da expressão de desiodases encontrado in vivo no cérebro

Para desenvolver um modelo in vitro do metabolismo dos HT e sua ação no cérebro, procuramos primeiramente identificar linhagens de células adequadas que imitariam o padrão da expressão das desiodases no cérebro, com a expressão de D2 em células gliais (14) e D3 em neurônios (23). Para este efeito, as células precursoras ricas em glia foram diferenciadas na presença de T₃, PDGF-el, b-FGF, ou FCS (24), mas a expressão de D2 não pode ser induzida nestas células (dados não mostrados). Ao mesmo tempo, o rastreamento de várias linhagens de células de glioma e microglia identificou as células de glioma H4 humano (figura 1A), que exibem atividade endógena de D2, mas não da D1 ou D3 (figura 1B). Exatamente como em astrócitos primários (dados não mostrados), as células H4 também expressam outros componentes-chave de transporte dos HT, MCT8, MCT10 e transportador 1c1 de ânion orgânico portador de soluto (SL01C1, também conhecido como OATPI4) e da ação dos HT (Trα, Trβ, neurogranina \[NRGN; também conhecida como RC3\] e ectonucleotídeo-pirofosfátase/fosfodiesterase 2 \[ENPP2\]) no cérebro (Figura 1C). Como parte deste modelo, escolhemos usar a linhagem de células de neuroblastoma SK-N-AS, cuja atividade de D3 já tinha sido caracterizada anteriormente (figura 1, A e B) (20). Os transportadores dos HT MCT8 e MCT10 foram expressos similarmente em ambas as linhagens celulares, enquanto que a expressão do transportador OATPI4 tendeu a ser mais alta nas células de neuroblastoma (figura 1C).

A seguir, fizemos testes para determinar se estas linhagens de células são responsivas a T₃, incubando-as em meio livre de qualquer metabólito (removidos por carvão ativado) ou 100 nM de T₃ (figura 1D). Estudamos dois genes previamente caracterizados como responsivos a T₃ no cérebro, RC3 e ENPP2 (2), tendo descoberto que somente as células SK-N-AS respondem consistentemente a T₃ de modo concentração e tempo-dependentes, aumentando-se a expressão de expressão de ENPP2 por aproximadamente cinco vezes (figura 1, D-F). Em contraste, as células da glia não responderam a T₃ quando estes mesmos genes foram testados (figura 1, D-F). Como a expressão do gene da proteína de desacoplamento 2 \(UCP2\) não se alterou com
a adição de T₃ ao meio de qualquer tipo de célula (figura 1D), este gene foi posteriormente usado como gene controle.

A co-cultura de células neuronais e da glia é necessária para sensibilidade a T₄.

Para testar a hipótese de que o T₃ derivado de glia atua em células neuronais, aproveitamos uma preparação in vitro projetada para a co-cultura de células em dois compartimentos adjacentes banhados com o mesmo meio de cultura (Sistema Transwell: figura 1E). Usando esta preparação, primeiramente incubamos ambos os tipos de células com T₃, tendo descoberto que a expressão do gene ENPP2 é estimulada em células SK-N-AS independentemente destas células serem cultivadas sozinhas ou com células H4; como sempre, as últimas não responderam à exposição a T₃ (figura 1, F e G). No próximo conjunto de experimentos, escolhemos incubar todas as células com T₄ em meio contendo BSA a 0,1% (ao invés de SFB), de tal maneira que a concentração livre de T₃ pudesse ser definida como descrita anteriormente (25). Sob estas condições, as células H4 e/ou SK-N-AS foram expostas a T₄ livre 20 pM (similar à concentração de T₄ livre no soro humano) por 48 horas. Embora tendo-se observado a indução de aproximadamente 2 vezes a expressão do gene ENPP2 no crescimento de células SK-N-AS isoladas, a coincubação com células H4 ampliou a expressão do gene ENPP2 induzida por T₄ em aproximadamente 7 vezes (figura 2A). O efeito de T₄ na expressão do gene ENPP2 seguiu um padrão responsive à dose, com incrementos proporcionais observados na faixa entre 0 e 48 pM (dados não mostrados). Em contraste, a coincubação de células não afetou a expressão do gene ENPP2 em células SK-N-AS quando T₄ não foi adicionado ao meio e a expressão do gene UCP2 permaneceu estável durante todo o experimento (figura 2A). Para determinar se a coincubação de células poderia afetar a conversão fracional de T₄ para T₃ e, portanto, a produção de T₃, a última foi monitorada por amostragem periódica do meio seguida por separação e quantificação de iodotironinas por UPLC, como descrito (26). A conversão de T₄ para T₃ aumentou de modo estável apenas quando as células H4 foram usadas e isto não foi afetado por coincubação com células SK-N-AS (figura 2B). A análise completa do metabolismo dos HT usando diferentes substratos radioativos confirmou que as células H4 metabolizam T₄ em T₃ e iodo via D2 (figura 2C), as células SK-N-AS metabolizam adicionalmente T₃ em T₂ e T₁ via D3 (figura 2D) e a co-cultura de H4 e SK-N-AS produz T₃ e seus esperados metabólitos (figura 2E).
Para verificar que a expressão do gene ENPP2 induzida por T4 em células SK-N-AS é devida à produção de T3 mediada por D2 em células H4, repetimos estes estudos de co-cultura na presença de 3,5,3’-triodotironina (rT3) 20 nM, uma ioddotironina inerte que inibe competitivamente D2 (27). Embora a incubação com concentrações crescentes de T4 aumentasse progressivamente a expressão do gene ENPP2 em células SK-N-AS, o mesmo não foi observado quando rT3 foi adicionado ao meio (figura 2F). Como um controle, rT3 não interferiu com a indução de ENPP2 quando T3 20 nM foi adicionado ao meio (figura 2F). Mais que 95% da inhibição de D2 foi verificada no fim do período de incubação avaliando a atividade de D2 em sonicados celulares (dados não mostrados). A seguir, para provar que T3 é o mediador da expressão do gene ENPP2 por T4, repetimos estes estudos na presença do antissoro de coelho α-T3, que imunoneutraliza o T3 no meio de células (26). Notadamente, a adição do antissoro α-T3 enfraqueceu a resposta de ENPP2 a T4 (figura 2G), sem afetar a conversão fracional de T4 para T3 (dados não mostrados).

Hipóxia e via de “hedgehog” diminuem a ação transcricional neuronal de T3 mediada por astrócitos num modelo experimental de derrame cerebral.

Uma vez que determinamos que a produção de T3 por células da glia (H4) define uma ação transcricional específica em células neuronais (SK-N-AS), decidimos verificar se mudanças programadas nas atividade das desiodases ativadas por vias relevantes do ponto de vista fisiológico e/ou fisiopatológico poderiam afetar este mecanismo. Testamos um modelo de acidente vascular cerebral, dado que a isquemia e a hipóxia induzem a expressão de D3 por meio de um mecanismo mediado por HIF-1α em situações de pós-infarto do miocárdio e durante insuficiência cardíaca hipertrófica (20). Para modelar tal sistema in vivo, nós primeiramente analisamos o padrão da expressão de D3 no cérebro. Em animais operados de modo simulado (“sham”), detectou-se forte imunorreatividade de D3 (IR) na área pré-óptica, núcleo da stria terminalis e hipotálamo, enquanto observou-se IR moderada no córtex e hipocampo e IR bastante baixa no tálamo (figura 3A). Em concordância com estudos prévios de hibridização in situ que mostram sinal forte de mRNA de D3 em camadas ricas em neurônios do córtex e do hipotálamo (15), a IR de D3 apareceu no pericário e nos dendritos apicais das células piramidais (figura 3B); também estavam visíveis axônios que emanam das áreas fortemente imunomarcadas da área pré-óptica e do hipotálamo (figura 3C). A oclusão da artéria
cerebral média (OACM) causou uma indução dramática de IR de D3 em neurônios no córtex somatossensorial ipsilateral (figura 3E) e hipocampo (figura 3G).

Como estes dados in vivo confirmam a importância da indução de D3 em tecido neuronal isquêmico, aproveitamos o sistema de co-cultura de células aqui caracterizado para estudar a ação transcriacional de T3 mediada por astrócitos sob condições hipóxicas, numa câmara contendo 2% de oxigênio. A hipóxia foi documentada pelo aumento progressivo nos níveis de mRNA de PFK em ambos os tipos celulares (figura 4A). Sob estas condições, houve um aumento de aproximadamente 6 vezes na atividade de D3 em células SK-N-AS e um aumento paralelo de aproximadamente 2 vezes na atividade de D2 nas células H4 (figura 4B). Juntamente com estas mudanças na expressão das desiodases, a expressão do gene EPPN2 induzida por T4 em neurônios foi reduzida em aproximadamente 50%, enquanto a hipóxia isoladamente não afetou os níveis basais de mRNA de ENPP2 (figura 5C) ou mRNA de UCP2 (dados não mostrados). A expressão de D3 é regulada positivamente por HIF-1α e, em consequência, utilizamos um quelante de ferro, desferrioxamina quelador a de ferro (DFO), um mimético de hipóxia que inibe a ubiquitinação de HIF e causa sua acumulação, mesmo sob condições normóxicas. Notavelmente, a exposição do sistema de co-cultura a 100 μM de DFO, produziu resultados muito similares à hipóxia, isto é, uma diminuição na sinalização dos HT em células SK-N-AS, o que confirma o envolvimento de HIF-1α nesta via (figura 4, D-F).

No cérebro, a proteína Hedgehog (Shh) é expressa em neurônios e considera-se que está implicada na remodelagem de lesão causada por derrame e hipóxia (28). Na preparação de nosso sistema de coincubação, a adição de 4 μg/ml de Shh ao meio de coincubação aumentou rapidamente a expressão do gene PATCHED 1 (PTCH1 ou PTC) em ambos os tipos de células, mas principalmente em células SK-N-AS, indicando que a via de sinalização de Shh está ativa nestas células (figura 5A). A adição de Shh aumentou dramaticamente a atividade de D3 em células SK-N-AS, enquanto houve perda progressiva de atividade de D2 nas células da glia (figura 5B). Mais importantemente, embora a adição de T4 induzisse progressivamente a expressão neuronal do gene ENPP2, as mudanças induzidas por Shh nas atividades das desiodases abrandaram o efeito de T4 sem afetar a expressão basal do gene ENPP2 (figura 5C) ou a expressão de UCP2 (figura 5D). Numa estratégia reversa, usamos ciclopamina, um antagonista de Shh, que diminuiu progressivamente os níveis de mRNA de PTC (figura 5E) e atividade de D3 e aumentou a atividade de D2 em aproximadamente 3,5 vezes.
(figura 5F). Notavelmente, a ciclopamina dobrou o efeito de T₃ na expressão do gene ENPP2, aumentando-a aproximadamente 7 a 15 vezes (figura 5G), enquanto somente pequenas alterações na expressão de UCP2 foram observadas (figura 5H).

**Inflamação induzida por LPS amplifica a ação transcrional neuronal de T₃ mediada por astrócitos num modelo de hipotiroidismo central.**

Ao mesmo tempo, também estudamos modelos fisiológicos e/ou fisiopatológicos nos quais mudanças na expressão das desiodases poderiam aumentar a sinalização dos HT. Por exemplo, é sabido que a administração intra-peritoneal de LPS em ratos induz a expressão de D2 em tanicitos, que está associada à supressão da expressão de TRH e hipotiroidismo central, mimetizando a situação encontrada na doença grave não-tiroidiana (18). Isto indicaria que um aumento na atividade de D2 nos tanicitos eleva a concentração local de T₃, regulando negativamente a expressão de TRH em neurônios específicos do núcleo paraventricular hipotalâmico. Para determinar a extensão até a qual a D2 está envolvida neste mecanismo, analisamos primeiramente os níveis de mRNA de TRH dos núcleos paraventriculares em camundongos WT e D2KO 12 a 24 horas após a administração sistêmica de LPS (figura 6). Embora uma redução esperada de mais de 50% no mRNA de TRH fosse observada nos animais WT após a injeção de LPS (figura 6, A, B e E), os animais D2KO não reduziram os níveis de mRNA de TRH (figura 6, C-E). Isto confirma que a D2 é um componente crítico desta via.

Atualmente não existem modelos celulares de tanicitos que ofereçam uma pureza celular suficientemente alta para estudos como aqueles. Assim, para modelar o interrelacionamento entre estas células e os neurônios expressando TRH no núcleo paraventricular, nós co-incubamos células SK-N-AS neuronais com células de glioma H4, as quais compartilham uma origem glial similar com os tanicitos. A responsividade de ENPP2 a T₄ foi então testada após a adição de LPS. Para garantir que os valores de tempo e concentração dos efeitos de LPS fossem compatíveis com o modelo de célula desenvolvido nos presentes estudos, nós usamos células HEK-TLR4-MD2-ELAM, que têm sinalização funcional de LPS-NF-κB por meio da expressão estável do receptor TLR4 e um repórter de Luciferasse responsável a NF-κB (29). Nestas células, a adição de 1 mg/ml de LPS ao meio causou a ativação de várias vezes do gene repórter de Luc (figura 6F) em 6 horas. Quando usado nas mesmas concentrações no sistema de células de co-incubação, o LPS ativou as principais mudanças na expressão das desiodases, como
documentado pelo aumento de aproximadamente 3 vezes no mRNA/atividade de D2 e diminuição de aproximadamente 30% no mRNA e atividade de D3 (figura 6, G-H). Consequentemente, existe um aumento dramático na indução mediada por T₄ na expressão do gene ENPP2 (figura 6I), enquanto o mRNA de UCP2 permaneceu grandemente não afetado (figura 6J).
DISCUSSÃO

Os dados apresentados neste estudo proporcionam uma nova visão para a compreensão do modo pelo qual as desiodases regulam a ação dos HT no cérebro durante a saúde ou a doença, fornecendo evidências diretas para um novo e peculiar paradigma de ação dos HT. Três etapas caracterizam este mecanismo parácrino: primeiramente o pró-hormônio T₄ é ativado nas células da glia (astrócitos e tanicitos) por meio da D2; a seguir, o T₃ resultante sai do compartimento glial e adentra nos neurônios adjacentes; finalmente, o T₃ estabelece sua ação transcripcional por meio de sua ligação aos TRs neuronais (figura 7). As implicações fisiológicas destes achados são consideráveis, uma vez que tanto a geração de T₃, como sua translocação são pontos potenciais de controle para a sinalização dos HT no cérebro. É notável que a magnitude das ações transcripcionais de T₃ mediada por D2 em neurônios não atue de modo constante; pelo contrário, podem ser moduladas por alterações coordenadas nas atividades de D2 e D3, amplificando ou mitigando a sinalização dos HT na dependência de que se necessite o favorecimento da ativação dos HT (via D2) ou a sua inativação (via D3) por meio de ações de moléculas-chave ligadas ao desenvolvimento e/ou ao metabolismo, tais como as proteínas hedgehog, HIF-1α e LPS.

A proposição de que as desiodases em células gliais e neurônios podem afetar a sinalização dos HT é derivada de sua distribuição anatômica e do papel que estas enzimas representam em outros sistemas celulares, tais como o tecido adiposo marrom e a glândula pituitária (2). As descobertas atuais fornecem uma explicação do mecanismo pelo qual isto acontece, demonstrando inequivocamente que uma quantidade suficiente de T₃ gerado por D2 atua de modo parácrino, causando suas ações transcripcionais em células neuronais (figura 2). A ressaltar, uma quantidade suficiente de T₃ é produzida, de tal modo que ultrapasse a ação da desiodase inativadora (D3) presente nas células neuronais. A significância destas descobertas é reforçada porque estes experimentos foram executados com quantidades fisiológicas de T₄ livre, isto é, 20 pM. A adição de T₄ ao sistema de co-cultura foi suficiente para mudar a expressão de um gene responsivo a T₃, ENPP2, somente na presença da linhagem de células de glioma H4 expressando D2. Este efeito dependente de T₄ é bloqueado por rT₃, um inibidor da D2, e também pelo antissoro α-T₃ altamente específico, o que confirma que o T₃ gerado por D2 é uma etapa crítica nesta via (figura 2).
É claro que as limitações de nosso sistema de co-cultura requerem que se tenha cuidado ao generalizar estes resultados para a fisiologia cerebral. Para demonstrar a alça parácrina conectando neurônios e glia (figura 7), nós desconstruímos a arquitetura do cérebro, mantendo propositadamente estes dois tipos de células fisicamente separados (Sistema Transwell). A situação in vivo é provavelmente mais complexa, uma vez que estes tipos de células (glia e neurônios) são adjacentes e fisicamente tocam-se entre si, elevando a possibilidade de um controle local ainda mais direto de sinalização dos HT do que o demonstrado em nossos estudos. Contudo, dada a conhecida co-expressão de D2 e D3 no córtex, hipocampo e hipotálamo (14, 15, 23, 30), é provável que tal mecanismo seja totalmente ativo nestas regiões; a dependência da indução mediada por LPS na expressão de mRNA de TRH na atividade de D2 nos tanícos (gliais) reforça fortemente esta hipótese (figura 6).

A hipóxia é um importante sinal de doença no cérebro e, na presente investigação, detectamos uma notável indução de D3 no cérebro de rato depois da isquemia causada por obstrução na artéria cerebral média (OACM) (figura 3). De acordo com este modelo, tal indução de D3 limitaria rapidamente a ação transcriacional de T3 nos neurônios, o que tornaria os neurônios que expressam D3 relativamente hipotiroidianos. Assim, nós aproveitamos o sistema de co-cultura para testar esta hipótese e constatamos que, de fato, a hipóxia induz aproximadamente 7 vezes a expressão de D3 e limita a ação transcriacional dependente de T3 nos astrócitos em pelo menos 50% (figura 4). Notavelmente, o agente mimetizador de hipóxia, DFO, causou efeitos similares (figura 4). Estes dados revelam um mecanismo que é intuitivamente adaptivo por natureza, dado que o hipotiroidismo mediado por D3 diminuiria o gasto energético e a demanda de O2 dos neurônios (20). A partir de uma perspectiva mais ampliada, é possível que tal indução de D3 seja parte do processo de lesão-cura, como observado em outros tecidos tais como miocárdio (31), músculo esquelético (32), nervos (33), pele (34) e fígado (35). O T3 tem um efeito supressivo no gene da ciclinaD1 (36) e, portanto, elaborou-se a hipótese de que uma diminuição na sinalização dos HT favoreceria a proliferação celular, o que contribui para o processo de cura. Tal mecanismo tem sido ligado à proliferação celular na placa de crescimento do desenvolvimento da galinha (21), na regeneração hepática (35) e no crescimento de carcinoma de células basais (22). É notável que tal fato também aconteça no cérebro lesionado, o que pode indicar que a promoção de hipotiroidismo específico por meio da indução de D3 é um mecanismo habitual comum a
muitos tecidos. Além disso, a possibilidade de que a indução de D3 também desempenhe um papel em outros tipos de lesões cerebrais é muito excitante, uma vez que ela abre potencialmente novas estratégias para se tratar pacientes com diversas doenças neurológicas.

A sinalização pela via “Hedgehog” ocupa um papel importante no desenvolvimento e também é induzida por estímulos fisiopatológicos como injúria e inflamação aguda no cérebro (37). A adição de Shh no sistema de co-cultura diminui notavelmente a sinalização dos HT por meio da indução de D3 e a supressão de atividade de D2 em astrócitos, que são sabidamente respondedores dos sinais de Shh (ref. (38) e figura 5). Shh diminuiu a ação transcriacional astrócito-dependente de T3 em neurônios, enquanto que a ciclosporina, um antagonista de Shh, provocou o efeito oposto (figura 5). A observação de que as vias de Shh e dos HT podem ser integradas no cérebro por meio de uma distribuição regional específica das suas moléculas de sinalização (39) deve ter um impacto em nossa compreensão do desenvolvimento do cérebro e sua responsividade a estímulos fisiológicos e patológicos. De fato, durante o desenvolvimento, a via Shh promove a proliferação e inibe a diferenciação de células progenitoras neurais e reforça a padronização dorso-ventral do sistema nervoso central (40, 41). Dado o impacto crítico dos HT no desenvolvimento e funções cerebrais, a presente investigação sugere que pelo menos parte dos efeitos de Shh poderiam ser mediados por mudanças na sinalização dos HT. As células-tronco neurais também existem nos sistemas nervosos adultos de todos os mamíferos e sua proliferação está sob controle de Shh (40, 41); desta forma, aqui também a sinalização dos HT pode estar envolvida, explicando seu papel em funções cognitivas superiores; foi sugerido que a neurogênese dos adultos mediada por Shh no giro dentado hipocampal pode desempenhar um papel na formação da memória (42).

Estes estudos também fornecem evidência que alterações na expressão das desiodases podem amplificar substancialmente a ação transcriacional de T3 estabelecida em neurônios por meio da desiodação de T4 em astrócitos. Especificamente no HMB, a D2 é expressa em células da glia especializadas, chamadas tanicitos. Também é conhecido que a administração sistêmica de LPS causa uma rápida indução da expressão de D2 e atividade em tanicitos, um efeito ligado à supressão da expressão de TRH nos neurônios do núcleo para-ventricular observados em estados em jejum e de doença (18). A observação que o mRNA de TRH no núcleo paraventricular não cai no
camundongo nocauteados para D2 (D2KO) depois da administração sistêmica de LPS sugere que a ativação de D2 é uma etapa crítica nesta via, reforçando um efeito parácrino do T₃ produzido no taníctico por meio da desiodação de T₄ na regulação neuronal de TRH (figura 6). Para determinar se isto era possível, nós usamos o sistema de co-cultura e constatamos que, de fato, a exposição ao LPS induziu a expressão de D2, com atividade 3 a 4 vezes maior e mais do que dobrou a sinalização dos HT para neurônios neste sistema (figura 6), enquanto não afetou os níveis de mRNA de UCP2.

O refinamento deste modelo parácrino da ação dos HT certamente irá requerer estudos adicionais, em particular dirigidos a compreender o comportamento dos transportadores, tais como MCT8, na resposta aos fatores identificados como moduladores da atividade das desiodases no sistema nervoso central. Outras moléculas e vias adicionais capazes de modular a atividade das desiodases existem, tais como compostos xenobióticos (43), insulina e tiazolidinedionas (44) e ácidos biliares (45). É interessante especular que estes e talvez outros sinais ainda não identificados possam afetar a ação dos HT no cérebro, o que traz implicações significativas para condições clínicas como depressão e perturbações do humor, que são conhecidamente afetadas pelos HT.
CONCLUSÕES

Os dados apresentados neste trabalho permite concluir que:

- Foi identificado o primeiro tipo de linhagem celular glial (H4) que possui atividade endógena de D2. Este tipo celular, juntamente com células derivadas de um neuroblastoma (SK-N-AS) imitam o padrão de expressão gênica das células encontradas in vivo no cérebro humano;

- O modelo de co-cultura celular com a H4 e SK-N-AS obtido com sucesso permitiu observar que a presença das células gliais e a tiroxina são necessários em conjunto para a ativação da expressão gênica responsiva a T3 nos neurônios. Essa ativação do hormônio na expressão gênica do neurônio é tempo/dose dependente;

- A situação de hipóxia e o estímulo pela via de “Hedgehog” atuaram de maneira semelhante à previamente descrita na literatura, seja pelo aumento da atividade da D3 (hipóxia) ou pelo aumento da degradação proteassômica da D2 (Shh). De maneira oposta, a ciclopamina proporciona uma maior atividade da D2 causando uma maior resposta na expressão gênica neuronal;

- O tratamento com LPS demonstrou um aumento celular local na atividade da D2 resultando no aumento da resposta na expressão gênica neuronal.
Figura 1

A

![Images of SK-N-AS and H4 cells]

B

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Deiodinase activity (nmol/min/mg protein)

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Relative mRNA levels (target gene/cyclophilin A)

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<td>ENPP2</td>
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Relative mRNA levels (target gene/cyclophilin A)

E

![Diagram of Transwell insert with compartments and membrane]
Sinalização de HT em linhagens de células neuronais (SK-N-AS) e da glia (H4). (A) Imunocitoquímica de SK-N-AS provada com α-tubulina e marcadas com DAPI. Ampliação original x20. (B) Atividade de desiodinase endogena em linhagens de células H4 e SK-N-AS (SK), nd, não detectável. (C) Padrão de expressão de RNA de genes envolvidos no metabolismo e ação do HT como determinado por RT-PCR em linhagens de células H4 e SK-N-AS. (D) Responsividade putativa de genes alvo T₃ (UCP2, RC3, e ENPP2) em linhagens de células H4 e SK-N-AS tratadas com meio contendo soro extraído com carvão ou T₃ 100 nM por 48 horas como indicado. 5x indica indução de 5 vezes da expressão. (E) Representação esquemática do Sistema Transwell no qual um inserto é colocado em uma placa de 6 poços e células (H4) são semeadas dentro do inserto; células SK-N-AS são semeadas no fundo da placa de 6 poços. Após as células serem semeadas, ambos os tipos de células são mantidas separadas durante a noite e então colocadas juntas na mesma placa multipoço como indicado. (F) A responsividade à dose do gene ENPP2 a T₃ em linhagens de células H4 e SK-N-AS incubadas sozinhas como em D ou coincubadas no sistema Transwell por 48 horas como indicada. (G) O mesmo que em F, exceto que esta é uma responsividade no tempo a T₃. Em todos os experimentos, os valores são médios ± DPM [desvio padrão da média] de 5-9 poços independentes; *P < 0,01 versus controle.
As células de astrócitos H4 ativam T₄ em T₃ e estabelecem uma resposta específica gênica de mRNA em células neuronais SK-N-AS. (A) Efeitos da adição de T₄ na expressão do gene ENPP2 ou UCP2 em culturas de SK-N-AS ou co-culturas de células SK-N-AS e H4 como indicado. As células passaram por cultura como na legenda da figura 1 e foram tratadas com T₄ (fração livre) 20 pM por 48 horas. (B) Conversão fraccional de T₄ para T₃ como medida após a adição de ¹²⁵I-T₄ e determinação da taxa de desiodação do anel
externo via a medição de $^{125}$I livre. As amostras do meio foram coletadas nos instantes indicados. (C) Cromatogramas de meio de células H4 nos instantes indicados após a adição de $^{125}$I-T4. Picos típicos de $^{125}$I-T3 e $^{125}$I são mostrados após 24 horas. (D) O mesmo que em C, exceto que $^{125}$I-T3 foi adicionado às culturas de células SK-N-AS e picos de $^{125}$I-T2 e $^{125}$I-T1 são visualizados. (E) O mesmo que em C, exceto que $^{125}$I-T4 foi adicionado às co-culturas de H4 e SK-N-AS e os picos indicados são visualizados. (F) O mesmo que em A, exceto que diferentes concentrações de T4 livre foram usadas e rT3 20 nM foi adicionado no início da incubação como indicado. O T3 indica que T3 100 nM foi adicionado no início da incubação. (G) O mesmo que em F, exceto que antissoro α-T3 foi adicionado no instante zero como indicado. Em todos os experimentos os valores são média ± DPM de 5-9 poços independentes; * $P < 0.01$ versus controle.

Figura 3:

A hipóxia induz D3 no cérebro e diminui a sinalização do HT. IR de D3 usando o antissoro α-D3 NBP1-5767 em seções coroais do cérebro de modelo de derrame de rato macho: (A-C) operado simuladamente (Sham); (D-I) OACM (MCAO em inglês) unilateral por 1 hora. (A) A micrografia de baixa potência de uma seção coronal do cérebro (bregma, -3,3 mm) mostra distribuição amplamente dispersa de IR de D3 em operação simulada. A intensidade da imunoreatividade de D3 exibe variações regionais; no córtex cerebral e hipotálamo de controles operados simuladamente, a IR é moderada e localizada primariamente no dendrito apical (pontas de setas) e no pericário (setas) de células piramidais (B), visto ser intenso nos axônios...
A hipóxia diminui a sinalização do hormônio tiroidiano mediada por astrócito em células neuronais SK-N-AS. (A) O sistema de co-cultura descrito na figura 2 foi colocado em uma câmara de hipóxia (1% de O₂) para os tempos indicados na presença de T₄ (fração livre) 20 pM. As células foram cultivadas nos tempos indicados e processadas para a atividade de desiodase ou RT-PCR, PFK, fosfofructoquinase. (B) O curso do tempo das atividades de D2 e D3 após a hipóxia ser iniciada. (C) Níveis de mRNA de ENPP2 em células SK-N-AS nos pontos do tempo indicados. (D-F) O mesmo que em A-C, exceto que as células
foram tratadas com DFO para os tempos indicados. Os valores são média ± DPM de 6-9 poços independentes versus controle.

**Figura 5:**

A sinalização com Shh limita a resposta específica gênica de mRNA mediada por astrócito de H4 em células neuronais S-N-AS. O sistema de co-cultura descrito na figura 2 foi exposto a Shh pelos tempos indicados na presença de T₄ (fração livre) 20 pM. As células foram colhidas nos instantes indicados e processadas para atividade de desiodase ou RT-PCR quantitativa. (A) Curso do tempo de expressão de PTC após a adição de Shh. (B) Curso do tempo das atividades de D2 e D3 após a adição de Shh. (C) Níveis de mRNA de ENPP2 em células SK-N-AS nos pontos do tempo indicados. (D) Níveis de mRNA de UCP2
em células SK-N-AS nos pontos do tempo indicados. (E-H) O mesmo que em A-D, exceto que as células foram tratadas com ciclopamina 10 μM para os tempos indicados. Os valores são médias ± DPM para 6-9 poços independentes versus controle.

**Figura 6:**

![Imagens de microscopia e gráficos de dados]
A sinalização com LPS amplifica a resposta específica gênica de mRNA mediada por astrócito em células neuronais. O efeito do LPS no mRNA de TRH em NPV de camundongos WT e D2KO. Note a aparente redução em mRNA de TRH em camundongos WT seguindo a administração sistêmica de LPS (A-B), mas a ausência de uma resposta em animais D2KO (C-D). Barra de escalas: 100 μm, III, terceiro ventrículo. 
(E) Quantificação do sinal de hibridização de mRNA de TRH de cada um dos 4 grupos de animais (n = 4 por grupo). (F) Atividade de luciferase em células TLR4-MD2-ELAM, as quais têm uma sinalização funcional de LPS-NF-κB via a expressão estável do receptor TLR4 e um repórter de Luciferase responsivo a NF-κB após a adição de 1 μg/ml de LPS (lipossacarídeo bacteriano). (G) O sistema de co-cultura descrito na figura 2 foi exposto a LPS como em F para os tempos indicados na presença de T4 (fração livre) 20 pM. As células foram colhidas nos instantes indicados e processadas para a atividade de desiodase ou RT-PCR quantitativa. São mostrados os curso do tempo dos níveis de mRNA ou atividades de D2 e D3 (H) após a adição de LPS. (I) Níveis de mRNA de ENPP2 em células SK-N-AS nos pontos do tempo indicados. (J) Níveis de mRNA de UCP2 em células SK-N-AS nos pontos do tempo indicados. Os valores são mediadas ± DPM de 6-9 poços independentes. *P < 0,01 versus controle.
Figura 7:

Modelo proposto de sinalização de HT no cérebro. 3 etapas caracterizam este mecanismo parácrino: primeira, o pró-hormônio T₄ é ativado em células da glia (astrócitos ou tanicitos) via D₂; a seguir, o T₃ resultante sai do compartimento glial e entra em neurônios adjacentes; e finalmente o T₃ estabelece uma resposta específica gênica transcripcional via a ligação a TRs neuronais. O transporte do HT é via o transportador MCT8; outros transportadores de HT, p.ex., OATP1C1, também são sabidos a operar no cérebro; o LPS ativa a transcrição de D2 e o Shh promove a inativação de D2 via ubiquitinação mediada por WSB-1; tanto hipóxia quanto Shh ativam a transcrição do gene de D3.


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Paracrine signaling by glial cell–derived triiodothyronine activates neuronal gene expression in the rodent brain and human cells

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Hypothyroidism in humans is characterized by severe neurological consequences that are often irreversible, highlighting the critical role of thyroid hormone (TH) in the brain. Despite this, not much is known about the signaling pathways that control TH action in the brain. What is known is that the prohormone thyroxine (T4) is converted to the active hormone triiodothyronine (T3) by type 2 deiodinase (D2) and that this occurs in astrocytes, while TH receptors and type 3 deiodinase (D3), which inactivates T3, are found in adjacent neurons. Here, we modeled TH action in the brain using an in vitro coculture system of D2-expressing H4 human glioma cells and D3-expressing SK-N-AS human neuroblastoma cells. We found that glial cell D2 activity resulted in increased T3 production, which activated TH receptors in the neonatal rodent brain, thereby identifying deiodinases as potential control points for the regulation of TH signaling in the brain during health and disease.

