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Antithyroid Drugs Inhibit Thyroid Hormone Receptor-Mediated Transcription

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Context: Methimazole (MMI) and propylthiouracil (PTU) are widely used as antithyroid drugs (ATDs) for the treatment of Graves' disease. Both MMI and PTU reduce thyroid hormone levels by several mechanisms, including inhibition of thyroid hormone synthesis and secretion. In addition, PTU decreases 5′-deiodination of T_4 in peripheral tissues. ATDs may also interfere with T_3 binding to nuclear thyroid hormone receptors (TRs). However, the effect of ATDs on the transcriptional activities of T_3 mediated by TRs has not been studied.

Objective: The present study was undertaken to determine whether ATDs have an effect on the gene transcription regulated by T_3 and TRs *in vitro*.

Methods: Transient gene expression experiments and GH secretion assays were performed. To elucidate possible mechanisms of the antagonistic action of ATDs, the interaction between TR and nuclear cofactors was examined.

Results: In the transient gene expression experiments, both MMI and PTU significantly suppressed transcriptional activities mediated by the TR and T_3 in a dose-dependent manner. In mammalian two-hybrid assays, both drugs recruited one of the nuclear corepressors, nuclear receptor corepressor, to the TR in the absence of T_3. In addition, PTU dissociated nuclear coactivators, such as steroid receptor coactivator-1 and glucocorticoid receptor interacting protein-1, from the TR in the presence of T_3. Finally, MMI decreased the GH release that was stimulated by T_3.

Conclusions: ATDs inhibit T_3 action by recruitment of transcriptional corepressors and/or dissociation of coactivators. This is the first report to show that ATDs can modulate T_3 action at the transcriptional level. (*J Clin Endocrinol Metab* **92: 1066–1072, 2007**)

THYROID HORMONES REGULATE growth, development, and critical metabolic functions. They exert these effects through complex biological pathways, which offer a wealth of opportunity to intervene pharmacologically in thyroid hormone signaling at numerous steps. These include biosynthesis, cell-specific uptake, or export of thyroid hormone as well as nuclear targeting and actions, which are exerted through thyroid hormone receptor (TR) binding and histone acetylation. Such processes represent potentially important pharmacological targets for the drug therapies of thyroid hormone abnormalities, especially hyperthyroidism.

Some compounds having thionamide structure, such as thiourea and thiouracil, inhibit thyroid function. Clinically used antithyroid drugs (ATDs) include methimazole (1-methyl-2-mercaptoimidazole; MMI), and propylthiouracil (6-propyl-2-thiouracil; PTU) to treat Graves' hyperthyroidism (Fig. 1). ATDs have intrathyroidal and extrathyroidal actions. The chief intrathyroidal actions include inhibition of iodine oxidation and organization and iodotyrosine coupling, among others. The main extrathyroidal action is inhibition of conversion of T_4 to T_3 by PTU, but not MMI (1, 2). Thus, the reduction in thyroid hormone production induced by the drugs is central to these actions.

Furthermore, ATDs are known to influence oxygen consumption, or peripheral metabolic suppression, although the mechanisms are not fully understood (3, 4). To date, a number of studies was performed to elucidate how ATDs suppress peripheral metabolism. ATDs can affect gene expression and modulate functions of some cell types (5). Although ATDs were not effective in the binding affinity of T_4 to serum thyroxine binding globulin, they inhibited T_3 binding to the hepatic nuclear extracts (6). However, the effect of ATDs on the transcriptional activities of T_3 mediated by TRs has not been studied in detail. The present study was undertaken to determine whether ATDs have an effect on the gene transcription regulated by T_3 and TRs *in vitro*.

Materials and Methods

Reagents

The chemical structures of PTU, MMI, and T_3 are shown in Fig. 1. ATDs were purchased from Sigma-Aldrich, Corp. (St. Louis, MO). T_3 was purchased from Nakalai Tesque Inc. (Kyoto, Japan).

Plasmid constructions

Expression vectors containing wild-type human TRβ1 (pCMX-hTRβ1) and human TRα1 (pCMX-hTRα1) were provided by K. Ume-

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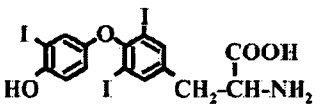
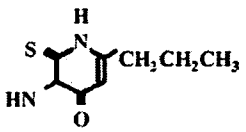
Abbreviations: AF, Activation function; ATD, antithyroid drug; CoA, coactivator protein; CoR, corepressor protein; DBD, DNA binding domain; FBS, fetal bovine serum; GRIP, glucocorticoid receptor interacting protein; LBD, ligand-binding domain; Luc, luciferase; MMI, methimazole; NCoR, nuclear receptor corepressor; PTU, propylthiouracil; SMRT, silencing mediator of retinoid and thyroid receptor; SRC, steroid receptor coactivator; TK, thymidine kinase; TR, thyroid hormone receptor; TRE, thyroid hormone response element.

A Triiodothyronine (T3)



B Propylthiouracil (PTU)



C Methimazole (MMI)

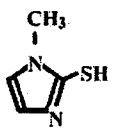


FIG. 1. The structure of T_3 and two antithyroid drugs, propylthiouracil and methimazole.

sono (Salk Institute, San Diego, CA) (7). The ligand-binding domain (LBD) of TRα1 or TRβ1 was fused to the DNA binding domain (DBD) of Gal4 in-frame in pSG424 (8). The Gal4-NCoR (residues 1552–2453), Gal4-SRC1 (residues 213-1061), and Gal4-GRIP1 (residues 480-1462) constructs contain the indicated TR interaction domains of these proteins (9). The VP16 construct for TRβ contains the LBD of the receptor downstream of the VP16 activation domain in-frame in pCMX (9). The reporter plasmids, TRE-TK-Luc (9) and ME-TK-Luc (10), contain two copies of a palindromic thyroid hormone response elements (TRE) and malic enzyme, respectively, upstream of the thymidine kinase (TK) promoter in the pA3-luciferase vector (Luc). TSHα-Luc contains 846 bp of the 5′-flanking sequence and 44 bp of exon I from the human glycoprotein hormone α-subunit gene in pA3-Luc (9). The Gal4 reporter plasmid, UAS-E1BTATA-Luc, contains five copies of the Gal4 recognition sequence (UAS) upstream of E1BTATA in pA3-Luc (11). The pRL-TK vector (Promega Corp., Madison, WI) comprised of the tk promoter and *Renilla* luciferase cDNA was used as an internal control.

