

Statins and Downstream Inhibitors of the Isoprenylation Pathway Increase Type 2 Iodothyronine Deiodinase Activity

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The type 2 iodothyronine selenodeiodinase (D2) is a critical determinant of local thyroid signaling, converting T_4 to the active form T_3 at the cytoplasmic face of the endoplasmic reticulum, thus supplying the nucleus with T_3 without immediately affecting circulating thyroid hormone levels. Although inhibitors of the cholesterol synthesis/isoprenylation pathway, such as hydroxy-methylglutaryl-coenzyme A reductase inhibitors (statins) have been shown to down-regulate selenoproteins via interruption of normal selenocysteine incorporation, little is known about the effect of statins on D2. Here, we report that statins and prenyl transferase inhibitors actually increase D2 activity in cells with endogenous D2 expression. Although we confirmed that lovastatin (LVS) decreases the activity of transiently expressed D2 in HEK-293 cells, the prenyl transferase inhibitors increase activity in this system as well. LVS treatment increases endogenous *Dio2* mRNA in MSTO-211H cells but does not alter transiently expressed *Dio2* mRNA in HEK-293 cells. The prenyl transferase inhibitors do not increase *Dio2* mRNA in either system, indicating that a posttranscriptional mechanism must exist. Cotreatment with LVS or the prenyl transferase inhibitors with the proteasome inhibitor MG-132 did not lead to additive increases in D2 activity, indirectly implicating the ubiquitin-proteasomal system in the mechanism. Finally, C57BL/6J mice treated with LVS or farnesyl transferase inhibitor-277 for 24 h exhibited increased D2 activity in their brown adipose tissue. These data indicate that statins and downstream inhibitors of the isoprenylation pathway may increase thyroid signaling via stimulation of D2 activity. (*Endocrinology* 153: 4039–4048, 2012)

An emerging concept in the field of thyroid hormone action is that metabolism can be regulated on a tissue-specific basis by local mechanisms controlling thyroid hormone signaling (1). Clinically, this concept is important because it reminds us that measurement of thyroid hormone levels in the blood, *i.e.* systemic thyroid hormone levels, may not reflect dynamic changes in the intensity of thyroid signaling occurring in specific tissues. The therapeutic implication is that pharmacologic regulation of local thyroid hormone signaling could be a potential strategy for the treatment of obesity and the metabolic syndrome.

The type 2 iodothyronine selenodeiodinase (D2) is a critical determinant of local thyroid hormone signaling, acting to convert T_4 to the active form T_3 at the cytoplasmic face of the endoplasmic reticulum, thus supplying the nucleus with T_3 without immediately altering circulating thyroid hormone levels (2). D2 is one of three iodothyronine deiodinases: the inactivating D3 decreases the intensity of local thyroid hormone signaling, and the bifunctional activating/inactivating D1 plays less of a role in local control (3). In recent years, an important metabolic role for D2 has emerged, most notably from a study dem-

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Abbreviations: AOSMC, Aortic smooth muscle cell; BAT, brown adipose tissue; D2, type 2 iodothyronine selenodeiodinase; FBS, fetal bovine serum; FTase, farnesyl transferase; FTI, FTase inhibitor; GGTase, geranylgeranyl transferase; GGTI, GGTase inhibitor; HMG-CoA, hydroxy-methyl-glutaryl-coenzyme A; LVS, lovastatin; MLL, mevalonolactone; PVS, pravastatin; sec-tRNA, selenocysteine tRNA; UCP-1, uncoupling protein-1; VDU-1, von Hippel-Lindau interacting deubiquitinating enzyme-1; VDU-2, von Hippel-Lindau interacting deubiquitinase-2.

onstrating that bile acids increase the energy expenditure of brown adipose tissue (BAT) via activation of the G protein-coupled receptor TGR5; the mechanism requires downstream transcriptional up-regulation of D2 (4). The thiazolidinedione drug pioglitazone has also been shown to up-regulate D2 in skeletal myocytes (5). Given that D2 is expressed in a number of metabolically important tissues, including BAT and vascular smooth muscle, these discoveries have established D2 as an important target in the effort to achieve pharmacologic tissue-specific control of metabolism (6–12).

D2 is dynamically regulated, with a short half-life on the order of 20–40 min secondary to tight control of activity and stability via the ubiquitin-proteasomal system (13–17). Current models suggest that D2 exists in a pool of active/unubiquitinated and inactive/ubiquitinated forms (18). The ubiquitination of D2 can be accelerated by increasing substrate concentration, *i.e.* increased D2 catalysis is linked to an accelerated rate of D2 ubiquitination; the covalent attachment of ubiquitin inactivates the enzyme and ultimately targets it for proteasomal degradation (15, 19, 20).

A largely unexplored potential mechanism for down-regulating D2 is by interruption of the isoprenylation pathway. The deiodinases are selenoproteins, each having the rare amino acid selenocysteine in their active center (3). Proper selenoprotein synthesis requires a specific selenocysteine tRNA (*sec*-tRNA), and for normal function, this *sec*-tRNA must undergo isoprenylation, *i.e.* addition of an isoprene to an adenine nucleoside near the anticodon (21). For proteins, the isoprenylation machinery attaches a farnesyl or geranylgeranyl moiety to a cysteine residue in the carboxy terminus. The pathways for farnesylation and geranylgeranylation are branches of the cholesterol synthesis pathway, with the rate-limiting enzyme being hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) reductase, which converts HMG-CoA to mevalonolactone (MLL), a precursor for both cholesterol and other isoprenoids (see figure 7 below). Inhibitors of HMG-CoA reductase, such as the “statin” class drugs, can therefore down-regulate the synthesis of selenoproteins, and this mechanism has been proposed as an explanation for some of their clinical effects (22, 23).