Introduction

One of the most devastating aspects of neonatal hypothyroidism is severe neurologic impairment and mental retardation, frequently associated with abnormal facies, deaf mutism, and motor spasticity (1, 2). These features are by and large irreversible, and a worldwide network of neonatal screening was put in place several decades ago to identify and treat the newborns with this condition. The adult brain is also sensitive to thyroid hormone (TH). Hypothyroid patients are frequently lethargic, have poor motor coordination, memory impairment, and a tendency toward depression and mood disorders. In turn, hyperthyroidism is associated with irritability, anxiety, and mood swings (3), and the efficacy of antidepressive agents is potentiated when associated with TH administration.

While these syndromes illustrate a critical role played by TH in the brain, not much is known about the signaling pathways that control TH action in this organ. In contrast with the majority of tissues, most TH in the brain is produced locally through activation of thyroxine (T4) to 3,5,3'-triiodothyronine (T3) via the action of the type 2 deiodinase (D2) (4). This has been studied directly through dual-labeling techniques and recently confirmed with the observation that mice with targeted disruption of the D2 gene exhibit half as much T3 in their brains as WT littersmates (5). Thus, it is widely accepted that D2 expression in discrete areas of the brain increases TH signaling, a mechanism that has been linked to important brain functions such as cell-cohesion development, the thyrotropin-releasing hormone/thyroid-stimulating hormone (TRH/TSH) feedback mechanism, and seasonal breeding in birds (6, 7). At the same time, the TH-inactivating type 3 deiodinase (D3) pathway is also active in discrete areas of the brain, damping TH action. Not surprisingly, the D2 and D3 pathways are inversely synchronized in a spatial and temporal fashion. Thus, it is assumed that the balance of these 2 pathways, i.e., D2 versus D3, and the less significant contribution of plasma T3, determine critical brain processes such as myelination, neuronal migration, glial differentiation, and neurogenesis (1, 8, 9).

Neurons express TH receptors (TRs) and are presumably the major target of T3 in the brain, but D2 is expressed in astrocytes, not neurons. This poses an anatomical question: does T3 generated in astrocytes reach TRs in neurons (2, 10, 11)? Furthermore, does the expression of D3 in the neurons limit this paracrine path-
way? Such a paracrine mechanism became more plausible with the discovery of active TH transport into neurons via transporters such as monocarboxylic TH transporter-8 (MCT8); mutations in MCT8 explain the molecular basis for the Allan-Herndon-Dudley syndrome (AHDS), a rare X-linked disorder characterized by neurological abnormalities including global developmental delay, central hypotonia, rotatory nystagmus, impaired hearing, and spasticity (12, 13). Another line of evidence supporting the concept of paracrine TH transport in the brain stems from studies of TH metabolism in the hypothalamus, where D2 is expressed in specialized glial cells located in the floor and infralateral wall of the third ventricle in the medio basal hypothalamus (MBH) called tanyocytes (14, 15). It has been suggested that T3 generation via D2 in tanyocytes could affect gene expression in TRH neurons, located in the paraventricular nucleus (PVN), thus explaining why T4 is critical in the negative feedback of TRH (16). At the same time, upregulation of D2 in tanyocytes has been demonstrated in a rodent model for nonthyroidal illness and fasting, suggesting that a relative local increase in TH action mediates the central hypothroidism frequently observed under these circumstances (17–19).

In the Japanese quail, the expression of D2 in the MBH is induced by light. Intracerebroventricular administration of T3 mimics the photoperiodic response, whereas the D2 inhibitor ipropanoic acid prevents gonadal growth, indicating that light-induced D2 expression in the medial basal hypothalamus (MBH) may be involved in the photoperiodic response of gonads in Japanese quail (7).

If this glial-D2/neuronal TR connection indeed exists, it could have the advantage of allowing for a much more sophisticated regulation of TH action in the brain, with control of glial or neuronal deiodination being a control point. A number of signaling pathways, which have recently been established to be relevant for deiodinases can be operant in the brain, for example, HIF-1a activation of D3 in hypoxic tissue (20) and hedgehog protein family-mediated inactivation of D2 and activation of D3 (21, 22).

Direct evidence of a deiodinase-mediated transcriptional TH footprint in neurons has not been available. Here we modeled this pathway in vitro by coculturing D2-expressing H4 glioma cells with neuronal cells that express D3, SK-N-AS. Using this system, we found that glial cell-generated T3 (via D2 activity) was able to act in a paracrine fashion to induce the expression of T3-responsive genes in cocultured neurons, in spite of the presence of D3 activity. Furthermore, we found that the system is regulated by signals including hypoxia, hedgehog proteins, and LPS-induced inflammation. In two studies using ischemia and LPS further validate the relevance of these findings. To our knowledge, these data represent the first direct evidence for a paracrine loop linking D2 in glial cells to TRs in neurons, identifying deiodinases as control points for the regulation of TH signaling in the brain during health and disease.

Results

H4 and SK-N-AS cells mimic a pattern of deiodinase expression found in vivo in the brain. In order to develop an in vitro model of TH metabolism and action in the brain, we first sought to identify suitable cell lines that would mimic the pattern of deiodinase expression in the brain, namely D2 expression in glial cells (14) and D3 in neurons (23). To that effect, glial-restricted precursor cells were differentiated in the presence of T3, PDGF-B, bFGF, or FCS (24), but D2 expression could not be induced in these cells (data not shown). At the same time, the screening of several glioma and microglia cell lines identified the human H4 glioma cells (Figure 1A) that exhibit substantial D2 activity but not type 1 deiodinase (D1) or D3 (Figure 1B). Just as in primary astrocytes (data not shown), H4 cells also express other key components of TH transport (monocarboxylic acid transporter 8 [MCT8], MCT10, and solute carrier organic anion transporter 1c1 [SLCO1C1; also known as OATP1A4]) and action (TRα, TRβ), neurogranin [NRGN; also known as RGC], and ectonucleotide pyrophosphatase/phosphodiesterase 2 [ENPP2]) in the brain (Figure 1C). As part of this model, we chose to use the SK-N-AS neuroblastoma cell line, which has D3 activity as previously characterized (Figure 1, A and B) (20). The TH transporters MCT8 and MCT10 were found to be expressed similarly in both cell lines, whereas the expression of OATP1A4 transporter tended to be higher in the neuroblastoma cells (Figure 1C).

Subsequently, we tested to determine whether these cell lines are responsive to T3 by incubating both lines in media containing charcoal-stripped serum or 100 nM T3 (Figure 1D). We looked at 2 previously characterized T3-responsive genes in the brain, i.e., RC3 and ENPP2 (2), having found that only SK-N-AS cells consistently respond to T3 in a concentration- and time-dependent fashion, increasing ENPP2 expression to a maximum of approximately 5-fold (Figure 1, D–F). In contrast, glial cells did not respond to T3 at all when these same genes were tested (Figure 1, D–F). As uncoupling protein 2 (UCP2) gene expression did not change with the addition of T3 in the media of either cell type (Figure 1D), this gene was subsequently used as control.

Coculture of neuronal and glial cells is required for sensitivity to T3. To test the hypothesis that glial-derived T3 acts in neuronal cells, we took advantage of an in vitro setup designed for coculture of cells in 2 adjacent compartments that are bathed with the same culture media (Transwell System; Figure 1E). Using this setup, we first incubated both cell types with T3, having found that ENPP2 gene expression is stimulated in SK-N-AS cells regardless of whether these cells are cultured alone or with H4 cells; as always, the latter did not respond to exposure to T3 (Figure 1, F and G). In the next set of experiments, we chose to incubate all cells with T4 in media containing 0.1% BSA (instead of FBS), so that the free concentration of T4 could be defined as described previously (25). Under these conditions, H4 and/or SK-N-AS cells were exposed to 20 nM free T4 (similar to free T4 concentration in human serum) for 48 hours. While approximately 2-fold induction of ENPP2 gene expression was observed in SK-N-AS cells grown alone, coincubation with H4 cells magnified the T4-induced ENPP2 gene expression by approximately 7-fold (Figure 2A). The effect of T4 on ENPP2 gene expression followed a dose-response pattern, with proportional increments observed in the range between 0 and 48 PM (data not shown). In contrast, cell coincubation did not affect ENPP2 gene expression in SK-N-AS cells when T4 was not added to the media, and UCP2 gene expression remained stable throughout the experiment (Figure 2A). To determine whether cell coincubation could be affecting the fractional conversion of T4 to T3, and hence T3 production, the latter was monitored by periodic sampling of the media followed by separation and quantification of iodothyronines by UPLC as described (26). The conversion of T4 to T3 increased steadily only when the H4 cells were used, and this was not affected by coincubation with SK-N-AS cells (Figure 2B). The complete analysis of TH metabolism using different radioactive substrates confirmed that H4 cells metabolize T4 into T3 and iodide via D2 (Figure 2C), SK-N-AS cells further metabolize T3 into T2 and T1 via D3 (Figure 2D), and the coculture of H4 and SK-N-AS produces T3 and its expected metabolites (Figure 2E).
Figure 1
TH signaling in neuronal (SK-N-AS) and glial (H4) cell lines. (A) Immunocytochemistry of SK-N-AS and H4 probed with α-tubulin and stained with DAPI. Original magnification, ×20. (B) Endogenous deiodinase activity in H4 and SK-N-AS (SK) cell lines. nd, not detectable. (C) RNA expression pattern of genes involved in TH metabolism and action as determined by quantitative RT-PCR in H4 and SK-N-AS cell lines. (D) Responsiveness of putative T3-target genes (UCP2, RC3, and ENPP2) in H4 and SK-N-AS cell lines treated with media containing charcoal-stripped serum or 100 nM T3 for 48 hours as indicated. 5x indicates 5-fold induction of expression. (E) Schematic representation of the Transwell System in which an insert is placed on a 6-well plate and cells (H4) are seeded inside the insert. SK-N-AS cells are seeded at the bottom of the 6-well plate. After cells are seeded, both cell types are kept separated overnight and then placed together in the same multwell plate as indicated. (F) Dose responsiveness of ENPP2 gene to T3 in H4 and SK-N-AS cell lines incubated alone as in D or coincubated in the Transwell system for 48 hours as indicated. (G) Same as in F, except that this is a time-responsive assay to T3. In all experiments, values are mean ± SEM of 5–9 independent wells.

*P < 0.01 versus control.
Figure 2
H4 astrocyte cells activate T4 into T3 and establish an mRNA footprint in neuronal SK-N-AS cells. (A) Effects of T4 addition on ENPP2 or UCP2 gene expression in cultures of SK-N-AS or cocultures of SK-N-AS and H4 cells as indicated. Cells were cultured as in legend to Figure 1 and treated with 20 nM T4 (free fraction) for 48 hours. (B) Fractional conversion of T4 to T3 as measured after addition of 125I-T4 and determination of outer ring disintegration via measurement of free 125I. Media samples were collected at the indicated times. (C) Chromatograms of H4 cell conditioned medium at the indicated times after addition of 125I-T4. Typical peaks of 125I-T9 and 125I-T2 are shown after 24 hours. (D) Same as in C, except that 125I-T9 was added to cultures of SK-N-AS cells and 125I-T2 and 125I-T1 peaks are visualized. (E) Same as in C, except that 125I-T4 was added to H4 and SK-N-AS cocultures and the indicated peaks are visualized. (F) Same as in A, except that different concentrations of free T4 were used and 20 nM rT3 was added at the beginning of incubation as indicated. (G) Same as in F, except that rT3 antiserum was added at time zero as indicated. In all experiments, values are mean ± SEM of 5–9 independent wells. *P < 0.01 versus control.

To verify that T4-induced ENPP2 gene expression in SK-N-AS cells is due to D2-mediated T3 production in H4 cells, we repeated these coculture studies in the presence of 20 nM 3,5,3'-triiodothyronine (rT3), an inert iodothyronine that competitively inhibits D2 (27). While the incubation with increasing concentrations of T4 progressively increased ENPP2 gene expression in SK-N-AS cells, the same was not observed when rT3 was added to the media (Figure 2F). As a control, rT3 did not interfere with the ENPP2 induction when 20 nM T3 was added to the media (Figure 2F). Greater than 95% inhibition of D2 was verified at the end of the incubation period by assaying D2 activity in cell sonicates (data not shown). Next, to prove that T3 is the mediator of ENPP2 gene expression by T4, we repeated these studies in the presence of a high-affinity α-T3 rabbit antiserum, previously shown to immunoneutralize T3
in cell media (26). Remarkably, addition of the α-T3 antisense significantly blunted the ENPP2 response to T4 (Figure 2G), without affecting the fractional conversion of T4 to T3 (data not shown).

Hypoxia and hodgepodge pathway decrease the astrocyte-mediated neuronal transcriptional T3 footprint in a model of brain stroke. Once we determined that T3 production by glial cells (H4) defines a specific transcriptional footprint in neuronal cells (SK-N-AS), we sought to verify whether programmed changes in deiodinase activity that were triggered by physiologically and/or pathophysiologically relevant pathways in the brain could affect this mechanism. A stroke model was tested, given that ischemia and hypoxia induce D3 expression via an HIF-1α-mediated mechanism in postinfarction myocardium and during hypertrophic cardiac insufficiency (20). To model such a system in vivo, we first analyzed the pattern of D3 expression in the brain. In sham-operated animals, strong D3 immunoreactivity (IR) was detected in the preoptic area, bed nucleus of the stria terminalis, and hypothalamus, while moderate IR was observed in the cortex and hippocampus, but rather low IR was present in the thalamus (Figure 3A). In concordance with previous in situ hybridization studies showing strong signals of D3 mRNA in neuron-rich layers of the cortex and hippocampus (15), the D3 IR appeared in the perikaryon and the apical dendrites of the pyramidal cells (Figure 3B) and immunoreactive axons emanating from the strongly immunolabeled areas of the preoptic area and the hypothalamus were also visible (Figure 3C). Occlusion of the middle cerebral artery (MCAO) resulted in dramatic induction of D3 IR in neurons in the ipsilateral somatosensory cortex (Figure 3E) and hippocampus (Figure 3G).

As these in vivo data confirm the importance of D3 induction in ischemic neuronal tissue, we took advantage of the cell culture system characterized here to study the astrocyte-mediated transcriptional T3 footprint under hypoxic conditions, in a chamber containing 2% oxygen. Hypoxia was documented by the progressive

Figure 3
Hypoxia induces D3 in the brain and decreases TH signaling. D3 IR using the NBPI-5767 α-D3 antisense in coronal brain sections of male rat stroke model: (A–C) sham operated; (D–I) unilateral MCAO for 1 hour. (A) Low-power micrograph of a coronal brain section (bregma, −3.3 mm) shows widespread distribution of D3 IR in sham. The intensity of D3 staining exhibits regional variations; in the cerebral cortex and hippocampus of sham-operated controls, the IR is moderate and localized primarily to the apical dendrite (arrowheads) and the perikaryon (arrows) of pyramidal cells (B), whereas it is intense in the axons (arrowheads) and cell bodies of the hypothalamic preoptic area (C). (D–E) Ischemia resulted in an increased D3 IR in the ipsilateral side (MCAO-II) compared with the control contralateral side (MCAO-III) of the occlusion shown for both the primary somatosensory cortex (E versus D) and the hippocampus (G versus F). (H–I) Prinocubation of the primary antibody with the peptide antigen (PEP) used for immunization resulted in complete loss of immunostaining demonstrated in neighboring hypothalamic sections. Scale bars: 3 mm (A); 50 μm (B); 100 μm (C); 1 mm (D–I). DG, dentate gyrus; POA, preoptic area; S1, primary somatosensory cortex; V, third ventricle.

increase in PFK mRNA levels in both cell types (Figure 4A). Under these conditions, there was an approximately 6-fold increase in D3 activity in SK-N-AS cells and a parallel approximately 2-fold increase in D2 activity in the H4 cells (Figure 4B). Along with these changes in deiodinase expression, the T4-induced ENPP2 gene expression in neurons was decreased by approximately 50%, while hypoxia alone did not affect basal ENPP2 mRNA levels (Figure 5C) or UCP2 mRNA (data not shown). D3 expression is upregulated by HIF-1α and thus we also used the iron chelator desferrioxamine (DFO), a hypoxia mimetic that inhibits HIF ubiquitination and leads to its accumulation even under normoxic conditions. Remarkably, exposing the coculture system to 100 μM DFO elicited results very similar to hypoxia, i.e., a decrease in TH signaling in SK-N-AS cells, confirming the involvement of HIF-1α in this pathway (Figure 4, D–F).

In the brain, sonic hedgehog (Shh) protein is expressed in neurons and has been implicated in injury remodeling caused by stroke and hypoxia (28). In the setting of our coculture system, addition of 4 μg/ml Shh to the coculture media rapidly increased patched (PTC) expression in both cell types, but mainly in SK-N-AS cells, indicating that the Shh signaling pathway is active in these cells (Figure 5A). Addition of Shh dramatically increased D3 activity in SK-N-AS cells, while there was progressive loss of D2 activity in the glial cells (Figure 5B). More importantly, while addition of T4 progressively induced neuronal ENPP2 gene expression, the Shh-induced changes in deiodinase activity blunted the effect of T4 without affecting basal ENPP2 gene expression (Figure 5C) or UCP2 expression (Figure 5D). In a reverse strategy, we used cycloamine, an antagonist of Shh, which progressively decreased PTC mRNA levels (Figure 5E) and D3 activity and increased D2 activity by approximately 3.5-fold (Figure 5F). Remarkably, cycloamine doubled the effect of T4 on ENPP2 gene expression, increasing it from approximately 7- to 15-fold (Figure 5G), whereas only minor changes in UCP2 expression were observed (Figure 5H).

LPS-induced inflammation amplifies the astrocyte-mediated neuronal transcriptional T3 footprint in a model of central hypothyroidism. At the same time, we looked at physiological and/or pathophysiological models in which changes in deiodinase expression could increase TH signaling. For example, it is known that in rats, the i.p. administration of LPS induces D2 expression in tanyocytes that is associated with suppression of TRH expression and central hypothyroidism.
mimicking the situation found in nonthyroidal illness (18). This would indicate that an increase in D2 activity in the tanyocytes augments local T3 concentration, negatively regulating TRH expression in discrete neurons of the hypothalamic PVN. To determine the extent to which D2 is involved in such a mechanism, we first analyzed PVN TRH mRNA levels in WT and D2-KO mice 12–24 hours after the systemic administration of LPS (Figure 6). While an expected reduction of over 50% in TRH mRNA was observed in the WT animals following LPS (Figure 6, A, B, and F), the D2-KO animals failed to reduce TRH mRNA levels (Figure 6, C–E). This confirms that D2 is a critical component of this pathway.

Currently there are no tanyocyte (cell) models that provide sufficiently high cell purity for studies such as these. Thus, to model the interrelationship between these cells and TRH-expressing neurons in the PVN, we coincubated neuronal SK-N-AS cells with H4 glioma cells, which share a similar glial origin with tanyocytes. The ENPP2 responsiveness to T4 was then tested after the addition of LPS. To ensure that the timing and concentration dependence of the LPS effects was compatible with the cell model developed in the present studies, we used HEK-TLR4-MD2-ELAM cells, which have functional LPS–NF-κB signaling via stable expression of the TLR4 receptor and an NF-κB-responsive Luciferase reporter (29). In these cells, addition of 1 μg/ml LPS to the media resulted in several-fold activation of the Luc reporter gene (Figure 6F) by 6 hours. When used at the same concentrations in the cocultivation cell system, LPS triggered major changes in deiodinase expression, as documented by the approximately 3-fold increase in D2 mRNA/activity and approximately 30% decrease in D3 mRNA and activity (Figure 6, G–H). Consequently, there was a dramatic increase in T4-mediated induction of neuronal ENPP2 gene expression (Figure 6I), whereas UCP2 mRNA remained largely unaffected (Figure 6J).

Discussion

These present studies give exciting insights, which we believe are new, into how deiodinases regulate TH action in the brain during health or disease, providing direct evidence for a unique paradigm of TH action. Three steps characterize this paracrine mechanism: first, the prohormone T4 is activated in glial cells (astrocytes and tanyocytes) via D2; next, the resultant T3 exits the glial compartment and enters adjacent neurons; and finally, the T3 establishes a transcriptional footprint via binding to neuronal TRs (Figure 7). The physiologic implications are considerable, since the generation of T3 and its translocation are both potential control points for TH signaling in the brain. It is remarkable that the magnitude of the glial D2-mediated T3 footprint in neurons is not constant; on the contrary, it can be modulated by coordinated changes in D2 and D3 activities, amplifying or minimizing the TH signaling depending on whether the changes favor TH activation (via D2) or inactivation (via D3) via the actions of key developmental and/or metabolic molecules such as hedgehog proteins, HIF-1α, and LPS.

That deiodinases in glial cells and neurons can affect TH signaling was proposed based on their anatomical distribution and on the role that deiodinases play in other cell systems such as brown adipose tissue and pituitary gland (2). The present findings provide a mechanistic explanation for how this happens, unequivocally demonstrating that enough D2-generated T3 acts in a paracrine fashion, establishing a transcriptional footprint in neuronal
Figure 5
Shh signaling limits the H4 astrocyte-mediated mRNA footprint in neuronal SK-N-AS cells. The coculture system described in Figure 2 was exposed to Shh for the indicated times in the presence of 20 nM T4 (free fraction). Cells were harvested at the indicated times and processed for deiodinase activity or quantitative RT-PCR. (A) Time course of PTC expression with addition of Shh. (B) Time course of D2 and D3 activities after addition of Shh. (C) ENPP2 mRNA levels in SK-N-AS cells at the indicated time points. (D) UCPI mRNA levels in SK-N-AS cells at the indicated time points. (E-H) Same as in A-D, except that cells were treated with 10 μM cyclosporine for the indicated times. Values are mean ± SEM of 6–9 independent wells; *P < 0.01 versus control.

cells (Figure 2). Of note, T3 is produced so that it bypasses the inactivating deiodinase (D3) present in neuronal cells. The significance of these findings is enhanced because the present experiments were performed with physiological amounts of free T4, i.e., 20 nM. Addition of T4 to the coculture system was enough to change the expression of the T3-responsive gene, ENPP2, only in the presence of the D2-expressing H4 glioma cell line. This T4-dependent effect is blocked by rT3, a D2 inhibitor, and also by the highly specific α-T3 antiserum, confirming that D2-generated T3 is a critical step in this pathway (Figure 2).

Of course, the limitations of our coculture system require one to exercise caution when generalizing the results obtained to the physiology of the brain. In order to demonstrate the paracrine loop connecting neurons and glia (Figure 7), we deconstructed the brain's architecture, purposely keeping these 2 cell types physically separated (Transwell System). The in vivo situation is likely to be more complex, since these cell types (glia and neurons) are adjacent and physically touch each other, raising the possibility of even more direct local control of TH signaling than seen in our studies. Nevertheless, given the known coexpression of D2 and D3 in the cortex, hippocampus, and hypothalamus (14, 15, 23, 30), it is likely that such a mechanism is fully active in these regions; the dependency of the LPS-mediated induction of neuronal TH mRNA expression on rT3 (glial) D2 activity strongly supports this hypothesis (Figure 6).

Hypoxia is an important disease signal in the brain, and in the present investigation we detected dramatic D3 induction in the rat brain after ischemia caused by MCAO (Figure 3). According to the model of TH action in the brain being developed here, such a D3 induction would rapidly limit the T3 footprint established in neurons, essentially rendering D3-expressing neurons relatively hypothyroid. Thus, we took advantage of the coculture system to test this hypothesis and found that, indeed, hypoxia induces D3 expression approximately 7-fold and limits the astrocyte-dependent T3 footprint in neurons by at least 50% (Figure 4). Notably, the hypoxic mimetic agent DFX pro-
LPS signaling amplifies the astrocyte-mediated mRNA footprint in neuronal cells. Effect of LPS on TRH mRNA in the PVN of WT and D2-KO mice. Note apparent reduction in TRH mRNA in WT mice following the systemic administration of LPS (A–B), but absence of a response in D2-KO animals (C–D). Scale bar: 100 μm. III. third ventricle. (E) Quantification of the TRH mRNA hybridization signal from each of the 4 animal groups (n = 4 per group). (F) Luciferase activity in HEK-TLR4-MD2-ELAM cells, which have a functional LPS–NF-κB signaling via stable expression of the TLR4 receptor and an NF-κB–responsive Luciferase reporter after the addition of 1 μg/ml LPS (bacterial lipopolysaccharide). (G) The coculture system described in Figure 2 was exposed to LPS as in F for the indicated times in the presence of 20 μM T4 (free fraction). Cells were harvested at the indicated times and processed for deiodinase activity or quantitative RT-PCR. Shown are the time courses of D2 and D3 mRNA levels or activities (H) after addition of LPS. (I) ENPP2 mRNA levels in SK-N-AS cells at the indicated time points. (J) UCP2 mRNA levels in SK-N-AS cells at the indicated time points. Values are mean ± SEM of 6–9 independent wells; *P < 0.01 versus control.
Figure 7
Proposed model of TH signaling in the brain. 3 steps characterize this paracrine mechanism: first, the prohormone T4 is activated in glial cells (astrocytes and tanyctyes) via D2; next, the resultant T3 exits the glial compartment and enters adjacent neurons; and finally the T3 establishes a transcriptional footprint via binding to neuronal TRs. TH transport is via the MCT8 transporter; other TH transporters, e.g., OATP1C1, are also known to operate in the brain; LPS activates D2 transcription and Shh promotes D2 methylation via WSB-1-mediated ubiquitination; both hypoxia and Shh activate D3 gene transcription.

possibility that D3 induction also plays a role in other types of brain injuries is very exciting, as it potentially opens new strategies for approaching patients with diverse neurological disease.

Hedgehog signaling is a known developmental player and is also induced by pathophysiological stimuli such as acute brain injury and inflammation (37). Addition of Shh to the coculture system decreased TH signaling dramatically via induction of D3 and suppression of D2 activity in astrocytes, which are known to respond to Shh signals (ref. 38 and Figure 5). Shh decreased the astrocyte-dependent T3 footprint in neurons, while cyclopamine, a Shh antagonist, caused the opposite effect (Figure 5). The observation that Shh and TH pathways may be integrated in the brain through region-specific distribution of their signaling molecules (39) should have an impact on our understanding of brain development and responsiveness to physiological and pathophysiological stimuli. In fact, during development, Shh promotes proliferation and inhibits differentiation of neural progenitors and supports dorso-ventral patterning of the CNS (40, 41). Given the critical impact of TH on brain development and function, the present investigation suggests that at least some of the Shh effects could be mediated through changes in TH signaling. Importantly, neural stem cells also exist in the adult nervous systems of all mammals and their proliferation is under Shh control (41). Here also TH signaling could be involved, explaining its role in higher cognitive functions; it has been suggested that Shh-mediated adult neurogenesis in the hippocampal dentate gyrus could play a role in memory formation (42).

The present studies also provide evidence that changes in deiodinase expression can substantially amplify the T3 footprint established in neurons via T4 deiodination in astrocytes. Specifically in the MBH, D2 is expressed in specialized glial cells, tanyctyes. It is known that systemic administration of LPS results in a rapid induction of D2 expression and activity in tanyctyes, an effect linked to the suppression of TRH expression in PVN neurons observed in fasting and disease states (19). The observation that TRH mRNA in the PVN did not fall in the D2-KO mouse following the systemic administration of LPS suggests that D2 activation is a critical step in this pathway, supporting a paracrine effect of tanyctye-produced T3 via T4 deiodination on hypothalamic TRH neuronal regulation (Figure 6). To determine whether this was possible, we used the cocultured system and found that, indeed, exposure to LPS induced D2 expression and activity by 3- to 4-fold and more than doubled TH signaling to neurons in this system (Figure 6), while not affecting UCP2 mRNA levels.

Refining this paracrine model of TH action will certainly require additional studies, in particular directed to understanding the behavior of the transporters such as MCT8 in response to the factors identified as modulating deiodinase activity in the CNS. Addi-
tional molecules and pathways capable of modulating deiodinase activity do exist, such as xenobiotic compounds (43), insulin and thiazolidinediones (44), and bile acids (45). It is exciting to speculate that these and perhaps other still-unidentified signals might affect TH action in the brain, with significant implications for clinical conditions such as depression and mood disorders, which are known to be affected by TH.

**Methods**

Reagents: T4, T3, forskolin, cyclopamine, PBS, ampicillin, gentamicin, and sodium selenite were purchased from Sigma-Aldrich. rt3 was purchased from Calbiochem. SN-NS cells were provided by S. Huang (Children's Hospital, Boston, Massachusetts, USA). H4 glioma cells were provided by M. LeVoie (Brigham and Women's Hospital), and HEK-TLR4-MD2-Ham cells were provided by M. Genes (Gaia Research Institute, Andover, Massachusetts, USA). The Transwell Insert was obtained from Corning Scientific. DMEM was purchased from Gibco. All reverse transcriptase materials, DNase, RNase out, DNTp mix, EDTA, SuperScript II, and DAPI and antibodies, anti-tubulin (mouse), Alexa Fluor 594-conjugated goat anti-mouse IgG, and Trizol were purchased from Invitrogen.

**Animals.** Adult male C57BL/6 WT or D2-KO mice and Sprague-Dawley rats were used as detailed in the sections describing the LPS infection and hypoxia animal models. The animals were housed under standard environmental conditions (light between 0600-1800 hours, temperature 22 ± 1°C, rat chow and water available ad libitum). Animals were kept and experiments were performed according to protocols approved by the Animal Care and Use Committees of Tufts University and University of Miami in compliance with NIH standards. Animal protocols followed the European Communities Council Directive of November 24, 1998 (86/609/EEC) and, when appropriate, were reviewed and approved by the Animal Welfare Committee at the Institute of Experimental Medicine, Hungarian Academy of Sciences.

**LPS infection model.** Experiments were performed on male C57BL/6 WT or D2-KO mice (C57BL/6 background; Jackson Laboratories) weighing between 20 and 30 g. The D2-KO animals have been extensively characterized previously (46). LPS treatment was performed as described (18) using 2.5 µg/100 g of body weight i.p. injection of bacterial LPS (O127: B8, Sigma-Aldrich) in saline. Twelve or 24 hours after treatment, mice were euthanized with an overdose of pentobarbital and perfused through the ascending aorta with 4% PFA in 0.1 M PBS-saline. Brains were postfixed for 4 hours in the same fixative, cryoprotected in 30% sucrose, and then processed for section hybridization for TRH as previously described (47).

**Brain hypoxia caused by surgical MCAO.** Cerebral ischemia in rats was induced (n = 10) by a transient 60-minute right MCAO under Nembutal anesthesia (60 mg/kg i.p.), as previously described (48, 49). Sham-operated animals (n = 10) underwent the same surgical procedure, but no filament was advanced to the internal carotid artery. Intracerebral neurochemical deficits were confirmed by detecting gut abnormalities characterized by circling or moving to the left in animals with successful MCAO. Animals showing no deficits were excluded from the study. After 60 minutes of ischemia, animals were perfused with 4% PFA solution in 0.1 M PBS-saline.

**Time course and Transwell System.** SN-NS and H4 were propagated in DMEM media with 10% FBS with 15 µg gentamicin and 50 µg ampicillin per liter of media. Cells were incubated overnight with 10% charcoal-stripped serum the night prior to the treatment with T4 or T3. Media for all cell types was supplemented with 10 mM sodium selenite. Cells were incubated in hypoxic- and normoxic conditions in closed hypoxia chambers (Billups-Rothenberg Inc.). For the Transwell System, 1.5 × 10^4 H4 cells were plated inside each Transwell insert, and 7.5 × 10^4 SN-NS cells were placed at the bottom of the 6-well plate (Figure 1). Cells were plated on different 6-well plates to avoid cell mixture and placed together on the next day with 10% charcoal-stripped media. The HEK-TLR4-MD2-Ham cells stably express the TLR4-MD2 complex. These cells were used to establish concentration and timing of the cellular responsiveness to LPS (29). The cultures were maintained in DMEM media with 10% FBS supplemented with 150 µg/mL Zeocin, 300 µg/mL G418, and 50 µg/mL hygromycin. The cells were treated in triplicate for 2 hours and 5 hours with 1 µg/mL LPS (026:B6, Sigma-Aldrich) or PBS as control. Mouse cortical primary astrocytes were isolated as described (50, 51) and kept in MEM supplemented with 10% FCS for 3–4 weeks with 1 passage. As indicated, a high-affinity rabbit polyclonal α-T3 antisemur was added to the medium at 1:5,000 to trap released T3, when appropriate, equal amounts of normal rabbit serum was added to control wells. For immunofluorescence studies, SN-NS and H4 cells were plated at a low density in 35 mm glass-bottom plates (MatTek Corp.) and, after 24 hours, fixation was performed with 10% formalin in PBS for 30 minutes. Cells were then washed in glycine buffer (100 mM glycine, pH 7.4), permeabilized with 0.1% Triton X-100 in PBS for 10 minutes, and incubated overnight with primary α-tubulin (Sigma-Aldrich). Secondary antibody was α-mouse IgG labeled with Alexa Fluor 594 (goat; Molecular Probes, Invitrogen). Samples were cured for 2 hours and visualized with a Zeiss LSM META 510 (Carl Zeiss) confocal microscope.