Cell culture

TSA201, a clone of human embryonic kidney 293 cells (12), human hepatoblastoma HepG2 cells, and rat pituitary GH3 cells were maintained in DMEM, containing penicillin (100 U/ml) and streptomycin (100 μg/ml) with 10% fetal bovine serum (FBS; Invitrogen, Corp., Carlsbad, CA). For hormone and drug treatment, the medium was changed to phenol red-free DMEM (Nikken Biomedical Laboratory, Kyoto, Japan) containing 10% charcoal-stripped FBS (13).

Transient expression assays

TSA201 and HepG2 cells were grown in phenol red-free DMEM (Nikken Biomedical Laboratory) with 10% charcoal-stripped FBS and were transfected using the calcium precipitation method (14). After exposure to the calcium phosphate-DNA precipitate for 8 h, phenol red-free DMEM with charcoal-stripped FBS was added, in the absence or presence of compounds and/or T_3. Cells were harvested for measurements of luciferase activity, according to the manufacturer's instructions (dual-luciferase reporter assay system; Promega). The transfection efficiencies were corrected with the internal control. Both firefly and *Renilla* luciferase activities were measured to monitor the transfection efficiency and cytotoxicity of the added materials. The firefly activity obtained by the T_3-specific promoter was divided by the *Renilla* activity obtained by the nonspecific promoter in each well. Results are expressed as the mean ± SD from at least three transfections, each performed in triplicate. Data were analyzed by ANOVA with *post hoc* Dunnett's tests to compare with the control.

GH secretion and assay

For GH assays, GH3 cells, derived from the rat pituitary tumor cell line, were seeded into 6-well plates at 1.5×10^4 cells/well. T_3 (1 nM) as a physiological concentration and/or 10 μM MMI was added on the day after the medium was replaced. In the case of 10 μM PTU, cell survival was inhibited and the assay was abandoned. Culture media were collected after 2 d of incubation and GH was measured by ELISA (rat GH enzyme-immunoassay system; GE Healthcare UK Ltd., Buckinghamshire, UK) according to the manufacturer's instructions. After harvesting the supernatant, cell numbers were counted to evaluate cell proliferation. Results are expressed as the mean ± SD from at least five experiments, each performed in quadricate. Data were analyzed by ANOVA with *post hoc* Dunnett's tests to compare with the control.

Results

ATDs suppressed transcriptional activities mediated by TRα1 and TRβ1

The chemical structure of T_3, PTU, and MMI are shown in Fig. 1. ATDs are known as thionamides, which contain a sulfhydryl group and a thiourea moiety within a heterocyclic structure. Takagi *et al.* (6) reported that ATDs inhibit specific binding of T_3 to its receptor, perhaps due to an interaction with cysteine residues of the receptor.

We examined whether ATDs antagonize T_3-induced TR activation. Transient expression experiments were performed using TSA201 cells, which are a derivative of human embryonic kidney 293 cells. The LBD of TRα1 or TRβ was fused to the DNA binding domain of the yeast transcription factor, Gal4, and was cotransfected with a Gal4 reporter gene, UAS-E1BTATA-Luc. We determined the effects of ATDs on various physiological concentrations of T_3. In the presence of 10 μM PTU, increasing amounts of T_3 were added to the medium, and transcriptional activity was measured. PTU had no significant effects on TR-mediated transcriptional activity in the absence of T3 (Fig. 2) and in the absence of Gal4-TR (see Fig. 6A). However, PTU suppressed the activity mediated by Gal4-TRα1 up to 36% and Gal4-TRβ up to 39% of the respective control levels in the presence of T_3. The maximum suppression by PTU was obtained at the concentration of 1 nM of T_3. Similar results were obtained using MMI (data not shown).

The inhibitory effects of ATDs were also examined in the context of native receptors. A T_3-responsive reporter gene, TRE-TK-Luc, was cotransfected with full-length TRs (Fig. 3A). ATDs did not affect the activity of TRE-TK-Luc alone without TR. In the presence of 1 nM T_3, dose-dependent inhibition of transcription mediated by TRα1 and TRβ1 was observed with both ATDs. PTU suppressed the TRα1-mediated activity by 45% and TRβ1-mediated activity by 39% and MMI suppressed TRα1-mediated activity by 45% and TRβ1-mediated activity by 53% of the respective T_3 effect.

In a reciprocal manner, another group of negatively regulated genes was stimulated by TRs in the absence of T_3 and was repressed in response to T_3 (14). The effects of ATDs on the TSHα promoter were examined as a model of a negatively regulated gene. As shown in Fig. 3B, although PTU did

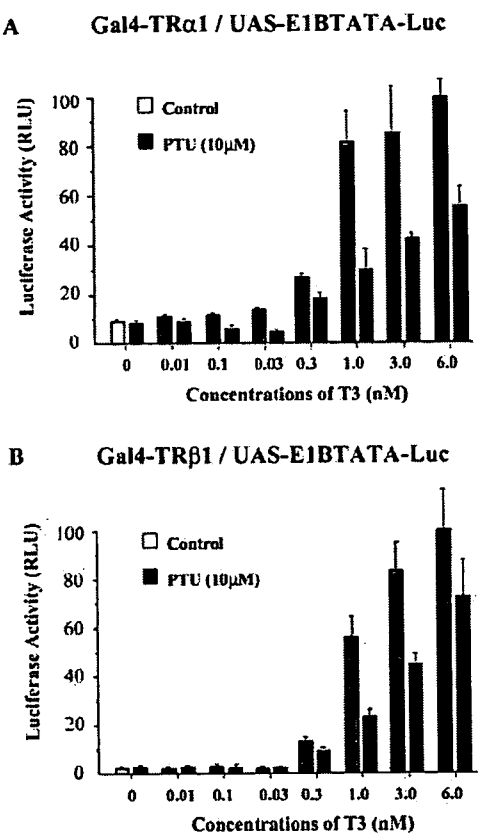


FIG. 2. ATDs suppress TR-mediated transcription in the presence of a physiological range of T_3. Gal4-TRα1 (A) or Gal4-TRβ1 (B; 50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, UAS-E1BTATA-Luc, in the absence or presence of 10 μM PTU. RLU, Relative light units. The firefly activity obtained by UAS-E1BTATA-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc.