At the same time, studies have suggested that inhibition of the isoprenylation pathway also leads to a decrease in proteasome activity (24–26). Because D2 is degraded in the proteasome, this might be expected to increase D2 activity; D2-expressing cells treated with the proteasome inhibitor MG-132 exhibit a large increase in D2 activity (17). One previous study examined the effect of statins on deiodinases, finding that lovastatin (LVS) interferes with the synthesis of transiently expressed D1 and D2 in Chi-

nese hamster ovary cells (21). Thus, the current model holds that isoprenylation inhibition decreases deiodinase activity.

In this study, we sought to further characterize the potential for statins to alter thyroid signaling via their effects on the deiodinases. Our preliminary findings were remarkable: both statins and downstream inhibitors of the isoprenylation pathway significantly increased, rather than decreased, the activity of endogenously expressed D2 in cultured MSTO-211H cells. We therefore undertook the investigations described herein to understand how D2 activity could be induced by isoprenylation pathway inhibitors.

Materials and Methods

Reagents

Unless otherwise specified, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). Outer ring-labeled ^{125}I -T₄ (specific activity 4400 Ci/mmol), ^{125}I -T₃ (specific activity 2200 Ci/mmol), and ^{125}I -rT₃ (specific activity 2200 Ci/mmol) were purchased from PerkinElmer (Boston, MA) and purified on LH-20 columns before use. LVS was converted to its active carboxylate form following published methods (27, 28). Briefly, 25 mg of the lactone form were dissolved in 500 μl of 100% ethanol, alkalinized by adding 250 μl of 0.6 M NaOH, incubated at 50 C for 2 h, neutralized with 0.4 M HCl (pH 7.5), and brought to 10 mM with H₂O.

Cell culture

Unless otherwise noted, cell lines were purchased from the American Type Culture Collection (Manassas, VA) and were cultured in a humidified atmosphere with 5% CO₂ at 37 C. HEK-293 (immortalized human embryonic kidney), HepG2 (human hepatocellular carcinoma), and SK-N-AS (human neuroblastoma) cells were cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS). MSTO-211H (human mesothelioma) cells were cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS. Primary human aortic smooth muscle cells (AOSMC) were purchased from Lonza (Basel, Switzerland). AOSMC were cultured using media and reagents from the manufacturer, supplemented with sodium selenite to 100 nM, and assayed before 15 doublings as per the manufacturer's instructions.

Transfection studies

Transient transfection was performed in HEK-293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Twenty-four hours before transfection, cells were plated in six-well dishes and allowed to reach 75% confluence. The ratio of DNA to Lipofectamine 2000 reagent used was 1:3. After a 12-h incubation, media were replaced by the cell propagation media (DMEM plus 10% FBS), and drug treatments were begun. Transfection efficiency was controlled via β -galactosidase cotransfection and ortho-Nitrophenyl- β -galactoside spectrophotometric assay as previously described (18).

For transient expression, constructs for full-length wild-type human *Dio2* coding sequence (with the rat Selenoprotein P selenocysteine insertion sequence) in mammalian D10 expression vector were kind gifts from Antonio C. Bianco (University of Miami) (for details, see reference Ref.17). For *Dio2* promoter studies, a 6.9-kb 5'-flanking region human *Dio2* construct with firefly luciferase reporter was also a gift from the Bianco lab and was performed as described previously (29). Luciferase activity was quantified in 1×10^5 cells using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) accordingly to the manufacturer instruction and detected by Veritas Microplate Luminometer (Turner BioSystems, Sunnyvale, CA). All experiments were conducted in triplicate.

RNA isolation and mRNA quantitation

Total cellular RNA was isolated using TRIzol reagent (Invitrogen) as per manufacturer's instructions. RNA was then treated with TURBO DNA-free (Ambion, Austin, TX) following the manufacturer's instructions. Reverse transcription of 1 μ g of total RNA was performed using the High Capacity cDNA kit (Applied Biosystems/Invitrogen, Carlsbad, CA). Abundance of specific mRNA molecules was analyzed via quantitative SYBR Green-based real-time PCR using the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Relative quantitation was determined using the standard curve method and the iCycler software and normalized for the expression of cyclophilin A. Relative mRNA quantitation was also confirmed using TaqMan gene expression assays following the manufacturer's instructions in a StepOnePlus real-time PCR system (Applied Biosystems/Invitrogen). Relative quantitation was analyzed via δ cycle threshold method (30).

Deiodinase assays

D1, D2, and D3 assays were performed as previously described (5, 30). D3 assays were performed as previously described using an ACQUITY system Ultra High Performance Liquid Chromatography (Waters Corp., Milford, MA) (31). Endogenous deiodinase activities for vehicle-treated cells ranged from 18 to 22 pmol/min[chmep]mg for D1 in HepG2 cells, 3 to 6 fmol/min[chmep]mg for D2 in MSTO-211H cells, 0.05 to 0.5 fmol/min[chmep]mg for D2 in AOSMC, and 0.3 to 0.6 fmol/min[chmep]mg for D3 in SK-N-AS cells. All deiodinase assays for *in vitro* studies were performed with each data point in duplicate, and with each experimental group having three to six samples. Mouse BAT D2 activity was approximately 0.1 fmol/min[chmep]mg in wild-type mice at room temperature.

