In Vivo Activity of the Thyroid Hormone Receptor β- and α-Selective Agonists GC-24 and CO23 on Rat Liver, Heart, and Brain

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Thyroid hormone analogs with selective actions through specific thyroid hormone receptor (TR) subtypes are of great interest. They might offer the possibility of mimicking physiological actions of thyroid hormone with receptor subtype or tissue specificity with therapeutic aims. They are also pharmacological tools to dissect biochemical pathways mediated by specific receptor subtypes, in a complementary way to mouse genetic modifications. In this work, we studied the *in vivo* activity in developing rats of two thyroid hormone agonists, the TRβ-selective GC-24 and the TRα-selective CO23. Our principal goal was to check whether these compounds were active in the rat brain. Analog activity was assessed by measuring the expression of thyroid hormone target genes in liver, heart, and brain, after administration to hypothyroid rats. GC-24 was very selective for TRβ and lacked activity on the brain. On the other hand, CO23 was active in liver, heart, and brain on genes regulated by either TRα or TRβ. This compound, previously shown to be TRα-selective in tadpoles, displayed no selectivity in the rat *in vivo*. **(*Endocrinology* 152: 1136–1142, 2011)**

The physiological actions of thyroid hormones are mediated in part by interaction of T_3 with nuclear receptors [thyroid hormone receptors (TRs)] and regulation of gene expression (for a recent review, please see Ref. 1). There are two receptor subtypes in mammals, TRα and TRβ, and three isoforms, TRα1, TRβ1, and TRβ2, encoded by two distinct genes, *Thra* and *Thrb*. Results from knockout mice lacking either receptor subtype have shown overlapping as well as specific functions *in vivo* (2–5). The different roles *in vivo* of TRα1 and TRβ are mostly due to differences in cellular or tissue expression, with some exceptions (6–8). In this respect, TRβ1 is the predominant receptor subtype in liver, whereas in the heart and brain, the predominant receptor subtype is TRα1, which accounts for up to 80–85% of total T_3 binding (2, 9).

Selective targeting of TRβ has great therapeutic interest, in view of the role that TRβ plays in lipid metabolism (10, 11). TRβ-selective agonists are able to reduce blood levels of cholesterol, triglycerides, and lipoprotein(a) and may also reduce body weight (BW), without affecting the heart. The metabolic actions of several compounds, such as GC-1, GC-24, KB-141, and KB-2115 (12–15), have been extensively studied, although little attention has been paid to their effects on the brain (8, 16–19). Given the involvement of TRα1 in behavior (20, 21), TRα1 targeting in the brain might be of value in the treatment of anxiety and other psychiatric disorders. T_3 analogs able to be transported and act on the brain would also be valuable agents in the treatment of patients with monocarboxylate transporter 8 (MCT8) mutations (22). The goal of this work

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Abbreviations: *Atp2a2*, Sarcoplasmic reticulum ATPase; BW, body weight; *Dio1*, type 1 deiodinase; *Gsta3*, glutathion-S-transferase; *Hr*, hairless; Mct8 or MCT8, monocarboxylate transporter 8; *Myh6*, myosin heavy chain α; *Myh7*, myosin heavy chain β; *Nrgn*, RC3/neurogranin; *Nr1d1*, RevErbAα; *Ntf3*, neurotrophin 3; P, postnatal day; *Rasd2*, Rhes; *Syt12*, synaptotagmin 12; TR, thyroid hormone receptor.

was to examine whether the TRβ-selective T_3 agonist GC-24 (12, 19, 23) and the TRα1-selective agonist, CO23 (19, 24, 25), are active on T_3 targets in the brain.

Materials and Methods

Wistar rats bred in our animal facilities were used. Animals were under light- and temperature-controlled conditions (12-h light, 12-h dark cycle and 22 ± 2 C) and had free access to food and water. Animal procedures were supervised by institutional Ethic Committee, and in agreement with directives from the European Union. Euthyroid, hypothyroid, and hypothyroid rat pups treated with T_3 or the analogs were used. Hypothyroidism was induced by administering 0.02% 2-mercapto-1-methylimidazol (Sigma Chemical Co, St. Louis, MO) and 1% $KClO_4$ in the drinking water to the dams from gestational d 9 until the end of the experiment on postnatal d (P)16. T_3 was administered to the hypothyroid pups at a daily dose of 22.5 pmol/g of BW (15 ng/g) in PBS containing 0.1% BSA. GC-24 and CO23 were administered to the pups in the same solution also as single daily ip injections at the doses specified for each experiment. Treatments were started on P10, and the last dose was administered on P15. Twenty-four hours after the last injection, the animals were anesthetized and decapitated for PCR assays or perfused with 4% paraformaldehyde in 0.1 M phosphate buffer for *in situ* hybridization.