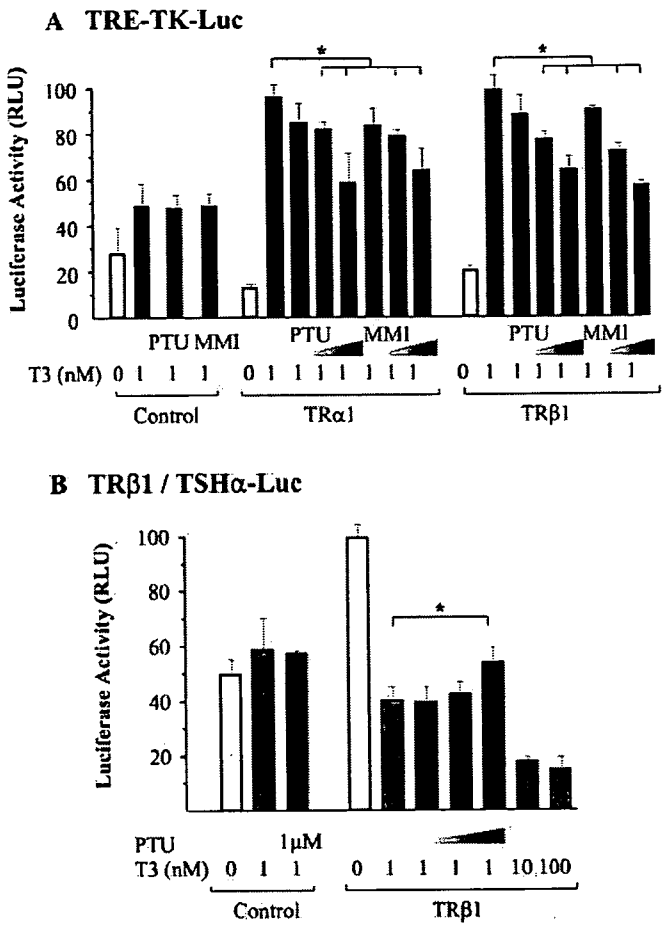


FIG. 3. The inhibitory effects of ATDs on the gene transcription mediated by native TR. A, The CMX, CMX-TRα1, or CMX-TRβ1 (50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, TRE-TK-Luc, in the absence or presence of 1 nM T_3 and increasing amount of ATDs. B, The CMX or CMX-TRβ1 (50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, TSHα-Luc, in the absence or the presence of 1 nM T_3 and increasing amount of PTU (1 nM, 100 nM, and 10 μM). RLU, Relative light units. The firefly activity obtained by TRE-TK-Luc or TSHα-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc. *, $P < 0.05$.

not affect the activity of TSHα-Luc alone without TR, PTU increased the transcriptional activity, which was suppressed by 1 nM T_3 up to the control level. Similar results were obtained using MMI (data not shown).

The inhibitory effects of PTU were also examined at high concentrations of T_3. In the presence of 100 nM of T_3, which is almost 20 times greater than concentrations found in blood samples of patients with severe hyperthyroidism, the effects of PTU were eliminated (Fig. 4). Similar results were obtained using MMI (data not shown).

ATDs suppressed transcriptional activities mediated by endogenous TRs

We next studied the effects of ATDs using a cell line that contains physiological amounts of endogenous TRs. The reporter gene regulated by the ME-TRE, ME-TK-Luc, was transfected into human hepatoblastoma, HepG2. No significant effects were observed by 48 h incubation with 10 μM PTU or MMI on the ME-TK-Luc activity in the absence of T_3. One nanomole T_3 stimulated the activity by 1.8-fold, compared with that without T_3, and ATDs inhibited its increase by 70% (Fig. 5).

ATDs recruit corepressor and dissociate coactivator

Transcriptional repression of the positively regulated genes by unliganded TR is mediated by interacting with corepressor proteins (CoRs) such as nuclear receptor corepressor (NCoR) (15) and silencing mediator of retinoid and thyroid receptors (SMRT) (16). CoRs might also be involved in the basal activation of negatively regulated genes (14). In the other hand, in the presence of T_3, liganded TR activates transcription of the positively regulated genes and inhibits that of the negatively regulated genes by interacting with coactivator proteins (CoAs) (17) such as steroid receptor coactivator (SRC)-1 (18) and glucocorticoid-interacting protein (GRIP)-1 (19). Using a mammalian two-hybrid assay, the effect of ATDs on the TR-CoR or TR-CoA interaction was examined. The TR interaction domain of CoRs or CoAs was fused to the Gal4-DBD. The LBD of TRβ was fused to the transcriptional activation domain of VP16 to allow detection of the interaction between the Gal4-CoR/CoA and VP16-TR.

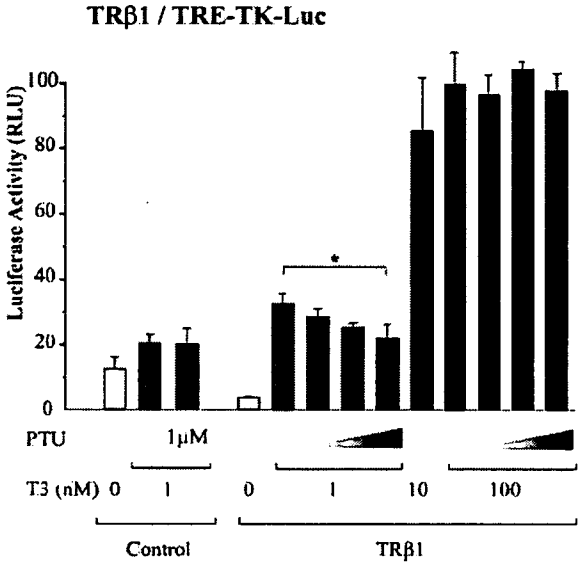


FIG. 4. High amount of T_3 blocks the inhibitory effects of ATDs on the gene transcription mediated by native TR. The CMX or CMX-TRβ1 (50 ng) was cotransfected into TSA-201 cells with 100 ng of the reporter gene, TRE-TK-Luc, in the absence or the presence of 1 or 100 nM T_3 and an increasing amount of PTU (1 nM, 100 nM, and 10 μM). RLU, Relative light units. The firefly activity obtained by TRE-TK-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc. *, $P < 0.05$.