**Sodium chromate chromatography using UPLC.** Cell cultures were incubated with about 250,000 cpm of [125I]-T4/mL or [125I]-T3/mL, totalizing 2 mL per well. Total free sodium chromate concentration was 20 µM. At the indicated times (24 hours), 100 µL of medium was mixed, with 100 µL of 1008 METOH, and loaded to an AgQ10 UPLC System (Waters). Fractions were automatically processed through a Flow Scilibration Analyzer Radiomatic 610TR (PerkinElmer) for radiation.

**RT-PCR.** Total RNA was extracted using the Trizol method. Reverse transcription was performed using 3.0 µg of total RNA in the SuperScript First-Strand Synthesis System. Quantitative real-time PCR was performed using iQ SYBR Green PCR kit (Bio-Rad) using primers designed for specific genes using Primer3 software (v. 0.4.0). Expression of cyclophilin A was used as a housekeeping gene. The primer sequences used were as follows: hCycloA (forward: GGCGGAATGCTGGGCAACTAC, reverse: TGCCATCTCCGGACCCAAAGG), hEPNP2 (forward: ACTCCCTGAAAGGGACAGAG, reverse: CAAAGATCGGGAGATGTGAC), hMCT8 (forward: AGCTGATCAATCATCTGGCT, reverse: ACAAGGAATACCCCTGCAATGG), hOATP14 (forward: AGAGCCGAGAGAAGGAGG, reverse: TGCCACACACACACAGAC), hRC3 (forward: TCAATGGCTCCGGAGGAAGA, reverse: CTAAGGTCCTGACCTGGCTA, hTRx (forward: GCCGTGGCTGTGAATGCTA, reverse: CGGAACGTGCTACCTCTG), hTRx (forward: AGCTGAAATGTCGGGTCTT, reverse: TCAGCTGGTGTTTCTGAGG), and hUCP2 (forward: TCTCTTTTCCCCACCTCTCC, reverse: AGGACAGAATGATCTGGTGA). The PCR cycle conditions were as follows: 2 minutes, 30 seconds at 95°C, 30 seconds at 95°C, 30 seconds at 60°C, 45 seconds at 72°C for 50 cycles followed by the melting curve protocol to verify the specificity of amplification. Gene expression was determined by generation of a standard curve as described (52). cDNA synthesis for semiquantitative RT-PCR on 2 separate cultures of cortical astrocytes was performed using standard procedures. In the minus reverse transcription controls, reverse transcriptase was replaced by water. The cDNA was amplified by Taq polymerase for 35 cycles using intron-spanning primers. In negative controls, Taq was replaced by water.

**D3 immunohistochemistry.** Brains were postfixed in 4% PFA overnight then infiltrated with 20% sucrose solution until they sank. Prior to cutting, brains were labeled by micropunches for subsequent identification. 25-µm-thick sections were cut on a freezing microtome. Every sixth section per brain from operated (MCAO) and sham-operated animals was processed in the same staining jar according to immunocytochemical procedures described earlier (53). Sections were incubated in 1 µg/mL rabbit polyclonal anti-D3 anti-
serum (NBPI-05767) raised against rat protein residues within aa 250-306; Novus Biologicals) for 36 hours at 4 °C, followed by biotinylated donkey anti-rabbit secondary antibody (1:1,000; Jackson Immunoresearch Laboratories) for 2 hours, and finally, peroxidase-conjugated avidin biotin complex (ABC, 1:3,000; Vector Laboratories) for 1.5 hours. Tissue were rinsed in PBS between immunohistochemical steps. The immunoreactive sites were visualized with 2.5% 3,3-diaminobenzidine and 0.15% nickel ammonium sulfate dissolved in TRIS buffer, pH 7.6. In some sections, the immunoreactive protein was silver intensified (33). Sections were then mounted, dehydrated, cleared in xylene, and coverslipped with DPX (Fluka).

The staining pattern obtained by the NBPI-05767 antiserum was comparable to that obtained with an antisera (NB110-9414 Novus Biologicals) recognizing a different epitope of the D3 protein within aa 1-100 (not shown) and to a single-hybridization signal for the rat D3 mRNA (G3). When increasing dilution of the antibody was used, the immunohistochemical signal decreased in each D3-positive site in the forebrain. No signal was detected when the primary antibody (NBPI-05767) was omitted from the immunohistochemical procedure or the working dilution of the antiserum was preincubated with its blocking peptide (NBPI-0769PEP, Novus Biologicals).

**Dioxygenase assays.** Analysis was performed using cell sonicates prepared using 0.1 M phosphate and 1 mM EDTA at pH 6.9 with 10 mM dithiothreitol and 0.25 M sucrose. D1, D2, and D3 assays were performed as previously described (20).

**Results.** Statistics were performed by 2-tailed Student's t test, and multiple comparisons were done by ANOVA followed by Student-Neuman-Keuls test. P < 0.05 was used to reject the null hypothesis.

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Impaired Metabolic Effects of a Thyroid Hormone Receptor Beta-Selective Agonist in a Mouse Model of Diet-Induced Obesity

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Background: The use of selective agonists of the thyroid hormone receptor isoform β (TRβ) has been linked to metabolic improvement in animal models of diet-induced obesity, nonalcoholic liver disease, and genetic hypercholesterolemia.

Methods: To identify potential target tissues of such compounds, we exposed primary murine brown adipocytes and skeletal myocytes for 24 hours to 50 nM GC-24, a highly selective TRβ agonist. GC-24 (17 ng/kg BW/day) for 36 days was also tested in a mouse model of diet-induced obesity.

Results: While the brown adipocytes responded to GC-24, with 17%–400% increases in the expression of 12 metabolically relevant genes, the myocytes remained largely unresponsive to GC-24 treatment. In control mice kept on chow diet, GC-24 treatment accelerated energy expenditure by about 15% and limited body weight gain by about 50%. However, in the obese animals the GC-24-mediated reduction in body weight gain dropped to only 20%, while energy expenditure remained unaffected. In addition, an analysis of gene expression in the skeletal muscle, brown adipose tissue, and liver of these obese animals failed to identify a conclusive GC-24 transcriptome footprint.

Conclusion: Feeding a high-fat diet impairs most of the beneficial metabolic effects associated with treatment with TRβ-selective agonists.

Introduction

Thyroid hormones is a highly metabolically active molecule that accelerates energy expenditure and reduces serum lipid concentrations at physiological concentrations (1). Nevertheless, its generalized use as a strategy to improve metabolic homeostasis has not been possible given its pleiotropic nature, with substantial effects in the heart, skeletal muscle, bone, and central nervous system to name a few. This concept has been evolving and frequently revisited, particularly in light of the understanding that thyroid hormone signaling is mediated through two thyroid hormone receptor isoforms (2), thyroid hormone receptor isoform α and β (TRα and TRβ), which conveniently exhibit diverse tissue distribution (3). While TRα expression predominates in the heart, skeletal muscle, bone, and brain, TRβ is preferentially expressed in the liver, with the adipose tissue expressing both TR isoforms.

The development of a TRβ-selective agonist (4) has prompted a number of studies addressing whether such molecules could be used to trigger the metabolic effects of thyroid hormones while preserving the TRα-expressing tissues (5–8). To a large extent, the findings have been encouraging, with a series of studies indicating that the use of TRβ-selective agonists can prevent or improve metabolic parameters and/or complications resulting from high-fat feeding, nonalcoholic liver disease (9), or genetic hypercholesterolemia (10). Because many of the biological effects attributed to TRβ-selective agonists are linked to lipid metabolism, it is well accepted that the liver is a major target of such molecules. In fact, tissue distribution analyses suggest that these molecules achieve TR selectivity by virtue of being...
concentrated predominantly in the liver, a tissue in which TRβ predominates (5).

At the same time, thyroid hormone is known for accelerating energy expenditure and decreasing the size of the white adipose tissue depot (1); thus, some beneficial effects of TRβ-selective agonists could be due to a decrease in adiposity. In this regard, treatment with GC-1, a TRβ-selective agonist, was shown to accelerate energy expenditure in rats (11), but the cellular and molecular basis underlying this metabolic effect remains unresolved. Given that the uncoupling protein 1 (UCP1) expression in the brown adipose tissue (BAT) is highly sensitive to triiodothyronine (T3) (12), it is thus possible that TRβ agonists act to stimulate BAT. In fact, in an early study UCP1 expression was shown to be induced by GC-1 (13). BAT is the main site of adaptive thermogenesis in small mammals, and recently its presence has been well documented in adult humans (14). BAT has the thyroid-hormone-activating type 2 deiodinase (D2), which is several fold stimulated during cold exposure, increasing tissue T3 concentration and the expression genes encoding key thermogenic proteins (15). Accordingly, mice with targeted disruption of the D2 gene are cold intolerant and shivering is activated to sustain thermal homeostasis (16,17).

Studies with GC-24, a highly selective TRβ agonist, indicate that BAT was the only clear GC-24 metabolic target identified in a rat model of diet-induced obesity, with only minimal alterations in gene expression observed in liver, white adipose tissue, and skeletal muscle (18). Thus, in this study we used an in vitro approach to evaluate the metabolic actions of GC-24 and specifically test whether this molecule can modify gene expression in primary cultures of murine brown adipocytes and skeletal myocytes. Our data indicate that while a number of metabolically relevant genes are rapidly upregulated in the brown adipocytes by GC-24, skeletal myocytes remain largely unresponsive under similar conditions. At the same time, while treatment with GC-24 accelerated energy expenditure and limited body weight gain in chow-fed mice, a similar treatment only slightly minimized body weight gain and did not affect energy expenditure in a mouse model of high-fat feeding. In addition, we failed to significantly measure a measurable miRNA footprint in liver, skeletal muscle, or BAT of the obese animals. We conclude that although brown adipocytes in culture continue being an important metabolic target of TRβ-selective agonists, in a mouse model of diet-induced obesity their effects are much less prominent and a major metabolic target tissue of these compounds remains to be identified.

Materials and Methods

Animals and treatment

Male C57BL/J6 mice about 5–6 weeks old were purchased from Jackson Laboratory. Mice were kept at 21°C ± 1°C, with a 12-hour dark–light cycle starting at 06:00 hours, and housed in standard plastic cages with four mice per cage. All procedures described were approved by the Institutional Animal Care and Use Committee. Animals were fed either chow diet (3.3 kcal/g, Teklad 7001; Harlan Teklad) or high-fat diet (HFD, 4.5 kcal/g, TD 95121; Harlan Teklad). After 20 days on the chow or the HFD, the animals started receiving daily subcutaneous injections of vehicle, T3 (30 ng/g BW/day) or equimolar doses of GC-24 (17 ng/g BW/day) for 36 days as indicated (Fig. 1). Food consumption and body weight were measured daily. Animals were subsequently euthanized using carbon dioxide (CO2). Tissue samples were obtained and immediately snap frozen for further analyses.

Indirect calorimetry

Mice were individually housed and acclimatized to the calorimeter cages for 2 days followed by 2 days of data collection of gas exchanges and food intake. Indirect calorimetry was performed with a computer-controlled open circuit calorimeter system (Oxymax; Columbus Instruments) comprised of six respiratory chambers equipped with a stainless steel elevated wire floor, water bottle, and food tray connected to a balance. Oxygen (O2) consumption and CO2 production were measured for each mouse at 14-minute intervals, and outdoor air reference values were determined after every 10 measurements. Gas sensors were calibrated daily with primary gas standards containing known concentrations of O2, CO2, and N2 (Airgas). A mass flow meter was used to measure and control airflow. O2 was measured by an electrochemical sensor based on a limited-diffusion metal air battery. CO2 was measured with a spectrophotometric sensor. The respiratory exchange rate was calculated as the ratio between CO2 production (liters) over O2 consumption (liters). Energy expenditure was calculated using the following formula: (3.815 + 1.232 × VO2/VO2) × VO2.

Primary cell cultures

Interscapular BAT and skeletal muscle (gastrocnemius) cells were immediately processed, and precursor cells were differentiated in vitro as previously described (19,20). Briefly, tissues were surgically removed from mice (8–10 mice per group) killed by CO2 asphyxiation. The dissected tissues were pooled, minced, and digested with collagenase (Sigma-Aldrich) dissolved in the medium containing Dulbecco’s modified Eagle’s medium, 10 mM HEPES, and antibiotics (25 μg/mL streptomycin, 25 μg/mL tetracycline, 25 μg/mL ampicillin, and 0.8 μg/mL Fungizone). Cells were strained to remove tissue debris, plated in BD 75-cm2 T-flasks (BD Biosciences), and incubated (37°C, 5% CO2) for 5–6 days in the same medium plus 10% (v/v) fetal bovine serum and 3nM insulin. Differentiation of preadipocytes into mature brown

![Diagram](https://example.com/diagram.png)
FIG. 2. Gene expression profile of primary brown adipocytes (A) and skeletal myocytes (B) exposed to vehicle, 50 nM T3 or 50 nM GC-24, for 24 hours. Results are expressed as ratio of test mRNA/cyclophilin mRNA and normalized to the levels observed in the vehicle-treated cells. The genes analyzed are grouped in different sets as indicated. Values are the mean ± SEM of three independent samples; gene abbreviations are as indicated in the Materials and Methods section; *p < 0.01 versus vehicle-treated cells; **p < 0.01 versus T3-treated cells. T3, triiodothyronine; SEM, standard error of the mean.

adipocytes was confirmed by the presence of multilocular lipiddrops in the cytosol by light microscopy. Cells were treated for 24 hours with 50 nM of T3 or GC-24, and dimethylsulfoxide was used as vehicle. Subsequently, cells were harvested and processed for RNA isolation, as described.

mRNA analysis

Total RNA was extracted from adipose tissue samples using the RNeasy kit (Qiagen) as previously described (21). The extracted RNA was analyzed by a NanoDrop spectrophotometer, and 2.5 μg of total RNA was reverse transcribed into cDNA by using High Capacity cDNA reverse transcription Kit (Applied Biosystem). Genes of interest were measured by RT-qPCR (BioRad iCycler iQ Real-Time PCR Detection System) using the IQ SYBR Green Supermix (BioRad) with the following conditions: 15 minutes at 94°C (Hot Start), 30-50 seconds at 94°C, 30-50 seconds at 55-60°C, and 45-60 seconds at 72°C for 40 cycles. A final extension at 72°C for 5 minutes was performed as well as the melting curve protocol to verify the specificity of the amplicon generation. Standard curves consisting of four to five points of serial dilution of mixed experimental and control group cDNA were prepared for each assay. Cyclophilin A was used as a housekeeping internal control gene. The coefficient of correlation (r2) was >0.98 for all standard curves, and the amplification efficiency ranged between 80% and 110%. Results are expressed as ratios of test mRNA/cyclophilin mRNA. The mRNA levels of the following genes were measured: nuclear respiratory factor 1 (Nrf1); sarcoplasmic/endoplasmic reticulum Ca2+ ATPase (Serca); phospholamban (Phl); myosin heavy chain alpha (Mia); myosin heavy chain beta (Mib); hyperpolarization-activated cyclic nucleotide-gated channel (Hcn); estrogen-related receptor (Erra); uncoupling protein 3 (Ucp3); glucose transporter (Glut4); forkhead box protein O1 (Fox O1); cyclophilin A (Cyclo A); peroxisome proliferator-activated receptor γ coactivator 1α (Pgc-1α); peroxisome proliferator-activated receptor γ coactivator 1β (Pgc-1β); carnitine palmitoyltransferase-1 (Cpt-1β); acetyl-coenzyme A carboxylase (Acac); murine medium-chain acyl-CoA-dehydrogenase (mMaCoA); murine long-chain acyl-CoA-dehydrogenase (mLCoA); peroxisome proliferator-activated receptors (Ppar), peroxisome proliferator-activated receptor δ (Ppar δ); cytochrome oxidase (Cox); ATP synthase, H+ transporting, mitochondrial F0 complex, subunit c1 (Atpsg1); uncoupling protein 1 (Ucp1); superoxide dismutase 1 (Sod-1); superoxide dismutase 2 (Sod-2); scavenger receptor class B (Srb); cholesterol 7α-hydroxylase (Cyp7a); type I deiodinase (D1); small heterodimer partner (Shp).

Statistical analysis

All data were analyzed using PRISM software (GraphPad Software) and are expressed as mean ± standard error of the
mean. One-way analysis of variance was used to compare more than two groups, followed by the Student–Newman–Keuls test to detect differences between two groups. The Student's t-test was used to compare the differences between two groups. p < 0.05 was used to reject the null hypothesis.

Results

Effects of GC-24 on gene expression in mouse primary myocytes and brown adipocytes

To evaluate the gene expression profile induced by the TRβ-selective agonist GC-24, brown adipocytes and primary skeletal myocytes were exposed in vitro to 50 nM GC-24 for 24 hours. Brown adipocytes were particularly sensitive to this molecule, with increases of 17%–400% observed in the expression of multiple genes, including PGC-1α, Errα, Nrf1, Ppar γ, Ppar δ, mTfα, Cpt1, Atp5g1, Ucp1, Sod1, and Acc (all p < 0.01; Fig. 2A). Other genes were not affected by treatment with GC-24, including Pgc-1α, CoxIV, Lspd, Mda5, Pdk-4, and SdhaA (Fig. 2A). As a comparison, other brown adipocyte cultures were treated with equinolar amounts of T3 and similar responses were observed, although less pronounced (Fig. 2A). On the other hand, in skeletal myocytes the changes in gene expression were minimal across 19 genes studied (Errα, Tfs, Ucp3, Nrf1, Pgc1a, Pgc1b, Cpt1b, Acc, Mda5, Lspd, Ppar γ, Ppar δ, Ppar δ, CoxIV, Atp5g1, Sod1, Sod2, Sd, Glut 4, and Pdk-4), with only Fox O1 increasing about 40% (p < 0.05; Fig. 2B). Treatment with T3 at the same concentration did not affect gene expression at all in these skeletal myocytes cells (Fig. 2B).

Metabolic effects of GC-24 in animals kept on a chow diet

On the basis of these findings that brown adipocytes are metabolic targets of GC-24, we next tested the hypothesis that BAT activation in vivo by GC-24 would trigger metabolic
effects in animals. Treatment with GC-24 (17 ng/[g BW/day]) for 36 days (Fig. 1A) cut by half the body weight gain (p < 0.05; Fig. 3A) while not affecting the daily caloric intake (Fig. 3B). At the same time, the rate of energy expenditure was accelerated by about 15% in GC-24-treated animals (p < 0.0001; Fig. 3C–E), which is compatible with the finding that brown adipocytes are activated by this molecule (Fig. 2A).

**Metabolic effects of GC-24 in animals kept on a HFD**

Next, we tested the hypothesis that BAT activation achieved through treatment with GC-24 can minimize the metabolic consequences of feeding with a HFD. To this end, mice were placed on a HFD for 3 weeks to induce obesity, and subsequently treated with GC-24 (Fig. 1B). On a HFD alone, these animals exhibited an approximately 25% increase in caloric intake (Fig. 4A) that resulted in about 13% (p < 0.01) increase in body weight gain (Fig. 4C). Subsequently, daily treatment with GC-24 (17 ng/[g BW/day]) or T3 (30 ng/[g BW/day]) was started, remaining for the next 5 weeks (Fig. 1B). During the treatment phase, the T3-treated animals exhibited a further increase in caloric intake (~23%; p < 0.001), whereas the same was not observed in the GC-24-treated animals (Fig. 4B). Notably, T3 treatment did not slow down the body weight gain associated with the high-fat feeding, whereas the effect of GC-24 dropped to only 20% that in control mice (p < 0.01; Fig. 4C). The body mass index (BMI) changed accordingly, increasing ~15% with high-fat feeding (p < 0.01; Table 1). Notably, combination of high-fat feeding with GC-24 treatment resulted in only a ~5% increase in BMI, whereas a similar combination with T3 was much less effective (11% increase in BMI; p < 0.01; Table 1). The individual analysis of heart, BAT, and kidney of these animals indicates that the combination of high-fat feeding and treatment with T3 increased their weights significantly by 11%–100% (p < 0.01), whereas GC-24 did not (Table 1). In the liver, both T3 and GC-24 had similar effects, decreasing organ weight when compared with high-fat feeding alone (Table 1). The indirect calorimetry indicates that between days 20 and 56 there was a significant acceleration in the rate of energy expenditure, compatible with continued feeding of a HFD. These rates were not affected by treatment with GC-24 (Fig. 4D).

**Effects of GC-24 on gene expression in animal tissues**

Given that brown adipocytes are a target of GC-24 and that treatment with GC-24 reduced body weight gain (Figs. 3A and 4C), we hypothesized that expression of metabolically relevant genes in the BAT of these animals would be upregulated by GC-24. In this regard, TRβ analogues are known to act in the liver and in the BAT while sparing the heart and bones (5, 22). Thus, we looked at the expression of key genes in BAT, skeletal muscle, heart, and liver, finding that changes in gene expression in these tissues were very mild in the animals kept on a HFD (Fig. 5A–D). On the other hand, clear cut GC-24-triggered effects were documented in other tissues of

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**FIG. 4.** Metabolic profile of animals kept on HFD and treated with GC-24. (A) Caloric intake. Food intake was measure daily. During the first 3 weeks of the experiment, animals were fed with either chow or HFD; \*p < 0.001 versus chow diet. (B) HFD mice in (A) were split into three different groups and treated with GC-24 or T3 as indicated. Shown is the average of daily caloric intake for each animal group during the treatment period. Each entry is the mean ± SEM of eight animals; \*p < 0.001 versus chow; \*\*p < 0.001 versus HFD. (C) Body weight gain during the experimental period. Animals were weighed daily and body weight gain (ΔBW) is shown. Each entry is the mean ± SEM of eight animals; \*p < 0.001 versus chow; \*\*p < 0.001 versus HFD. (D) Energy expenditure of HFD animals treated with vehicle or GC-24.
the same animals. In the heart, HCN mRNA levels fell to almost undetectable levels in the animals treated with GC-24, whereas equimolar doses of T3 increased its mRNA levels by about fourfold (Fig. 5B), an effect that was reported previously (5). Similarly, PNPLA mRNA levels in the heart decreased with both GC-24 and T3 treatments (Fig. 5B). Notably, the only GC-24-induced change in mRNA levels detected in skeletal muscle was a decrease in Ucp3 mRNA levels (Fig. 5A), while the BAT there was a marked decrease in Cyp7a mRNA (Fig. 5C). In the liver, the proposed main target of TRβ-selective agonists, treatment with GC-24 was limited to increasing Cyp7a mRNA levels by approximately threefold and reducing Sreb1 mRNA levels by 50% (Fig. 5D). Despite these changes, it is difficult to ascertain how much of these effects are direct or due to an improvement in the overall metabolic profile of these animals caused by GC-24.

### Discussion

The use of TRβ analogs has been shown to have promising metabolic effects in animals fed chow diet (23) and in animal models of nonalcoholic liver disease (9) or genetic hypercholesterolemia (10), with only minimal repercussions in heart (5,23), bone (22), brain (24), or perturbations of thyroid hormone homeostasis (13). In this regard, a striking effect of TRβ-selective agonists is to accelerate the basal metabolic rate with a resulting decrease in adiposity and body weight (11), as also documented in the present study (Fig. 3). Thus, a logical next

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**Table 1. Effect of GC-24 or Triiodothyronine Treatment on Body Mass Index and Tissue Weights**

<table>
<thead>
<tr>
<th>Group</th>
<th>BMI (kg/m²)</th>
<th>Liver (g)</th>
<th>Heart (g)</th>
<th>BAT (g)</th>
<th>Kidney (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.88 ± 0.01</td>
<td>1.23 ± 0.04</td>
<td>0.13 ± 0.01</td>
<td>0.08 ± 0.004</td>
<td>0.16 ± 0.01</td>
</tr>
<tr>
<td>HF</td>
<td>3.32 ± 0.01</td>
<td>1.45 ± 0.03</td>
<td>0.14 ± 0.01</td>
<td>0.08 ± 0.01</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>HF + T3</td>
<td>3.21 ± 0.11</td>
<td>1.36 ± 0.04</td>
<td>0.17 ± 0.01</td>
<td>0.17 ± 0.01</td>
<td>0.19 ± 0.01</td>
</tr>
<tr>
<td>HF + GC-24</td>
<td>3.01 ± 0.08</td>
<td>1.26 ± 0.02</td>
<td>0.12 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.15 ± 0.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± standard error of the mean of eight animals.

* p < 0.05 versus control; † p < 0.05 versus HF; ‡ p < 0.05 versus T3.

BAT, brown adipose tissue; BMI, body mass index; HFD, high-fat diet; T3, triiodothyronine.
step and the scope of the present investigation was (i) to identify the site(s) where TRβ-selective agonists, for example, GC-24, are acting to produce their metabolic effects, and (ii) whether these effect can be harnessed to prevent obesity in animals kept on a HFD.

On the basis of data obtained with GC-1, another TRβ-selective agonist, one would expect that BAT is a primary target of GC-24 (13). In fact, brown adipocytes in culture respond to GC-24 by increasing expression of 11 metabolically relevant genes that were tested (Fig. 2A). Further, animals kept on chow diet had their energy expenditure rate accelerated by treatment with GC-24 (Fig. 3C–E) without affecting their caloric intake (Fig. 3B), limiting their body weight gain over time (Fig. 3A). However, in the present mouse model of obesity, expression of a number of BAT genes was not affected at all by treatment with a dose of GC-24 that is the molar equivalent to 10 times the physiological replacement dose of T3 (Fig. 5C). Despite this, a small reduction in body weight gain (Fig. 4C) and sizable modifications in gene expression in the heart of the same animals were observed (Fig. 5B). Interestingly, treatment with GC-24 did not affect gene expression in the skeletal muscle of the obese mice (Fig. 5A), nor did it in the primary cultures of skeletal myocytes (Fig. 2B), making it unlikely that muscle is the site at which TRβ-selective agonists trigger their main effects. In the liver, a bona fide target of TRβ-selective agonists (25), treatment with GC-24 only induced Cyp7a1 and lowered Srebl, whereas expression of other genes remained unaffected (Fig. 5D). Although induction of Cyp7a1 is compatible with the cholesterol-lowering effect of TRβ-selective agonists, it is puzzling the lack of a major foot print left by GC-24.

These observations raise two important questions: (i) Is thyroid hormone (or GC-24) signaling reduced in obesity and/or models of high-fat feeding? (ii) What is the mechanism by which treatment with GC-24 prevents body weight gain in the present mouse model of obesity? Addressing the first question, recent studies indicate that thyroid hormone signaling is likely to be impaired in humans with fatty liver, after a large gene set of positively regulated T3-responsive genes was found to be downregulated in surgical liver biopsies from obese subjects (26). Further, T3-induced expression of this set of genes in the mouse liver was abolished by feeding a HFD, indicating that impaired thyroid hormone action contributes to altered patterns of gene expression in fatty liver. This study supports these recent observations to the extent that the GC-24-induced acceleration of energy expenditure in mice fed a Chow diet was diminished in the obese animals (Fig. 3C–E vs. Fig. 4D), as was the reduction in body weight gain (Fig. 3A vs. Fig. 4C). In addition, BAT and liver of high-fat-fed mice did not respond to treatment with GC-24 (Fig. 5C, D). The impairment in TRβ-mediated thyroid hormone signaling was quite remarkable because treatment with GC-24 started after the animals had been on a HFD for 3 weeks (Fig. 1B). A less pronounced impairment in GC-24 signaling by high-fat feeding was also observed in a rat model in which the administration of GC-24 was split into two daily injections versus one single injection in the present study (18). A mechanistic explanation for such impairment in thyroid hormone (GC-24) signaling is unknown, but as discussed by Pihlajamäki et al. (27), it possibly involves a reduction in the Fg-1 levels, a well-known TR corepressor.

These observations have important clinical implications given that the development of TRβ-selective agonists is aimed at treating the metabolic consequences of obesity and dyslipidemia. If confirmed in a clinical setting, the present findings would indicate that relatively higher doses of TRβ-selective agonists should be used in individuals on a HFD, obese or with liver steatosis. It is possible that feeding a HFD somehow accelerates thyroid hormone/GC-24 catabolism, which would explain the decreased efficiency of these molecules under such settings. However, this hypothesis is unlikely as seen by the modifications in gene expression in the heart of the present animals (Fig. 5B), indicating that GC-24 and T3 maintain their biological effects in certain tissues despite the high-fat feeding.

The second point raised by the present findings has to do with the site of action of GC-24, which remains poorly characterized. Our present data confirm that brown adipocytes are a target of GC-24 (Fig. 2A) and that energy expenditure is accelerated in nonobese animals (Fig. 3C–E). However, the lack of acceleration in energy expenditure in obese animals (Fig. 4D) and the fact that BAT gene expression was not affected by treatment with GC-24 (Fig. 5C) indicate otherwise. Of course, it is possible that BAT activation is so limited that cannot be detected by the present analysis, or it does not involve aerobic pathways, or it takes place through a different metabolic pathway not involving the eight key genes studied. The first possibility is more likely given that in obese rats treated with GC-24, some gene induction was observed in BAT (18). Similar arguments could be used to analyze the involvement of other metabolically relevant tissues, including skeletal muscle, liver, and adipose tissue. While we presently found no evidence that skeletal muscle is a target of GC-24, there are other studies indicating that TRβ-selective agonists spare the skeletal muscle tissue (28). At the same time, white adipose tissue is unlikely to be a target of GC-24 in a setting that is similar to the present studies (18).

A novel observation brought to light with our data is that treatment with GC-24 does not increase food intake, a well-known effect of T3 (28). In fact, a administration of GC-24 failed to increase further the caloric intake of mice placed on a HFD (Fig. 4B). It is well accepted that T3 increases caloric intake as a result of direct actions in the medial-basal hypothalamus (28) and also indirectly, as a result of the increased energy expenditure (29). At face value, the present observation indicates that this T3 pathway is mediated via a TRα mechanism.

Given that the brain is a predominantly TRα-expressing tissue (30), it is indeed likely that these central (metabolic) effects of T3 are mediated by a TRα-dependent mechanism. At the same time, it is unknown whether the blood-brain barrier could be playing a role in this pathway selectivity as well. In the periphery, given that tissues such as BAT, skeletal muscle, and liver were not activated in the GC-24-treated animals, it would seem unlikely that indirect effects triggered by GC-24 could increase caloric intake.

Conclusions

Administration of the TRβ-selective agonist GC-24 failed to accelerate energy expenditure (Fig. 4D) and activate gene expression in BAT, skeletal muscle, and liver of obese mice (Fig. 5), although significant changes were noticed in nonobese mice (Fig. 3C–E) and cultures of brown adipocytes
(Fig. 2A). These data would indicate that the model of high-fat feeding results in impaired thyroid hormone (GC-24) signaling, disrupting the ability of such molecules to promote metabolic homeostasis as reported in other animal models, including humans. An objective explanation for the small effect of GC-24 on preventing body weight gain is still lacking. Perhaps very important is the observation that, as opposed to T3, GC-24 did not increase food consumption in this animal model. The metabolic impact of this observation should be evaluated in further studies since it could very well represent a major mechanism by which TRβ-selective agonist mediates metabolic homeostasis.

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Disclosure Statement

The authors declare that they have no competing financial interests exist.

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PROJETOS REALIZADOS DURANTE A PÓS-GRADUAÇÃO


Ubiquitination-Induced Conformational Change within the Deiodinase Dimer Is a Switch Regulating Enzyme Activity

G. D. Vivek Sagar, Balaázs Gereben, Isabelle Callebaut, Jean-Paul Mornon, Anikó Zeöld, Wagner S. da Silva, Cristina Luongo, Monica Dentice, Susana M. Tente, Beatriz C. G. Freitas, John W. Harney, Ann Marie Zavacki, and Antonio C. Bianco

Esta publicação, no início da minha pós-graduação marcou o começo dos meus estudos investigando as vias de degradação da D2. Trabalhando no laboratório da Thyroid Section (Boston, MA) sob orientação do professor Dr. Antonio Carlos Bianco durante minha iniciação científica, busquei em um projeto de dois híbridos de levedura possíveis proteínas de interação com a D2 (Curcio-Morelli e cols., 2003). Com esse sistema encontrei a proteína WSB-1, que posteriormente foi identificada como uma E3 ligase que participa do processo de ubiquitinação da D2 diretamente (Dentice e cols., 2005). Com a identificação dessa proteína o laboratório iniciou projetos que visavam estudar mais detalhadamente essa interação da WSB-1 com a D2 e as maneiras de regular o processo de ubiquitinação da D2. Neste projeto participei no estudo de novas tecnologias com fluorescência, chamados de BRET (do inglês Bioluminescence Resonance Energy Transfer) conforme ilustrado na figura 1 desta publicação. A idéia principal era mostrar a localização dos domínios protéicos da D2 na membrana com sua dimerização. Através do BRET confirmei os resultados apresentados nos estudos de FRET mostrando que a dimerização ocorre no domínio transmembrana e globular.
Ubiquitination-Induced Conformational Change within the Deiodinase Dimer Is a Switch Regulating Enzyme Activity

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Ubiquitination is a critical posttranslational regulator of protein stability and/or subcellular localization. Here we show that ubiquitination can also regulate proteins by transiently inactivating enzymatic function through conformational change in a dimeric enzyme, which can be reversed upon deubiquitination. Our model system is the thyroid hormone-activating type 2 deiodinase (D2), an endoplasmic reticulum-resident type 1 integral membrane enzyme. D2 exists as a homodimer maintained by interacting surfaces at its transmembrane and globular cytosolic domains. The D2 dimer associates with the Hedgehog-inducible ubiquitin ligase WSB-1, the ubiquitin conjugate UBC-7, and VDU-1, a D2-specific deubiquitinaise. Upon binding of T4, its natural substrate, D2 is ubiquitinated, which inactivates the enzyme by interfering with D2’s globular interacting surfaces that are critical for dimerization and catalytic activity. This state of transient inactivity and change in dimer conformation persists until deubiquitination. The continuous association of D2 with this regulatory protein complex supports rapid cycles of deubiquitination, conjugation to ubiquitin, and enzyme reactivation by deubiquitination, allowing tight control of thyroid hormone action.