Animal studies

Animal studies were approved by the Institutional Animal Care and Use Committee of the University of Miami in compliance with National Institutes of Health standards. Mice were treated with a standard mouse chow diet and water *ad libitum* under a 12-h light, 12-h dark cycle. All experiments were conducted in 8- to 10-wk-old male C57BL/6J mice obtained from The Jackson Laboratory (Bar Harbor, ME). Activated LVS and farnesyl transferase (FTase) inhibitor (FTI)-277 were diluted in PBS and administered via ip injection at a dose of 10 mg/kg body weight, with a second dose given 12 h after the first. At the end of the 24-h treatment period (12 h after the second dose), mice were euthanized by CO₂ asphyxiation. BAT samples were col-

lected and immediately stored in liquid nitrogen. Frozen samples of BAT samples were processed for D2 activity assay or mRNA analysis as previously reported (32). Protease inhibitor cocktail (catalog no. S8820–20TAB; Sigma-Aldrich) was added to the assay buffer before processing. Experimental groups had four mice, and the experiment was performed three times.

Statistical analysis

Data were analyzed by Student's *t* test or one-way ANOVA with Newman-Keuls *post hoc* testing when multiple comparisons were made, using GraphPad PRISM 5 software (GraphPad, La Jolla, CA). Significance was held at $P < 0.05$ (two-tailed).

Results

Regulation of endogenously expressed deiodinase activities by LVS

To characterize the effect of statins on endogenous deiodinase activities, we exposed cells endogenously expressing D1 (HepG2), D2 (MSTO-211H), or D3 (SK-N-AS) to 10 μ M LVS for 8 h and then measured the appropriate deiodinase activity (Fig. 1). Remarkably, endogenous D2 activity was significantly increased, whereas endogenous D1 and D3 activities were not significantly altered compared with vehicle. Given the longer half-lives of D1 and D3, we repeated these assays at 16 and 24 h but found no differences compared with vehicle (data not shown). These data demonstrate that LVS has a stimulatory effect on endogenous D2 activity that would not have been predicted based on previous studies in which D2 was transiently expressed.

Characterization of the stimulatory effect of LVS on D2 activity

To further characterize the positive effect of LVS on D2 activity, we performed dose-response and time-course studies in MSTO-211H cells (Fig. 2). Addition of LVS (2.5–10 μ M) for 8 h resulted in a marked dose-dependent

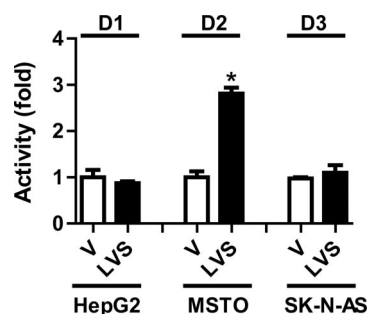


FIG. 1. Effect of LVS treatment on endogenous deiodinase activities. D1 activity in HepG2 cells, D2 activity in MSTO-211H, or D3 activity in SK-N-AS cells was measured after 8 h of incubation with LVS (10 μ M) or vehicle (V). Bars represent mean fold activity relative to vehicle \pm SEM; *, $P < 0.05$ vs. vehicle. Typical experiments are shown out of five experiments for D1, 10 experiments for D2, and six experiments for D3.

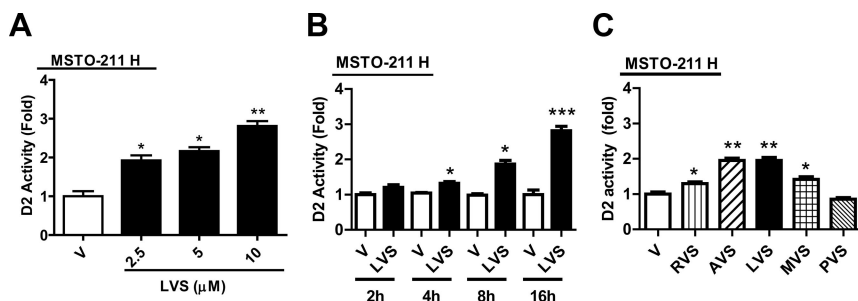


FIG. 2. Characterization of LVS effect on D2 activity in MSTO-211H cells. A, Dose response of D2 activity in cells treated with LVS (μM as indicated, 8 h). B, Time course of D2 activity in cells treated with 10 μM LVS (hours as indicated). C, Effect of various statins (10 μM , 8 h treatment) on D2 activity. Data presented as mean fold activity relative to vehicle \pm SEM; *, $P < 0.05$ vs. vehicle (V); **, $P < 0.01$ vs. vehicle; ***, $P < 0.01$ vs. all other groups. RVS, Rosuvastatin; AVS, atorvastatin; MVS, mevastatin; PVS, pravastatin. All experiments performed in triplicate; typical experiments shown.

increase in D2 activity (Fig. 2A). The increase in D2 activity was detectable in 4 h with 10 μM LVS (Fig. 2B). Incubations longer than 16 h were not associated with further increases in activity (data not shown). To determine whether this was a statin class effect, we tested other statins (10 μM for 8 h), including atorvastatin, mevastatin, rosuvastatin, and pravastatin (PVS) (Fig. 2C). With the

exception of PVS, the other statins also increased D2 activity, indicating that this is a class effect.

The isoprenylation pathway and D2

Given that statins inhibit HMG-CoA reductase, we hypothesized that the isoprenylation pathway is relevant with regards to the stimulatory effect on D2. To test this hypothesis, we exposed MSTO-211H cells to LVS with or without the downstream product of HMG-CoA reductase, MLL (100 μM), for 8 h (Fig. 3A). Cotreatment with LVS and the downstream product of the enzyme (MLL) led to a loss of the stimulatory effect on D2 activity, indicating that the upstream blockade of HMG-Co reductase leads to downstream effects important for D2 regulation.