As shown in Fig. 6A, both PTU and MMI had no significant effects on the activity mediated by Gal4-DBD and VP16-TR. PTU enhanced TR-NCoR interaction but not TR-SMRT interaction in a dose-dependent manner (Fig. 6B). In contrast, PTU inhibited TR-SRC1 interaction and, to a lesser degree, TR-GRIP1 interaction in a dose-dependent manner (Fig. 6D). Similarly, MMI enhanced TR-NCoR interaction but not TR-SMRT interaction and inhibited TR-GRIP1 interaction but not TR-SRC1 interaction (Fig. 6, C and E).

ATDs inhibited endogenous hormone secretion induced by T_3

T_3 stimulates endogenous GH gene transcription mediated by endogenous TRs in rat pituitary tumor cells (20–22).

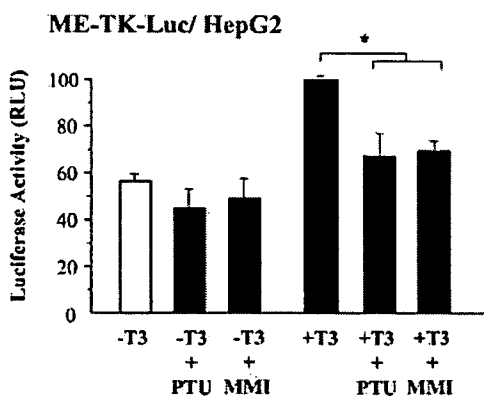


FIG. 5. ATDs suppress transcriptional activities mediated by endogenous TRs. The ME-TK-Luc (100 ng) was transfected into HepG2 cells and incubated with or without 1 nM T_3 and/or 10 μM PTU or MMI for 48 h. RLU, Relative light units. The firefly activity obtained by ME-TK-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc. *, $P < 0.05$.

The production of GH was measured by ELISA in the culture media of GH3 cells. Forty-eight-hour incubation with 1 nM T_3 stimulated the GH production by 2.7-fold (Fig. 7). Addition of 10 μM MMI significantly decreased the GH release to 82% of that with 1 nM T_3 alone. In the case of 10 μM PTU, cell death was induced (data not shown).

Discussion

The ATDs have been a mainstay of treatment of patients with Graves' hyperthyroidism (23). The ATDs are heterocyclic compounds known as thionamides that contain a thioureylene group (Fig. 1). Two kinds of drugs of this type are available at present: one is MMI and carbimazole (1-methyl-2-thio-3-carbethxy-imidazole), which is rapidly metabolized to MMI, and the other is PTU. These drugs cause goiter in animals, which is due to the stimulation of the thyroid by the pituitary, consequent to pharmacological inhibition of thyroid hormone production (24, 25). Thus, derivatives of thiourea and thiouracil have been used as antithyroid drugs for more than 60 yr.

The main effect of ATDs is to inhibit the synthesis of T_4 and T_3 as an intrathyroidal action, including inhibition of iodine oxidation and organization, inhibition of iodotyrosine coupling, possible alteration of thyroglobulin structure, and inhibition of thyroglobulin biosynthesis. As an extrathyroidal action, PTU but not MMI inhibits conversion of T_4 to T_3. During the last 3 decades, reports have been accumulating on extrathyroidal actions of the ATDs, especially of the thionamide group, causing several undesirable side effects. Bandyopadhyay *et al.* (5) reviewed extrathyroidal actions of antithyroid thionamides in animals and humans. Thionamides inhibit lactoperoxidase, which contributes to the antibacterial activities of a number of mammalian exocrine gland secretions that protect a variety of mucosal surfaces. These drugs stimulate both gastric acid and pepsinogen secretions, thereby augmenting the severity of gastric ulcers and preventing wound healing. Severe abnormalities may develop in blood cells and the immune system after thionamide therapy. They may cause agranulocytosis, aplastic anemia, and purpura along with immune suppression. Olfactory and auditory systems are also affected by these drugs. Thionamide affects the sense of smell and taste and may also cause loss of hearing. Thionamide also affects gene expression and modulate the functions of some cell types.

After administration of ATDs to patients with Graves' disease, it usually takes 2 or 3 d to start decreasing serum level of T_4 and T_3. However, O_2 consumption or other peripheral metabolic indexes indicated that ATDs exerted an immediate effect *in vivo* (4, 5). Besides a specific effect of PTU as a 5′-deiodinase inhibitor effectively preventing T_3 generation, those actions were reported to persist in the presence of both of ATDs. Because thyroid hormones directly activate the expression of the human and mouse uncoupling protein-3 genes through a thyroid response element in the proximal promoter region, peripheral metabolism was controlled most partly by thyroid hormone level (26). An immediate effect of both of ATDs might be involved in the inhibition of transcriptional activities by thyroid hormone.