Thyroid hormone receptors are ligand-dependent transcription factors that can either activate or repress transcription. Deiodination is a critical process that can convert the minimally active T4 molecule to the ligand for thyroid hormone receptors, T3. While there are three known deiodinases, the type 2 deiodinase (D2) is the key enzyme necessary for intracellular T3 production, a process shown to be critical in development (8, 16, 24), neuroendocrine gonadal control (33), and energy homeostasis (27, 31). Given the fact that D2 catalyzes intracellular T3 production, it is not surprising that changes in D2 expression can modulate the thyroid hormone signaling pathway in a tissue-specific fashion, without affecting serum T3 concentrations (5).

The D2 pathway, and hence tissue-specific thyroid hormone signaling, is regulated by ubiquitination, a critical posttranslational regulator of protein stability (11, 25). D2 ubiquitination is accelerated in proportion to T4 concentration, thus creating a feedback loop controlling T3 production. D2 is inactivated upon conjugation to ubiquitin and, like other ubiquitinated proteins, targeted to the proteasome system (18, 20). The physiological relevance of this pathway is illustrated in the thyroid growth plate of developing chickens, where ubiquitination of D2 and thus thyroid hormone signaling are under the control of the Hedgehog signaling cascade. In response to Hedgehog signaling, the D2-specific ubiquitin ligase WD repeat and SOCS box-containing 1 (WSB-1) is induced in perichondrial cells, thus accelerating D2 ubiquitination (16). The resulting decrease in D2 activity and local T3 concentration is thought to contribute to the Hedgehog induction of parathyroid hormone-related peptide (PTHrP) (16). The link between D2 activity and PTHrP has been further substantiated by studies showing that pharmacologic acceleration of D2 ubiquitination via non-Hedgehog pathways also results in induction of this peptide (16). Alternatively, ubiquitinated D2 can be reactivated by von Hippel-Lindau protein-interacting deubiquitinating enzymes 1 and 2 (VDU-1 and VDU-2, respectively), rescuing D2 from proteasomal degradation. Thus, in response to sympathetic signaling, VDU-1 is induced in brown adipocytes, accelerating D2 deubiquitination and local T3 production, playing a role in brown adipose tissue adaptive thermogenesis (14).

D2 is an endoplasmic reticulum-resident type 1 integral membrane enzyme (2, 3, 12) that exists as a homodimer as determined by nondenaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and communoprecipitation studies (13, 23). A current model for D2 ubiquitination consists of a ubiquitinating catalytic core complex modeled as elongin BC-Cul5-Rbx1 (ECSWSB-1) assembled in close association with the D2-D2 dimer (16). In order to gain insight into the molecular mechanisms through which ECSWSB-1-mediated D2 ubiquitination inactivates D2, we sought to characterize the structural determinants of the D2-D2 dimer, as well as its relationships with the ECSWSB-1 catalytic core complex during D2 ubiquitination and deubiquitination. Our results indicate that while ubiquitination can target the D2 protein for degradation, it also regulates D2
activity at another level by mediating a transient conformational change within the D2-D2 dimer, thus identifying a novel molecular underpinning of cell-specific thyroid hormone activation.

MATERIALS AND METHODS

Reagents. Unless otherwise specified, all reagents were obtained from Sigma (St. Louis, MO) or Calbiochem (La Jolla, CA). T4, obtained from Sigma (St. Louis, MO), was dissolved in 40 mM NaOH. Outer ring labeled 3,5,3'-T4 (specific activity, 4,800 Ci/mol) was from NEN Life Science Products (Boston, MA). All other chemicals were from either Sigma (St. Louis, MO) or Calbiochem (La Jolla, CA). Lipopolysaccharide (LPS) from Salmonella typhosa was from List Biological Laboratories, Inc. (Beverly, MA). Lipofectamine 2000 was from Invitrogen (Carlsbad CA), and MG132 was from Calbiochem (San Diego, CA).

DNA constructs. All DNA fragments were generated with Vent PCR on templates containing the coding region of human D2 with a Cys-mutated activation center. Decidinase fragments were fused to the carboxyl end of yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP), in vector pEYFP-C1 or pCFP-C1 (Clontech), respectively, while pEYFP-N1 or pCFP-N1 was used to fuse D2 to the amino end of YFP or CFP. A similar strategy was used to fuse the N-FLAG-tagged and transmembraneless version (lacking the first 42 amino acids [aas]) of D2 to the N terminus of YFP. The D2 fragment was also fused to the N terminus of humanized Revilin luciferase (RLuc) by replacing CFP with RLuc in pEYFP-C1. To fuse mouse WSB-1 to the N terminus of YFP, the pEYFP-N1 vector was used. The WSB-1 SOCS box from aa 242 to the C terminus was also attached to the C terminus of YFP, yielding mWSB-1–YFP-SOCS. To fuse the human VDU-1 fragment (from aa 29 to the C terminus) to the C terminus of CFP or YFP, we replaced enhanced green fluorescent protein of the plGFPC5-4VVDU1-4Knap-Acpad construct (kindly donated by Zhao L.) with enhanced CFP or enhanced YFP (EYFP), resulting in CFP–VDU1-4 and YFP–VDU1-4, respectively. The mouse UBC7 was fused to the C or N terminus of YFP (using the pEYFP1-C1 or pCFP-C1 N vector), resulting in VDP–UBC7 and mUCP–YFP, respectively. The mouse UBC7 was also fused to the N terminus of CFP in pEYFP-C1 by a similar strategy, resulting in mUCB–CB7.

Cell culture, transfections, and D2 assay. HEK 293 cells were plated in 60-mm plates and transfected with Lipofectamine 2000, and when appropriate, human serum conditions were used to maintain cell viability and transfection efficiency (30). The amounts and types of plasmids transfected in each experiment are indicated in the respective figure legends. In most experiments, 48 h after transfection, the cells were washed twice in phosphate-buffered saline (PBS) and live-cell fluorescent resonance energy transfer (FRET) imaging was performed as described below. In some cases cell sonicates were prepared, D2 activity was assessed, as described earlier (12) in the presence of 20 μM diiodotyrosine and 1 μM T3-T4, and results were reported as fmoI/min/mg protein.

FRET data and image acquisition. We used confocal microscopy based FRET detection by acceptor photobleaching, which ensures a reduction of energy transfer when the acceptor is photobleached and an increase in the donor fluorescence (21). Numerical data were obtained and images were acquired with the use of a Zeiss LSM 510 confocal microscope (Carl Zeiss, Inc., Thornwood, NY). A 25 mW argon laser tuned to lines at 458, 477, 488, and 514 nm, with a tube current of 61.4 A, was used. Live HEK 293 cells in PBS were viewed and examined, and images were taken with an Achromat 63–×0.9-W water objective (2× zoom). Typically, a 2-μm optical slice was used to visualize a cell expressing the constructs of interest, tagged with CFP and YFP. Dual excitation of CFP and YFP was achieved using an argon laser with a 466-nm/514-nm dichroic mirror (21). Optimized images were collected at 12-bit resolution over 512 × 512 pixels with a pixel dwell time of 1.6 μs (20). A cell was selected as the region of interest, which was then irradiated with the 514-nm laser line (100% intensity), and the number of iterations was varied, although the goal was to photo bleach YFP as quickly as possible. The general goal has been to obtain around 65% photobleaching (9) for an effective FRET. To better appreciate the occurrence of FRET, caution was taken not to overexpose the region of interest. Photobleached images were acquired immediately following acceptor (YFP) photobleaching. FRET was present when YFP photobleaching yielded an increase in CFP fluorescence intensity (20). FRET efficiency was calculated by using the following equation:

\[ \text{FRET efficiency} = \frac{\text{CFP photobleach fluorescence intensity} - \text{CFP photobleach fluorescence intensity}}{\text{CFP photobleach fluorescence intensity}} \times 100\% \]

A minimum of at least 10 cells to a maximum of 90 cells per condition were evaluated. Results were calculated as a percentage of the signal measured in cells expressing a CFP-YFP positive-control fusion protein. In these positive-control cells, an approximately 20% increase in CFP fluorescence after YFP photobleaching is typically observed (data not shown).

Bioluminescence resonance energy transfer (BRET) assay. Briefly, HEK 293 cells were cultured and transfected as described above, with the following differences: 1.5 μg each of D2 and D2-YFP constructs was used per 60-mm plate for a single experiment. Forty-eight hours posttransfection, cells were washed twice with PBS, detached in PBS containing 2 mM EDTA, centrifuged, and resuspended in PBS containing 0.1% glucose. About 100,000 cells were dispensed into each well of a 96-well plate with clear bottoms and black walls to minimize noise caused by autofluorescence. Studies in cell sonicates were done by resuspending the cell pellet in 250 μl of PBS containing 0.1% glucose followed by brief sonication. Protein was measured by the Bio-Rad Protein assay, and equal amounts of protein were added to each well.

To activate RLuc, 1 μM of its substrate (coelenterazine) was added in the same buffer and read after 10 min in a Fluostar Optima fluorimeter (BMG Lab Technologies, Offenburg, Germany), programmed with the appropriate software and filters: 475 to 30 nm for RLuc and 355 to 30 nm for YFP at a preset gain. BRET ratios were calculated using the following formula:

\[ \frac{\text{Emission at 355 nm}}{\text{Emission at 475 nm}} = \frac{\text{RLuc (475 to 30 nm)}}{\text{RLuc (475 to 30 nm) + YFP (475 to 30 nm)}} \]

The asterisk marks the sample where the SLAC construct is expressed alone.

Sequence analysis and structure modeling. Further sequence analysis of the deiodinase iodothyronine-like insertion has been conducted using hydrophobic cluster analysis (17, 18), as previously reported for the construction of the thionine fold model of deiodinase (6) or for the structural insights deduced to the D2 ubiquitin ligase (19). These dimers were modeled and built based on the Swiss pdb viewer tool (19).

RESULTS

D2 dimerization surfaces exist in the transmembrane and globular domains. When transiently expressed in HEK 293 cells, D2-CFP and D2-YFP display endoplasmic reticulum distribution (Fig. 1A), as seen in endogenous expression (12, 15, 18, 22). The YFP photobleaching approach to measuring FRET revealed that the D2-D2 FRET signal reached about 75% of that detected in positive-control cells expressing a well-characterized CFP-YFP fusion protein with a high degree of FRET (10) (Fig. 1B and data not shown), and no significant energy transfer was observed in cells expressing CFP, YFP, or both (Fig. 1B). D2-D2 FRET was detected regardless of whether both chromophores were fused to the amino or to the carboxyl end of D2 (Fig. 1B), but FRET was absent if the chromophores were placed in opposite orientations (Fig. 1B). This specificity was confirmed when D2-D2 dimerization was monitored by an alternative approach that used BRET (Fig. 1B and data not shown) in cells expressing D2 fused to RLuc and D2-YFP. Coexpression of D2 with the structurally related D1 enzyme fused to the appropriate chromophores did not result in measurable energy transfer by FRET or BRET (data not shown).

We next sought to characterize the structural basis of the D2-D2 dimer interaction. The single D2 transmembrane segment starts near C22 and ends near L41 (Fig. 1C). This is a typical transmembrane helix containing potentially charged residues (D, E, K, and R as well as H) that would normally achieve stability in the hydrophobic membrane environment by dimerization. In this way, charged residues could be neutralized via intermolecular interaction of residue couples such as D9/K55 as well as direct contacts between polar residues such as H36-K34, compatible with modeling of the two D2 transmembrane segments in an α hexameric architecture (data not shown). To study the role of this segment in D2-D2 dimeriza-
FIG. 1. D2 holoenzyme is a homodimer. (A) Photomicrography of an individual HEK-293 cell transiently coexpressing D2-CFP and D2-YFP, pre- and post-YFP photobleaching. (B) Quantification of D2-D2 FRET in cells expressing D2-CFP and/or D2-YFP fused at the indicated termini of D2. The location of the chromophores in the D2 molecule is indicated by N (amino) or C (carboxyl) for YFP and CFP, respectively. Results were calculated as a percentage of the signal measured in cells expressing a CFP-YFP positive-control (+) fusion protein. In these positive-control cells, an approximately 20% increase in CFP fluorescence after YFP photobleaching is typically observed (data not shown). Also shown (right axis) is the D2-D2 BRET ratio in cells expressing D2-RLuc and D2-YFP. The location of the chromophores in the D2 molecule is indicated by N (amino) or C (carboxyl) for YFP and RLuc, respectively. Data are means ± standard deviations of at least 10 data points. (C) Partial sequence alignment of human D1, D2, and D3, denoting the transmembrane and N-linker regions (6). Hydrophobic residues are green, loop-forming residues are depicted by white letters on a pink background, and the neutral amino acid H is shown on a purple background; the putative transmembrane segment (20 aa) is shown on a light green background, with amino acids likely to be in tight contact in the dimer interface shown in orange. Red, acidic; blue, basic; yellow, cysteine. (D) Western blot analysis with anti-FLAG antibody of cytosol (C) or microsomal (M) subcellular fractions of cells transiently expressing a truncated D2 without the transmembrane domain (ΔD2), full-length D2 (D2), or empty D10 vector.

A truncated D2 molecule missing the first 42 aa (ΔD2) was expressed and found to be a cytosolic protein (Fig. 1D) that does not homodimerize (Fig. 2A). While this indicates a critical role for the transmembrane domain in dimerization, it does not exclude a possible role of the globular domain as well. In fact, coexpressing ΔD2-YFP and a full-length D2-CFP molecule produced about 60% of the FRET detected in the full-length D2-D2 dimer (Fig. 2A), and this occurred only if the chromophores were fused to the C terminus of the two molecules, confirming that the interaction is through the globular domain (Fig. 2A). Based on these data, we sought to characterize the D2 globular dimerization interface. Since D2 is known to be a thioredoxin fold-containing protein (6), this interface was tentatively modeled by comparison to the human thioredoxin fold, which shows a clear propensity to dimerize via a large interface formed by the alignment of two β strands that constitute a small β sheet (32). Due to the high sequence identity around the canonical thioredoxin β1α2β2 motif, the D2 globular dimerization model could be fitted on the crystal structure of dimeric oxidized thioredoxin (PDB identifier 1eru) with no evident clash (Fig. 2C). In subsequent studies we failed to identify additional potential dimerization interfaces in the long and well-conserved segment S42 to K76, which connects the transmembrane and globular domains of D2, modeled as a miniglobule with all hydrophobic amino acids clustered into an internal core (Fig. 2D). Taken as a whole, these studies suggest that the native D2-D2 dimer is formed by interactions at both the transmembrane and the globular domains (Fig. 2D). This model reveals a region of negative electrostatic potential around the active sites that could act as an attracting field for the hormone and a large positive region for the rest of the molecules (Fig. 2E).

Ubiquitination transiently induces a conformational change within the D2-D2 dimer. Having established the dimeric nature of the D2 molecule, we next wished to investigate the role of dimerization in enzymatic activity. In initial studies using increasing concentrations of urea to promote conformational changes in the D2-D2 dimer, we observed a correlation between loss of BRET signal and enzymatic activity (data not shown). Taking advantage of the ΔD2-D2 dimerization (Fig. 2A), we looked at how dimerization affects enzyme activity by coexpressing a full-length catalytically inactive D2 molecule (with Ala replacing the critical selenocysteine [Sec] residue in the active center of the enzyme [Ala133D2]) with the Sec-containing ΔD2. While the Sec-containing ΔD2 molecule is inactive when expressed alone (Fig. 2B), when it is expressed in combination with Ala133D2, D2 catalytic activity is readily measurable (Fig. 2B). This indicates that dimerization of the globular domains is sufficient for D2 catalytic activity. The fit between ΔD2 and Ala133D2 molecules seems to position the
active center in ΔD2 in its natural conformation, as the apparent enzyme $K_m$ of T4 is indistinguishable from that of wild-type D2 protein (data not shown). However, the fact that in cells coexpressing ΔD2-Ala133D2 the FRET signal is not different from that of control cells [(C)ΔD2CFP+(C)ΔD2YFP] while the D2 activity is markedly reduced suggests that the transmembrane domain could modulate catalytic activity.

Based on the relationship between dimerization and D2 enzymatic activity, we asked whether ubiquitin-mediated enzyme inactivation induced by exposure to T4 (18) could be a
FIG. 3. Properties of D2-D2 dimer during catalysis. In all experiments cells were treated with 0, 0.1, 1, 5, or 10 μM T4 (from left to right) in 10% charcoal-stripped fetal bovine serum-containing medium for 4 h immediately prior to harvesting or FRET studies. (A) D2 activity in cells coexpressing full-length D2-CFP and D2-YFP, chromophores were placed at the carboxyl (C) terminus of D2; VDU-1 or WSB-1 interfering RNA (iRNA) plasmids were also used as indicated. (B) D2-D2 FRET signal in cells transfected as in panel A. (C) D2-D2 FRET signal in cells coexpressing D2-CFP and D2-YFP, chromophores were placed at the amino (N) terminus of D2. (D) Same as in panel A, except that ΔD2 was used. (E) ΔD2-D2 FRET signal in cells transfected as in panel D. Data are means ± standard deviations of at least 10 data points.

product of conformational changes within the D2-D2 dimer. This was tested by exposing D2-expressing cells to progressively higher concentrations of T4 (Fig. 3A to E). Initially we looked at FRET between the globular domains using D2 molecules containing chromophores fused to the carboxyl ends. Remarkably, exposure to T4 resulted in progressive loss of globular FRET signal in the D2-D2 dimer (Fig. 3B). This is presumably due to T4-induced ubiquitination and not overall loss of D2 protein, since the FRET signal being monitored is the fractional increase in CFP intensity after 90 seconds of YFP photobleaching. To test this hypothesis, similar studies were performed using small interfering RNA for WSB-1, under conditions that we have shown elsewhere to knock down WSB-1 expression and prevent D2 ubiquitination (16) (data not shown). Under these conditions, exposure to increasing concentrations of T4 only minimally inactivated D2 (Fig. 3A) and failed to promote the conformational change within the D2-D2 dimer (Fig. 3B), further suggesting that conjugation to ubiquitin interferes with the D2 globular dimerization interface. The T4-mediated dimer conformational change was the same in cells treated with the proteasome inhibitor MG132 (data not shown), indicating that this is not mediated by the proteasomes.

Next, we sought to determine whether these ubiquitination-induced changes in the D2-D2 dimer are reversible or lead to a terminal disassembly of the D2 holoenzyme. This was studied by coexpressing the D2-specific deubiquitinas enzyme VDU-1 (14). Under these conditions, exposure to increasing concentrations of T4 did not result in loss of D2 activity (Fig. 3A) or conformational changes within the D2-D2 dimer (Fig. 3B). These data support the reversibility of these T4-induced changes in D2-D2 dimer conformation-caused ubiquitination. The transient nature of this D2-D2 dimer interference suggests that other interacting surfaces in the dimer are also preventing its irreversible disassembly. To test this possibility, FRET studies were repeated using D2 molecules containing chromophores fused to their amino ends (Fig. 3C). Remarkably, from this perspective, ubiquitin-mediated conformational change within the D2-D2 dimer was almost nonexistent, indicating that the transmembrane-interacting surfaces remain largely unaffected during the ubiquitination/deubiquitination cycle. This model is further supported by the findings that the T4-induced changes within the ΔD2-D2 dimer are irreversible, given that normal dimerization cannot be reestablished by accelerating D2 deubiquitination with VDU-1 coexpression (Fig. 3D and E). Only WSB-1 knockdown can prevent such changes in the ΔD2-D2 dimer (Fig. 3D and E). Taken together, these studies indicate that T4-induced D2 ubiquitination promotes a conformational change within the globular domains, while the transmembrane domains remain largely unaffected by ubiquitin conjugation.

The ECS<sup>WSB-1</sup> catalytic core complex is continuously assembled around D2. In the next set of experiments, we wished to evaluate the relationships of the D2-D2 dimer with the ECS<sup>WSB-1</sup> catalytic core complex and VDU-1, a D2-specific deubiquitinase. This was done by coexpressing appropriate chromophore-labeled D2, WSB-1, UBC-7, and/or VDU-1. A strong FRET signal, equivalent to that of the D2-D2 dimer, was obtained from D2 interactions with WSB-1, UBC-7, and VDU-1. Remarkably, significant energy transfer was detected even when D2 ubiquitination was minimized, i.e., in the absence of T4, suggesting a continuous association between ECS<sup>WSB-1</sup> catalytic core complex and D2 (Fig. 4A and B). Coexpression studies indicate that each of these D2-interacting proteins attaches to distinct and
FIG. 4. D2 interaction with UBC-7, WSB-1, and VDU-1. (A) FRET studies in HEK-293 cells transiently coexpressing D2-CFP and UBC-7-YFP, WSB-1-YFP, or VDU-1-YFP. As indicated, some studies were performed in cells with WSB-1 knockdown (interfering RNA [siRNA]) or coexpressing WSB-1. The positions of the chromophores in the D2 molecule are indicated as amino (N) or carboxyl (C) termini. (B) Same as panel A, except that the interactions of ΔD2 with UBC-7, VDU-1, or WSB-1 were studied in the absence of or during full-length D2 coexpression. In the experiments for panels C to F, cells were treated with 0, 0.1, 1, 5, or 10 μM T4 (from left to right) in 10% charcoal-stripped fetal bovine serum-containing medium for 4 h immediately prior to FRET studies. (C to E) D2 interaction with WSB-1 (C), with VDU-1 (D), and with UBC-7 (E), with or without VDU-1 or WSB-1. (F) Same as in panel C, except that ΔD2 was used. Data are means ± standard deviations of at least 10 data points.

independent interactive surfaces on the D2-D2 dimer, as WSB-1 knockdown or coexpression only minimally interfered with D2–UBC-7 or D2–VDU-1 interactions (Fig. 4A). Additionally, coexpression of UBC-7 and VDU-1 did not interfere with either protein’s interaction with D2 or with the D2–WSB-1 interaction (data not shown). Cross-binding and dimerization were minimal and observed only between WSB-1 and UBC-7 and between UBC-7 and UBC-7, respectively, and both were disrupted by D2 coexpression (data not shown). While we cannot exclude the possibility that the ECSWSB-1 catalytic core complex interacts with D2 as a monomer, ΔD2 did not interact with WSB-1 except when
full-length D2 was simultaneously coexposed (Fig. 4B), indicating that WSB-1 binds the D2-D2 dimer. This also indicates that the N linker and/or the globular domains in D2 are sufficient to mediate WSB-1 recognition. UBC-7, on the other hand, did not bind to ΔD2 under any circumstance, and VDU-1 binding to ΔD2 was minimal and not affected by full-length D2 coexpression (Fig. 4B). The latter also provides an alternate explanation as to why VDU-1 coexpression failed to rescue the loss of ΔD2 D2imerization upon exposure to T4 (Fig. 3D and E).

The finding of an association between D2 and UBC-7 independent of WSB-1 is notable. As the cullin/Rbx1 heterodimer is generally believed to recruit E2s for ubiquitination (34), knockdown of WSB-1 would intuitively be expected to disrupt the interaction between D2 and UBC-7, but this was not the case (Fig. 4A). In fact, we have previously shown that human D2 specifically associates with UBC-7 (but not UBC-6) in reticulocyte lysates as well as when it is transiently expressed in HEK-293 cells, with localization to a 169 to 234 of D2, independent of WSB-1 (22). These data are consistent with our present results and also suggest a possible role played by UBC-7 in E3complex-1 assembly.

Given that exposure to T4 promotes D2 ubiquitination and a marked conformational change in the D2-D2 dimer, our next
question was whether such effects could modulate D2 interaction with the key proteins in the ECS\(^{-}\) WB-3 catalytic core complex or VDU-1 (Fig. 4C to F). Notably, exposure to T4 increased the D2-WB-1 association while decreasing the interactions of D2-VDU-1 and D2-UBC-7 (Fig. 4C to E). These changes are likely to result from D2 ubiquitination, given that they are minimized with coexpression of VDU-1 or by WB-1 knockdown. However, our studies do not address the timing with which these changes occur. Thus, it is difficult to speculate whether the conformational change within the D2-D2 dimer is a cause or result of such changes or whether after ubiquitination WB-1 could occupy VDU-1 or UBC-7 binding sites in the D2-D2 dimer. In contrast, the association between the \(\Delta D2\)-D2 dimer and WB-1 does not increase with exposure to T4 (Fig. 4F), suggesting that perhaps the \(\Delta D2\)-D2 dimer is terminally disassembled upon ubiquitination.

Human D2 contains 15 Lys residues. To identify which one is ubiquitinated by the ECS\(^{-}\) WB-3 catalytic core complex, 13 Lys residues were replaced with Arg in different combinations, while the carboxyl-terminal 267KK268 pair was truncated. After extensive analysis, a combined K237R/K244R catalytically inactive D2 mutant was identified as being resistant to WB-1-mediated ubiquitination in vitro (Fig. 5A). In fact, based on our D2-D2 model (Fig. 2C to E), K244 lies within the dimer interface, near the Sec residue of the catalytic site, and its ubiquitination would be expected to interfere with the D2-D2 conformation. In addition, K237 is in the vicinity of K244, within the hinge area between the thioredoxin-folded domain and the N-linker small domain, and thus also in a position to affect overall dimer stability. When expressed in cells, the K237R/K244R D2 mutant remained in the endoplasmic reticulum (data not shown) but accumulated about fivefold above wild-type levels as a result of having a much longer half-life in \(^{13}\)S methionine metabolic labeling studies (Fig. 5B). Interestingly, the isolated K237R or K244R D2 mutants is active and behaves normally in all aspects studied (data not shown), suggesting that WB-1-mediated D2 ubiquitination can take place in either one of these residues. In subsequent studies, the K237R/K244R D2 mutant was found to be a dimer that, unlike the wild-type molecule, is stable upon exposure to T4 (Fig. 5C). Exposure to T4 also failed to change the interaction between the double Lys mutant and UBC-7, VDU-1, or WB-1, indicating that these processes depend on conjugation to ubiquitin (Fig. 5C).

It has already been established that T4 must interact with the Sec residue in the D2 active center in order to increase D2 susceptibility to ubiquitination (28), but it is not known whether catalysis, i.e., T3 production, is necessary. To address this question, D2-expressing cells were treated with kaempferol, a flavonol that at 40 \(\mu\)M specifically prevents D2-mediated T4-to-T3 conversion by interfering with the endogenous cofactor for D2 (Fig. 3A) (15). In control cells, exposure to increasing concentrations of T4 resulted in T3 production (up to 1.0 femol T3/h/mg protein) and -50% loss of D2 activity due to ubiquitination (Fig. 5G). At the same time, in kaempferol-treated cells, exposure to T4 was associated with only minimal T3 production (up to 0.05 femol T3/h/mg protein) but the loss in D2 activity was similar (Fig. 5G). Thus, in the absence of substantial T4 catalysis, the same D2-D2 modifications were observed as when T3 is produced, i.e., loss of D2 catalytic activity (Fig. 5G) and of changes in D2-D2 dimer conformation (Fig. 5H), as well as a decrease in the D2-UBC-7 and D2-VDU-1 associations and an increase in D2-WB-1 association (Fig. 5I to K). These data indicate that binding of T4 to the D2 active center is sufficient to trigger D2 ubiquitination, presumably by inducing conformational changes within the D2-D2 dimer that result in exposure of the critical K237R/K244R to UBC-7.

Lastly, we evaluated the relative contributions of changes in dimer conformation versus D2 proteolysis to the overall loss of D2 activity during exposure to T4. Monitoring D2-YFP signal as an index of intracellular D2 levels in parallel with D2 activity and D2-D2 FRET signal in live cells addressed this question. Upon exposure to T4, there was a rapid and progressive loss in D2 activity that was predominantly due to the modification within the D2-D2 dimer. At the same time, D2 degradation as assessed by loss of D2-YFP signal contributed much less to the loss in D2 activity (Fig. 5L).

**DISCUSSION**

The data obtained in the present studies describe details of the mechanism through which conjugation to ubiquitin can regulate deiodinase function and thyroid hormone activation. Our model system is based on an integral membrane protein that exhibits catalytic activity only in a dimeric conformation (Fig. 1 and 2). In this system, substrate-induced ubiquitination inactivates the D2 enzyme by transiently interfering with its dimeric conformation (Fig. 3). While the FRET and BRET studies were performed in cells transiently expressing the different D2 fusion proteins, the intrinsic properties of these proteins such as subcellular localization, turnover rate, and responsiveness to T4, D2's natural substrate, are indistinguishable from those of endogenous D2 (12). Under these conditions, it is intriguing that the WB-1- and VDU-1-based catalytic core complex that regulates D2-D2 ubiquitination is continuously associated with dimeric D2 (Fig. 4), instead of being assembled at every cycle of deiodination, ubiquitination, and deubiquitination.

The picture that emerges from the present study is that D2 has two dimerization surfaces: one involves its transmembrane domain while the other involves its globular catalytic domain. In addition, it is clear that globular homodimerization is sufficient for catalytic activity (Fig. 2A and B). As \(\Delta D2\) is inactive and does not homodimerize (i.e., \(\Delta D2\)-\(\Delta D2\) [Fig. 2A and B]), the interaction between these two truncated molecules containing only the globular domain does not mediate stable dimerization. However, \(\Delta D2\) still dimerizes with full-length D2, which results in catalytic activity (Fig. 2A and B). While it is unclear why there is no interaction between two \(\Delta D2\) globular domains in the cytosol, the finding that \(\Delta D2\)-\(\Delta D2\) globular domains do dimerize indicates that insertion in the endoplasmic reticulum membrane of at least one of the dimer counterparts is required to accommodate globular dimerization.

FRET decreases as the physical distance between the chromophores increases. Thus, one interpretation of the T4-induced loss of D2-D2 FRET signal is that ubiquitination promotes a change within the D2-D2 dimer that increases the physical separation of the two chromophores. WB-1-mediated D2 ubiquitination takes place at K237 and/or K244 (Fig. 5).
Given that K244 lies within the dimer interface near the Sec residue of the catalytic site (6), its ubiquitination and/or that of its neighbor K237 provides a possible molecular mechanism for such conformational change to occur. Regardless of the mechanism, it is remarkable that such interference with the D2-D2 dimer is restricted to the globular domain of the enzyme, which contains the active center, but does not affect the transmembrane domains (Fig. 3). This sets the stage for a mechanism in which deubiquitination restores D2 catalytic activity by reversing ubiquitin-mediated change in D2-D2 dimer conformation.

The fact that pharmacological inhibitors of the proteasome prolong D2 half-life (28, 29) suggests that some D2 is eventually degraded through this system, although it is not clear what determines the fate of ubiquitinated D2. The fact that the D2-D2 dimer is continuously associated with WS1-1, UBC-7, and VDU-1, even under conditions of minimal D2 ubiquitination (Fig. 4), suggests that D2 molecules can undergo multiple cycles of ubiquitination and deubiquitination before proteasomal degradation. This view is supported by the finding that the loss in D2 activity caused by T4 is predominantly due to conformational changes within the dimer, while proteasomal degradation contributes to a lesser extent (Fig. 5L). In fact, the constant association between D2 and these proteins contrasts with the situation for most proteins, in which modifications such as phosphorylation trigger binding to the ubiquitin ligase adaptor and the assembly of the catalytic core complex.