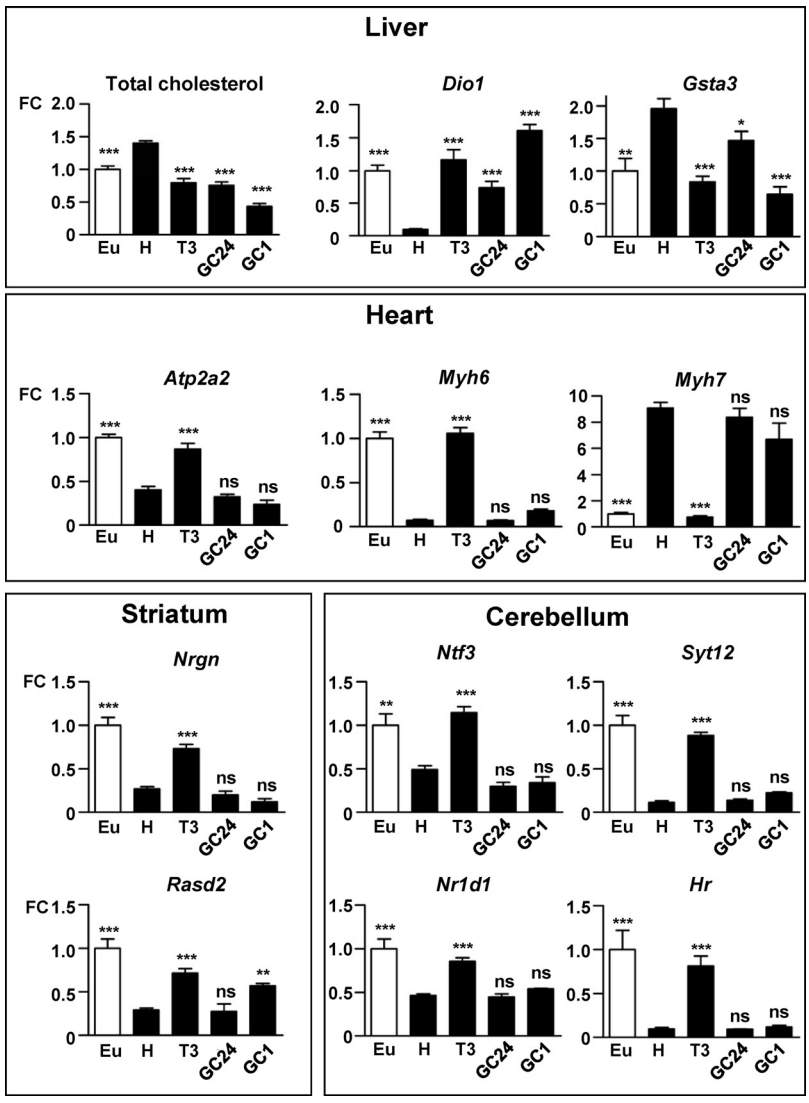

FIG. 1. Effects of T_3 and the TR synthetic agonists GC-24 and GC-1 on liver, heart, striatum, and cerebellum. *Black bars*, Each compound was administered to hypothyroid rats (H) as one daily injection of 22.5 pmol/g from P10 to P15. *Open bar*, Euthyroid animals. On P16, several end points of thyroid hormone action were measured. Typical TRβ responses, such as plasma cholesterol and liver *Dio1* and *Gsta3* mRNAs, were sensitive to hypothyroidism and drug administration. TRα-mediated responses, such as heart *Atp2a2*, *Myh6*, and *Myh7*, were sensitive to T_3 but insensitive to GC-1 or GC-24 treatment. Thyroid hormone-regulated striatal and cerebellar genes were insensitive to GC-24. Statistical comparisons are made to the hypothyroid group, so as to evaluate the difference between euthyroid (*open bar*) and hypothyroid animals and the effect of treatments *vs.* the untreated hypothyroid animals. See Supplemental Table 1 for multiple cross comparisons. Eu, Untreated euthyroid rats (n = 5); H, untreated hypothyroid rats (n = 5); T_3, T_3-treated H rats (n = 5); GC-24, GC-24-treated H rats (n = 7); GC-1, GC-1-treated H rats (n = 4). *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. ns, Not significant; FC, fold change.