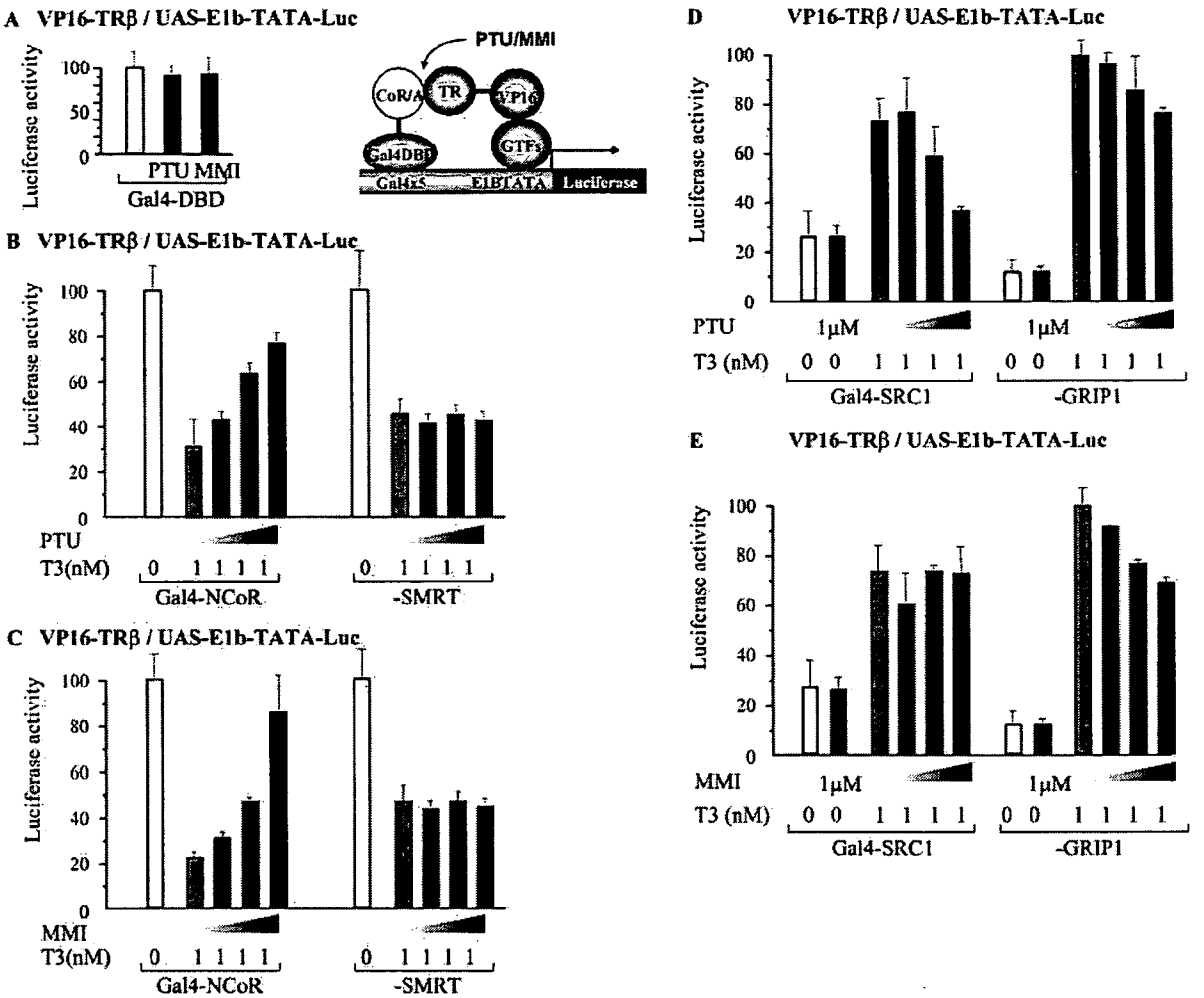


FIG. 6. The effects of ATDs on the interaction between TR and cofactors. The format of the mammalian two-hybrid experiment is shown *between panels A and D.* Gal4 fusion plasmids of indicated cofactors (50 ng) were cotransfected into TSA-201 cells with 100 ng of VP16-TRβ together with 100 ng of the reporter gene, UAS-E1BTATA-Luc, in the absence or presence of 1 nM T3. Increasing amounts (1 nM, 100 nM, and 10 μM) of PTU or MMI were added. A, Gal4-DBD. B and C, Gal4-NCoR and Gal4-SMRT. D and E, Gal4-SRC1 and Gal4-GRIP1. RLU, Relative light units. The firefly activity obtained by UAS-E1BTATA-Luc was normalized to the *Renilla* activity obtained by PRL-TK-Luc. GTF, General transcription factor.

Induction of cell proliferation by mitogen or growth factor stimulation leads to the specific and sequential expression of a large number of genes. To date several reports showed that ATDs change mRNA expression level of certain genes. MMI and PTU increase thyroglobulin gene expression and increase thyroid-specific mRNA concentration in human thyroid FRTL-5 cells (27–29). The stimulatory effects of MMI and PTU can be suppressed by iodide and do not occur when protein synthesis is inhibited by cycloheximide. MMI and PTU increased thyroid peroxidase mRNA in cultured porcine thyroid follicles (30). MMI can suppress the interferon-γ-induced increase in HLA-DRα gene expression (31). Fas ligand expression is induced by MMI in follicular cells of thyroid glands obtained from Graves' patients and cultured thyrocytes, resulting in Fas ligand-dependent apoptosis of thyrocytes (32). In the thyroid, the TR expression has been reported (33–35).

ATDs are mainly concentrated in the thyroid gland but more than 70% of ATDs are unevenly distributed in the whole body (36). Therefore, the transcriptional inhibition by ATDs in the periphery may be dependent on the concentration of ATDs in each tissue. The peak serum concentrations of MMI are in the range of 300 ng/ml (2.6 mM) after a 15-mg oral dose (37), and those of PTU are about 3 mg/ml (18 mM) after a 150-mg oral dose (38). As we have shown here, inhibition of T_3 action was observed for GH expression (20–22) in rat pituitary GH3 cells and for malic enzyme expression (10) in human hepatic HepG2 cells. However, the inhibitory effects on cell proliferation of these cells were also seen under the therapeutic concentrations of ATDs.

Takagi *et al.* (6) demonstrated that thionamides inhibit specific T_3 binding to the hepatic nuclear receptor. MMI and PTU at a pharmacological dose (2 mM) reduced specific T_3 binding to chromatographed nuclear receptors by 84 and 85%, respectively. Scatchard analyses indicated that neither MMI nor PTU significantly altered the affinity constant, whereas they both

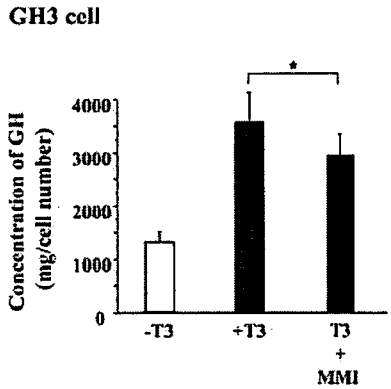


FIG. 7. The inhibitory effects of ATDs on the GH secretion induced by T_3. GH3 cells were incubated with or without 1 nM T_3 and/or 10 μM MMI for 48 h. Culture media were collected and GH was measured by ELISA. *, $P < 0.05$.

decreased maximal binding capacity. We obtained similar results using rat liver nuclear extract (data not shown) (13). Thereafter, there is no study of the actions of ATDs on the transcriptional regulation mediated by T_3 and TR.