The sensitivity to the proteasome inhibitors could also be interpreted as supporting the existence of an alternative pathway for D2 ubiquitination. The observation that D2 accumulates in a doa10A yeast strain indicates that D2 might also be the target of the more general degradation process based in the endoplasmic reticulum, traditionally known to be associated with the proteasome (26). If this is also the case in vertebrates, an interesting possibility is that D2 would be dually regulated, first via the Hedgehog signaling pathway via the ECSW38-1 catalytic core complex in which deubiquitination is possible and second via TE84, the mammalian ortholog of doa10, targeting it for proteasomal degradation.

D2 contains the rare amino acid Sec in its active center. Decoding the Sec-encoding UGA codon as a signal for cotranslational insertion of Sec, not termination, decreases translation efficiency 20- to 400-fold (4). Thus, due to this intrinsic inefficiency of the selenoprotein synthesis, the availability of a reversible ubiquitination-dependent mechanism to control the activity of D2 constitutes a biochemical and physiological advantage that allows for an additional control of thyroid hormone activation.

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Expression patterns of WSB-1 and USP-33 underlie cell-specific post-translational control of type 2 deiodinase (D2) in the rat brain


Neste trabalho de minha co-autoria sob orientação do professor Bianco em Boston, o papel das enzimas envolvidas no processo de degradação da D2 e sua distribuição tecidual foi investigado através da análise de expressão gênica, com ênfase no sistema nervoso central (SNC). Com análises in silico dos níveis de expressão gênico destas enzimas (WSB-1, USP33 entre outras) em camundongos disponível no site do Genomics Institute of the Novartis Research Foundation (GNF SymAtlas - biogps.gnf.org), conseguimos identificar uma correlação na expressão de WSB-1 com a USP33 (VDU-1) indicando a presença de vias de ubiquitinação e desubiquitinação da D2 nestes tecidos. Essa expressão concomitante destas duas enzimas ocorre com maior intensidade no SNC onde esta correlação é ainda mais acentuada. Juntamente com essa análise in silico criamos um modelo animal buscando a modulação da expressão dessas proteínas no SNC em diferentes concentrações hormonais (hiper-; eu- ou hipotiroidismo). A diferença na expressão dos genes envolvidos na degradação da D2 não apresentou mudanças na maioria dos tecidos estudados, apenas no hipotálamo médio basal e hipófise. Os resultados demonstram que a WSB-1 não é específica para a D2, podendo existir outros substratos ainda não identificados para essa enzima.
Expression Patterns of WSB-1 and USP-33 Underlie Cell-Specific Posttranslational Control of Type 2 Deiodinase in the Rat Brain


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The type 2 deiodinase (D2) activates thyroid hormone and constitutes an important source of 3,3',5'-triiodothyronine in the brain. D2 is inactivated via WSB-1 mediated ubiquitination but can be rescued from proteasomal degradation by USP-33 mediated deubiquitination. Using an in situ analysis of published array data, we found a significant positive correlation between the relative mRNA expression levels of WSB-1 and USP-33 in a set of 56 mouse tissues (r = 0.08; P < 0.04). Subsequently, we used in situ hybridization combined with immunocytochemistry in rat brain to show that in addition to neurons, WSB-1 and USP-33 are differently expressed in astrocytes and tanyocytes, the two main D2 expressing cell types in this tissue. Tanyocytes, which are thought to participate in the feedback regulation of TSH neurons express both WSB-1 and USP-33, indicating the potential for D2 ubiquitination and deubiquitination in these cells. Notably, only WSB-1 is expressed in glial fibrillary acidic protein-positive astrocytes throughout the brain. Although developmental and environmental signals are known to regulate the expression of WSB-1 and USP-33 in other tissues, our real-time PCR studies indicate that changes in thyroid status do not affect the expression of these genes in several rat brain regions, whereas in the mediobasal hypothalamus, changes in gene expression were minimal. In conclusion, the correlation between the relative mRNA levels of WSB-1 and USP-33 in numerous tissues that do not express D2 suggests that these ubiquitin-related enzymes share additional substrates besides D2. Furthermore, the data indicate that changes in WSB-1 and USP-33 expression are not part of the brain homeostatic response to hypothyroidism or hyperthyroidism.

The main product secreted by the thyroid gland is T₄, a prohormone that must be converted to T₃ via outer ring deiodination to gain full biological activity. Although in most tissues thyroid hormone action is predominantly determined by plasma T₃ concentration, the brain expresses deiodinases that can modulate thyroid hormone signaling locally, relatively independent of plasma T₄ and T₃ levels (1–3). In fact, Gallione et al. (4) recently demonstrated that brain T₃ content is substantially reduced in mice with targeted disruption of the type 2 deiodinase (D2) encoding Dhdo2 gene. D2 is a thioredoxin fold-containing dimeric selenoenzyme that in the brain is predominantly expressed in two types of glial cells: the tanyocytes lining the wall of the third ventricle, and the astrocytes (5–7). D2 expression and activity are under complex regulation, including transcriptional and posttranscriptional mechanisms. In contrast to the other two deiodinases (D1 and D3), D2 has a short half-life (8) due to substrate-induced ubiquitination and selective proteolysis via the ubiquitin/26S proteasome pathway (9, 10). Both UBC6 and UBC7 ubiquitin conjugating (E2) enzymes play a role in D2 ubiquitination (11, 12). The D2-ubiquitinating catalytic core complex has been modeled as Elongin BC-Cullin RBX1 (ECS3/5WSB³), with WSB-1 implicated as a D2-specific E3 ubiquitin ligase adaptor subunit (13). WSB-1 (also known as SWIP-1) is a hingebox-inducible SCOS-box-containing WD-40 protein (14) that interacts with D2 through a specific instability loop present in D2 (15, 16).

Upon binding of T₄, D2 is ubiquitinasa, which inactivates the enzyme by interfering with D2’s globular interacting surfaces that are critical for dimerization and catalytic activity (16). Ubiquitinated D2 is catalytically inactive, but it is not immediately up taken by the proteasomes. A pair of D2-binding deubiquitinating enzymes (USP-33 and USP-20; also known as von Hippel-Lindau interacting deubiquitinating enzymes VDU-1 and VDU-2) can reactivate D2 through deubiquitination, rescuing it from proteasomal degradation (17). The continuous association of D2 with this regulatory protein complex supports rapid cycles of deiodination, conjugation to ubiquitin, and enzyme reactivation by deubiquitination, allowing tight control of thyroid hormone action (16).

Although components of the ubiquitinating pathway for en-
doplasmic reticulum resident proteins are generally ubiquitously expressed, the E3 ubiquitin ligase adaptors, which provide substrate recognition to the catalytic core complex, have more selective expression and, of course, must be coexpressed with the target protein in the same cell (18). To find out more about the expression of WSB-1 and USP-33, we first used data mining and in silico analyses of publicly available data sets of mouse tissues. Second, we used in situ hybridization combined with immunocytochemistry to analyze the expression of WSB-1 and USP-33 in the rat brain, finding that although WSB-1 is coexpressed with D2 in astrocytes and tanyocytes, USP-33 coexpression with D2 is limited to tanyocytes. This indicates that in the brain, USP-33-mediated reactivation of ubiquitinated D2 is a mechanism limited to the medial basal hypothalamus (MBH).

Materials and Methods

Gene expression data

The data of the relative gene expression of the different genes analyzed were obtained from the Genomics Institute of the Novartis Research Foundation website (http://synapsis.gnf.org). These data consist of a transcriptional profile from 56 mouse tissues created with Affymetrix GNF3, a custom microarray (19). For our analysis, em-
**TABLE 1.** Correlation analyses of the relative expression levels of Dio2, WSB-1, USP-33, and other ubiquitin-related genes in 56 mouse tissues

<table>
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Values are the relative gene expression analyzed using SyntGene. *r* is the correlation coefficient, and *P* indicates the level of significance by linear regression analysis.

Bryonic and fetal tissues were excluded, leaving 56 mouse tissues. Samples for microarray hybridization in the SyntGene were run in duplicate made from a pool of individual tissues. The reporter selected for each gene were: WSB-1, gfnfmr26736_a_at; USP-33, gfnfmr69397_a_at; USP-20, gfnfmr28848_a_at; USP-21, gfnfmr01851_a_at; UBE2C2, gfnfmr08314_a_at; USP-48, gfnfmr9416_a_at; UBE3C, gfnfmr11602_a_at. Only data processed using Affymetrix Microarray Suite (MAS5, Affymetrix, Santa Clara, CA) were analyzed in the present study.

**Animals**

Experiments were performed on male Wistar rats (TCOL-COOP-KFT, Budapest, Hungary and Harlan, Indianapolis, IN) weighing between 200 and 300 g. The animals were housed under standard environmental conditions (light between 0600 and 1800 h, temperature 22 ± 1°C, rat chow and water available ad libitum). Animals were kept and experiments were performed according to protocols approved by the Animal Care and Use Committees of the Institute of Experimental Medicine of the Hungarian Academy of Sciences and Harvard Medical School in compliance with National Institutes of Health standards. To generate hypothyroidism, the rats were given 0.1% methimazole and 0.5% sodium perchlorate in drinking water for 13 d. The hyperthyroid group received 10 µg T3/100 g body weight ip for 3 d. Non treated euthyroid animals of the same age were used as controls. At the end of the experimental period, animals were anesthetized with 50 mg/kg body weight sodium pentobarbital, killed by exsanguination, and rapidly

**Fig. 2.** A–C and E–I, A series of low-power images illustrates the distribution of WSB-1 mRNA at eight rostral-caudal levels of the forebrain. J, Distribution of the WSB-1 mRNA in the cerebellum. D, No hybridization signal was detected using a sense WSB-1 probe. Scale bar, 2000 µm.
GTCAACTTCTTCA. The region corresponds to bases 277-122 of GenBank XM_001080019. The fragment was cloned into pGemT vector (Promega Corp., Madison, WI) and confirmed by sequencing. NcoI digestion followed by transcription with SfI polymerase was used to generate the antisense cRNA probe in the presence of 35S-UTP, whereas NcoI digestion and T7 polymerase was used for the sense probe.

The 808-bp long rat WSB-1 fragment corresponding to bases 152-959 of XM_220736 was amplified on rat MBH cDNA using the oligos as indicated: sense, CAGAGGTCACAGGAGAAAAGAGAT; and antisense, GACCGAGTGACTGATGATGCT. The fragment was cloned into pHoney vector, confirmed by sequencing and transcribed the same way as indicated for USP-33 previously.

**Single-label in situ hybridization**

The rats were decapitated. The brains were removed quickly from the skull, quickly frozen on dry ice, and stored at -80°C until used. Serial 12-μm thick coronal sections were cut on a cryostat (Leica Microsystems GmbH, Wetlar, Germany), mounted on Superfrost Plus slides (Fisher, Hampton, N.B.), and dried at 42°C overnight, as described (20). On the day of hybridization, the sections were fixed with 4% paraformaldehyde in PBS (pH 7.4) for 1 h, washed in 2× saline-sodium citrate (sodium chloride) (2× SSC) containing 0.2% acetic anhydride in 0.9% triethanolamine for 20 min, and then treated in graded solutions of ethanol (70%, 85%, and 100%), chloroform, and a decending series of ethanol (100% and 96%) for 5 min each, and hybridized with the aforementioned rat WSB-1 or USP-33 single-stranded 35S-UTP labeled cDNA probes. The hybrids were performed under plastic coverslips in a buffer containing 50% formamide, 2-fold concentration of SSC (2× SSC), 10% dextran sulfate, 0.5% sodium dodecyl sulfate, 250 μg/ml denatured salmon sperm DNA, and the 35S-UTP radiolabeled probe for 16 h at 56°C. The slides were washed in 1× SSC for 15 min and then treated with RNase (25 μg/ml) for 1 h at 37°C, followed by additional washes in 0.1× SSC (2× 40 min) at 60°C. After dehybridization in graded dilutions of ethanol, the slides were dipped into Kodak NTB autoradiography emulsion (Eastman Kodak, Rochester, NY), and the autoradiograms were developed after 6-wk exposure at 4°C. The specificity of hybridization was confirmed using sense probes that resulted in the complete absence of hybridization signal in the brain.

**Double-labeling in situ hybridization and immunocytochemistry for WSB-1, USP-33, and glial fibrillary acidic protein (GFAP)**

Brain sections were prepared and hybridized for WSB-1 and USP-33, respectively, as described previously. After post-hybridization washes, the

**TABLE 2. Effects of hypothyroidism and hyperthyroidism on the expression WSB-1, USP-33, and D2 in the cortex, cerebellum, hippocampus, MBH, and pituitary**

<table>
<thead>
<tr>
<th></th>
<th>D2</th>
<th>WSB-1</th>
<th>USP-33</th>
<th>Cycle A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypo</td>
<td>1.1 ± 0.50 (4)</td>
<td>1.2 ± 0.24 (4)</td>
<td>1.0 ± 0.58 (4)</td>
<td>1.04 ± 0.29 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>1.4 ± 0.65 (4)</td>
<td>1.3 ± 0.35 (4)</td>
<td>1.5 ± 0.41 (4)</td>
<td>1.00 ± 0.73 (4)</td>
</tr>
<tr>
<td>Cerebellum</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypo</td>
<td>0.91 ± 0.26 (4)</td>
<td>1.45 ± 0.21 (4)</td>
<td>1.3 ± 0.05 (4)</td>
<td>1.27 ± 0.34 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>0.96 ± 0.13 (4)</td>
<td>1.9 ± 0.44 (4)</td>
<td>1.6 ± 0.32 (4)</td>
<td>1.28 ± 0.49 (4)</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypo</td>
<td>0.73 ± 0.07 (4)</td>
<td>1.45 ± 0.21 (4)</td>
<td>1.0 ± 0.80 (4)</td>
<td>1.17 ± 0.50 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>1.1 ± 0.53 (4)</td>
<td>nd</td>
<td>0.75 ± 0.18 (4)</td>
<td>1.8 ± 0.93 (4)</td>
</tr>
<tr>
<td>MBH</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypo</td>
<td>1.1 ± 0.39 (4)</td>
<td>nd</td>
<td>0.86 ± 0.31 (4)</td>
<td>0.91 ± 1.99 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>1.0 ± 0.25 (4)</td>
<td>nd</td>
<td>0.97 ± 0.38 (4)</td>
<td>1.29 ± 0.34 (4)</td>
</tr>
<tr>
<td>Pituitary</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypo</td>
<td>0.95 ± 0.21 (4)</td>
<td>0.74 ± 0.03 GP*</td>
<td>0.90 ± 0.17 (4)</td>
<td>1.06 ± 0.18 (4)</td>
</tr>
<tr>
<td>Control</td>
<td>2.6 ± 0.60 (4)*</td>
<td>1.0 ± 0.15 (4)</td>
<td>1.2 ± 0.19 (4)*</td>
<td>1.98 ± 0.17 (4)</td>
</tr>
</tbody>
</table>

Values represented are the mean ± SD. The number of animals per group is indicated in parentheses. Cycle A, Cyclophillin A; Hypo, hypothyroidism; Hypo, hyperthyroidism; nd, not determined.

* P < 0.05 vs. the corresponding D2, WSB-1, or USP-33 control by ANOVA, followed by the Newman-Keuls test.

* P < 0.01.

* P < 0.001.
sections were treated with the mixture of 0.5% Triton X-100 and 0.5% H2O2 for 15 min, and then with 1% BSA in PBS for 20 min to reduce nonspecific antibody binding. The sections were incubated with a mouse monoclonal antibody against GRP (1:100; Boehringer Mannheim, Vienna, Austria) at 150 dilution in 1% BSA containing PBS overnight at 4°C. After washes in PBS, the sections were incubated in donkey antimouse IgG (1:1500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h and ABC Elite (1:1000; Vector Laboratories, Burlington, CA) for 1 h. The immunoreactivity was detected with 0.05%, 3,3'-diaminobenzidine containing 0.003% H2O2 in 0.06 M Tris buffer (pH 7.5). After several washes in PBS, the sections were dehydrated in graded dilutions of ethanol, slides were dipped into Kodak NTB autoradiography emulsion, and autoradiograms were developed after 6-week exposure at 4°C. A series of sections were then counterstained with 0.1% Fast Green FCF (Sigma-Aldrich, St. Louis, MO) dissolved in 1% acetic acid.

Imaging

Images were captured using a Zeiss Axioskop hot microscope (Zeiss, Vienna, Austria) equipped with a Real Time Spot Digital Camera (Diagnostic Instruments, Sterling Heights, MI). The single-labeled images were captured under dark-field illumination, whereas bright-field illumination was used to image the double-labeled preparations.

Real-time PCR (qPCR)

Frozen tissues, including cortex, cerebellum, hippocampus, and pituitary, were processed for isolation of total RNA using Trizol Reagent from Invitrogen (Carlsbad, CA). After quantification, RNA was fractionated by agarose electrophoresis to ensure integrity. The reverse transcriptase reaction was performed using superscript II (Invitrogen) and oligo-dT. qPCR was performed as described previously by Zarnecki et al. (21). Primers used in the real-time PCR for USP-33 were previously described by Curcio-Morelli et al. (17), and the D2 and WSB-1 are the same as in the study by Dentice et al. (13). All values for the qPCR were normalized using cyclophilin A mRNA as an internal control.

Statistical analysis

Multiple groups were compared using one-way ANOVA, followed by a Newman-Keuls post hoc test (Prism 4; GraphPad Software, San Diego, CA). In silico correlation analysis was performed by linear regression, whereas data obtained in Ta T1 cells were analyzed by t test (Prism 4).

Results

Tissue distribution of WSB-1 and USP-33

The expression of WSB-1 and USP-33 genes in mouse tissues was analyzed in situ using data previously published (19) and available through the SymAtlas web site (http://symatlas.nig.msf.gov/SymAtlas/). Both genes are expressed in all tissues analyzed, and there is a general tendency for USP-33 to have higher expression levels in the central nervous system (CNS) when compared with all other tissues (Fig. 1). WSB-1 is also highly expressed in the CNS, which is compatible with the fact that both USP-33 and WSB-1 were identified as a D2-interacting protein in a yeast two-hybrid screening of a human brain library (13, 17). Although WSB-1 is highly expressed in the preoptic area, hypothalamus, lower spinal cord, umbilical cord, large intestine, B cells, and thymus, USP-33 is found predominantly in
the amygdala, frontal cortex, nucleus trigeminius, cerebral cortex, hypothalamus, lower and upper spinal cord, substantia nigra, prostate, and eye (Fig. 1).

It is notable the tendency for coexpression between WSB-1 and USP-33, particularly in the CNS. At the same time, no clear overall relationship with D2 expression is observed (Fig. 1). Performing correlation analyses between the respective relative mRNA levels revealed a positive relationship between WSB-1 and USP-33 relative expression. The overall correlation is weak but reached the statistical significance level ($r = 0.08; P < 0.04$; Table 1). When only CNS regions were analyzed, the correlation became much stronger, reaching 0.35 ($P = 0.05$). To test how specific the WSB-1 and USP-33 relationship is, we also looked at the gene expression of other ubiquitin-related proteins and their correlation with WSB-1 and USP-33, i.e., the ubiquitin-activating enzyme UBE1 C, two ubiquitin conjugates UBC-6 and UBC-7, two deubiquitases USP-20 and USP-48, and two ubiquitin ligases SKP-1 and SKP-2. Although no significant correlations were observed between the relative expression of WSB-1 or USP-33 gene expressions with that of the ubiquitin-activating and conjugating enzymes, there was a positive correlation between WSB-1 and that of USP-20 and USP-48. The relative expression of USP-33 also correlated significantly with that of SKP-1 (Table 1).

D2 expression is also predominant in the CNS (Fig. 1) (22). However, no significant correlation was identified between relative D2 mRNA levels and those of WSB-1 or USP-33 (Table 1).

**Distribution of the WSB-1 and USP-33 mRNA in the rat brain**

Given the predominant distribution of D2 and USP-33 in the CNS and the fact that the expression of both genes is positively correlated, we used in situ hybridization to assess the distribution of mRNA corresponding to both genes in the rat brain. WSB-1 mRNA was widely expressed in all regions of the rat brain, including the hypothalamus (Fig. 2 and see Fig. 4, C-G) cortex (Fig. 2 and see Fig. 4B), hippocampus (Fig. 2 and see Fig. 4A), and cerebellum (Fig. 2 and see Fig. 4H). WSB-1 is expressed in both neurons, including the pyramidal cells of the cortex (see Fig. 6B) and the CA1 region of the hippocampus (see Fig. 6D), as well as in GFAP-positive astrocytes (see Fig. 6C). In addition to these two cell types, WSB-1 mRNA was also detected in ependymal cells lining the wall of the third ventricle between the rostral pole of the median eminence and the mamillary recess. In the ependyma, the signal was localized to the floor of the third ventricle at the rostral pole of the median eminence, whereas more caudally, WSB-1 expressing cells covered the ventral half to two thirds of the ventricular wall (see Fig. 4C-G). Two regions of WSB-1 expressing ependymal cells were reminiscent of the distribution of third ventricular tanyocytes. WSB-1 expression was not detected in other regions of the third ventricular wall.

USP-33 mRNA was also widely expressed in all brain regions studied (Fig. 3 and see Fig. 5). Although similar to WSB-1, the USP-33 hybridization signal was observed over the vast majority of the fast green FCF-stained neurons (see

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**Fig. S.** Medium-power magnification images of USP-33 mRNA distribution in the hippocampus (A), cortex (B), ependymal cells lining the wall of the third ventricle (C), C and D), in the cerebellum (E). A very dense hybridization signal is observed in the hippocampus (A) over the pyramidal layer (CA1–CA3) and the granular layer of the dentate gyrus (GrDG). Only rare cells were labeled in the oriens layer (Or), stratum radiatum (Rad), and lacunosum molecular layer of the hippocampus (LMol), and in the polymorph layer of the dentate gyrus (PoDG). A dense USP-33 hybridization signal was observed in the two to five layers of the cortex (E), whereas no hybridization signal was absent from the first layer of cortex (arrow). All regions of the hypothalamus were labeled densely with a USP-33 hybridization signal (C and D). A USP-33 hybridization signal was also detected over ependymal cells lining all regions of the wall of the third ventricle (arrow) (C and D). A very dense hybridization signal was observed over the granular layer of the cerebellum (GrO) (E). A moderate density hybridization signal was observed over the molecular layer of the cerebellum (CMO) (E). No hybridization signal was observed over the white matter (WM) (E). Scale bar on B, 200 μm, corresponding to A, B, and E. Scale bar on D, 200 μm, corresponding to C and D. Mol, Molecular layer of gyrus dentatus; MR, mammillary recess.
Fig. 6. Cell-type specificity of WSB-1 and USP-33 expression in the brain. A WSB-1 hybridization signal is observed over the majority of cortical GFAP-positive astrocytes (brown) as well as many GFAP-negative cells (A). Medium-power magnification images illustrate a WSB-1 expressing GFAP-positive astrocyte and several WSB-1 expressing neurons labeled with Fast Green FCF in the third layer of the cortex (B). The majority of astrocytes express WSB-1 in the oris (Or), pyramidal layers, and stratum radiatum (Rad) of the hippocampus (D, arrows). Medium-power micrograph shows a WSB-1 mRNA-containing GFAP-positive astrocyte and WSB-1 positive pyramidal cells in the CA1 region of the hippocampus (D). A WSB-1 hybridization signal is observed over the majority of astrocytes in the dentate gyrus (MoD (arrows)) (G). Non-GFAP positive cells also express WSB-1 in these regions. Low-power images illustrate that the USP-33 hybridization signal is absent from the majority of GFAP-positive astrocytes in the cortex (I), hippocampus (J), and dentate gyrus (N), but the USP-33 hybridization signal is observed over numerous non-GFAP positive cells. Medium-power images illustrate the absence of a USP-33 hybridization signal in GFAP-positive astrocytes (brown), and USP-33 expression in neurons of the third layer of cortex (J, green cells) and pyramidal cells of the CA1 region of the hippocampus (G, green cells). Scale bar on K, 25 μm, corresponding to B, D, I, and K. Scale bar on N, 50 μm, corresponding to A, C, E-H, J, and L-N. GR, Granular layer of cerebellum; GrDG, granular layer of the dentate gyrus; MR, mammillary recess; PoDG, polymorph layer of the dentate gyrus; WM, white matter.

Fig. 6, I and K: only scattered GFAP-immunoreactive astrocytes were labeled with the USP-33 hybridization signal. The USP-33 hybridization signal was also detected in ependymal cells lining the wall of the lateral and third ventricles, including the regions where tanyocytes line the wall of the third ventricle (see Fig. 5, C and D).
Expression of WSB-1 and USP-33 during hypothyroidism and hyperthyroidism

WSB-1 and USP-33 mRNA levels were measured by qPCR in specific brain regions of rats made systemically hypothyroid or hyperthyroid. WSB-1 mRNA levels were not significantly affected by changes in thyroid status in all brain regions studied, except for the MBH (Table 2). In this area, WSB-1 mRNA levels were decreased both in hypothyroid and hyperthyroid rats, but changes were restricted to a maximum of 50%. On the other hand, there was a general tendency for the USP-33 mRNA levels to be reduced in the brain of hypothyroid rats, and statistical significance was achieved in the pituitary gland, in which the reduction reached approximately 30% (Table 2). As a reference, D2 mRNA levels were also measured in this set of experiments, and, given that D2 regulation is mostly posttranscriptional, its mRNA levels were only slightly affected by both conditions (22–24). The pituitary gland was the only tissue in which changes in D2 mRNA levels reached statistical significance, with an approximately 3-fold increase in hypothyroid rats (Table 2).

A possible regulation of WSB-1 and USP-33 expression by thyroid hormone was also tested in Ta T1 cells, a thyrotropic cell line that expresses D2 and is known to be responsive to T3 by decreasing TSHβ mRNA levels (25). However, whereas exposing these cells to stripped serum-containing media almost doubled D2 activity, no changes in WSB-1 or USP-33 mRNA levels were detected (see Fig. 7).

Discussion

The present studies found in a large data set of murine tissues a positive correlation between the relative expression of the genes encoding two critical components of the D2-related ubiquitination machinery, WSB-1 and USP-33 (Fig. 1 and Table 1). We are certainly aware of the limitations imposed by measuring specific mRNA and not protein levels. However, in the present study, this is offset by the gain of studying large data sets otherwise unavailable for protein levels. The lack of correlation between the relative expression level of these two genes and that of other genes that are general components of the ubiquitination machinery, i.e., UBE1-C, UBC-6, and UBC-7 (Table 1), suggests a high degree of specificity in this finding, and a possible functional linkage between WSB-1 and USP-33. Although Dio2 mRNA levels did not correlate with WSB-1 or USP-33 relative expression levels, the mRNA signal of both genes were found in D2-expressing cells of the adult rat brain. WSB-1, which inactivates D2 through conjugation to ubiquitin, is expressed in tanyocytes and astrocytes, the two major D2-expressing cell types in the brain. At the same time, USP-33 is only coexpressed with D2 in the tanyocytes, indicating that in the CNS, there are alternative pathways regulating D2-mediated T3 production.

Given that WSB-1 and USP-33 have opposite roles in the conjugation of ubiquitin to proteins, the finding of a positive correlation between the basal relative expressions of both genes is fascinating. One could speculate that by keeping a balance between the expressions of both genes constitutes a limiting mechanism to how much WSB-1 ubiquitinated substrates are effectively taken up by the proteasomes. This is clearly the case with ubiquinated D2, which can be rescued from proteasomal destruction by USP-33. Notably, despite significant correlation in their relative basal expression levels (Table 1), both WSB-1 and USP-33 do have specific regulation independent from each other. For example, WSB-1, but not USP-33, is downstream of the hedgehog pathway in different cell types, whereas only USP-33, but not WSB-1, is induced by cold stimulation in brown adipocytes (13, 14, 17) (Dentice, M., and A.C.B., unpublished data). Revealing more upstream regulators of WSB-1 and /or USP-33 would shed light on key players in fine-tuning the balance between D2 ubiquitination and reactivation.

The finding that D2 is coexpressed with WSB-1 and USP-33 in tanyocytes (Figs. 4 and 5) is not unexpected, and indicates that the cycle of D2 ubiquitination-deubiquitination takes place in tanyocytes as well. Such assumption is based on what has been previously characterized in other cells and tissues. WSB-1 and USP-33 are expressed in brown adipocytes, and deubiquitination has been suggested to play a role in acute T3 activation of brown fat during cold exposure (17, 19). In the case of the chicken developing growth plate, WSB-1 mediated D2 deubiquitination increases parathyroid hormone-related peptide expression and, thus, promotes chondrocyte proliferation (13). USP-33 is also expressed in the perichondrial cells, in which D2 and WSB-1 are also coexpressed, indicating that D2 deubiquitination also plays a role in this location. Furthermore, D2 plays a critical role in TSH feedback regulation, and a murine thyrotropic cell line was shown to coexpress D2, WSB-1, and USP-33 (25). Of note, the coexpression of sonic hedgehog (Shh) signaling components (Shh, Ptc, and Smo) in the median eminence and in the ventromedial wall of the third ventricle (26, 27), the exact location of the D2 expressing tanyocytes, suggests that local Shh signaling might regulate T3 production in tanyocytes. Given that WSB-1 and USP-33 are coexpressed in all the other D2-expressing cells studied to date, the finding that USP-33 is not coexpressed with WSB-1 and D2 in astrocytes is unexpected (Fig. 6). The scattered expression of USP-33 in astrocytes indicates that D2 deubiquitination is not an active pathway in these cells. This of course is based on the assumption that no other D2-deubiquitinating activity exists in these cells. In this regard, the expression of USP-20 in the brain as well as in isolated astrocytes is well below the median (19) and, thus, would be predicted to result in a shorter T3 half-life when compared with USP-33 containing cells. In fact, D2’s reported half-life in astrocytes is the lowest reported to date, approximately 20 min in the presence of fetal bovine serum-containing media (28).

WSB-1 is induced by the hedgehog-signaling pathway, and it is notable that WSB-1 is coexpressed with D2 in brain areas influenced by Shh. In these areas, Shh acts as a mitogen on precursor cells, regulating the fate of neural stem cells (29) in areas such as the spinal cord and hippocampus (29–31). Based on the studies performed in the developing tibia growth plate of chickens, it is likely that the Shh-induced WSB-1 would accelerate D2 ubiquitination and decrease T3 production. This microenvironment of relative hypothyroidism would certainly favor the pro-proliferation effects of Shh to an extent that remains to be determined.

The high-expression levels of WSB-1 in cerebellar astro-
cytes (Fig. 6F) is notable because the cerebellum is a known target of Shh in the adult brain and expresses D2 in the granular layer (6). In the cerebellum, Shh is secreted by Purkinje cells, preventing differentiation and inducing a potent, long-lasting proliferative response of granule cell precursors (52), the most abundant type of neuron in the brain. Shh also regulates proliferation of CNS precursor cells in the hippocampus (29), and D2 is expressed in the molecular layer astrocytes (6), which we also found to express WSB-1.

The expression levels of WSB-1 and USP-33 were largely unaffected by changes in thyroid status (Table 2). In the pituitary gland, there was a significant decrease in USP-33 mRNA levels during hypothyroidism, but the significance of this change to TSH regulation is questionable, given that similar findings were not observed in Tu1 cells (Fig. 7). In addition, limited changes in WSB-1 mRNA levels were observed in the M1E of hypothyroid and hyperthyroid rats (Table 2), which are hard to interpret from a thyroid perspective alone. Perhaps this could be related to the existence of additional substrates for the WSB-1 USP-33 pair. In fact, the observation that WSB-1 and USP-33 are expressed in a number of tissues, not just those of which do not express D2, strongly suggests that WSB-1 and USP-33 have additional substrates. Alternatively, one could speculate that our finding of WSB-1 and USP-33 expression in neurons is reminiscent of D2 expression in neural progenitor cells, which decreases during progenitor differentiation (53). Further studies will determine the identity of these additional substrates, and whether such proteins are shared by WSB-1 and USP-33, such as in the case of D2.