Next, we tested whether direct inhibitors of the major downstream branch-point enzymes FTase and geranylgeranyl transferase (GGTase) would also increase D2 activity (Fig. 7). Dose-dependent increases in D2 activity were found for inhibitors of FTase, FTI-277 and FPT-II, with maximal increases at 10 μM (Fig. 3B). The GGTase inhibitor (GGTI)-298 did not significantly increase D2 activity in MSTO-211H cells, but GGTI-2147 did significantly increase D2 activity at 20 μM . Given that FTI-277 and GGTI-2147 had strong positive effects, subsequent MSTO-211H studies used only these prenyl transferase inhibitors. These data confirm that downstream inhibition of the isoprenylation pathway is sufficient to induce D2 in MSTO-211H cells and establish that blockade of either pathway is sufficient.

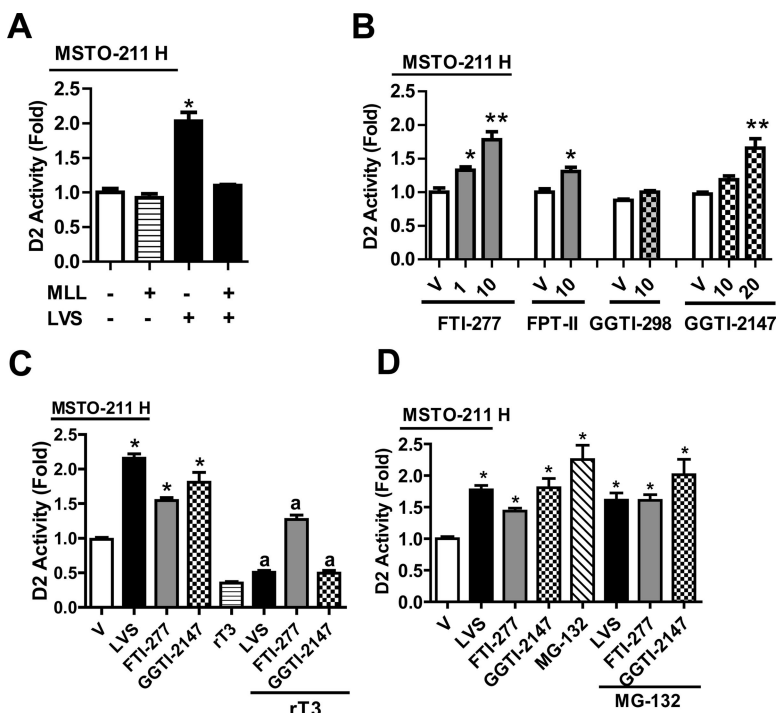


FIG. 3. Cholesterol-synthesis/isoprenylation pathway inhibition and D2 activity in MSTO-211H cells. A, D2 activity in cells treated with 10 μM LVS, 100 μM MLL, or a combination of both drugs for 8 h. B, Dose response of D2 activity in cells treated for 8 h with FTI-277, FPT-II, GGTI-298, or GGTI-2147 (dose in μM). C, D2 activity after 8 h of treatment with pathway inhibitors (10 μM) with or without rT_3 (1 μM) as indicated. D, D2 activity after 8 h of treatment with pathway inhibitors (10 μM) alone and in combination with 1 μM MG-132 as indicated. A–D, Data presented as mean fold activity relative to vehicle (V) \pm SEM; *, $P < 0.05$ vs. vehicle; **, $P < 0.05$ vs. vehicle and lower doses of same drug; ^a, $P < 0.05$ vs. rT_3 alone and drug alone. All experiments performed in triplicate; typical experiment shown.

Ubiquitin-proteasomal regulation of D2 vs. isoprenylation inhibitors

Next, we investigated whether the inhibitors disrupt the ubiquitination and/or proteasomal degradation of endogenously expressed D2. We first examined ubiquitination by treating MSTO-211H cells with the inhibitors in the presence or absence of 1 μM rT_3 ; this dose of rT_3 has been previously shown to strongly promote substrate-accelerated ubiquitination and thus inactivation of D2 (33). As expected, rT_3 was associated with strong suppression of

D2 activity (Fig. 3C). When rT_3 was combined with LVS or GGTI-2147, the net D2 activity was intermediate between the levels seen with each drug alone and also lower than vehicle (being about ~25% higher than with rT_3 alone). When rT_3 was combined with FTI-277, D2 activity was also intermediate compared with either drug alone, although in this case, the net activity was still slightly higher than vehicle. These equivocal results do not rule out partial inhibition of substrate-accelerated ubiquitination by the isoprenylation inhibitors. We measured mRNA expression of the deubiquitinases von Hippel-Lindau interacting deubiquitinating enzyme-1 (VDU-1) and von Hippel-Lindau interacting deubiquitinase-2 (VDU-2) after treatment with the inhibitors, finding no significant change in MSTO-211H-treated cells relative to vehicle (LVS, 0.9 ± 0.3 VDU-1 and 1.19 ± 0.3 VDU-2; FTI-277, 1.48 ± 0.5 VDU-1 and 1.56 ± 0.4 VDU-2; and GGTI-2147, 0.53 ± 0.5 and 0.57 ± 0.5 ; all not significant).