RNA preparation and real-time PCR was performed as described (26), using TaqMan probes (Applied Biosystems, Foster City, CA) for genes previously shown to be sensitive to thyroid hormones: type 1 deiodinase (*Dio1*, Rn00572183_m1), glutathion-S-transferase (*Gsta3*, Rn00579867_m1), sarcoplasmic reticulum ATPase or sarco/endoplasmic reticulum calcium transporting ATPase (*Atp2a2*, Rn00568762_m1), myosin heavy chain α (*Myh6*, Rn00568304_m1) and myosin heavy chain β (*Myh7*, Rn00568328_m1), hairless (*Hr*, Rn00577605_m1), RevErbAα (*Nr1d1*, Rn00595671_m1), neurotrophin 3 (*Ntf3*, Rn00579280_m1), reelin (*Reln*, Rn00589609_m1), synaptotagmin 12 (*Syt12*, Rn00593706_m1), RC3/neurogranin (*Nrgn*, Rn00480741_m1), and ras homolog enriched in striatum (*Rasd2*, Rn00592054_m1). TRβ was also measured using a TaqMan probe (*Thrb*, Rn00562044_m1), and TRα1 was measured using SYBRGreen quantitative PCR with the forward primer 5′AGCTGCTGATGAAGGTGACTGA3′ and reverse primer 5′TGAGGCTTTAGACTTCCTGATCCT3′.

Total plasma cholesterol was measured using the Infinity Cholesterol kit supplied by Thermo Electron (Thermo Fisher Scientific, Auburn, AL). *In situ* mRNA hybridization analysis for *Nrgn*, *Rasd2* in the cerebrum, and *Hr* in the cerebellum was performed using methods previously described in detail (8). Primary neuronal cultures were established as previously described (26).

Data were analyzed with the GraphPad Prism 5 software (GraphPad, San Diego, CA). For the comparisons between groups, we used one-way ANOVA with Tukey′s test as *post hoc* test. All results are expressed as mean ± SEM. Statistical comparisons shown in most panels are made to the hypothyroid group, so as to evaluate the effect of hypothyroidism *vs.* the control and the effect of treatments *vs.* the hy-

pothyroid. Results on multiple comparisons of all groups are provided in Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://endo.endojournals.org.

Results

GC-24 is a TRβ-selective compound *in vivo* without effects on the brain

T_3 or GC-24 was administered to hypothyroid pups at the dose of 22.5 pmol/g, and thyroid hormone responses were studied in liver, heart, striatum, and cerebellum (Fig. 1). In agreement with the TRβ selectivity, GC-24 was active in liver but not in heart. First, plasma cholesterol, which in hypothyroid pups was elevated by 40% with respect to controls, decreased by 45% after treatment with T_3 or GC-24. For comparison, we also tested the TRβ selective compound GC-1 at the same molar dose. Treatment of hypothyroid rats with this compound reduced plasma cholesterol by 70%. As T_3 target genes, we measured the expression of *Dio1*, an up-regulated gene (27), and *Gsta3*, a down-regulated gene (28). *Dio1* was decreased in hypothyroid animals to less than 10% of control values. It then increased after treatment of hypothyroid rats with T_3 (12-fold), GC-24 (8-fold), or GC-1 (16-fold). Conversely, *Gsta3* was increased almost 2-fold in untreated hypothyroid livers and was decreased by T_3 (58%), GC-24 (25%), and GC-1 (68%). We found that GC-1 was more potent than T_3 or GC-24 on liver targets.