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The E3 ubiquitin ligase TEB4 mediates the degradation of the type 2 iodothyronine deiodinase (D2)

Ann Marie Zavacki, Rafael Arrojo e Drigo, Beatriz C. G. Freitas, Mirra Chung, John W. Harney, Péter Egri, Gábor Wittmann, Csaba Fekete, Balázs Gereben, Antonio C. Bianco

A mesma busca pelo sistema de dois híbridos de levedura que levou a identificação das enzimas envolvidas na degradação da D2, WSB-1 e USP33 entre outras, identificou posteriormente a proteína TEB4, uma E3 ligase como a WSB-1. O objetivo deste trabalho é a caracterização funcional dessa enzima na degradação da D2 além do estudo da correlação de sua expressão comparada com a da WSB-1 em diferentes tecidos de camundongos. Neste trabalho participei realizando a mesma análise in silico usada no estudo anterior, obtida no site da GNF SymAtlas identificando os níveis da expressão tecidual de camundongos da WSB-1 e TEB4. Esta análise mostrou que ambas enzimas são amplamente expressas no tecido animal, sendo que a TEB4 tem uma expressão mais acentuada nos tecidos do sistema imune e testículos. Por outro lado a WSB-1 predomina no cérebro, ovário e útero. Os estudos apresentados nessa publicação demonstram que a identificação de uma nova ligase para ubiquitinação da D2 ilustra a complexidade da homeostase desta enzima que tem como principal função a ativação da tiroxina intracelular.
The E3 Ubiquitin Ligase TEB4 Mediates Degradation of Type 2 Iodothyronine Deiodinase

Ann Marie Zavacki,† Rafael Arrojo e Drigo,† Beatriz C. G. Freitas, Mirra Chung, John W. Harney, Péter Egri, Gábor Wittmann, Csaba Fekete, and Antonio C. Bianco

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The endoplasmic reticulum resident thyroid hormone-activating type 2 deiodinase (D2) is inactivated by ubiquitination via the hedgehog-inducible WSB-1. Ubiquitinated D2 can then be subsequently taken up by the proteasomal system or be reactivated by USP-33/20-mediated deubiquitination. Given that heterologously expressed D2 accumulates in Saccharomyces cerevisiae lacking the E3 ligase Doa10, we tested whether the human Doa10 ortholog, TEB4, plays a role in D2 ubiquitination and degradation. In a setting of transient coexpression in HEK-293 cells, TEB4 and D2 could be communoprecipitated, and additional TEB4 expression decreased D2 activity by ~50% (P < 0.05). A highly efficient TEB4 knockdown (>90% reduction in mRNA and protein levels) decreased D2 ubiquitination and increased D2 activity and protein levels by about fourfold. The other activating deiodinases, D1, or a truncated D2 molecule (A18-D2) that lacks a critical instability domain was not affected by TEB4 knockdown. Furthermore, TEB4 knockdown prolonged D2 activity half-life at least fourfold, even under conditions known to promote D2 ubiquitination. Neither exposure to 1 μM of the proteasomal inhibitor MG132 for 24 h nor RNA interference WSB-1 knockdown resulted in additive effects on D2 expression when combined with TEB4 knockdown. Similar results were obtained with MSTO-211 cells, which endogenously express D2, after TEB4 knockdown using a lentivirus-based transduction strategy. While TEB4 expression predominates in the hematopoietic lineage, both WSB-1 and TEB4 are coexpressed with D2 in a number of tissues and cell types, except the thyroid and brown adipose tissue, where TEB4 expression is minimal. We conclude that TEB4 interacts with and mediates loss of D2 activity, indicating that D2 ubiquitination and degradation can be tissue specific, depending on WSB-1 and TEB4 expression levels.

3,5,3'-Triiodothyronine (T3) modulates gene expression through the ligand-dependent transcription factor thyroid hormone receptor (15). Despite the presence of T3 (~20%) in the thyroid secretion, most T3 in the body is generated outside the thyroid parenchyma via deiodination of thyroxine (T4), the main secretory product of the thyroid gland (29). The type 2 iodothyronine deiodinase (D2) is the key thyroid hormone-activating deiodinase, while the type 3 deiodinase (D3) plays the opposite role, inactivating both T3 and T4 also via specific deiodinase reactions, terminating thyroid hormone action. While the D2 pathway is a major source of plasma T3, contributing about 30% in small rodents and at least double that in humans, thyroid hormones are cleared predominantly through the D3 pathway (2). Combined, the roles played by D2 and D3 provided an elegant homeostatic mechanism by which coordinated reciprocal changes in their activities ensure adaptation to iodine availability in the environment (reviewed in reference 24).

Deiodinase expression can also regulate thyroid hormone signaling on a temporal and cell-specific basis. D2 expression results in an additional intracellular source of T3 that ultimately increases the nuclear concentration of T3 and regulates the expression of T3-responsive genes. On the contrary, D3 expression decreases availability of intracellular T3, reducing T3-dependent gene expression (18). The relevance of such mechanisms is illustrated in brown adipose tissue (BAT), where a potent D2 induction increases T3 production during cold exposure (3), and in models of resistance to diet-induced obesity (21, 36). In the chicken developing growth plate, inactivation of D2 is part of the mechanism by which Indian hedgehog induces FTH1, thereby regulating chondrocyte differentiation (11).

D2 is an endoplasmic reticulum (ER) resident protein (1) that is assembled in a dimeric formation (9) and has a half-life of about 20 min, which can be further reduced by exposure to T4 (25, 31). This substrate-dependent downregulation provides a potent feedback mechanism to efficiently control T3 production (24). Ubiquitination is a key element of D2's posttranslational regulation which inactivates the enzyme and targets it for proteasomal degradation (17). While both UBC6 and UBC7 can function as ubiquitin-conjugating enzymes in D2

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ubiquitination (4, 22), the SOCS box-containing protein WS-B-1 has been established as an E3 ligase for D2, forming a ubiquitinating catalytic core complex along with elongin B, elongin C, Cul5, and Rhx1 (ECS[SWI]-1) (11). Furthermore, ubiquitinated D2 (Ub-D2) can be rescued and reactivated from proteasomal degradation by the von Hippel-Lindau protein (pVHL)-interacting deubiquitinating enzyme-1 (VDU1/USP33) (10). In addition to mediating ubiquitination of D2, WS-B-1 has recently been shown to be an E3 ligase for homeodomain protein kinase 2 (HPK2), a member of the nuclear protein kinase family, which induces both p53- and CtBP-mediated apoptosis (6).

Notably, both WS-B-1 and USP33 are physically associated with the D2 protein simultaneously, thus allowing rapid regulatory cycles of ubiquitination and deubiquitination to occur in a dynamic manner (27). WS-B-1-mediated D2 ubiquitination takes place at K237 and/or K244 after the substrate (T4 or 3',5'-triiodothyronine [T3]) binds to the enzyme active center. Given that K244 lies within the D2 dimer interface near the catalytic site, its ubiquitination and/or that of its neighbor K237 provides a putative molecular mechanism for enzyme inactivation to occur (27).

Substantial knowledge about the mechanistic details of ubiquitination was obtained by heterologously expressing human D2 in Saccharomyces cerevisiae (4). In this setting, deletion of Dna10 but not Hrd1, two E3 ligases that target proteases for ER-associated degradation, increases the accumulation of D2 protein (26). The ER-associated degradation system is involved in a number of human diseases, most notably cystic fibrosis (35), and recently has also been reported as a major player in the regulation of metabolism (28). TEB4 (MARCH-VI) is the mammalian homolog of the yeast Dna10 protein (23). This ER resident ubiquitin ligase contains 14 transmembrane helices and a conserved RING-CH finger domain at the N terminus and ubiquitates ER-associated proteins with a cytoplasmic domain in a UBC7-dependent manner (5, 23, 33), such as Mps2, Ubc6, and Stre (5, 6, 20, 26, 34). Additionally, as with other RING-finger-containing E3 ligases, TEB4 regulates its own UBC7-mediated degradation (19).

In the present work, we investigated the role of TEB4 in the proteosomal degradation of D2 in mammalian cells. We conclude that TEB4 can mediate loss of D2 activity under both basal and substrate-induced D2 ubiquitination, indicating that control of D2 activity and expression via conjugation to ubiquitin are under dual control by both WS-B-1 and TEB4. Given that most T3 in the brain is produced via the D2 pathway (16, 24), these findings could have important consequences for our understanding of the C judiciary syndrome, a neurodegenerative disorder associated with a chromosome region containing the TEB4 gene (19).

MATERIALS AND METHODS

DNA constructs. A total of 0.1 μg of a mammalian expression vector encoding wild-type rat D2 cloned into a CINa vector (G21) or human D2 cloned into a D19 vector (D19/CINa) was used for transfection in experiments that involved measurement of deiodinase activity. When measuring deiodinase protein levels, cells were transfected with 0.5 or 1 μg of D19/D19/CINa expression vectors encoding human Sol23/CINa/D2 fused to a FLAG tag at the carboxyl end (Flag D2) (10). The green fluorescent protein (GFP)-TEB4 vector was generously provided by Mark Hochstrasser (26). The WS-B-1 RNA interference (RNAi) vector and the ΔB-D2 mutant have been previously described (11). The following sequences were cloned using T4 ligase into the HindIII and BamHI site of a plasmid 21-1A neo (AmpliON, Austin, TX) to generate the TEB4 RNAi vector: RNAi 1 (up strand), 5'-GGATGGGCACGCAACTACGAT-3'; RNAi 2 (up strand), 5'-GGATCCAGATTTCATACCGA-3'.

Reagents. T4 and T3 were obtained from Sigma (St. Louis, MO) and dissolved in 40 mM NaOH. Outer-ring-labeled [3,5-3H]T4 and [3,5-3H]T3 (specific activities of ~4,400 Ci/mmol and ~7,400 Ci/mmol, respectively) were obtained from NEN Life Science Products (Boston, MA), and GH123 was obtained from Calbiochem (San Diego, CA). Anti-Flag M2 was obtained from Sigma.

Cell culture and transfections. HEK-293 cells were maintained in serum-free dishes and transfected using Lipofectamine and Plus reagents (Invitrogen, Carlsbad, CA) as described previously (13). To correct for efficiency of transfection, cells were cotransfected with 0.5 μg of vectors constitutively expressing either luciferase or β-galactosidase (11). Cells were harvested 48 h after transfection and immediately processed or stored at −80°C. MTSO-211 cells endogenously express D2 activity (30) and were used in TEB4 knockdown experiments (see below). For all TEB4 RNAi studies of the HEK-293 cell line, cells were transfected with 0.3 μg of each RNAi vector or 0.6 μg of non-specific RNAi vector.

Western blotting and IP. All cell samples used for Western blotting were sonicated in 0.5 M sucrose-phosphates-EDTA buffer (pH 7.4), and gel was stained. Gel GTP-TEB4 and 2 ng Flag-D2, treated with 1 μM MGI2 24 h prior to harvest in order to stabilize TEB4 and D2 proteins, and subsequently processed, as described previously, in a buffer containing 0.25 M Tris (pH 7.5), 0.5% Triton X-100, and 500 mM NaCl plus protease inhibitors (Roche, Indianapolis, IN). Cell tissues were incubated with goat anti-GFP (1:500, Abcam, Cambridge, MA), and complexes were pulled down using protein A-agarose beads (Oncogene Research Products, San Diego, CA). After precipitation, Flag detection was performed as described above with mouse anti-horseradish peroxidase (1:25,000; Roche, Indianapolis, IN). For ubiquitin-specific bait-based precipitation, HEK-293 cells transiently expressing Flag-D2 were transfected with 2 μg of non-luciferase RNAi vector or 0.6 μg of TEB4 RNAi vectors 1 and 2, as described above, and subsequently processed using the ubiquitinated protein enrichment kit (Calbiochem, CA) following the manufacturer’s recommendations. After precipitation, samples were Western blotted, and detection of Flag-D2 was performed as described above.

Dual domains assays. D1 and D2 assays were performed with cell stimulation as described previously (9) using 500 nM T3 as the substrate for D1 and 0.1 M T4 as the substrate for D2.

Lentivirus production and transfection of MTSO-211 cells. Lentiviruses generating an RNAi vector targetted against either TEB4 or a non-specific sequence were produced using the Expression Vector (GFP, lentiviral system (Open Bio- systems, Huntsville, AL), and the V2L.HS 12421 and V2L.HS 192026, clones, respectively. Lentiviruses were produced following the manufacturer’s recommendations, following transfection HEK-293 cells with 0.5 μg of the pGIPZ vector of interest along with 2.5 μg of pMD.2.G(enh), 2.5 μg pVSV.G, 2.5 μg pCMV.RsRed, and 2.5 μg pGIPZ-puro G418 via “10-cm” dishes. Forty-eight hours after transfection, virus was harvested and stored at −70°C and then titrated in U2OS cells. MTSO-211 cells were infected at a multiplicity of infection of 1:200 ng/ml of Polybrene (Invitrogen). Forty-eight hours after infection, cells were selected using puromycin at 0.5 μg/ml for 2 weeks prior to use in experiments. Photos of GFP-expressing cells were taken using an Olympus CKX-41 microscope, using QCapture Pro software (Media Cybernetics, Inc., Bethesda, MD).

Real-time qPCR. Total RNA was extracted from HEK-293 or MTSO-211 cells, using Trizol, following the manufacturer’s recommendations (Invitrogen). Real-time quantitative PCR (qPCR) was performed as described previously (7) using a five point standard curve and the following primers for TEB4, sense, 5' TCG TCTCCTCAAGTGCCGAC-3', antisense, 5' GATCCTGTGGAGATGTTCTCGA-3'; for cyclophilin A, sense, 5' GGC AACAATCTGAGGAAAC-3', and antisense, 5' GGC TACAGGCTGACACAG-3'; for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), sense, 5' GACGAGTTCACTCCATATCATAGTAC-3', and antisense, 5' ACAAGACTCCAGGGCTTCTT-3'; for cyclophilin A, sense,
and H2O, for 15 min and then incubated with 1% bovine serum albumin in phosphate-buffered saline (PBS) for 20 min. Sections were then incubated with a mouse GAD monoclonal anti-GFAP (1:50; Roche Molecular Biochemicals GmbH, Vienna, Austria) dilution in 1% bovine serum albumin containing PBS overnight at 4°C. Slides were washed with PBS and then incubated in donkey anti-mouse immunoglobulin G (1:500 Jacksonville Immunoresearch Laboratories, Inc., West Grove, PA) for 2 h and ABC Elite (1:1000; Vector Laboratories, Burlingame, CA) for 1 h. Immunoreactivity was detected with 0.025% 3,3’-diaminobenzidine containing 0.0036% H2O2 in 0.05 M Tris buffer (pH 7.6). After several washes in PBS, the sections were dehydrated, dipped in emulsion, and exposed as described above.

Statistical analysis. Statistical significance was determined by using an unpaired Student t test when two groups were compared or one-way analysis of variance (ANOVA) with a Newman-Keuls post hoc test when more than two groups were compared, using Prism 3.0 (GraphPad Software, San Diego, CA). In all figures, data shown are means ± the standard error of the mean.

RESULTS

TEB4 interacts with D2 and mediates loss of D2 activity and protein. To assess the potential interaction between TEB4 and D2, we used a system in which HEK-293 cells transiently coexpress a GFP-tagged TEB4 (GFP-TEB4) and a Flag-tagged D2 (Flag-D2). In this heterologous system, D2 activity is equivalent to that found in cells that endogenously express this enzyme, and Flag-D2 has been previously shown to display ER distribution and other properties shared by endogenously expressed D2 (27). After harvesting, cells were processed for IP with anti-GFP to pull down TEB4. Subsequent Western blot analysis of the IP pellets with anti-Flag revealed Flag-D2, indicating that both proteins are associated together within the cell (Fig. 1A). Next, their functional relationship was tested by increasing the amount of coexpressed GFP-TEB4. This resulted in a loss of approximately 50% of Flag-D2 activity, and Western blot analysis indicated a progressive loss of Flag-D2 protein that reached 35% of control values (Fig. 1B).

TEB4 knockdown increases D2 activity and protein levels. To further investigate the role of TEB4 in D2 expression, we generated two vectors that produce two distinct RNAi vectors targeted against TEB4 (TEB4 RNAi vector 1 and TEB4 RNAi vector 2). Cotransfection of GFP-TEB4 and either TEB4 RNAi vector 1 or 2 in HEK-293 cells markedly decreased GFP-TEB4 protein levels, while cotransfection of both RNAi vectors together decreased GFP-TEB4 protein by >90% (Fig. 2A). In subsequent experiments, real-time qPCR also indicated that endogenous TEB4 mRNA levels were decreased to 63% of control levels when both TEB4 RNAi 1 and 2 vectors were cotransfected (Fig. 2A). The less-efficient knockdown of TEB4 mRNA versus TEB4 protein presumably reflects the transfection efficiency in these cells, which is typically about 50%.

Cotransfection of D2 with either RNAi vector alone increased D2 activity by up to 50% in a dose-dependent manner (see Fig. S1A in the supplemental material), while TEB4 knockdown using both RNAi vectors together increased D2 activity.
FIG. 2. TEB4 knockdown and D2 expression. (A) Validation of RNAi constructs targeting TEB4 mRNA. HEK-293 cells transiently expressing GFP-TEB4 and either the TEB4 RNAi vector(s) or a nonspecific RNAi vector as indicated, along with a β-galactosidase expression vector, were treated with 1 μM MG132 for 24 h prior to harvesting to stabilize and increase TEB4 protein levels. GFP-TEB4 levels were detected by Western blotting with anti-GFP, with loading of cell lysates normalized to β-galactosidase levels. For real-time qPCR, endogenous levels of TEB4 in HEK-293 cells were measured in the presence of either both TEB4 RNAi vectors or a nonspecific RNAi vector as indicated. TEB4 mRNA levels were normalized by cyclophilin A (cyclo A) levels. (B) Effects of TEB4 knockdown in D2 protein levels and activity. HEK-293 cells transiently expressing Flag-D2 and the indicated RNAi vectors were Western blotted as described in the legend for Fig. 1B. For D2 enzyme activity, HEK-293 cells transiently expressing D2 and increasing equimolar amounts of TEB4 RNAi vectors or a nonspecific RNAi vector, as indicated, were used. Enzyme activity was normalized to β-galactosidase levels. (C) TEB4 knockdown affects D2 ubiquitination. Sonicates of HEK-293 cells transiently expressing Flag-D2 were processed using the ubiquitin enrichment kit, and the resulting pellet was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with anti-Flag (C1). Negative controls are indicated. Ub-D2 bands (>250 kDa) are indicated and present only in cells expressing D2. In a separate experiment (C2), high-molecular-mass Ub-D2 bands were visualized with decreased intensity during TEB4 knockdown. The effect of TEB4 knockdown on D2 protein levels is shown in panel C3. (D) TEB4 knockdown specifically affects D2. For enzyme activity, HEK-293 cells transiently expressing both D2 and D1 with or without nonspecific RNAi or TEB4 RNAi vectors, as indicated, and a β-galactosidase vector were assayed as described previously (9), and enzyme activity was normalized to β-galactosidase levels. Deiodinase activity is expressed relative to nonspecific RNAI levels. Values are mean ± SEM; n = 3. ** indicates P < 0.001, # indicates P < 0.05, and NS indicates nonsignificant by ANOVA. The results shown are those from typical experiments that were repeated at least once.
**FIG. 3. TEB4 knockdown interferes with D2 proteasomal degradation.** (A) Loss of substrate-induced D2 degradation with TEB knockdown. HEK-293 cells transiently expressing D2 with or without TEB4 RNAi vectors and a β-galactosidase vector were treated with 100 nM T4 or 100 μM cycloheximide for 2 h prior to harvest, as indicated. Deiodinase activity was measured as described in the legend for Fig. 2B, with activity levels normalized to β-galactosidase levels. (B) HEK-293 cells transiently expressing D2 with or without TEB4 RNAi vectors and a β-galactosidase vector were treated with 1 μM MG132 24 h prior to harvest, as indicated, and deiodinase activity was measured as described in the legend for Fig. 2B, with activity levels normalized to β-galactosidase levels. (C) D2 activity in the absence of TEB4 and/or WSB-1. HEK-293 cells transiently expressing D2 with or without TEB4/WSB-1 RNAi vectors and a β-galactosidase vector were treated with 100 nM T4 or 100 μM cycloheximide for 2 h prior to harvest, as indicated. Deiodinase activity was measured as described in the legend for Fig. 2B, with activity levels normalized to β-galactosidase levels. (D) Relative enzyme activity of HEK-293 cells transiently expressing D2 with or without TEB4 RNAi vectors, and a β-galactosidase vector were treated with 1 μM MG132 24 h prior to harvest, as indicated, and deiodinase activity was measured as described in the legend for Fig. 2B, with activity levels normalized to β-galactosidase levels. (E) D3 activity in the absence of TEB4 and/or WSB-1. HEK-293 cells transiently expressing D3 with or without TEB4 RNAi vectors and a β-galactosidase vector were treated with 100 nM T4 or 100 μM cycloheximide for 2 h prior to harvest, as indicated. Deiodinase activity was measured as described in the legend for Fig. 2B, with activity levels normalized to β-galactosidase levels. (F) D3 activity in the absence of TEB4 and/or WSB-1. HEK-293 cells transiently expressing D3 with or without TEB4 RNAi vectors and a β-galactosidase vector were treated with 1 μM MG132 24 h prior to harvest, as indicated, and deiodinase activity was measured as described in the legend for Fig. 2B, with activity levels normalized to β-galactosidase levels. (G) D3 activity in the absence of TEB4 and/or WSB-1. HEK-293 cells transiently expressing D3 with or without TEB4 RNAi vectors and a β-galactosidase vector were treated with 100 nM T4 or 100 μM cycloheximide for 2 h prior to harvest, as indicated. Deiodinase activity was measured as described in the legend for Fig. 2B, with activity levels normalized to β-galactosidase levels.

**D2 ubiquitination by TEB4**

A combined IP-Western blot analysis using ubiquitin-binding beads and anti-Flag (identified Ub-D2 as higher molecular mass bands, as previously reported (17)). Notably, TEB4 knockdown substantially reduced the intensity of these Ub-D2 bands (Fig. 2C) while increasing Flag-D2 protein levels (Fig. 2C, bottom panel). Next, to test the specificity of the D2-TEB4 interaction, we knocked down TEB4 in cells transiently expressing both the type 1 deiodinase (D1), which is not subject to degradation by the ubiquitin-proteasomal system, and D2 (17). While D1 activity remained unaffected in these cells, D2 activity was increased 2.5-fold (Fig. 2C).

**TEB4 knockdown interferes with D2 proteasomal degradation.** We next evaluated the role of TEB4 in the proteasomal degradation of D2. Previously, we have shown that D2 interaction with its natural substrate, T4, promotes conformational changes in the D2 molecule that favors D2 ubiquitination and proteasomal degradation, resulting in loss of D2 activity (27). Accordingly, treatment with either cycloheximide or T4 for 2 h decreased D2 activity by 25% (Fig. 3A). However, with TEB4 knockdown, D2 activity almost doubled and was not affected by either of these treatments (Fig. 3A). Additionally, we have shown that treatment with the proteasomal inhibitor MG132 blocks D2 degradation, leading to increased D2 activity (30). Thus, MG132 treatment alone increased D2 activity by about fivefold (Fig. 3B). At the same time, while TEB4 knockdown increased D2 activity by about threefold, a combination of both MG132 and TEB4 knockdown elevated D2 activity only to the same level as that seen with MG132 treatment alone (Fig. 3B).

It has been previously established that WSB-1 is an E3 ligase adaptor that is involved in both the ubiquitination and subsequent proteasomal degradation of D2 (11). When WSB-1 is knocked down, D2 activity is increased 1.5-fold, while with TEB4 knockdown, D2 activity is increased twofold (Fig. 3C). Interestingly, knockdown of both TEB4 and WSB-1 elevates D2 to levels that are comparable to those seen with TEB4 knockdown alone (Fig. 3C). A destabilization loop of 18 amino acids, from position 92 to 109 in D2, is necessary for WSB-1-mediated ubiquitination of D2 (11, 38). To test if this loop is involved in TEB4-mediated D2 ubiquitination, we transiently expressed the Δ18-D2 mutant, in which the destabilization loop had been deleted, in HEK-293 cells with TEB4 knockdown. While D2 activity increased about 1.5-fold, the D2 mutant (Δ18-D2) remained unaffected (Fig. 3D).

**TEB4 knockdown increases endogenous D2 activity.** The effects of TEB4 knockdown were also assessed in the mesothelioma cell line MSTO-211 that endogenously expresses D2 (8), without TEB4/WSB-1 RNAi vectors as indicated were assayed for D2 activity as described in the legend for Fig. 2B. Enzyme activity levels were normalized to β-galactosidase levels. (D) TEB4 knockdown does not affect a mutant D2 lacking the instability loop. HEK-293 cells transiently expressing either D2 or Δ18-D2 with or without TEB4 RNAi vectors, as indicated, were assayed for D2 activity as described in the legend for Fig. 2B. Enzyme activity was normalized to nonspecific RNA levels. Values are mean ± SEM; n = 3, * indicates P < 0.01, # indicates P < 0.05, and NS indicates nonsignificant by ANOVA (A to O) or unpaired Student’s t test (D). The results shown are those from typical experiments that were repeated at least once.
FIG. 4. TEB4 knockdown increases endogenous D2 activity. (A) HEK-293 cells transiently expressing GFP-TEB4 and either the lentiviral TEB4 RNAi vector or the lentiviral nonspecific RNAi vector (lentivector), as indicated, were Western blotted for GFP-TEB4 levels, as described in the legend for Fig. 2A. (B) Photos of MSTO-211 cells transfected with either the TEB4 or nonspecific RNAi encoding lentivirus. GFP marker expression in the lentivirus-infected MSTO-211 cells with either the nonspecific lentiviral RNAi vector (left panels) or TEB4 RNAi vector (right panels) after 2 weeks of puromycin selection is shown. Phase contrast images are shown in the bottom row to illustrate the total number of cells. Images were taken with 400 magnification. (C) Decreased endogenous TEB4 mRNA levels in MSTO-211 cells. TEB4 mRNA levels of the cells shown in panel B were determined by real-time qPCR, as described in the legend for Fig. 2A, and normalized to cyclophilin A levels. (D) D2 activity in MSTO-211 cells ± lentiviral TEB4 knockdown was measured as described in reference 9. Values are mean ± SEM, n = 3. ** indicates P < 0.001, and # indicates P < 0.05 by ANOVA. The results shown are those from typical experiments that were repeated at least once.

Here, we used a lentivirus-mediated transduction strategy to knock down TEB4 expression. Initially, a vector that encodes sequences to generate an RNAi targeting either TEB4 or a nonspecific control sequence, along with genes conferring puromycin resistance and a GFP marker, flanked by lentiviral long-terminal-repeat sequences was obtained from Open Biosystems. To validate the efficacy of the RNAi targeted against TEB4, HEK-293 cells transiently expressing GFP-TEB4 were also transfected with either the vector producing an RNAi against TEB4 or a nonspecific control sequence, as shown in Fig. 2A. Western blotting indicated that GFP-TEB4 expression was reduced to undetectable levels compared to cells transfected with the nonspecific control, confirming the effectiveness of this vector (Fig. 4A). We first transfected MSTO-211 cells with the RNAi-generating vector, and because the transfection efficiency is low, GFP-positive cells were sorted by flow cytometry, and D2 activity was measured. Under these conditions, TEB4 knockdown increased endogenous D2 activity by about threefold (see Fig. S1B in the supplemental material). Second, lentivirus was produced and used to infect MSTO-211 cells, which were subsequently selected with puromycin. After 2 weeks of treatment, it appeared that a majority of the MSTO-211 cells were expressing the GFP marker and consequently also the RNAi of interest (Fig. 4B). Real-time qPCR confirmed that TEB4 mRNA levels were decreased by 75% in the MSTO-211 cells infected with the TEB4 RNAi-producing virus (Fig. 4C). Remarkably, D2 activity was doubled in the same MSTO-211 cells (Fig. 4D).

WSB-1 and TEB4 have distinct patterns of tissue expression. In order to establish the pattern of tissue expression of TEB4 and WSB-1, RNA was extracted from a variety of rat tissues, and the relative amounts of TEB4 and WSB-1 were determined using semiquantitative reverse transcriptase PCR (Fig. 5). Most of the tissues examined expressed TEB4, whereas liver had substantially lower levels of TEB4 mRNA, and the D2-expressing brown adipose tissue (BAT) had almost undetectable levels of TEB4 mRNA. WSB-1 mRNA is also well expressed in most tissues, with somewhat smaller amounts being found in kidney. Unlike TEB4, WSB-1 is well expressed in BAT, and neither WSB-1 nor TEB4 is expressed in the rat thyroid.

Expression of both WSB-1 and TEB4 was found in the rat brain, not only in neurons but also in glia and astrocytes, the two cell types that predominantly express D2 in the brain (13). Thus, we used in situ hybridization to study TEB4 expression in
FIG. 6. TEB4 mRNA distribution in the brain. Dark-field images of TEB4 mRNA distribution in the ependymal cells lining the wall of the third ventricle (3V) in the mediobasal hypothalamus (A), hippocampus (B), cerebellum (C), and cerebral cortex (D) of rats. Arrows indicate TEB4 hybridization signals detected over ependymal cells lining the floor and wall of the third ventricle (A). In the hippocampus (B), a very strong hybridization signal was present over the pyramidal layer (CA1 to CA3) and the granular layer of the dentate gyrus (GrDG). A very intense hybridization signal was also observed over the granular layer of the cerebellum (Gr) (C). TEB4 hybridization signal was detected in the second to sixth layers of the cerebral cortex (D). Scale bars for panels A, B, and D represent 500 μm, and the scale bar for panel C represents 1,000 μm.

the rat brain and found its mRNA to be expressed in most brain regions, including the hypothalamus, hippocampus, cerebellum, and cortex (Fig. 6). Intense TEB4 signal was observed in the floor and lateral wall of the third ventricle, between the rostral pole of the median eminence and the mammillary recess, and in the ependymal cells lining the wall of the third ventricle, which is reminiscent of that of third ventricular tanyocytes (Fig. 6A). In control slides prepared in parallel, hybridization with the sense probe resulted in the complete absence of signal (data not shown). Using GFAP staining as a marker for astrocytes, TEB4 and GFAP signal overlapped only rarely in the cortex and hippocampus, suggesting that in these regions, TEB4 is primarily expressed by neurons. In the stratum granulosum of the cerebellum, TEB4 was found in both neurons and GFAP-positive astrocytes (Fig. 7). In contrast to the rather ubiquitous WSB-1 expression in astrocytes (13), TEB4 expression in astrocytes exhibited a region-specific pattern, with only a few scattered GFAP-positive astrocytes containing TEB4 mRNA in the cortex and hippocampus, which is in contrast to the pattern found in the cerebellum (Fig. 7).

Given this diverse expression pattern of TEB4 in the rat tissues, we next studied the relative expression of WSB-1 and TEB4 in human tissues in silico, using data from the SymAtlas website (http://symatlas.gnf.org/SymAtlas/) (32). It is clear that TEB4 expression predominates over WSB-1 expression in the immune system (cells and tissues), whereas WSB-1 expression predominates in the central nervous system, with the exception of pons, globus pallidus, amygdala, and cerebellum (see Fig. S2 in the supplemental material). In the other tissues, the relative expression of WSB-1 and TEB4 is variable, without clear tissue-specific predominance.

DISCUSSION
The present investigation provides evidence that D2 and the ER resident E3 ligase TEB4 interact within the cell. First,
these two proteins were communoprecipitated (Fig. 1A), suggesting that they either share common interacting surfaces or are part of a larger multiprotein complex. Second, their functional relationship is evident by the fact that increased TE84 expression reduces D2 activity and protein levels in a dose-dependent manner (Fig. 1B), while TE84 knockdown increases D2 activity and protein levels by more than 50% (Fig. 2B), while reducing Ub-D2 levels (Fig. 2C). These effects are also observed in cells endogenously expressing D2 (Fig. 2D).

Third, the T4-induced ubiquitination and subsequent loss of D2 activity are greatly reduced in cells with TE84 knockdown, illustrating that this E3 ligase mediates substrate-induced D2 proteolysis (Fig. 3A). These effects seem to be highly D2 specific, given that D1, the other thyroid hormone-activating deiodinase (17), is not affected by TE84 knockdown, even when coexpressed with D2 in the same cell (Fig. 2D). Taken together, these results indicate that TE84 mediates the posttranslational degradation of D2 in cells that coexpress both proteins at endogenous levels.

In view of the well-established role played by WSB-1 as an E3 ligase adaptor for D2 (11), the current findings that TE84 can also mediate D2 degradation illustrates that a critical step in thyroid hormone activation can be regulated by at least two ubiquitin ligases. A similar situation is found with Eps15, a protein that regulates EGF receptor internalization, the ubiquitination of which is mediated by the HECT domain E3 ligase Nedd4 and parkin, a RING finger-containing E3 ligase (12, 37). While we currently do not understand if or how these two pathways interact to regulate D2, it is puzzling that no additional protective effect on D2 activity was noted when both WSB-1 and TE84 were knocked down at the same time (Fig. 2C). This suggests that both pathways are not necessarily exclusive and in fact could even contribute to D2 ubiquitination in a sequential fashion. While both WSB-1 and TE84 function in association with UBC7 (11, 19), these ligases differ in their structures and substrate-processing mechanisms. WSB-1 is a small sonic hedgehog-inducible WD40-SOCS box-containing protein that is part of the LC3WSB-1 complex and remains associated with the ER membrane (11). Ubiquitination of D2 via this pathway results in an inactive D2 that remains fully assembled in the ER membrane and can be easily reactivated by USP33/20 deubiquitinses (10, 27). On the other hand, TE84 is a large CH4C RING finger-containing protein that spans the ER membrane multiple times (19, 23) and is thought to associate with Sec61α and β-translocation units and mediate retrotranslocation of multiple ubiquitinated ER proteins to the proteasome complex (19). Thus, given the decrease in Ub-D2 and accumulation of D2 protein during TE84 knockdown (Fig. 2C), it is likely that ubiquitination of D2 through this pathway is followed by terminal protein disassembly.