To test whether the inhibitors increase D2 activity via inhibition of the proteasome, MSTO-211H cells were incubated with these drugs in the presence of the proteasome inhibitor MG-132. Exposure to MG-132 alone increased D2 activity as expected (Fig. 3D). Combined treatment with the inhibitor drugs and MG-132 resulted in no further increase in endogenous D2 activity (Fig. 3D). The absence of either an additive or synergistic effect provides indirect support for the hypothesis that both statins and the downstream isoprenylation inhibitors increase D2 activity by interfering with proteasomal degradation.

Effect of isoprenylation inhibitors on transiently expressed D2 in HEK-293 cells

The only previous study of statins and deiodinases reported that LVS decreases the activity of transiently expressed D2, but no previous data exists regarding the downstream inhibitors of isoprenylation and D2 (21). Thus, we characterized the effects of LVS, FTI-277, GGTI-298, and GGTI-2147 in HEK-293 cells transiently expressing human D2. Consistent with the previous study, LVS treatment lead to a decrease in transiently expressed D2 activity (Fig. 4A); this stands in contrast to the results with cells endogenously expressing D2. FTI-277 ($10 \mu\text{M}$), GGTI-298 ($10 \mu\text{M}$), and GGTI-2147 (at $20 \mu\text{M}$) treatment all increased transiently expressed D2 activity, indicating that downstream inhibition of the pathway still has the same positive effect even for transiently expressed D2.

HEK-293 cells were also used to test whether the inhibitors could activate the *Dio2* (D2 gene) promoter: when cells transiently expressing a human *Dio2*-promoter/luciferase reporter construct were treated with the inhibitors, no promoter activation was seen (Fig. 4B). To ensure that the stimulatory effect of the inhibitors in HEK-

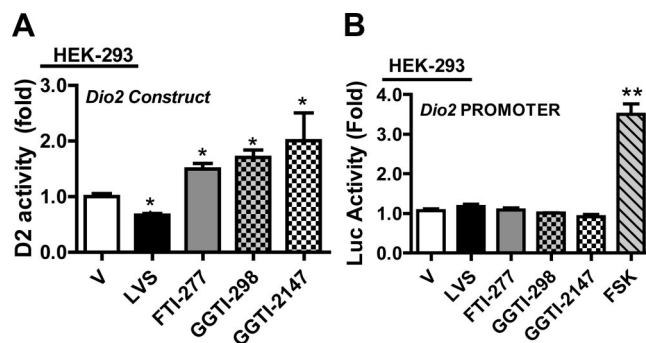


FIG. 4. Effect of isoprenylation inhibition on transiently expressed D2 in HEK-293 cells. A, D2 activity in HEK-293 cells transiently expressing D2 after 8 h of treatment with drugs as indicated ($10 \mu\text{M}$). B, Luciferase activity in HEK-293 cells transiently expressing a human *Dio2*-promoter/luciferase reporter construct after 8 h of treatment with the drugs as indicated ($10 \mu\text{M}$) or forskolin (FSK) positive assay control. A and B, Data presented as mean fold activity normalized for transfection efficiency via β -galactosidase activity \pm SEM; *, $P < 0.05$ vs. vehicle (V); **, $P < 0.01$ vs. vehicle. All experiments performed in triplicate; typical experiments are shown.

293 cells was not related to a direct effect on the D2/luciferase vector, HEK-293 cells transiently expressing a luciferase reporter in the same vector as used for the D2 studies were treated with the inhibitors, finding no increase in luciferase activity (Supplemental Fig. 1A, published on The Endocrine Society's Journals Online web site at <http://endo.endojournals.org>). Furthermore, there was no overt effect of the inhibitors on the β -galactosidase transfection control (Supplemental Fig. 1B). These data suggest that the dominant mechanism by which the downstream prenyl transferase inhibitors increase D2 is posttranscriptional.

Endogenous D2 in human primary AOSMC

To determine whether the effects of the isoprenylation inhibitors were limited to immortalized cells, we investigated their effects on endogenous D2 in human primary AOSMC (Fig. 5). Treatment with LVS ($10 \mu\text{M}$) for 8 h significantly induced D2 activity, although the magnitude of increase was smaller than that seen in MSTO-211H (Fig. 5A). Treatment with GGTI-298 increased D2 activity (GGTI-2147 did not, data not shown). FTI-277 strongly increased D2 activity in AOSMC. The time course of stimulation was more rapid in AOSMC, with significant differences being observable within 2 h (Fig. 5B). When AOSMC were treated with inhibitors combined with rT_3 , the net D2 effects were intermediate to those seen with rT_3 or the drugs alone (Fig. 5C). When the inhibitors were combined with MG-132, net D2 activity was not higher than MG-132. *In toto*, these data indicate that the stimulatory effect of the isoprenylation inhibitors on D2 is relevant for primary cells and is not limited to immortalized cells.