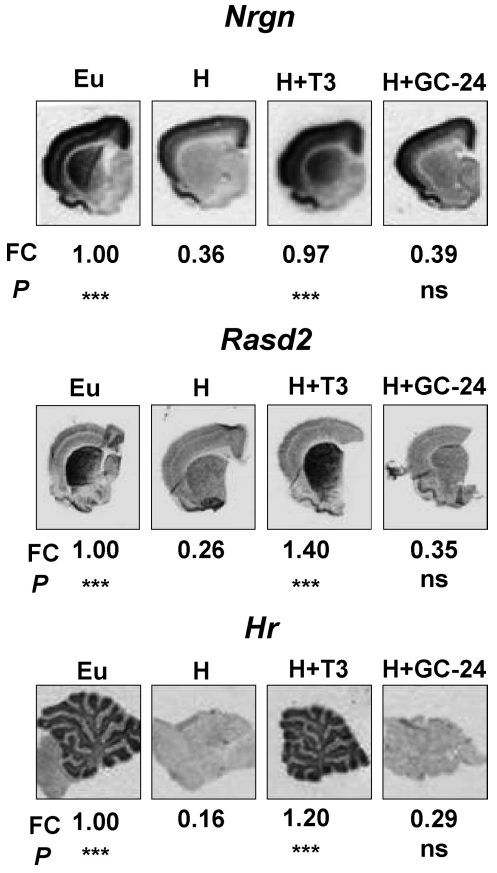

FIG. 2. *In situ* hybridization for *Nrgn*, *Rasd2* in the cerebrum, and *Hr* in the cerebellum in euthyroid rats, hypothyroid rats (H), and hypothyroid rats treated with one daily injection of 45 pmol/g BW T_3 or GC-24 for 6 d. The fold change (FC) and statistical significance (*P*) of differences respect to the hypothyroid sample are given after the quantification of slices from three animals per group. Eu, Untreated euthyroid; ns, not significant.

In the heart, the thyroid hormone responsive genes *Atp2a2* (29) and *Myh6* (30) were decreased by hypothyroidism by 60 and 90%, respectively. T_3 treatment of hypothyroid rats induced these genes by 2- and 15-fold, respectively, but neither GC-24 nor GC-1 were active. *Myh7*, which increased 9-fold after hypothyroidism, decreased to euthyroid levels after T_3, but GC-1 nor GC-24 were active. As for the brain, we measured the effects on the striatum and the cerebellum. In the striatum, hypothyroidism reduced the expression of *Nrgn* and *Rasd2* by 75%, and T_3 administration resulted in a 2.5-fold increase of both genes, as shown before (8). Also, as reported previously (8), GC-1 increased *Rasd2* expression but was without effect on *Nrgn*. In contrast, GC-24 was without effect on either gene. These results confirm previous findings that regulation of *Nrgn* by T_3 was mediated by TRα1, whereas *Rasd2* was also under the influence of TRβ (8).

Known target genes of thyroid hormone in the cerebellum, such as of *Ntf3* (31), *Syt12*, *Hr* (32), and *Nr1d1* (8), were not influenced by treatment with GC-1 or GC-24. The lack of effect of GC-24 in comparison with T_3 was also confirmed by *in situ* hybridization (Fig. 2) and by analyzing its effect on migration of cerebellar granule cells (data not shown).

In view of the lack of effect of GC-24 on *Rasd2*, in contrast to GC-1, we checked the effect of higher doses. GC-24 was administered to the hypothyroid pups in incremental doses of 22.5, 45, 90, and 225 pmol/g for 6 d and compared with 22.5 pmol/g of T_3. The results are shown in Fig. 3. GC-24 induced liver responses, such as cholesterol reduction, *Dio1* up-regulation, and *Gsta3* down-regulation at all the doses, whereas it had no effect on *Nrgn* and *Rasd2* in the striatum or *Hr* in the cerebellum. In addition, cerebellar *Rln* mRNA, which is down-regulated by T_3 through TRβ (8), was unaltered by GC-24 treatment (Supplemental Fig. 1). Despite the lack of action *in vivo*, GC-24, as well as GC-1, was able to induce Hr in cerebral cortex neurons in culture (Fig. 4).