Interaction of D2 with its natural substrate, T4, is thought to promote conformational changes that expose critical lysine residues (K237 and K244) in D2 that then can undergo ubiquitination (27). This is lost in Δ18-2D, a mutant D2 lacking an 18-amino-acid loop required for interaction with WSB-1 (11). A similar mechanism is likely to take place with TE84, given that substrate-induced loss in D2 activity and processing of the Δ18-2D molecule are both greatly minimized in cells with TE84 knockdown (Fig. 3A and D). It is remarkable that despite two very different pathways for D2 ubiquitination, the intrinsic mechanisms regulating its conjugation to ubiquitin still seem to be the same in both ubiquitin ligases, WSB-1 and TE84, to process D2 would gain additional relevance if these proteins had different expression patterns and/or regulation. However, based on data publicly available (http://synatlas.gaf.org/SynAtlas; see also Fig. 5A in the supplemental material) and our own reverse transcriptase PCR studies (Fig. 5), it is clear that both WSB-1 and TE84 are widely expressed in human and rodent tissues. At the same time, quantitative analyses reveal that TE84 predominates in cells of the immune system, testes, and some splanchic tissues, whereas WSB-1 predominates in the brain, ovary, and uterus (see Fig. S2B in the supplemental material). Of particular interest is the isolated expression of WSB-1 in BA1, which normally expresses high levels of D2 (Fig. 5). In the brain, in situ hybridization studies revealed that TE84, similar to WSB-1, is expressed in tanyctyes, while astrocytes in the hippocampus and cortex express WSB1 but not TE84 (Fig. 1 and 7) (13).

In conclusion, our studies have linked TE84 to D2 ubiquitination and posttranslational degradation, suggesting that it may be a key regulator of thyroid hormone activation in a number of tissues, including those of the brain. Given the recognized importance of thyroid hormone during brain development, the presence of TE84 in a chromosomal locus linked to the Cri-du-chat syndrome (19) raises the possibility that patients with this genetic disorder might have altered D2 regulation and thyroid hormone signaling during critical periods of brain development.

ACKNOWLEDGMENTS

We thank Mark Hochstrasser for providing the GFP-TE84 vector. This work was supported by NIDDK grants DK58558 and DR076113, Hungarian Scientific Research Fund Grant OTKA T049081, the János Bolyai Research Scholarship of the Hungarian Academy of Sciences, and the 6th EU Research Framework Program LSHM-CT-2005-513041.

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A TRbeta-selective agonist confers resistance to diet-induced obesity high-fat diet-induced obesity in rats


Durante minha iniciação científica na Universidade Presbiteriana Mackenzie participei de um projeto visando o estudo do agonista seletivo para TRβ, o GC-24 sob orientação da professora Miriam O. Ribeiro. Este trabalho foi seguido após o termo de minha graduação por sua aluna Beatriz Amorim. Durante minha pós graduação colaborei com a professora Miriam ainda nesse projeto, realizando as análises gênicas dos tecidos animais submetidos a dieta hipercalórica e tratamento com T₃ ou GC-24. O objetivo do trabalho é a possível utilização isolada do receptor do hormônio tiroidiano β através de um agonista específico para obtenção dos resultados benéficos da administração de do hormônio em uma dieta hipercalórica. O trabalho demonstrou que a administração crônica deste composto diminui os efeitos de uma dieta hipercalórica, como seu efeito na massa gorda, intolerância a glicose e hipertrigliceridemia. O tecido que apresentou maior diferença na expressão gênica em resposta a esse tratamento foi o tecido adiposo marrom, provavelmente o maior responsável pelo aumento no gasto energético desses animais. Infelizmente esse composto não solucionou o problema gerado pelo nível elevado do colesterol nos animais sob dieta, ilustrando a ineficácia desse composto para utilização neste fim.
A TRβ-selective agonist confers resistance to diet-induced obesity

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Abstract

Thyroid hormone receptor β (TRβ) also listed as THRβ on the MGI Database-selective agonists activate brown adipose tissue (BAT) thermogenesis, while only minimally affecting cardiac activity or lean body mass. Here, we tested the hypothesis that daily administration of the TRβ agonist GC-24 prevents the metabolic alterations associated with a hypercaloric diet. Rats were placed on a high-fat diet and after a month exhibited increased body weight (BW) and adiposity, fasting hyperglycemia and glucose intolerance, increased plasma levels of triglycerides, cholesterol, nonesterified fatty acids and interleukin-6. While GC-24 administration to these animals did not affect food ingestion or modified the progression of BW gain, it did increase energy expenditure, eliminating the increase in adiposity without causing cardiac hypertrophy. Fasting hyperglycemia remained unchanged, but treatment with GC-24 improved glucose tolerance by increasing insulin sensitivity, and also normalized plasma triglyceride levels. Plasma cholesterol levels were only partially normalized and liver cholesterol content remained high in the GC-24-treated animals. Gene expression in liver, skeletal muscle, and white adipose tissue was only minimally affected by treatment with GC-24, with the main target being BAT. In conclusion, during high-fat feeding treatment with the TRβ-selective agonist, GC-24 only partially improves metabolic control probably as a result of accelerating the resting metabolic rate.


Introduction

Thyroid hormone is a highly metabolic molecule. When given to animals and humans, it rapidly increases energy expenditure (Klitgaard et al. 1952), while lowering serum cholesterol (Hansson et al. 1983) and triglycerides (Abrahams et al. 1981) levels. However, side effects resulting from the pleomorphic actions of thyroid hormone, such as cardiac arrhythmia (Klein & Ojamaa 2001), bone loss (Ross 1994, Murphy & Williams 2004, Galliford et al. 2005), nervousness, and anxiety (Placidi et al. 1998), to name a few, prevent it from widespread clinical use. Ideally, one would want to harness the beneficial metabolic effects of thyroid hormone mediated at the liver, adipose tissue, and skeletal muscle, while sparing the myocardium, bone, brain, and other tissues.

Our current understanding of thyroid hormone action allows for the development of such strategies. Tri-iodothyronine (T3) effects are mediated by thyroid hormone receptors (TRs), which are ligand-dependent transcription factors that regulate the expression of different sets of genes involved not only in metabolic control, but also in development and growth (Yen 2001). The fact that the TR encoding genes are differentially expressed in various tissues indicates that the T3 effects can be TR isoform specific. Also, studies in patients with syndrome of resistance to T3, and studies in mice with targeted disruption of TRα1, TRβ (listed as THRα and THRβ on the MGI Database) or both, have illustrated selective functions of TRs, and some actions that are preferentially triggered by a specific TR isoform (Hsu & Brent 1998, Brent 2000, Bassett et al. 2007). As an example, studies using knockout and knockin mouse models have shown that TRβ is involved in mediating the T3 effects on liver metabolism such as reducing plasma cholesterol and triglycerides (Sidow et al. 2003, Fugier et al. 2006, Shin et al. 2006).

Thus, it makes perfect logic to use thyroid hormone analogs capable of tissue specificity either by selective
uptake and/or by selective binding to the two TR isoforms (Chellini et al. 1998, Ocasio & Scanlan 2006). Activation of TRβ1 with a selective thyroid hormone analog (GC-1 compound) in rats results in the induction of UCP1 gene, while only minimally mediating synergism between thyroid hormone and the sympathetic nervous system (Bilbeiro et al. 2001). In fact, the use of GC-1 or other TRβ-selective agonists in rodents and primates has recently been shown to increase energy expenditure, decrease fat mass and plasma levels of cholesterol (Grover et al. 2004), while sparing the heart (Trost et al. 2000) and the skeletal system (Freitas et al. 2003). Also, in the db/db mouse model, the administration of different TRβ agonists improves glucose homeostasis (Bryzgalo et al. 2008). It is still not clear, however, how much of the effects of these molecules is due to TRα selectivity as opposed to liver-specific uptake (Trost et al. 2000).

In the present study, we tested the hypothesis that chronic TRβ activation minimizes the metabolic consequences of feeding a high-fat diet. To such end, we used GC-24, a second generation TRβ-selective molecule, which binds to TRβ1 with 40-fold higher affinity than TRα (Borgmøse et al. 2003, Miyabara et al. 2005). Our results indicate that the use of GC-24 prevents much of the metabolic alterations associated with feeding a high-fat diet, while not affecting the overall body and cardiac weights. Notably, changes in gene expression triggered by GC-24 were primarily detected in the brown adipose tissue (BAT), while only minimally affecting liver, skeletal muscle, and white adipose tissue.

Materials and Methods

Drugs and reagents

All drugs and reagents were purchased from Sigma Chemical Co, unless otherwise specified. GC-24 was kindly provided by Dr Thomas Scanlan.

Animals and treatments

Male Wistar rats weighing 150–200 g were purchased from University of Sao Paulo Medical School (FMUSP, Sao Paulo, Brazil) and maintained on a 12 h light:12 h darkness cycle at 25 °C with food and water ad libitum.

In one set of experiments, animals were fed either chow (~1.8 kcal/g) or high-fat diet (~4.5 kcal/g), consisting of 42% carbohydrate, 24% proteins, and 23% fat. After 10 days on the high-fat diet, the animals were started on a treatment with T3 (30 ng/g body weight (BW) per day) or equimolar doses of GC-24 (17 ng/g BW per day) for 3 weeks. Administration was via daily i.p. injections. At the end of the experimental period, animals were studied for resting metabolic rate (RMR), glucose tolerance test (GTT), and insulin tolerance test (ITT). Animals were subsequently killed by exsanguinations under urethane anesthesia, and blood processed for plasma isolation and tissues samples were collected and immediately frozen for further analyses. Carcasses were frozen at −80 °C for further processing.

On a second set of experiments, animals fed chow diet were treated with T3 (15 ng/g BW per day) or equimolar doses of GC-24 (8.5 ng/g BW per day) for 45 days. Administration was via daily i.p. or s.c. injections. At the end of experimental period, animals were killed by exsanguinations under urethane anesthesia, and blood processed for plasma cholesterol determination.

Oxygen consumption

RMR was estimated by measuring oxygen consumption (VO2) in an open circuit respirometer system (O2-10, Sable System, Las Vegas, NV, USA) as previously described (Withren 1977, Cucio et al. 1999). The experiments were carried out over a period of 30 min in the afternoon (1400–1800 h) at room temperature (25 °C) in animals fed ad libitum. Animals were maintained in their normal experimental conditions until immediately before the measurements. The data were collected and analyzed by the Sable Systems software. The results are expressed as milliliters of O2/min per g BW.

Glucose tolerance test

Animals were fasted overnight, and glucose (2 g/kg) was administered by i.p. injection between 0900 and 1000 h. Blood samples were collected from the tail at various times after the glucose load, as indicated, and glycemia was immediately determined on a glucose analyzer (LifeScan, Inc., Milpitas, CA, USA).

Insulin tolerance test

Food was removed 6 h before the experiment, which was carried out between 1400 and 1500 h. Blood samples were collected from the tail at various times after insulin (0.5 U/kg) was administered by i.p. injection, as indicated, and glucose serum levels determined immediately using a glucose analyzer.

Blood chemistry

Plasma cholesterol and triglycerides were assessed by colorimetric method using a commercial kit (Roche Molecular Biochemicals). Plasma NEFA was assessed by colorimetric method using a commercial kit (WACO NEFA C kit, Wako Chemical Industries USA, Inc., Richmond, VA, USA). The plasma concentrations of tumour necrosis factor α (TNFα) and interleukin-6 (IL6) were measured by commercial ELISA kits (TNFα, IL-6, R&D System, IL-6, R&D System,
Belgium), according to the manufacturer’s instructions (Yu et al. 2007). The protein concentration was determined by the method of Bradford (1976).

Liver chemistry

Lipids were extracted from the liver by disrupting ~200 mg frozen liver samples in 2 ml isopropanol alcohol with a Potter Elvehjem homogenizer (model MA 199; Marconi, Piracicaba, SP, Brazil). Homogenates were maintained at 37°C for 30 min and then at 4°C overnight. Total cholesterol and triacylglycerols were determined by enzymatic methods (Roche Diagnostics) in a supernatant aliquot. Protein concentration was determined according to the method of Lowry et al. (1951) in liver samples (200 mg) previously homogenized in 4 ml of water.

mRNA analysis

Total RNA of liver, epididymal white fat and interscapular BAT was extracted using the TriZol (Life Technologies Inc.), according to the manufacturer’s instructions, and quantified by spectrophotometry. For the reverse transcriptase reaction, 0.8 μg of total RNA was used in the SuperScript First-Strand.

Table 1 Effects of tri-iodothyronine (T3) (10X) or equimolar doses of GC-24 in animals placed on a high-fat diet. Values indicated as mean ± E.M.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HF</th>
<th>HF+T3</th>
<th>HF+GC-24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart (mg/g)</td>
<td>3 ± 0.1</td>
<td>3 ± 0.2</td>
<td>4 ± 0.16*</td>
<td>3 ± 0.1</td>
</tr>
<tr>
<td>Fasting glucose (mg/dl)</td>
<td>65 ± 7.8</td>
<td>64 ± 2.8</td>
<td>50 ± 3.8</td>
<td>97 ± 7.9</td>
</tr>
<tr>
<td>TC (mg/dl)</td>
<td>67 ± 6</td>
<td>62 ± 5</td>
<td>60 ± 15*</td>
<td>70 ± 6.9</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>83 ± 8</td>
<td>125 ± 21*</td>
<td>65 ± 5.6</td>
<td>99 ± 7.5</td>
</tr>
<tr>
<td>NEFA (mg/dl)</td>
<td>6 ± 0.3</td>
<td>12 ± 0.5*</td>
<td>14 ± 0.3*</td>
<td>14 ± 1.6</td>
</tr>
<tr>
<td>IL-6</td>
<td>44 ± 3.6</td>
<td>61 ± 3.8*</td>
<td>47 ± 3.1*</td>
<td>59 ± 2.7*</td>
</tr>
<tr>
<td>TNFα</td>
<td>2 ± 1.1</td>
<td>10 ± 0.5*</td>
<td>9 ± 0.8*</td>
<td>10 ± 0.9*</td>
</tr>
<tr>
<td>Liver cholesterol (mg/mg)</td>
<td>1 ± 0.2</td>
<td>2 ± 0.7*</td>
<td>1 ± 0.3*</td>
<td>2 ± 0.5</td>
</tr>
<tr>
<td>T3 (mg/dl)</td>
<td>62 ± 7.8</td>
<td>62 ± 2.6</td>
<td>52 ± 1.9</td>
<td>66 ± 11.9</td>
</tr>
<tr>
<td>T4 (μg/L)</td>
<td>3 ± 0.4</td>
<td>4 ± 1.9</td>
<td>&lt;1</td>
<td>&gt;31 ± 0.7</td>
</tr>
</tbody>
</table>

*P<0.01 versus control; **P<0.001 versus control; ***P<0.05 versus high fat (HF); ****P<0.01 versus high fat; *****P<0.05 versus control. High fat + T3 (HF+T3); High fat + GC-24 (HF+GC-24); High fat + T3 + GC-24 (HF+T3+GC-24).
Table 2: Effects of triiodothyronine (T3) (10X) or equimolar doses of GC-24 in animals placed on a high-fat diet. Values indicated as mean±SEM.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>HF</th>
<th>HF+T₃</th>
<th>HF+GC-24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total water (ml/kg BW)</td>
<td>274±13</td>
<td>260±23</td>
<td>236±7*</td>
<td>274±19</td>
</tr>
<tr>
<td>Lean body mass (%)</td>
<td>85±2.2</td>
<td>79±9</td>
<td>91±3.7</td>
<td>88±2.4</td>
</tr>
</tbody>
</table>

*P<0.05 versus HF and P<0.06 versus T₃+GC-24.

Synthesis System for real-time (RT)-PCR (Invitrogen) on Roche Light Cycler (Stratagene, La Jolla CA, USA). About 120 ng cDNA was used for amplification. Quantitative RT-qPCR (RT-PCR) was performed using IQ SYBR Green PCR kit (BioRad) on iCycler thermal cycler machine (Bio-Rad). Primers were designed with the help of Beacon Designer 3.0 (Premier Biosoft Intl., Palo Alto, CA, USA), and the housekeeping gene cyclophilin A used as an internal reference. Primer sequences are available upon request. The cycle conditions were: 5 min at 94 °C (hot start); 30 s at 94 °C, 30 s at 58 °C, and 45 s at 72 °C for 50 cycles followed by the melting curve protocol to verify the specificity of amplicon generation. Gene expression was determined by ΔΔCₚ and all values were expressed using cyclophilin A mRNA as an internal control (Christoffolete et al. 2004).

RIA: Total thyroxine (T₄) and T₃ serum levels were measured in 25 μl serum samples in duplicate using specific RIA (Coat-A-Count T₄ Uptake Test Kit and Coat-A-Count T₃ Uptake Test Kit, DPC, Los Angeles, CA, USA).

Fat mass measurement

Carasses were thawed overnight at 4 °C, weighed, chopped in small pieces, and then, using a motorized blender (Kinetica AG, Lucerne Switzerland), thoroughly homogenized in a volume of distilled water that equals the weight of the carcasses. Aliquots of homogenates were used for measurements of water, protein, and fat content. The water content was calculated as the weight variance after 24 h at 100 °C, as previously described (Bier et al. 1998). Fat content was determined as previously described (Folch et al. 1957, Hartsock & Henningberger 1963, Azain et al. 1998), after lipid extraction with a 2:1 chloroform:methanol solution. The lipid-containing chloroform layer was separated and dried to constant weight. The protein content was determined as described by Bradford (1976).

Histological examination

After careful dissection, tissues were immersed in buffered formalin solution and fixed for 24 h. Paraffin-embedded tissues were sectioned and processed as described (Kett et al. 1995). Analyses were performed after hematoxylin–eosin staining. The area of the white adipocytes was estimated by analyzing pictures taken at 100X magnification. Picture printouts were cut, and the area of at least 40 adipocytes per animal was estimated.

Statistical analysis

Results are expressed as the mean±SEM throughout the text, tables, and figures. Multiple comparisons were performed by one-way ANOVA, followed by Student–Newman–Keuls test.

Figure 2: Effects of GC-24 on GTT and ITT. Rats were placed on a high-fat diet for 4 weeks and treated with T₃, 10X (30 ng/g BW per day) or GC-24 in equimolar dosage. (A) Glycemic levels (mg/dl). After fasting overnight, animals were injected with 2 g/kg of glucose, and plasma glucose levels were measured in 30 min intervals via tail bleeding. *P<0.01 versus all other groups. *P<0.06 versus other groups. (B) Glycemic levels in arbitrary units. After fasting for 6 h, animals were injected with 0.5 U/kg of insulin and glucose blood levels were measured in 15 min intervals via tail bleeding. *P<0.001 versus control and high fat; *P<0.05 versus control and high fat; *P<0.001 versus high fat + T₃. Entries are mean±SEM; n=5.
Results

Feeding a high-fat diet resulted in ~30% compensatory reduction in food intake as early as the first experimental week (Fig. 1A). Despite this, at the end of the 4-week period, the animals on the high-fat diet gained significantly more BW (~30%; Fig. 1B), and accumulated more fat in absolute and relative terms (~20% and ~50%; Fig. 1C and D). Concomitant treatment with GC-24 (1-7 μg/100 g BW per day) did not affect serum T3 levels or the T3/T4 ratio in the serum (Table 1). At the same time, it did reduce body fat to levels below those found in animals fed chow diet (~23%; Fig. 1C and D), but did not affect total body water nor prevent the accelerated increase in BW (Fig. 1B; Table 2). High-fat feeding caused visible fat deposition in liver, which was prevented by treatment with GC-24 (Fig. 1E). A similar, but much less dramatic, pattern was observed with the size of the fat cells (Fig. 1E and F). The estimated adipocyte area was doubled with the high-fat diet, while treatment with GC-24 only partially reduced it, to levels still above controls (~40%; Fig. 1E and F). Notably, this effect of GC-24 took place without changes in food ingestion (Fig. 1A) or cardiac weight (Table 1), a sensitive index of thyroid hormone effects in the heart. As a reference, other animals were placed on a high-fat diet and treated with equimolar amounts of T3 (3 μg/100 g BW per day), in a dosage equivalent to 10 times the daily T3 replacement dose. Such animals gained less BW (~45%), had less total fat content (~70%), less total body water (~15%; Table 2), while at the same time exhibited cardiac hypertrophy (~23%; Table 1).

Rats placed on a high-fat diet developed the expected fasting hyperglycemia (86 vs 65 mg/dl) and glucose intolerance with a 30-min peak after glucose load reaching 281 mg/dl (Fig. 2A); at least in this setting, these animals did not exhibit resistance to insulin (Fig. 2B). Whereas the GC-24-treated animals still had significant fasting hyperglycemia (96-5 mg/dl), the maximum 30-min glucose peak was ~30% reduced (Fig. 2A). Insulin sensitivity was increased at early time points after insulin administration in GC-24-treated animals (Fig. 2B). Likewise, treatment with T3 mimicked the effects of GC-24 (Fig. 2A and B).

Animals placed on a high-fat diet exhibited significantly higher plasma levels of triglycerides, total cholesterol, NEFA, and IL-6 (Table 1). At the same time, treatment with GC-24
Figure 4: Effects of GC-24 on resting metabolic rate (RMR). Rats were placed on a high-fat diet for 4 weeks and treated with (A) 10X 30 mg/kg BW per day) or GC-24 in equimolar dosage. (a) Normalized RMR (ml/min per kg BW); *P<0.001 versus control; #P<0.01 versus high fat. (b) Total RMR (ml/min per rat); *P<0.001 versus control; #P<0.01 versus high fat. Entries are mean ± SEM; n=6.

Discussion

Recent studies indicate that the administration of TRβ-selective agonists has beneficial metabolic effects, e.g., increase in energy expenditure, while lowering serum cholesterol and triglycerides (Trost et al. 2000, Grover et al. 2004, Johansson et al. 2005, Villieux et al. 2007). In the present study, we expanded these findings and have shown that the administration of GC-24, a novel and even more selective TRβ agonist, prevented some of the metabolic abnormalities associated with feeding a high-fat diet, namely increase in fat mass (Fig. 1C and D), glucose intolerance (Fig. 2A), and hypertriglyceridemia (Table 1), while it did improve sensitiviy to insulin (Fig. 2B). At the same time, other parameters that were elevated by high-fat feeding were only partially or not affected at all by treatment with GC-24, such as IL6, NEFA, and cholesterol levels (Table 1).

It is remarkable that in rats fed a high-fat diet, a substantial improvement in key metabolic parameters, i.e. plasma TG, body fat, glucose tolerance, and insulin sensitivity, is achieved with GC-24 treatment without affecting cardiac weight. One consideration is that GC-24 activates a number of well-known T3-responsive pathways, e.g. lipolysis, β-oxidation, which combined would improve the overall metabolic status.
of these animals. For example, it has been shown that T₃ administration improves glucose tolerance in db/db mice via TRβ activation (Bryzgalova et al. 2008), while it up-regulates GLUT4 expression (Cana et al. 1990, Torrance et al. 1997).

At the same time, a more comprehensive explanation could simply be that GC-24 markedly accelerates the RMR by mimicking T₃ actions (Fig. 4). An accelerated RMR would balance the increased energy intake, limit fat accumulation, and improve glucose homeostasis. This second hypothesis is supported by previous findings that a major contribution of TRβ-activating effects in energy expenditure (Grover et al. 2004, 2005, Villasoe et al. 2007).

Given the predominance of TRβ in the liver (Schwartz et al. 1992) and the substantial effects of TRβ-selective agonists as cholesterol-lowering agents, it is assumed that the bulk of the effects of these molecules take place in the liver (Grover et al. 2004, Erion et al. 2007). However, our analysis of gene expression in liver, skeletal muscle, white fat and BAT indicates that the latter was predominantly affected.

In the BAT, there was a significant increase in Cpt1, Sd, and Act gene expression by GC-24, indicating the activation of this tissue (Fig. 5). In turn, this would explain the increase in metabolic rate by GC-24.

At the same time, a number of other metabolic parameters were not restored or affected by GC-24, namely increased IL6 and NEFA plasma concentrations, fasting hyperglycemia as well as hypercholesterolemia and liver cholesterol content. Persistently elevated IL6 and NEFA indicate that despite...
improvements, these animals remain metabolically challenged due to the elevated fat intake. Fasting hyperglycemia remains despite increased insulin sensitivity most likely as a result of higher hepatic glucose production via stimulation of PEPCK gene expression (Loose et al. 1985, Kliewer et al. 2008).

The suboptimal effects of GC-24 on cholesterol metabolism are particularly notable. Thus, treatment with GC-24 only minimized, but not normalized, the increase in plasma cholesterol levels resulting from the high-fat feeding, while liver cholesterol content remained high (Table 1). Because TRβ-selective agonists were shown to be effective cholesterol-lowering agents (Trost et al. 2000, Grover et al. 2003, 2004, Johansen et al. 2005, Miyabara et al. 2009), one explanation is that GC-24/T₃ signaling could be decreased in the liver of high-fat fed animals (Crunckhorn & Pati 2008). This is supported by the observation that in rats kept on chow diet, GC-24 did reduce plasma cholesterol (Fig. 3C). However, the liver expression of Dio1, Sdr, and Ppata was all increased by GC-24 in high-fat fed animals, indicating that GC-24/T₃ signaling seems to be preserved in these animals. In addition, in such animals, both plasma cholesterol and liver cholesterol concentrations were normalized by treatment with equimolar doses of T₃ (Table 1). While it is not clear what the mechanisms interfering with GC-24 actions in liver is, reduced effectiveness of the TRβ-selective agonist as a cholesterol-lowering agent when combined with a high-fat diet is an important finding, which could have substantial impact on their planned therapeutic utilization.

Most studies with TRβ-selective agonists so far involved either oral gavage or i.p. administration. Because both routes involve a first passage through the liver, it has been suggested that this anatomic aspect contributes to the liver selectivity of these compounds. Our data, however, indicate that this is not the case given that animals kept on chow diet while receiving s.c. injections of GC-24 had their plasma cholesterol levels lowered (∼10% by) and gained less BW (∼37% by) (Fig. 3). In conclusion, the present studies show that administration of a highly TRβ-selective agonist to rats during feeding with a high-fat diet prevented a number of metabolic alterations typical of this condition such as increase in fat mass (Fig. 1C and D), glucose intolerance (Fig. 2A), and hyperglycemia (Table 1). The overall mechanism seems to be acceleration in the RMR, which takes place in BAT. However, GC-24 treatment did not restore hypercholesterolemia, increased hepatic cholesterol content, elevated NEFA and IL6 levels, indicating that these metabolic pathways are less sensitive to activation of TRβ signaling during feeding with a high-fat diet. These findings should have important repercussions to the potential usage of TRβ-selective agonists as cholesterol-lowering agents.

Declaration of interest

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research here reported.

Funding

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TRH activation prevents obesity

B S Aamor, C B Ueta and others

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Nos últimos anos de minha pós-graduação, trabalhando em colaboração com a professora Janete M. Cerutti, fui convidada para participar desta revisão sobre marcadores tumorais do câncer folicular tiroidiano. Nesta revisão buscamos com base na literatura disponível, a apresentação de todas as tecnologias disponíveis para a detecção de genes e técnicas histológicas que visam a diferenciação do tecido normal do tecido canceroso.
Review

Genetic markers differentiating follicular thyroid carcinoma from benign lesions

Beatriz C.G. Freitas, Janete M. Cerutti

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2 Division of Endocrinology, Department of Medicine, Federal University of São Paulo, SP, Brazil

Abstract

Thyroid nodules are commonly encountered during routine medical care. The main problem established by a discovery of a thyroid nodule is to discriminate whether a thyroid nodule is malignant or benign. Fine-needle aspiration (FNA) is the most widely used and cost-effective preoperative test for initial evaluation of a thyroid nodule. While the overall accuracy of FNA is 90%, it has limited accuracy with follicular lesions. Patients with a cytological report of indeterminate follicular lesions are referred to surgery for a more accurate diagnosis. A more acute molecular-based test for thyroid nodules is needed not only to improve treatment decisions, but also to potentially reduce the long-term health costs. Several studies have looked into biologic markers that could be used as an adjuvant to distinguish the benign from malignant nodules. This review will focus on those biomarkers that are potentially useful in the diagnosis of thyroid lesions commonly classified as indeterminate.

Contents

1. Introduction
2. PAX8/PPARγ rearrangements: a potential marker to discriminate FNA from FTC
3. Candidate gene approach vs. genome-wide expression profiling
4. Identification of four-marker panel using digital transcript profiling
5. The potential diagnostic applications of microRNA biomarkers
6. Can mutational profile help the preoperative diagnosis of nodules classified as indeterminate?
7. Problems and future directions

Acknowledgements
References

1. Introduction

Thyroid nodules are commonly encountered in clinical practice. Their prevalence largely depends on the method of screening and population evaluated. In North America, they are discovered by palpation in 3–7% in the general population (Tan and Gharib, 1997). With the widespread use of ultrasound, incidentally discovered thyroid nodule has become increasingly common and it is now recognized that nodules are present in up to 67% of the general population (Ezzat et al., 1994; Tan and Gharib, 1997). The increasing rate of detection of non-palpable thyroid nodules demands a careful preoperative evaluation of a thyroid nodule, since it may represent a thyroid malignancy.

Currently fine-needle aspiration (FNA) is the most reliable, widely used, and cost-effective preoperative test for initial evaluation of thyroid nodules. As a result of better patient selection, FNA reduces the number of surgeries and the overall cost of medical care (Mazzaferrri, 1993).

Although FNA is the gold standard for the preoperative identification of thyroid malignancy, the accuracy is mainly related to the histological subtype that is being evaluated and the experience of the physician. In centers with experience, papillary thyroid carcinoma (PTC) is readily identified with FNA. When correlated with the histology of the final surgical specimen, diagnosis is cor-
pect for FTC in approximately 90–100% of FNA specimens. FNA accuracy is much lower for both the benign follicular adenoma (FTA) and the malignant follicular carcinoma (FTC), since they have similar cytological appearance. Therefore, even for the most experienced pathologist, differentiating the benign from malignant nodules represents a diagnostic challenge. As a result, the specimens of follicular tumors are commonly interpreted as indeterminate or follicular-patterned lesions with diagnosis of malignancy requiring demonstration of capsular or vascular invasion on final histology (Yeh et al., 2004).

A further dilemma of preoperative diagnosis for thyroid nodules is the fact that FNA has little diagnostic value when evaluating Hürthle cell adenoma (HCA) and Hürthle cell carcinoma (HCC), which are considered variants of follicular thyroid tumors. Thyroid nodules diagnosed as indeterminate by FNA cytology may also represent hyperplasia (HN) or follicular variant papillary thyroid carcinoma (FVPTC) on final histological follow-up (Baloch et al., 1999; 2002). In fact, a high percentage in this category was found to be FVPTC (Baroluzzi et al., 2000; Logani et al., 2000). The lack of consensus among experts may add to the high percentage of thyroid nodules classified as indeterminate by FNA.

To improve the accuracy rate, some reports have suggested that nodules classified as indeterminate can be stratified according to risk of malignancy. The National Cancer Institute (NCI) recently sponsored the NCI Thyroid Fine-Needle Aspiration State in Science (Layfield et al., 2000). The diagnostic terminology and morphological criteria for the cytological diagnosis of thyroid lesions committee has proposed to classify the indeterminate group in a three-tiered category scheme, according with the predicted probability of malignancy: (1) follicular lesion of undetermined significance, (2) neoplasm (e.g., follicular neoplasm and Hürthle cell neoplasm) and (3) suspicious for malignancy (Baloch et al., 2002; Layfield et al., 2003).

Although this system represents a major step forward in the standardization of the FNA report and, therefore, allows a more appropriate triage of patients that need surgery (Nayar and Javovici, 2002), we still have a gray zone in FNA-cytology. Therefore, it is clinically important to have molecular markers that, in conjunction with FNA, can identify the subset of patients who have a malignant nodule.

Table 1

<table>
<thead>
<tr>
<th>Reference</th>
<th>HCA</th>
<th>FTA</th>
<th>FTC</th>
<th>PTC</th>
<th>HCC</th>
<th>FVPTC</th>
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<td>0/14</td>
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</tr>
<tr>
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</tr>
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</tr>
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<td>0/15 (63%)</td>
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<td>0/10 (0%)</td>
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<tr>
<td>Di了解到ovetti et al. (2005)</td>
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<td>0/2 (0%)</td>
<td>17/17 (100%)</td>
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</tr>
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<td>0/10 (0%)</td>
<td>0/10 (0%)</td>
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<td>0/10 (0%)</td>
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<tr>
<td>Total</td>
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<td>23/283 (8%)</td>
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<td>2/109 (1%)</td>
<td>1/109 (0.9%)</td>
</tr>
</tbody>
</table>

FTA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; HCA, Hürthle cell adenoma; HCC, Hürthle cell carcinoma; PTC, classic variant of papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma.