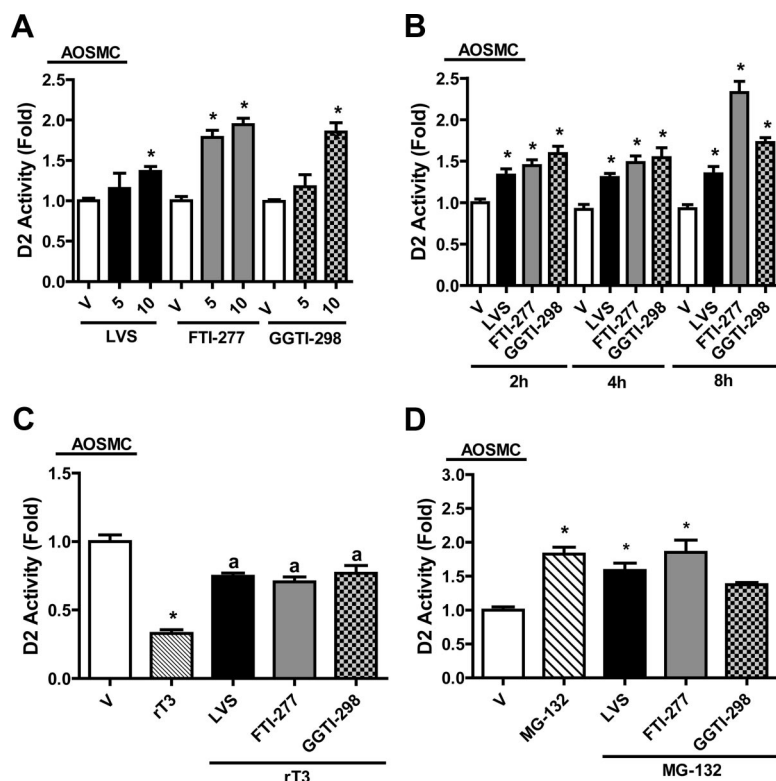


FIG. 5. Effect of isoprenylation inhibitors on endogenous D2 activity in human primary AOSMC. A, Dose response of D2 activity in AOSMC treated with LVS, FTI-277, and GGTI-298 (μM as indicated, 8 h of treatment). Data for LVS are pooled from six independent cell preparations, for FTI-277 and GGTI-298 from five different cell preparations. B, Time course of D2 activity in cells treated with isoprenylation inhibitors as indicated (10 μM) typical experiment of three shown. C, Effect of cotreatment with isoprenylation inhibitors and rT3 (1 μM). D, Effect of cotreatment with MG-132. A–D, Bars represent mean fold activity \pm SEM; *, $P < 0.05$ vs. vehicle; ^a, $P < 0.05$ vs. T₃ and vehicle. C and D, Typical of three experiments is shown.

Effects of the inhibitors on *Dio2* mRNA

Next, we analyzed the expression of *Dio2* gene in cells treated with LVS and the prenyl transferase inhibitors that increased D2 activity. In MSTO-211H cells, we found that LVS significantly increased *Dio2* mRNA at 8 h by 2-fold relative to vehicle (2.0 ± 0.26 , $P < 0.02$). However, neither FTI-277 (1.16 ± 0.11 , ns) nor GGTI-2147 (1.81 ± 0.6 , ns) significantly changed *Dio2* mRNA. In HEK-293 cells, mRNA of the transfected human *Dio2* was not significantly changed by any of the inhibitors (LVS, 0.66 ± 0.3 ; FTI-277, 1.19 ± 0.1 ; GGTI-298, 1.26 ± 0.3 ; and GGTI-2147, 1.03 ± 0.15 ; all ns). In AOSMC, we did not find a significant increase in *Dio2* mRNA with any of the drugs (LVS, 0.9 ± 0.09 ; FTI-277, 1.1 ± 0.09 ; and GGTI-298, 0.76 ± 0.09 ; all ns). These data indicate that although LVS may increase D2 transcriptionally to some extent in MSTO-211H, the predominant mechanism(s) must be posttranscriptional.

Isoprenylation of D2 and/or its associated proteins

We enquired whether D2 itself could be isoprenylated. However, the human *Dio2* amino acid sequence lacks the

classical carboxy-terminal “C-A-A-X” (cysteine-aliphatic-aliphatic-any amino acid) isoprenylation domain (34). Similarly, we analyzed the human amino acid sequences of known D2 interacting proteins for the CAAX motif but found no matches (Table 1).

LVS and D2 activity in murine BAT

To determine whether the effects seen *in vitro* could be extended to the *in vivo* setting, we treated 8- to 10-wk-old C57BL/6J mice with LVS for 24 h (10 mg/kg body weight, two ip doses given 12 h apart), and then measured D2 activity in BAT. LVS administration was associated with an approximately 2.75-fold increase in BAT D2 activity (Fig. 6). *Dio2* mRNA was increased by approximately 1.4-fold (1.42 ± 0.09 , $P < 0.02$). The T₃-sensitive gene uncoupling protein-1 (*UCP-1*) mRNA increased by approximately 1.9-fold ($\sim 1.86 \pm 0.3$, $P < 0.05$). When the experiment was repeated with FTI-277 (10 mg/kg body weight, two ip doses given 12 h apart), again D2 activity was increased (~ 1.4 -fold), albeit to a lesser extent than seen with LVS. For FTI-277, *Dio2* mRNA was not increased (1.01 ± 0.3 -fold) nor was *UCP-1* (0.95 ± 0.3 -fold). These data establish that the effects of LVS and FTI-277 on D2 are not limited to the *in vitro* setting but can also be recapitulated in living animals.

TABLE 1. Carboxy-terminal amino acid sequences for D1, D2, and proteins associated with D2

Protein	NP no.	C terminus (15 amino acids)
Dio1	NP000783.2	Y N P E E V R A V L E K L H S
Dio2	NP000784.2	E K N F S K R U K K T R L A G
WSB-1	NP056441.6	E L P L P S K L L E F L S Y R
VDU-1	NP055832.3	I L Q A E E K L E V E T R S L
VDU-2	NP001008563	N L H G E Q K L E A E T R A V
TEB-4	NP005876.2	G K Q G S S P P P P Q S S Q E
UBE2J2	NP919296.1	F A Y T V K Y V L R S L A Q E
UBE2G2	NP003334.2	F Y K I A K Q I V Q K S L G L
Elongin B	NP009039.1	K P Q D S G S S A N E Q A V Q
Elongin C	NP005639.1	E I A L E L L M A A N F L D C
CUL5	NP003469.2	I R R D E S D I N T F I Y M A
RBX1	NP055063.1	C P L D N R E W E F Q K Y G H

U denotes selenocysteine. Dio 1, Type 1 Deiodinase; Dio 2, type 2 deiodinase; WSB-1, WD repeat and SOCS box containing-1; TEB-4, membrane-associated ring finger (C3HC4) 6; UBE2J2, ubiquitin conjugating enzyme E2, J2 homolog; UBE2G2, ubiquitin conjugating enzyme E2G2; CUL5, cullin 5; RBX1, ring-box 1.