CO23 acts through TRα and TRβ *in vivo* and is active in the brain

In preliminary experiments (data not shown), a dose of CO23 of 22.5 pmol/g had no effect on plasma cholesterol,

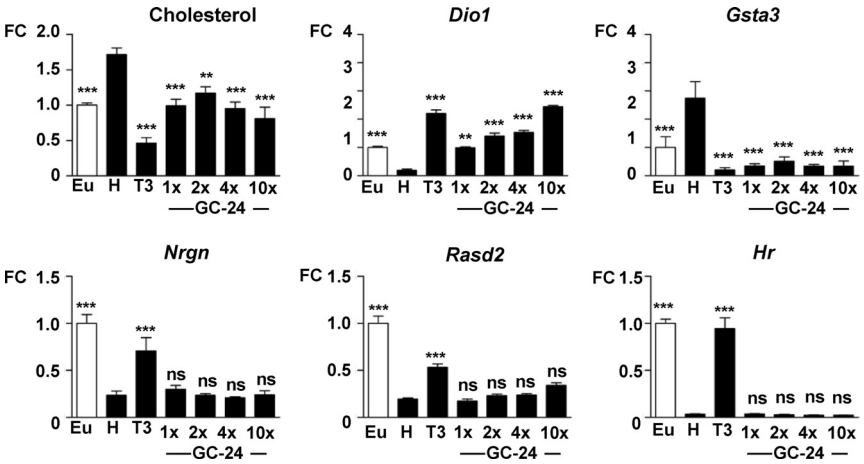

FIG. 3. Effects of high doses of GC-24. *Black bars*, GC-24 was administered to hypothyroid rats (H) as single daily injections for 6 d at the doses of 22.5, 45, 90, and 225 pmol/g BW. These doses represent 1×, 2×, 4×, and 10× the T_3 dose used, 22.5 pmol/g BW. Although GC-24 was active on the liver, there was no effect of on striatal or cerebellar genes. Statistical comparisons are made to the hypothyroid group, so as to evaluate the difference between euthyroid (*open bar*) and hypothyroid animals, and the effect of treatments *vs.* the hypothyroid animals. See Supplemental Table 1 for multiple cross comparisons. Eu, Untreated euthyroid rats; H, untreated hypothyroid rats. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; n = 6 in all groups. ns, Not significant; FC, fold change.

liver *Dio1* and *Gsta3*, heart *Myh6*, *Myh7*, and *Atp2a2*, or cerebellum *Hr* and *Ntf3*. The same dose of T_3 normalized all these parameters, in a similar way as described in Fig. 1. The relative activity of CO23, compared with T_3, was then evaluated in cerebellar granule neurons in primary cultures (Fig. 4). Addition of 10 nM T_3 to the cultures increased *Hr* mRNA almost by 20-fold and *Ntf3* by 3-fold. CO23 was used at incremental doses of 10, 100, and 500 nM. Significant responses were obtained with the two higher doses. The results suggested that CO23 was at least 50 times less active than T_3 on isolated neurons.

Based on the above data, we used CO23 for *in vivo* experiments at doses of 0.04, 0.8, 2.5, and 5.0 nmol/g, and the results are shown in Fig. 5. After administration to hypothyroid rats, cholesterol was not changed by the lower dose, whereas the 0.8 nmol/g dose had a similar effect as 22.5 pmol/g T_3 (≈50% cholesterol reduction). Higher CO23 doses were equally effective. T_3 increased Dio1 mRNA 10-fold after administration to hypothyroid rats. CO23 had no effect at the lowest dose of 0.04 nmol/g and increased Dio1 mRNA 5-, 10-, and 15-fold at the doses of 0.8, 2.5, and 5 nmol/g, respectively. Therefore, for liver *Dio1*, a dose of 2.5 nmol/g of CO23 was equivalent to 22.5 pmol/g of T_3. The effect on *Gsta3* was similar than on cholesterol, with the dose of 0.8 nmol/g having a similar effect as 22.5 pmol T_3 (65% Gsta3 mRNA reduction over hypothyroid levels).

In the heart, 2.5 nmol/g of CO23 was equivalent to 22.5 pmol/g of T_3 on *Atp2a2* induction (1.8-fold in each case), and the effect was still higher (2.4-fold) with the highest dose. For the myosin heavy chains, 2.5 nmol/g of CO23 was also equivalent to 22.5 pmol/g T_3 in the induction of *Myh6* (14-fold). T_3 treatment decreased by 95% the elevated levels of Myh7 mRNA of hypothyroid rats. Although 0.8 nmol/g of CO23 induced a 65% decreased, it required the highest dose to elicit a similar effect than 22.5 pmol T_3.