2. PAX8/PPARγ rearrangements: a potential marker to discriminate FTA from FTC?

In 2000, Kroll et al. reported an intrachromosomal translocation involving chromosome regions 2q13 and 3p25 in human tumors arising from thyroid follicular epithelial cells. The authors described an in-frame fusion of PAX8 (2q13) to the PPARγ (peroxisome proliferator-activated receptor γ) gene (3p25). The predicted PAX8–PPARγ fusion protein is composed of the paired (PAX) and partial homeobox DNA binding domains (HD) of PAX fused to the DNA binding, ligand binding, RRX dimerization, and transactivation domains (A–F) of PPARγ. Different PAX8–PPARγ isoforms (molecular mass 87–98kDa) can be found as a result of alternative splicing of PAX8 (Kroll et al., 2000).

Importantly, this recurrent translocation reported in five FTCs was not detected in any benign thyroid lesion or PTC (Table 1). Considering its specificity, the authors suggested that PAX8–PPARγ may aid in the differential diagnosis of FTC from benign lesions in FNA (Kroll et al., 2000). This finding corroborates with previous analyses that have suggested the presence of a putative tumor suppressor gene on chromosome 5p, since the loss of heterozygosity on the short arm of chromosome 3 was described in the majority of the evaluated FTC (Grebe et al., 1997; Herrmann et al., 1991). Moreover, 3p25 aneusomy was described as an early genetic event associated with pathogenesis of FTC (French et al., 2003).

Although this rearrangement was initially associated with FTC, it was also described in 0–5% of FTA s (for references, see Table 1). It has been suggested that the adenomas harboring this translocation may be in situ FTC in which the invasion was overlooked by the pathologist (Nikiforova et al., 2003). Importantly, the overall prevalence of this rearrangement in FTC is lower than the initially reported by Kroll et al., with cumulative prevalence of 8% (0–62%: Table 1). The reason for the discrepancy in the reported prevalence is not clear. Since RT-PCR has been used in most of the studies and it is considered the "gold standard" methodology for the detection of this rearrangement, it is unlikely the explanation for the observed differences. Geographical diversity, tumor heterogeneity and diagnostic difficulty in the distinction of follicular thyroid tumors may affect the end results.
Overall, if PAX8-PPARγ rearrangements are less specific than originally thought, the PAX8-PPARγ fusion protein is insufficient to promote tumorigenesis and an additional event is needed to develop a malignant phenotype. This initial diagnostic of FTA is inaccurate or if the FTA with PAX8-PPARγ rearrangement is in fact a carcinoma in situ, it is still uncertain.

This scenario raises the question on the potential use of PAX8-PPARγ as a preoperative diagnostic marker. Since PAX8-PPARγ rearrangement was negative in a high percentage of FTC and in most of malignant thyroid lesions commonly classified as indeterminate such as FVPTC and CCH, and false-negative findings are worrisome as results under treatment, the use of this marker in clinical practice has limited value. However, while PAX8-PPARγ pathogenic significance remains undefined, removing thyroid nodules with PAX8-PPARγ rearrangement is recommended, since it may represent a carcinoma in situ. Regarding the feasibility of testing PAX8-PPARγ in routine clinical practice, this will be discussed later in this review.

3. Candidate gene approach vs. genome-wide expression profiling

Traditionally, scientists have examined only one or a small number of candidate markers at a time. Using this strategy, a number of candidate biomarkers were identified. Among the genes that are thought to discriminate the benign from the malignant thyroid carcinoma is telomerase reverse transcriptase (TERT). When FNA report was suspicious for a nonfollicular neoplasm and when samples with lymphocytic infiltration were excluded, TERT was a perfect descriptor of malignancy. However, when FNA report was suggestive of a follicular neoplasm, TERT expression alone predicted malignancy in only 71% of cases (Umbrich et al., 2004). Others have shown that TERT expression was not only identified in benign lesions and inflammatory thyroid conditions but also in normal thyroid tissue (Hansen et al., 1997; Iwan et al., 2003; Sebesta et al., 2001; Yoshima et al., 1997). Considering its overall performance, TERT may not be an useful diagnostic marker for nodules classified as indeterminate by FNA.

Galectin-3, LGALS3, was initially described as a marker that could discriminate benign from well differentiated thyroid carcinomas with high sensitivity and specificity (Orlandi et al., 1998; Xu et al., 1995). There have been some controversial results in the follow-up studies. Some studies confirmed the initial findings (Bartolazzi et al., 2001; Orlandi et al., 1998; Saggiorato et al., 2001, 2005) while others have found LGALS3 expression in normal thyroid, FTC, and benign lesions (Cvejic et al., 1998; de Matos et al., 2005; Martini et al., 2002; Nascimento et al., 2001; Tokano et al., 2003). Additionally, a negative result for LGALS3 can exclude thyroid malignancy since a considerable percentage of FTC, UICC, and FVPTC was negative for LGALS3 (Table 2) (Bartolazzi et al., 2006; Bartolazzi et al., 2008; Cvejic et al., 1998; de Matos et al., 2005; Fernandez et al., 1997; Oler et al., 2008; Prasad et al., 2005). Accordingly, the overall specificity and sensitivity is lower when applied to tumors commonly classified as indeterminate nodules by FNA. It has been suggested that the reasons for this discrepancy might be related to the differences in technical procedures and antibody clone used (Bartolazzi et al., 2008). It has been suggested that LGALS3 needs formalin fixed and paraffin-embedded cytological preparations, biotin free detection method and monoclonal antibodies (for detail see Bartolazzi et al., 2003; Sanabria et al., 2007).

In a recent multicenter study, the utility of LGALS3 as a screening test was assessed in follicular thyroid nodules classified as indeterminate on FNA-cytology (Bartolazzi et al., 2008). In a total of 544 patients were evaluated. The overall sensitivity of the LGALS3 test was 78% and the specificity was 93%. If the option of surgery would be based on LGALS3 expression it would have missed 29 (22%) of cancers, although the authors declared that 71% of unnecessary thyroid surgical procedures could be avoided. Since there is a significant association between LGALS3 and Hurthle cells and it is not clear whether Hurthle cell adenomas were included in the validation set, we speculate how LGALS3 would perform in a larger set including HCA? While in this prospective multicenter study, operative and immunostaining protocols considered "gold standard" for LGALS3 staining were provided in advance to all centers, technical failure was still reported. It has been suggested that specific training and workshops is needed to lower the false-negative results. Considering the implications of false-negative findings, which have been reported in several studies, the use of LGALS3 alone in clinical practice should be interpreted cautiously. Given that follicular-patterned lesions may include a wide range of tumor subtypes, it will be unlikely that a single marker can make a clear-cut distinction between benign and malignant thyroid lesions.

Prasad et al. (2005) tested whether LGALS3 expression in combination with other four markers, may be useful to improve the diagnostic accuracy of FNA. The panel comprised LGALS3, Fibroblastin-1 (FN1), CITED-1, Cytokeratin-19 (CK19) and HMBE1. LGALS3, CK19 and CITED-1 were less specific, 55%, 31% and 24% respectively. Given that LGALS3 was more sensitive and CK19 and CITED-1 did not provide any additional discriminatory information, they were not included in the final panel. The final panel, comprising LGALS3, FN1 and/or HMBE1, detected more efficiently all follicular-cell derived carcinomas, with a false-positive rate of 24%.

Many studies have used tested the recommended markers (Barocea et al., 2008; de Matos et al., 2005; Saggiorato et al., 2005). While the panel detects most carcinomas, due to its false-positive rate, it has been suggested that caution should be taken in the interpretation of the results.

Technological development, especially in genomics and proteomics, has turned much easier to examine a large number of potential markers at once. Microarrays analysis has been used to assess the expression of thousands of genes in parallel. Barden et al. (2003) conducted a microarray-based study to determine the genetic profiles of FTC and TFA. The group described a 105-gene expression classifier that differentiated between the two entities with high accuracy, although their model still failed to identify minimally invasive FTCs.

Weber et al. (2005) performed gene expression profile of 12 FTC and 12 FTA. A new classification model, based on the expression levels of three genes, PCSK2, CDN2 and PLAB, allowed the authors to discriminate between FTA and FTC with a high sensitivity of 100% and specificity of 96.7%. PCSK2 and CDN2 were highly expressed in FTA whereas PLAB was highly expressed in FTC. These markers may be less sensitive in detecting typical variant or Hurthle cell adenomas since they were not included in their training or validation set of adenomas.

A comprehensive approach for expression profiling is Serial Analysis of Gene Expression (SAGE), which provides digital information on transcript levels. The ability for evaluating the expression pattern of thousands of genes in a quantitative fashion, without prior sequence information, is one of the most attractive features of SAGE (Velculescu et al., 1995). SAGE produces a comprehensive profile of gene expression and can be used to search for candidate tumor markers (Cerutti et al., 2003). This approach has also been used to identify diagnostic and prognostic markers in thyroid (Cerutti et al., 2001, 2004; Lack et al., 2000). SAGE produces a comprehensive profile of gene expression and can be used to search for candidate tumor markers (Cerutti et al., 2003). This approach has also been used to identify diagnostic and prognostic markers in thyroid (Cerutti et al., 2001, 2004; Lack et al., 2000). SAGE produces a comprehensive profile of gene expression and can be used to search for candidate tumor markers (Cerutti et al., 2003). This approach has also been used to identify diagnostic and prognostic markers in thyroid (Cerutti et al., 2001, 2004; Lack et al., 2000).
Table 2
LGALS3 expression in benign and malignant thyroid carcinomas tested by immunohistochemistry.

<table>
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<tr>
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<th>Nodular Goiter</th>
<th>FFA</th>
<th>FTC</th>
<th>HCA</th>
<th>HCC</th>
<th>PTC</th>
<th>FVPTC</th>
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<td>7/10</td>
<td>17/19</td>
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<td>(52%)</td>
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<td>30/29</td>
<td>-</td>
</tr>
<tr>
<td>Saggiorato et al. (2001)</td>
<td>Monoclonal GAL3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3/15</td>
<td>16/10</td>
<td>0/7</td>
<td>13/14</td>
<td>30/29</td>
<td>-</td>
</tr>
<tr>
<td>Bartolazzi et al. (2001)</td>
<td>Colectin H Thyrotropin Kit</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3/15</td>
<td>16/10</td>
<td>0/7</td>
<td>13/14</td>
<td>30/29</td>
<td>-</td>
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</tbody>
</table>

Total

<table>
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<tr>
<th>Normal</th>
<th>HIN</th>
<th>Nodular Goiter</th>
<th>FFA</th>
<th>FTC</th>
<th>HCA</th>
<th>HCC</th>
<th>PTC</th>
<th>FVPTC</th>
</tr>
</thead>
<tbody>
<tr>
<td>0/16</td>
<td>4/176</td>
<td>(2%)</td>
<td></td>
<td>76/465</td>
<td>125/157</td>
<td>15/48</td>
<td>30/43</td>
<td>39/127</td>
</tr>
</tbody>
</table>

FFA, follicular thyroid adenoma; FTC, follicular thyroid carcinoma; HCA, Hurthle cell adenoma; HCC, Hurthle cell carcinoma; PTC, classic variant of papillary thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; HIN, hyperplasia.

* Other variants of FTC were not included.
* Tested in FVPTC samples. FTC and FVPTC were grouped together.
* Tested in HIN samples. FTC and FVPTC were grouped together.
* Tested in FTC samples. FTC and FVPTC were grouped together.
* Tested in FTC samples. All variants of FTC.
diagnosis of FTC (Takano and Yamada, 2009). As down-regulation of TFF3 was mainly observed in widely invasive FTC and in minimally invasive FTC with distant metastasis, the authors suggested that TFF3 could help to classify thyroid tumors into two groups: (1) benign tumors with TFF3 expression and (2) possibly malignant tumors with down-regulation of TFF3 expression (Takano and Yamada, 2009). Only the latter should undergo surgery. Noteworthy is that TFF3 was down regulated in a high percentage of FTC (16%) while it was expressed in 28% of minimally invasive FTC cases. Therefore, due to its low accuracy in detecting minimally invasive FTCs, the combination of TFF3 mRNA expression, along with other molecular markers, may improve diagnosis of thyroid nodule.

In fact, a panel of markers consisting of TPS and other recently identified markers (ONX, PLAB, PC32, HGD1, BAG31, LGALS1, ST1, ADAM3 and TG) have been proposed and tested. Of these markers TPS, PLAB, HG31, HGD1 and LGALS1 allowed the best prediction of cancer (sensitivity of 80% and specificity of 100%) (Krause et al., 2008). Additionally, TPS, along with TEP1, PAEPRPA, CITD1 and EGR2 effectively predict high-risk FTC with high specificity and sensitivity. The authors also propose a second classifier (TPS and TERT) that predicted malignancy with high specificity (Foutakis et al., 2009).

Recently, however, TPS3 expression was evaluated by immunohistochemistry. Although TPS3 was highly expressed in normal thyroid compared to thyroid tumors, no difference was reported among FTC, PTC, FTC and RTVPTC (Panel et al., 2009).

Comparative genome-wide expression profile is one of the most widely used techniques for identifying molecular markers. As a consequence, there has been a flood of new markers. Although very promising classifiers have been described, a common criticism, however, is the disagreement between studies. One possible explanation is that studies rely on different platforms and laboratory procedures are much more likely to introduce differences between data sets. Additionally, analysis has been performed in small set of samples.

In 2009, Griffith et al. performed a meta-analysis of thyroid cancer biomarkers identified through gene expression profiling studies (Griffith et al., 2009). A review of the top 12 cancer-specific cancer markers revealed genes previously validated such as MET, TFF3, SFRP1, TIS11, TSPAN1 and RPOD and new candidate markers such as TGF, TEFF1, KIAA1, PCA3, FGFR4, EPIT and PCDH10. Whether a panel including the list of found genes using this approach will help preoperative evaluation of a thyroid nodule requires further analysis in a large multicenter study. Importantly, tumors included in the studies covered a wide range of samples subtypes, given that they were designed to answer different scientific questions. As an example, from 21 studies included in this analysis; the majority compared PTC to normal thyroid or benign lesions. Only six studies compared tumors classified as indeterminate on FNA cytology (Barden et al., 2003; Gara et al., 2004; Cheville et al., 2004; Finley et al., 2004; Takano and Amino, 2000; Weber et al., 2005).

4. Identification of four-marker panel using digital transcript profiling

We previously performed gene expression profiling of FTC, FTA and normal thyroid. The complete transcriptional dataset is available at the SAGE Genie and SAGE map websites (http://cgap.nci.nih.gov/SAGE (Boon et al., 2002)). A combination of the mRNA expression levels of four genes (DDIT3, TM1, AR2, COF2) had a predictive accuracy of 81% for distinguishing between FTA and FTC (Gara et al., 2004). Two of these predictive markers, DDIT3 and AR2, were also diagnosed at the protein level and the sensitivity and specificity were 85 and 90%, respectively.

An accuracy of 80% is not sufficient to avoid surgery for the benign nodule or for ensuring that patient with a malignant nodule should undergo surgery. To improve the accuracy of our test, we validated all markers in a wide range of thyroid tumors commonly classified as indeterminate by FNA. The markers distinguished benign thyroid lesions (FTTA and HN) from malignant thyroid tumors (FTC, HCC, FVPTC, and PTC) with a sensitivity estimate of 100%. By using the new antibodies, we achieved a substantially higher accuracy: 85% for HCC and FTA and 93% for FTC and PTC along with other follicular thyroid lesions (Gara et al., 2005; Gara et al., 2007). The two best markers were COF2 and ITM1 for detecting malignancy, although they had the drawback of few false-positives cases for benign lesions with Hurthle components. Importantly, these antibodies can effectively be used in FNA specimens (Gara et al., 2006). Further analysis testing these markers in a greater number of FNA specimens will clarify whether or not our markers can effectively help the preoperative evaluation of nodules classified as indeterminate and, therefore, be incorporated in most laboratories for the routine evaluation of thyroid nodules.

Another potential application of these markers is the postoperative diagnosis, since the difficulty in diagnosing FTC and FVPTC is not restricted to FNA cytology. In fact, nearly 15% of FVPTC can show capsular or vascular invasion and distant metastases to lungs and bones and can be mistaken for FTC. The large majority, however, is encapsulated tumors with lack of any invasive characteristics. There is still controversy about how the diagnosis of FVPTC is made in cases that are encapsulated and when no invasion is observed. Discrepancies were also observed in minimally invasive FTC, even among experienced pathologists (Frank et al., 2003; Lloyd et al., 2004). Accordingly, a diagnostic marker that could be used in both FNA and paraffin-embedded sections should improve the consistency in diagnosing.

Interestingly, the finding that AR2 was identified among the up-regulated tumor-related genes identified in a mouse model that spontaneously developed FTC through the progression of hyperplasia, capsular and vascular invasion, anaplasia and eventually metastasis to distant organs (Ying et al., 2003), suggests that ARG2 may play a role in carcinogenesis.

DDIT3 was one of the genes up-regulated in FTCs and Hurthle tumors (Barden et al., 2003; Bardin et al., 2004). Recently, the use a multi-gene marker panel which included DDIT3 was tested in FTC, FVPTC and FTA. The authors reported a sensitivity of 90%, although they found a specificity of 21%. One possible explanation for the decrease in specificity is that adenomas tested may have Hurthle cell components, given that our false-positive cases were FCA. Also, one can not exclude that the antibody dilution used resulted in a significant number of technical false positives cases (Bryson et al., 2008). This sample set also showed very low sensitivity and specificity for both CD2 and TFF3.

Matsuura et al. (2006) also found that COF2 expressed in malignant thyroid tumors and in benign lesions scattered cells with Hurthle métaplasia.

Although our analysis shows promises, further validation in a larger set of FNA sample is required in order to determine the potential utility of this panel for guiding treatment decisions.

5. The potential diagnostic applications of miRNA biomarkers

Several published reports have shown deregulation of microRNAs (miRNAs) in thyroid tumors compared with normal tissues, suggesting that they might play a role as oncogenes or tumor suppressors. The genome-wide miRNA expression profile revealed that two miRNAs (miR-197 and miR-346)
are over-expressed in FTC compared to FTA (Weber et al., 2006). Additionally, two specific miRNA (miR-221 and miR-222) have been found to be consistently over-expressed in FTC (He et al., 2005; Visone et al., 2007; TzCARD et al., 2007; Pallante et al., 2006; Nikiforov et al., 2008). When cytological specimens were analyzed, a much higher expression of these miRNAs was detected in carcinoma samples with respect to the normal thyroid cells (Pallante et al., 2006). Functional analysis substantiates the initial findings and suggests a critical role of these miRNAs in thyroid carcinogenesis (Pallante et al., 2006; Visone et al., 2007).

Additionally, Weber et al. found miRNA differences between FTA and FTC with four miRNAs over-expressed in FTC when compared to FTA. Two of them (miR-197 and miR-164) were further validated in a set of FA and FTC (Weber et al., 2006).

Recently, it was suggested that a set of seven miRNAs (miR-187, miR-221, miR-222, miR-146b, miR-155, miR-224, and miR-197) can be used to detect thyroid cancer in the surgical and preoperative FNA samples (Nikiforov et al., 2008). It is still not clear whether the measurement of the miRNA levels is a suitable tool for the preoperative diagnosis of thyroid nodules classified as indeterminate by FNA. A potential problem one might face is the small RNA fraction obtained from FNA biopsy. It is still not clear what is the amount of starting material needed for miRNA analysis and, therefore, whether this approach is technologically feasible or not.

Not only these miRNAs but also their target genes may help to improve preoperative diagnosis of a thyroid nodule. Although the analysis of a larger number of samples is needed.

6. Can mutational profile help the preoperative diagnosis of nodules classified as indeterminate?

There has been an extensive effort over the last few years to identify cancer-associated mutations that may improve the preoperative diagnosis of a thyroid nodule. It has been suggested that mutation in thyroid hormone receptor b (TRb) may play a role in thyroid tumorigenesis (Cheng, 2003). In fact a knock-in mouse that harbors a germline mutation of the TRb gene (TRb(WT)) spontaneously develops a thyroid carcinoma which is similar to human FTC beginning with hyperplasia, invasion through tumor capsule and vascular invasion with progression to distant metastasis and undifferentiated thyroid carcinoma (Suzuki et al., 2002). Further analysis demonstrated that single mutation in the TRb gene cause FTC (Kato et al., 2004). This mouse model has been used to dissect pathways that may be associated with metastasis of FTC. Analysis of FTC samples, however, has demonstrated that this it is not a common event associated with pathogenesis of FTC (Nishizuka et al., 2007). Hypermethylation of the gene TRb may be the main mechanism associated with pathogenesis of thyroid tumors (Joseph et al., 2007).

In addition to PAX8/PPARγ rearrangement, the activating mutation in RAS and inactivating mutation or loss of PTEN expression were associated with pathogenesis of FTC (Dahiya et al., 1997; Vaske et al., 2003). Since PTEN-Kis/Met/tor1 pathway is antagonized by PTEN, it has been suggested that pathways may play a role in the pathogenesis of thyroid tumors (Garcia-Roxan et al., 2005; Ricarte-Filho et al., 2006; Wu et al., 2008). Liu et al. investigated genetic alterations in genes along the PTEN/AKT and PI3K/AKT pathways in FTC and undifferentiated thyroid carcinoma (UTC). Mutations and copy number gain were commonly found in UTC, although they were much less common in FTC. Copy gains of PXKCB (45%) and mutation in RAS (20%) were the most common genetic alteration found in FTC (Liu et al., 2008). PXKCB copy number gain may help to identify a number of malignant lesions, even if this genetic variation should be further investigated in FTC samples. Although this series did not include benign lesions, RAS mutation has been previously identified in FTC (Vaske et al., 2003). If a benign lesion with RAS mutation represents, in fact, FTC carcinoma, is still a matter of debate.

Several groups have determined if BRAF V600E, RAS and RET/PTC mutation analysis can be an useful adjunct technique in indeterminate cytologic diagnoses (Rowe et al., 2007; Sapiro et al., 2007; Xing et al., 2009).

Nikiforov et al. recently conducted a prospective study to assess both feasibility and significance of testing a panel of tumor-specific mutations to improve the preoperative diagnosis of a thyroid nodule. The authors reported that these events were highly specific for detecting malignancy but less sensitive, mainly in nodules classified as indeterminate or follicular neoplasia and suspected for malignancy. These findings were expected given that none of the tested mutations were exclusively associated with FTC or PFTC (Nikiforov et al., 2009). While this diagnostic approach has low sensitivity for indeterminate nodules (Nikiforov et al., 2009; Rowe et al., 2007), the preoperative detection of these mutations, particularly for BRAF mutation, may help to identify patients at high risk of recurrence and death (Xing et al., 2009). Beyond the impact in patient management it will probably have an impact in therapeutics (Ober et al., 2009).

7. Problems and future directions

The existing clinical management of thyroid nodules classified as indeterminate leads to an onerous medical cost for both patients and the health system. The development of a reliable preoperative test that can distinguish between benign and malignant thyroid nodules and that bypass the pitfalls of a non-diagnosed nodule is mandatory. An increasing number of publications have described new potential molecular markers for diagnosis of thyroid nodules. However, very few markers have been validated in multiple studies and when validated the results are not always interchangeable. One potential explanation is that the studies not always have the same reagents and protocols. In fact, even for the two most commonly used thyroid carcinoma marker (Lgals3), standard methodologies have recently been suggested (Bartolazzi et al., 2009). From the technical standpoint, one can predict that that will happen for all markers discussed here. Moreover, the type of assay may influence the results. Assays based on RNA are a challenge for routine clinical pathology laboratories, due to contamination with non-malignant cells. In fact, for our panel of markers, immunohistochemistry performed in paraffin-embedded sections showed to be more robust than quantitative PCR performed in snap-frozen samples (Corulli et al., 2006). mRNA yields from FNA samples are variable and may not provide enough material for mutigene analysis.

It has been suggested that mutational profile can be effectively performed in FNA material (Nikiforov et al., 2009). Although very specific, potential limitations for mutation-based diagnostic test are tumor heterogeneity, amount of DNA isolated from FNA samples and possible contaminations with non-tumor cells, all of which may reduce the sensitivity. Additionally, some mutations potentially associated with pathogenesis of FTC may be missed by Sanger sequencing, as recently suggested (Ricarte-Filho et al., 2003). Finally, since intra- and interobserver variability may lead to differences in interpretation, the results should be clear enough to tailor medical management. Semi-quantitative antibody-based assays, which are observer-dependent, should be avoided.

Therefore, before the development of a preoperative diagnostic test that can be used routinely, we need to take into account several issues: (1) FNA specimens will contain non-tumor cells and blood cell. Assays based on quantitative level of expression (mRNA or protein) are not desirable; (2) PCR-based assays may not be easily
applied to routine; (3) although immunohistochemistry-based assays are more economical and is more readily adapted to any pathology laboratory, a minimal number of genes is required since cellular material is not always abundant in FNA; (4) the markers should be able to distinguish all lesions frequently classified as indeterminate on FNA; (5) large multi-institutional trials on FNA specimens are needed before implement to the routine.

In conclusion, what do we really need for nodules classified as follicular-patterned lesion on FNA material and what is available today? We need marker(s) that will reliably distinguish every single lesion that may be a source of diagnostic error on FNA. No single marker seems to be accurate enough to be introduced in the routine. Since it has been suggested that FTC may develop through at least two distinct and not overlapping molecular pathways (Niforoff et al., 2001), it is unlikely that a "universal marker" can correctly classify thyroid nodules classified as follicular-patterned lesion on FNA. We believe that a tumor marker panel may represent a helpful adjunct to the FNA cytology in order to rule in malignancy as a possibility, and in the selection of patients who might benefit from thyroidectomy. We do have a panel of candidate markers that need to undergo through large trials on FNA material before applying them into routine practice.

Disclosure statement

The authors declare that they have no affiliations that would constitute a financial conflict of interest relative to the subject matter of this study.

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*ABI3* ectopic expression reduces cell growth properties *in vitro* and *in vivo* and induces senescence in cancer cells

Flavia Roche Latini, **Beatriz C. G. Freitas**, Jefferson Pessoa Hemerly, Gisele Oler, Gregory Joseph Riggins, and Janete Maria Cerutti

Dando continuidade aos meus trabalhos de colaboração com a professora Janete Cerutti, realizei alguns experimentos necessários para a finalização da publicação iniciada durante a tese de doutorado de sua aluna, Flavia Latini com a linhagem celular ARO. Seu projeto tem como objetivo a caracterização funcional da proteína ABI3 na carcinogênese e sua correlação com ABI3BP (Latini e cols. 2008). Neste trabalho colaborei realizando os mesmos ensaios feitos na célula do tipo ARO com a linhagem celular WRO, sendo estes ensaios de senescência, apoptose e viabilidade celular; expressão gênica de genes relacionados a ciclo celular (*p21* e *E2F1*) e a visualização da diminuição da fosforilação de ERK em células contendo ABI3. Meus resultados colaboraram para elucidar que o possível papel do ABI3 em induzir a senescência celular e regredir a tumorigênese o que pode colaborar futuramente em estratégias terapêuticas para o câncer.
ABI3 ectopic expression reduces cell growth properties

in vitro and in vivo and induces senescence in cancer cells

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Running Title: Functional role of ABI3 in cancer

Key Words: ABI3, ABI3BP, thyroid carcinoma, senescence.

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Abstract

Although previous reports have indicated that ABI family member 3 (ABI3) functions as a suppressor of tumor metastasis, little is known about the molecular mechanisms by which ABI3 acts. The present study investigated ABI3 expression in thyroid benign and malignant tumors and explored a possible correlation between the expression of ABI3 and ABI3-binding protein (ABI3BP). We have recently showed that ABI3BP plays a role in tumorigenesis for thyroid cancer. We not only observed that ABI3 expression is reduced or lost in carcinomas but also that there is a positive correlation between ABI3 and ABI3BP expression. We next explored the biological effects of ABI3 ectopic expression in two carcinoma cell lines. Forced expression of ABI3 led to a lower transforming activity, reduced tumor growth properties in vitro and in vivo while increased cellular senescence. These responses were accompanied by the up-regulation of the cell cycle inhibitor p21 and reduced ERK phosphorylation and E2F1 expression. Moreover, ABI3 expression reduces cell invasion and migration. Overall, our results suggest that ABI3 loss of expression contributes to pathogenesis and progression of some cancers and suggests that targeting ABI3 or its pathway might be a treatment strategy worthy of further investigation.

Loss of function of human \textit{NKX2-5} are associated with thyroid ectopy: a study of 157 patients with thyroid disgenesis


Sob orientação do professor Rui Maciel trabalhei no primeiro estudo translacional em seu laboratório de um gene associado à um fenótipo de ectopia tiroidiana. O gene NKX2-5 já foi apontado como um gene envolvido na morfogênese cardíaca e tiroidiana, além disso, o grupo italiano de Dentice e colaboradores em 2006 identificou três mutações neste gene, associados à disgenesia tiroidiana (DT). O objetivo do trabalho foi averiguar a presença de possíveis mutações em pacientes com cardiopatias e DT, ou um grupo de pacientes apenas com DT comparado com um grupo de indivíduos normais. Este trabalho deu continuidade aos trabalhos clínicos apresentados na tese de doutorado do primeiro autor dessa publicação, Helton E. Ramos, onde foram identificadas quatro novas mutações no gene do NKX2-5 associadas a ectopia tiroidiana. Minha participação neste projeto foi a parte do estudo funcional das proteínas mutadas com sua super-expressão em células HeLa e seu estudo pelo ensaio de gene repórter da luciferase. Realizei um estudo de transativação gênica, utilizando diferentes concentrações dos plasmídeos para tentativa de uma ativação gênica próxima da forma nativa da proteína. Além disso, fiz um ensaio de co-dominância para averiguar a funcionalidade desses mutantes quando co-transfectados com a forma nativa da proteína visando a identificação do grau de comprometimento destes mutantes na formação do dímero de NKX2-5. O paciente que apresentou duas mutações (Ala80Gly/Asp105Glu) foi quem apresentou um maior comprometimento tanto na ativação gênica como no ensaio de luciferase e no de transativação e co-dominância. O mutante 16 (Asp16Glu) teve sua função similar ao apresentado pela forma nativa da proteína sugerindo que o posicionamento da mutação nos domínios protéicos tem influência no resultado da ativação gênica do NKX2-5.
LOSS OF FUNCTION OF HUMAN NKX2-5 ARE ASSOCIATED WITH THYROID ECTOPY: A STUDY OF 157 PATIENTS WITH THYROID DISGENESIS


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ABSTRACT

Context: Congenital hypothyroidism (CH) is due to Thyroid Dysgenesis (TD) in 80-85% of the cases. Initially, the homeobox transcription factor NKX2-5 was thought to be only required for the organogenesis of the heart tube; however, NKX2-5−/− embryos exhibited a smaller outgrowing thyroid bud. Indeed, several loss of function mutations in NKX2-5 have been described in patients with Congenital Heart Disease (CHD), and an Italian study has identified mutations with patients with TD. Objectives: To address if genetic abnormalities of NKX2-5 could be associated with patients presenting isolated TD or in association with CHD. Methods: A mutational screening was performed in 17 patients with both cardiac and thyroidal phenotype, in 140 patients presenting isolated TD, and then, compared with the results of 50 normal controls. Next, we examined DNA binding affinity using the electrophoretic mobility shift assay (EMSA). The transcriptional activation function was investigated by the dual-luciferase reporter assay system. Results: Three infants with thyroid ectopy presenting four novel NKX2-5 mutations were found; in addition, we have also identified another patient with ectopy harboring a mutation already described. Therefore, all mutations described in NKX2-5 are in patients of ectopy, which reveals an association between ectopy and NKX2-5 mutations (P=0.03). Furthermore, none affected individuals harboring NKX2-5 mutations presented CHD. The mutations identified in our study are predicted to result in codon change in conserved domain (Asp16Glu, Ala80Gly, Asp105Glu, Ala119Ser and Leu245Lle). One of the patients with a ectopic thyroid gland presented two of the novel mutations: c.239C>G (Ala80Gly) and c.315C>G (Asp105Glu). All mutants constructs had normal DNA binding activity but significantly reduced transcriptional activation function. As expected, the variant Ala80Gly/Asp105Glu, described in the same patient exhibited additional documented functional impairment in vitro. Three novel nonsynonomous SNP were found along with three already known common SNPs. Conclusion: Novel NKX2-5 mutations are associated with one particular TD form, ectopy, a finding that reinforces the role for NKX2-5 in thyroid embryogenesis. Parallel to the heart development, NKX2-5 might have an essential role in thyroid morphogenesis and is likely to be a component of the genetic circuit controlling thyroidal cell specification and migration.