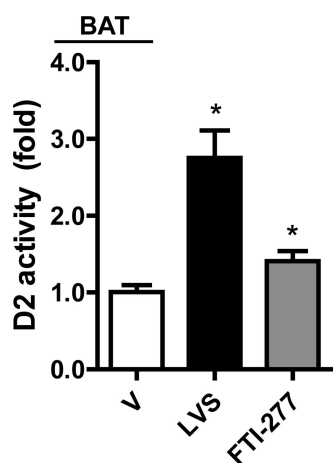


FIG. 6. Effect of LVS or FTI-277 injection on murine BAT D2 activity. D2 activity in BAT of 8- to 10-wk-old C57BL/6J mice treated with LVS (10 mg/kg body weight, two doses over 24 h), or FTI-277 (10 mg/kg body weight, two doses over 24 h). Data presented as mean fold activity relative to vehicle (V) \pm SEM; *, $P < 0.05$ vs. vehicle-treated mice, $n = 4$ mice per group. Typical experiment out of three for each drug is shown.

Discussion

The major findings of this study are that endogenous D2 activity in cultured immortalized or primary cells is significantly increased by inhibitors of the isoprenylation pathway. We also performed a “proof of concept” experiment showing an increase in BAT D2 activity and mRNA in mice treated with LVS or FTI-277, extending the potential significance of the data to the *in vivo* realm. This stimulation of D2 activity by statins and prenyl transferase inhibitors is unexpected, considering the previous finding

of Warner *et al.* (21) that LVS decreases the activity of transiently expressed D2, as well as the known dependence of selenoprotein synthesis on isoprenylation of the sec-tRNA (22, 23).

Significant regulatory complexity appears to exist in the relationship between isoprenylation pathway inhibitors and D2. Of note, various statins did not increase D2 in direct proportion to their potency with regards to inhibition of HMG-CoA reductase or low-density lipoprotein reduction (Fig. 2C); for example, rosuvastatin is the most potent HMG-CoA reductase inhibitor but only weakly increased D2. One could speculate that as the inhibition of cholesterol synthesis increases beyond a certain threshold, it might negatively impact D2 activity. On the other hand, molecule-specific factors, such as hydrophobicity, may play role, because PVS (the most hydrophobic statin tested) (35) had no stimulatory effect.

The finding that the FTase and GGTase inhibitors, when used at concentrations at which they exhibit high enzyme specificity, can individually increase D2 activity indicates that both the farnesylation and geranylation downstream branches of the pathway are mechanistically relevant. Although there is often redundancy between the prenyl transferases in terms of their protein targeting, it would seem that in this setting both pathways must be operating normally, else D2 activity increases. We ruled out *in silico* that human D2 or its known associated proteins contain the classical targeting sequence for isoprenylation (Table 1), so barring nonclassical isoprenylation, there must be an isoprenylated protein, nucleoside, or other molecule (or molecules) important for D2 activity. The list of known suspects includes more than 100 proteins that are thought to be isoprenylated, having a vast array of functions spanning the gamut from proliferation to oxidative stress (36, 37). Our review of the known isoprenylated targets suggests many potential mechanisms but no obviously favored mechanisms to explain the increase in D2 activity. Thus, future studies utilizing a systematic approach to identify connections between D2 and known isoprenylated factors will be required.

Even without knowing the identity of the exact isoprenylated factor affecting D2 activity, mechanistic details have emerged from the current data. The observation that the inhibitor drugs cannot increase D2 activity beyond levels seen with MG-132 in the cotreatment studies in either MSTO-211 or AOSMC indirectly supports the hypothesis that inhibition of isoprenylation interrupts normal ubiquitin-proteasomal degradation of D2 (Figs. 3D and 5D). It should be noted that mechanism may not be specific to D2; indeed, any drug or process that inhibits the proteasome might increase D2 activity by augmenting the pool of ubiquitinated D2.

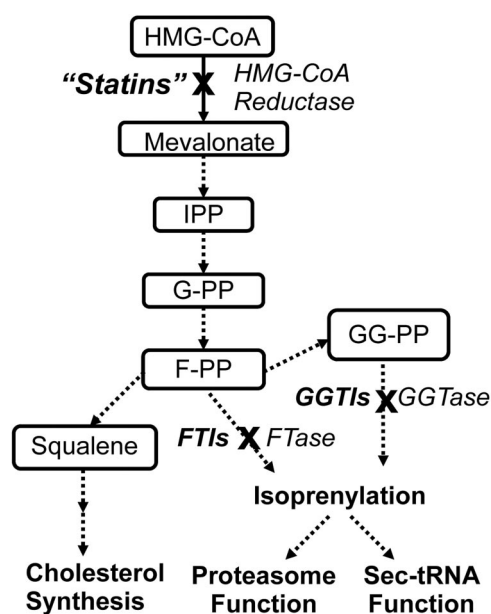


FIG. 7. The cholesterol synthesis and isoprenylation pathway. IPP, Isopentenyl pyrophosphate; G-PP, geranyl pyrophosphate; F-PP, farnesyl pyrophosphate; GG-PP, geranylgeranyl pyrophosphate.