In this study we demonstrated that ATDs could impair thyroid hormone action by suppressing its transcriptional activity. Gene suppression is attributed partly to the recruitment of NCoR and dissociation of GRIP1 or SRC1. In the case of positively regulated promoter, TRE-TK, the effect of T_3 was supposed to be inhibited by recruiting NCoR and dissociating GRIP1 or SRC1 by ATDs. In contrast, in the negatively regulated promoter, TSHα, the opposite effect was observed because corepressors may be involved in basal stimulation in the absence of T_3 (14) and coactivators may be involved in T_3-dependent inhibition in the presence of T_3 (17). A number of nuclear cofactors have been cloned, but most of their specific functions are unclear (39). These cofactors were initially studied in the context of the TR and other nuclear hormone receptors. However, every cofactor has multiple interaction domains comprised of subtly distinct LXXLL motif and its combination and seems to interact with multiple receptors in a different way. Each specific ligand also contributes conformational change of the receptor and modulates the cofactor binding to the receptor as an agonist or an antagonist. Indeed, PTU and MMI enhanced recruitment of NCoR but not SMRT to the TR and both drugs dissociated GRIP1, but dissociation of SRC1 occurred solely by PTU. NCoR preferentially bind TR homodimer over TR-RXR heterodimer (40) and TR prefers to recruit NCoR, and retinoid acid receptor prefers to recruit SMRT (41). These preferences are likely due to sequence differences in interacting domains of corepressors (42).

Conformational change of TR induced by ATDs may be subtly different from that induced by T_3, and it may enhance the interaction with specific domain of NCoR. The functional specificity is also reported among SRC family members. For example, progesterone receptor interacts preferentially with SRC1, which recruits cAMP response element-binding protein (CREB) binding protein (CBP) and enhances acetylation of histone H4, whereas glucocorticoid receptor interacts preferentially with SRC2 (GRIP1), which recruits p300/CBP-associated factor and results in histone H3 acetylation (43). PTU and MMI may have some different effect on the interaction between TR and SRC family members via their subtly distinct LXXLL motifs. Every receptor also has more than one activation domain [activation function (AF)-1 and AF-2]. Coactivators may preferentially use specific activation domains, depending on the receptor or activation function (AF-1 *vs.* AF-2) that is mediating the response to hormone (44). AF2 is conserved among nuclear hormone receptor superfamily as ligand-inducible transcription factors (45). We also tested the effects of ATDs on transcription mediated by other nuclear hormone receptors such as estrogen receptor-α and -β. ATDs had no effects on estrogen action (data not shown).

In summary, our findings demonstrate that ATDs, which are the most prevalent drugs to treat Graves' diseases, suppress transcriptional activity by modulating the cofactor recruitment to the TR. Although the clinical and therapeutic significance of our findings remains to be established, our data provide a mechanistic basis for one of the extrathyroidal actions of ATDs. It might be helpful in designing new therapeutic compounds with modifications of existing ATDs to enhance transcriptional inhibition against T_3 action.

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Augmentation of 11β-hydroxysteroid dehydrogenase type 1 in LPS-activated J774.1 macrophages – Role of 11β-HSD1 in pro-inflammatory properties in macrophages

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Abstract Macrophage infiltration in obese adipose tissue provokes local inflammation and insulin resistance. Evidence has accumulated that activation of 11β-HSD1 in adipocytes is critically involved in dysfunction of adipose tissue. However, the potential role of 11β-HSD1 in macrophages still remains unclear. We here demonstrate that a murine macrophage cell line, J774.1 cells expressed 11β-HSD1 mRNA and reductase activity, both of which were augmented by lipopolysaccharide (LPS)-induced cell activation. Three kinds of pharmacological inhibition of 11β-HSD1 in LPS-treated macrophages significantly suppressed the expression and secretion of interleukin 1β, tumor necrosis factor α or monocyte chemoattractant protein 1, thereby highlighting a novel role of 11β-HSD1 in pro-inflammatory properties of activated macrophages.

Keywords: 11β-HSD1; Macrophage; Inflammation; Metabolic syndrome; Inhibitor; Adipocyte

1. Introduction

Functional abnormalities in adipose tissue have been implicated in the central pathophysiology of the metabolic syndrome [1]. Glucocorticoids regulate adipocyte differentiation, function and distribution, and in excess, cause visceral fat obesity and convergence of metabolic diseases [2]. Two iso-enzymes of 11β-HSD, 11β-HSD1 and 11β-HSD2 catalyze the interconversion between hormonally active cortisol and inactive cortisone within cells. 11β-HSD1 is abundantly expressed in adipose tissue and liver [2,3] and reactivates cortisol from cortisone (oxo-reductase) [4]. Previous reports have demonstrated that 11β-HSD1 predominates in adipocytes compared to 11β-HSD2 [2], which inactivates cortisol to cortisone (dehydrogenase). Targeted overexpression of 11β-HSD1 selectively in adipose tissue in mice exemplified metabolic derangements including visceral fat obesity, insulin resistance, dyslipidemia and hypertension [5,6]. Conversely, systemic 11β-HSD1 knockouts as well as adipose-specific 11β-HSD2 overexpressors are completely protected against metabolic diseases under overnutrition [7–9]. These data suggest that increased level of adipose 11β-HSD1 plays a crucial role in dysfunction of adipose tissue and resultant metabolic disorders.

On the other hand, recent studies have demonstrated that infiltration of macrophages in obese adipose tissue provokes local and systemic inflammation and insulin resistance [10–12]. It has been shown that matured adipocytes abundantly express 11β-HSD1 which is critically involved in dysfunction of adipose tissue [2,5]. However, the potential role of 11β-HSD1 in macrophages remains largely unclear. In this context, we explored the expression and regulation of 11β-HSD1 in a murine reticular cell sarcoma-derived J774.1 macrophages [13]. To our knowledge, this is the first to demonstrate that 11β-HSD1 is critically involved in pro-inflammatory properties of activated macrophages.