In the brain, the lowest effective dose of CO23 was 0.8 nmol for *Syt12* induction (3-fold). For *Nrgn*, *Rasd2*, *Ntf3*, and *Nr1d1*, the lowest dose that resulted in significant induction was 2.5 nmol/g. The least sensitive gene was *Hr*, which required the highest dose of CO23 with about half the effect of T_3 (5.5- *vs.* 9.7-fold). In the cerebellum, *Rln* was insensitive to CO23 (Supplemental Fig. 1).

Finally we considered the possibility that some of the observations related to the effects of T_3 analog administration to hypothyroid pups was at least partially influenced by altered expression of the TRs by hypothyroidism. Therefore, we measured the relative amounts of TRα1 and TRβ mRNAs in the striatum of euthyroid and hypothyroid pups. Hypothyroidism increased TRα1 mRNA by 48% ($P = 0.02$) and decreased TRβ mRNA by 25% ($P = 0.013$) in relation to the euthyroid values (Supplemental Fig. 2).

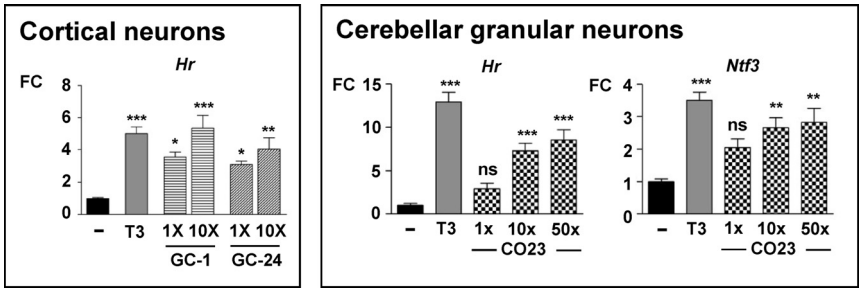

FIG. 4. Effects of agonists of TR on primary cultures of cortical and cerebellar neurons. Primary cultures were established from neonatal rat cortex (*left panel*) or cerebellum (*right panel*) and incubated in the absence or presence of T_3 1× (10 nM), GC-1, GC-24, or CO23 at the concentrations indicated expressed as fold over the T_3 concentration. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; n = 6 in all groups. ns, Not significant; FC, fold change.

Discussion

In this work, we compared the pharmacological activity of two thyroid hormone analogs, GC-24 and CO23, previously described as being TRβ- and TRα1-selective compounds, respectively (12, 23–25). Our primary inter-