Dissecting out where in the ubiquitination-proteasomal degradation pathway the inhibitors may be working will require further studies. Keeping in mind that ubiquitination (which deactivates D2) and proteasomal degradation are linked but separate processes, the interpretation of the intermediate D2 activities seen when inhibitors were combined with rT_3 (Figs. 3C and 5C) is complex: if the statins/prenyl transferase inhibitors induce D2 activity via total inhibition of substrate-accelerated ubiquitination, one would predict that the negative effect of rT_3 would be lost, but this was not seen. Thus, the results of the experiment were equivocal: the intermediate D2 activities are consistent either with partial inhibition of substrate-accelerated ubiquitination, or with the summation of a positive ubiquitination-independent effect of the inhibitors and the negative ubiquitination-mediated effect of rT_3 . An additional possibility that remains to be explored is whether isoprenylation inhibitors could somehow increase the expression or activity of deubiquitinases that reactivate ubiquitinated D2. However, if this were the case, one would expect that cotreatment with the inhibitors and MG-132 would increase D2 activity higher than with MG-132 alone (38), but this was not seen, nor was VDU-1 or VDU-2 mRNA increased. *In toto*, the current data support a mechanism by which an unidentified isoprenylated factor or factors act to decrease the overall ubiquitin-mediated proteolysis of D2, but whether the critical issue is ubiquitination, translocation of ubiquitinated D2 to the proteasome, deubiquitination, or proteasomal degradation of ubiquitinated D2 itself remains to be determined.

Why LVS does not increase transiently expressed D2 is intriguing (Fig. 4A). One issue may be that the transcriptional increase seen in MSTO-211H cells was not present in HEK-293 (Fig. 4B). This may relate to the nonnative promoter and the 3' untranslated region used in the construct. Although we could not detect a significant LVS-mediated increase in *Dio2* mRNA in AOSMC myocytes, the magnitude of D2 induction was also lower in these cells, leaving open the possibility that a small increase in *Dio2* mRNA was present. Another possibility is that HEK-293 may be more sensitive to intracellular cholesterol depletion; note that the prenyl transferase inhibitors would be expected to affect cholesterol synthesis less than the statins, and this might explain some of the differences seen between LVS and the downstream inhibitors in all the cell types. LVS may therefore invoke multiple mechanisms increasing D2 activity, overlapping with the proteasomal mechanism that predominates for the downstream inhibitors.

Cell type-specific differences among the prenyl transferases are notable in our studies, *e.g.* GGTI-298 and 2147

work differently in MSTO-211H *vs.* AOSMC. However, these differences are not unexpected given previous studies showing that different cell types have unique patterns of response to these drugs (39, 40). The fact that different FTase and GGTase inhibitors increase endogenously expressed D2 activity lends confidence that the mechanism is universal. A multitude of hypothetical explanations have been proposed to explain these cell line-specific differences, *e.g.* different pharmacologic kinetics of the drugs in the cell lines, or different expression patterns of critical isoprenylation targets. As more is learned about these drugs in other studies, unexpected clues as to D2 biology may also arise.

It is tempting to speculate as to whether patients taking statins exhibit any subtle changes in thyroid hormone signaling in their D2-expressing statin-exposed tissues. It should be remembered that such changes would not definitively be reflected by changes in plasma thyroid hormone levels or TSH, because D2 is capable of inducing tissue-specific changes. However, the low micromolar concentrations of statins (and prenyl transferases) used in these experiments must be considered a significant caveat. Although standard in the experimental literature (41–43), these concentrations are much higher than what is typically achieved in human plasma, except in rare patients receiving chemotherapeutic doses (44, 45). Thus, additional studies will be required before the clinical significance of these findings can be validated. The murine BAT data (Fig. 6) can be considered a first step. One could take the fact that *Dio2* mRNA was induced slightly with LVS but not FTI-277 to be indirect evidence that the same mechanisms apply in mice as in cells. The changes in *UCP-1* mRNA may relate to the extent of D2 stimulation, but it must be noted that the sensitivity of *UCP-1* to changes in D2-generated T_3 would be expected to be low in room-temperature animals (as opposed to in the cold, in which case *UCP-1* mRNA increases by many fold under the primary control of cAMP) (46). The next steps will be to extend the findings in mice, with a full characterization of thyroid hormone status in the plasma and in individual D2-expressing tissues under various statin and prenyl transferase inhibitor-exposure conditions, in particular examining chronic low-dose treatment effects.

In conclusion, the data indicate that the relationship between isoprenylation and D2-mediated regulation of thyroid hormone signaling is considerably more complex than once believed. The predominant mechanism appears to be largely posttranscriptional, likely involves the proteasome, and, for endogenously expressed D2, can outweigh any concomitant negative effect on D2 protein synthesis caused by impairment of sec-tRNA function.

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- Abbreviations: HMG-CoA hydroxymethylglutaryl coenzyme A, IPP isopentyl pyrophosphate, G-PP geranyl pyrophosphate, F-PP farnesyl pyrophosphate, GG-PP geranylgeranyl pyrophosphate, FTase farnesyl transferase, FTIs farnesyl transferase inhibitors, GGTase geranylgeranyl transferase, GGTIs geranylgeranyl transferase inhibitors, Sec-tRNA selenocysteine tRNA.



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