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Abbreviations: 11β-HSD1, 11β-hydroxysteroid dehydrogenase type 1; 11β-HSD2, 11β-hydroxysteroid dehydrogenase type 2; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; IL-1β, interleukin 1β; IL1R1, interleukin 1 receptor type 1; TNF-α, tumor necrosis factor α; TNFR1, tumor necrosis factor receptor type 1; MCP-1, monocyte chemoattractant protein 1; CCR2, chemokine (C–C motif) receptor 2; GR, glucocorticoid receptor; SVF, stromal-vascular fraction; inhibitor A, 3-(1-adamantyl)-5,6,7,8,9,10-hexahydro[1,2,4]triazolo[4,3-α]azocine trifluoroacetate salt; inhibitor B(BVT-3498), 2,4,6-trichloro-*N*-(5,5-dimethyl-7-oxo-4,5,6,7-tetrahydro-1,3-benzothiazol-2-yl)benzenesulfonamide; CBX, carbenoxolone; DEX, dexamethasone

2. Materials and methods

2.1. Reagents and chemicals

All reagents were of analytical grade unless otherwise indicated. Carbenoxolone [4,14,15], a non-selective inhibitor for 11β-HSD1 and 11β-HSD2 was obtained from Sigma (St. Louis, MO, USA). Recently-developed 11β-HSD1 selective inhibitors, 3-(1-adamantyl)-5,6,7,8,9,10-hexahydro[1,2,4] triazolo [4,3-α] azocine trifluoroacetate salt (WO03/065983, Merck Co., USA) (inhibitor A for short, unless otherwise indicated) [16] and 2,4,6-trichloro-*N*-(5,5-dimethyl-7-oxo-4,5,6,7-tetrahydro-1,3-benzothiazol-2-yl) benzenesulfonamide (BVT-3498, Biovitrum, Sweden) (inhibitor B for short, unless otherwise indicated) [17,18] were synthesized according to the patent information.

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2.2. Cell culture

Mouse reticulum cell sarcoma-derived J774.1 cells [13] were obtained from Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Japan. Cells were cultured in RPMI 1640 plus 10% heat-inactivated FBS at 37 °C in 5% CO_2.

2.3. Quantitative real time PCR

Total RNA was extracted using Trizol Reagent (Invitrogen, USA) and cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad, USA) according to the manufacturer's instruction. The sequence of probes and primers are summarized in Table 1. Taqman PCR was performed using ABI Prism 7700 Sequence Detection System as instructed by the manufacture (Applied Biosystems, USA). Level of mRNA was normalized to those of 18S mRNA.

2.4. 11β-HSD1 enzyme activity assay

11β-HSD1 primarily acts as a reductase and reactivates cortisol from cortisone in viable cells. On the other hand, under some conditions such as tissue homogenate or microsome fraction, 11β-HSD1 acts as a dehydrogenase and inactivate cortisone to cortisol [2]. 11β-HSD1 reductase activity in intact cells was measured as reported [2]. Briefly, cells were incubated for 24 h in serum-free RPMI 1640, adding 250 nM cortisone including tritium labeled tracer [1,2-^{3}H]$_2$ cortisone for reductase activity and 250 nM cortisol with [1,2,6,7-^{3}H]$_4$ cortisol for dehydrogenase activity. Cortisol and cortisone were extracted using ethyl acetate, evaporated and resuspended in ethanol, separated by thin-layer chromatography in chloroform:methanol (95:5) and quantified by autoradiography.

To validate inhibitory potency of compounds against 11β-HSD1, using FreeStyle 293 cells transiently transfected with human 11β-HSD1, the enzyme activity assay was carried out with 20 mM Tris–HCl, pH 7.0, 50 μM NADPH, 5 μg protein of microsomal fraction, 300 nM ^{3}H-cortison for 2 h. The reaction was stopped by 18β-Glycyrrhetinic acid. The labeled cortisol product was captured by mouse monoclonal anti-cortisol antibody, bound to SPA beads coated with protein A, and quantified in a scintillation counter.

2.5. Measurement of IL-1β, TNF-α and MCP-1 concentrations in cultured media

Interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) concentrations in J774.1 cell (1×10^6 cells/ml) cultured media were measured by enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers instructions (R & D Systems, USA).

2.6. Statistical analysis

Data are expressed as the means ± S.E.M. of triplicate experiments. Data were analyzed using one-way analysis of variance (ANOVA), followed by Student's *t*-tests for each pair for multiple comparisons. Differences were considered significant if $P < 0.05$.

3. Results

3.1. 11β-HSD1 mRNA level and reductase activity were substantially increased in activated J774.1 macrophages

Lipopolysaccharide (LPS) is a potent ligand for toll-like receptor (TLR) 4 and a representative activator of macrophages [19,20]. When J774.1 cells were treated with LPS (100 ng/ml) for 24 h, mRNA level of IL-1β, TNF-α and MCP-1 was markedly increased compared to control (approximately 1500-fold, 3-fold, 220-fold, respectively, $P < 0.01$). LPS (100 ng/ml, 24 h) also substantially augmented 11β-HSD1 mRNA level in J774.1 macrophages (~20-fold vs. control. $P < 0.01$) (Fig. 1A). 11β-HSD1 reductase activity was concomitantly augmented by LPS (100 ng/ml) compared to LPS-free control (2.9 ± 1.2-fold vs. control. $P < 0.05$) (Fig. 1B). Notably, 11β-HSD1 dehydrogenase activity was under-detectable not only at the basal but with LPS treatment (Fig. 1C).

3.2. Effect of pharmacological inhibition of 11β-HSD1 on inflammatory properties in J774.1 macrophages

To explore the potential role of 11β-HSD1 in cytokine release from activated macrophages, we examined the effect of pharmacological inhibition of 11β-HSD1 on gene expression of LPS-treated J774.1 cells. In the present study, two

Table 1
Sequences of TaqMan primers and probes

Gene	GenBank accession no.	Probe (FAM-5′ → 3′-TAMRA)	Primer (5′ → 3′)
Hsd11b1	NM008288	TCCGAGTTCAAGGCAGCGAGACACTACC	f CCAGGTCGGAGGAAGGTCTC r CCAGCAATGTAGTGAGCAGAGG
Hsd11b2	NM008289	TCAAGCCTGGCTGCTCCAAGACAG	f TCCCTGGGGTATCAAGGTCAG r CTCCCAGAGGTTCACATTAGTCAC
Il1b	NM008361	CATGGCACATTCTGTTCAAAGAGAGCCTG	f TCGCTCAGGGTCACAAAGAAA r CCATCAGAGGCAAGGAGGAA
Tnf	NM013693	CCCGACTACGTGCTCCTCACCCA	f TCTCTTCAAGGGACAAGGCTG r ATAGCAAATCGGCTGACGGT
Ccl2	NM011333	CCCCACTCACCTGCTGCTACTCATTCA	f TTGGCTCAGCCAGATGCA r CCAGCCTACTCATTGGGATCA
Il1r1	NM008362	CCTGACTTCAAGAATTACCTCATCGG	f GCATGTGCAGTTAATATACC r CAATTGTAGCCGTGAGGATG
Tnfrsf1a	NM011609	AGTGAGTGCGTCCCTTGCAGCC	f GCAGGGTTCTTTCTGAGAG r GGCACAACTTCATACACTCC
Ccr2	NM009915	CTCTGTCACCTGCATGGCCTGGTCT	f ATGAGTAACTGTGTGATTGACAAGCA r GCAGCAGTGTGTCATTCCAAGA
Nr3c1	NM008173	CCTGCTATGCTTTGCTCCTGATCTGATT	f ATCATACAGACAAGCAAGTGGAA r AGGGTAGAGTCATTCTCTGCTC
Tlr4	NM021297	CATGCCTTGTCTTCAATTGTTTCAA	f CTTCAGTGGCTGGATTTATC r GAGGTGGTGTAAGCCATG

Forward primers are designated by f and reverse primers by r.