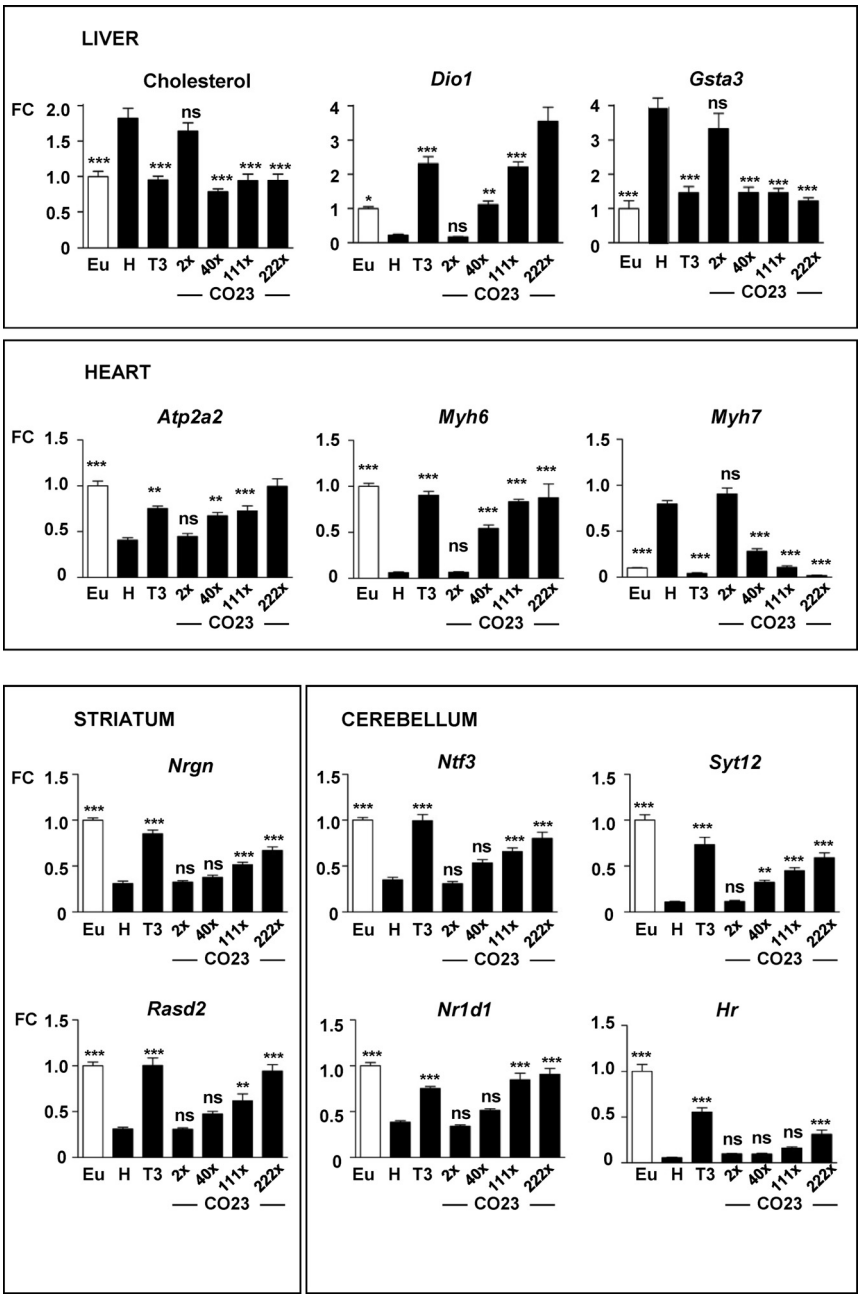

FIG. 5. Effect of CO23 on liver, heart, striatum, and cerebellum. *Black bars*, Hypothyroid rats were given one daily dose of 22.5 pmol/g BW T_3 for 6 d or incremental doses of CO23: 0.04, 0.8, 2.5, and 5.0 nmol/g BW (2×, 40×, 111×, and 222×, respectively, the molar dose of T_3 used in the experiment). Statistical comparisons were made to the hypothyroid group, so as to evaluate the difference between euthyroid (*open bar*) and hypothyroid animals and the effect of treatments *vs.* the hypothyroid animals. See Supplemental Table 1 for multiple cross comparisons. Eu, Untreated euthyroid rats; H, untreated hypothyroid rats; n = 8 in all groups. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. ns, Not significant; FC, fold change.

est was to analyze the effects of these compounds in the rat brain, which is often neglected in studies on the biological activity of thyroid hormone analogs.

As previously reported, GC-24 was found to have TRβ selectivity *in vivo*. Thyroid hormone-sensitive liver responses, such as plasma cholesterol, and expression of *Dio1* and *Gsta3* genes were sensitive to GC-24. In contrast to the liver, the heart genes *Atp2a2*, *Myh6*, and *Myh7* were not sensitive to GC-24 or GC-1, as was expected, because heart responses to thyroid hormone are mediated in large part by TRα1 (33).

In contrast to the liver, GC-24 was not active in the brain. We have previously shown that GC-1 has limited activity in brain (8, 17, 18), especially on genes that in the cerebellum are expressed in the granular cells, which express predominantly TRα1. GC-24 was also not active on cerebellar genes, but GC-1 and GC-24 differed in the induction of striatal *Rasd2*. In an earlier study (8), we found that GC-1 was able to induce *Rasd2* in the striatum but not *Nrgn*, even if these two genes are expressed in the same kind of cells, the medium γ-aminobutyric acid-ergic interneurons. We confirmed this result and show here that in contrast to GC-1, GC-24 was not active on *Rasd2*. In addition, GC-24 had no activity on cerebellar *Rln*, a gene also sensitive to GC-1 (8). The results indicate that GC-24 has no activity in the brain, which is likely due to restricted entry through the blood-brain barrier. Another factor that may contribute to the lack of activity of TRβ-selective compounds in the brain is the increase of the TRα1/TRβ ratio that occurs in hypothyroidism. An increase of TRα1 expression of 40% and decrease TRβ of 25%, as found in this work, may facilitate TRα1 responses, especially in the setting of restricted brain accumulation of the agonists.

As reported previously by Ocasio and Scanlan (24), CO23 showed no preference for TR subtype in a competitive ^{125}I-T_3 binding assay *in vitro*. Despite this, it displayed TRα-selective properties in cultured cells and *in vivo*. In cultured cells, it had 5-fold higher activity through TRα over TRβ in the activation of a direct repeat 4 thyroid responsive element in U2OS and HeLa cells (24). *In vivo* studies on the comparative actions of T_3, GC-1, and CO23 on metamorphosis showed that CO23 had similar effects as T_3 on hind limb growth but required 7-fold higher concentrations (16, 24). CO23 was much less active in tail, gills, and head resorption. CO23 had also similar effects as T_3 on cell proliferation in the ventric-

ular and subventricular zones during neurogenesis, whereas GC-1 and GC-24 had no effect (16). It was also observed that the combination of CO23 and GC-1 was able to mimic T_3 action completely. These observations support the TRα selectivity of CO23.

Despite its TRα subtype selectivity on tadpoles, when administered to rats, CO23 was active in tissues expressing preferentially either TRα1 or TRβ. For example, CO23 was found to activate thyroid hormone-responsive liver and heart genes in a similar way as T_3, unlike the TRβ-selective GC-1, which acts similarly to T_3 in the liver and lacks activity on the heart. These differences in TR selectivity might be related to differences in tissue distribution of the T_3 analogs. For example, the accumulation of GC-1 in liver is 17-fold of that in the heart, and this contributes to the *in vivo* organ selectivity (10, 11). High doses of CO23 needed to be used *in vivo*, due to the fact that the relative potency of CO23, in comparison with T_3, was relatively low. In cultured cerebellar granular cells, it was estimated that the potency of CO23 was more than 50-fold lower that of T_3. *In vivo*, high doses 100- to 200-fold of that of T_3 had to be used. Therefore, it may be argued that by using high doses of the agonist, we increase the chance of having an effect in liver through activation of TRα1, if CO23 accumulates preferentially in this organ.

We have no data on tissue CO23 distribution. However, it seems unlikely that the effects of CO23 on plasma cholesterol and liver *Dio1* are due to preferential accumulation of CO23 in the liver and subsequent activation of TRα1. The cholesterol response to thyroid hormone is totally dependent on TRβ. TRβ knockout mice are resistant to T_3-induced reduction in cholesterol, a defect that cannot be rescued by overexpressing TRα1 by 6-fold in the liver (34). On the other hand, *Dio1* expression shows a zonal distribution in the liver, with low mRNA concentration around the periportal area and increasing toward the pericentral area, where it reaches the highest level. This pattern of expression follows closely that of TRβ1 (35). In addition, only extremely high doses of T_3 administered to TRβ-deficient mice were able to weakly induce *Dio1* (36). Therefore, we think that the liver responses to CO23 are due to TRβ1 stimulation.

Taken together, the response of liver, heart, and brain suggests that CO23 was similarly active on TRα1 and TRβ, but a subtle preference for TRα1 cannot be discarded. In fact, the lack of response of *Rln* may suggest that CO23 entry into the brain is not as efficient as in the liver, precluding the activation of TRβ. Despite this, our results indicate that CO23 was able to be transported through the blood-brain barrier and to cross the neuronal cell membrane. Although we did not identify the specific transporter involved (37), it is unlikely that it uses the Mct8 transporter, which has extremely tight selectivity for T_4 and T_3. The reason for the apparent lack of receptor subtype selectivity of CO23 in rodents as compared with metamorphosing tadpoles is unknown at present. The lack of TR selectivity in mammalian tissues would make this compound less interesting as a therapeutic agent. On the other hand, its ability to cross the blood-brain barrier would make it worth to investigate its capacity to act on the brain in the absence of the specific T_4 and T_3 transporter Mct8 (22).

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