Abbreviations: FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine. Hsd11b1, hydroxysteroid 11-beta dehydrogenase 1; *Hsd11b2*, hydroxysteroid 11-beta dehydrogenase 2; *Il1b*, interleukin 1 beta; *Tnf*, tumor necrosis factor; *Ccl2* (=monocyte chemoattractant protein:MCP-1); chemokine (C–C motif) ligand 2, *Il1r1*; interleukin 1 receptor, type I, *Tnfrsf1a* (=TNFR1), tumor necrosis factor receptor superfamily member 1a; Ccr2, chemokine (C–C motif) receptor 2; *Nr3c1* (=glucocorcoid receptor, GR); nuclear receptor subfamily 3, group C, member 1; *Tlr4*, toll-like receptor 4.

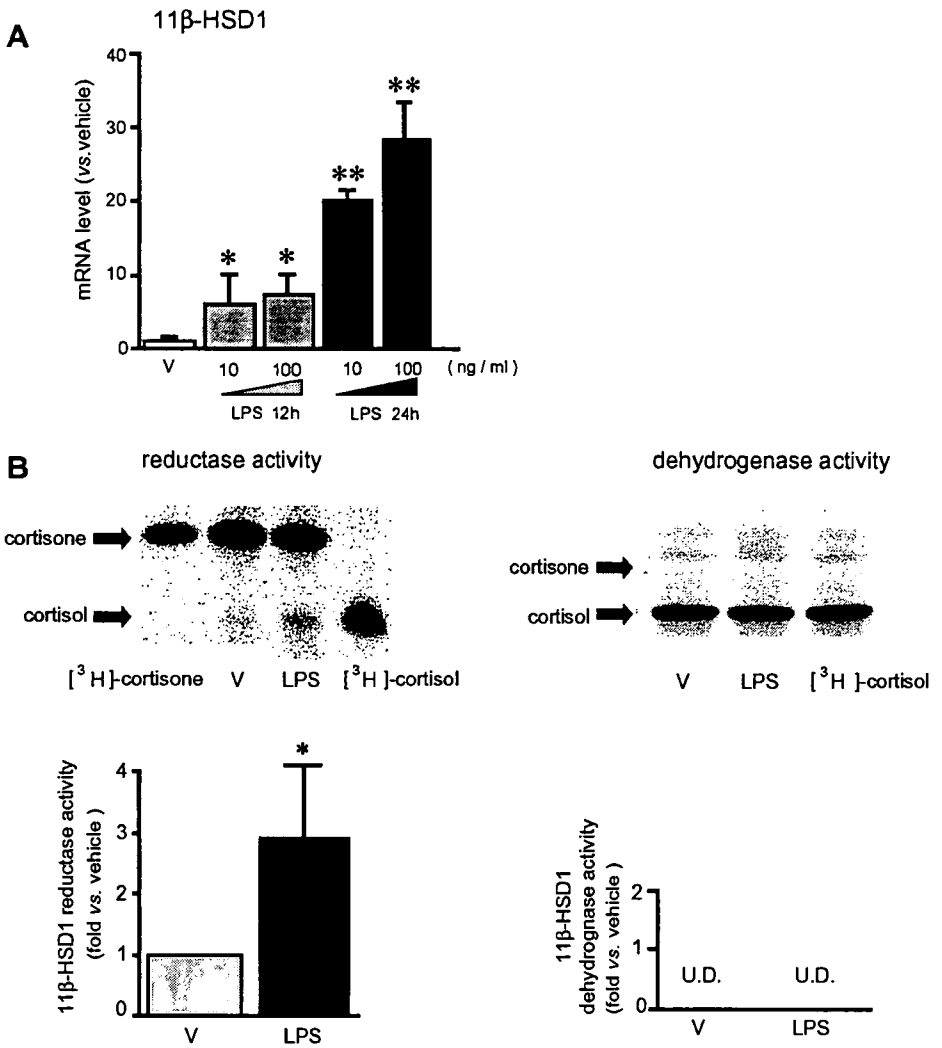

Fig. 1. 11β-HSD1 mRNA expression and reductase activity were induced by LPS in J774.1 macrophages. J774.1 cells (1×10^6 cells/ml, not-confluent) were treated with LPS ($10–10^3$ ng/ml, 12 or 24 h). (A) Level of mRNA for 11β-HSD1 was determined by real-time PCR. Level of each mRNA was normalized to that of 18S rRNA. (B) (left): 11β-HSD1 reductase activity (expressed as conversion ability of cortisone to cortisol) and (right): 11β-HSD1 dehydrogenase activity (expressed as conversion ability of cortisol to cortisone) were assessed in the media of J774.1 cells treated with 10 ng/ml LPS for 24 h. Reference corresponds to [^{3}H] cortisone or [^{3}H] cortisol used as a size marker. A representative autoradiograph of thin-layer chromatography (TLC) in 11β-HSD activity assay (upper) and quantification (lower). Intensities of signals at cortisol and cortisone correspond to the enzyme activity of reductase and dehydrogenase, respectively. U.D.; under detectable. Data are expressed as mean ± S.E.M. of triplicate experiments. *$P < 0.05$ vs. vehicle (V) treated group.

kinds of 11β-HSD1 selective inhibitors: inhibitor A and inhibitor B were synthesized and employed (as mentioned in materials and methods). 11β-HSD1 and 11β-HSD2 non-selective inhibitor, carbenoxolone (CBX) was also utilized. Based on our data that 11β-HSD2 mRNA (data not shown) and corresponding enzyme activity (dehydrogenase) [2,3] were under detectable in J774.1 cells even with LPS treatment (Fig. 1), CBX was considered to act virtually as a specific inhibitor against 11β-HSD1.

In the microsome fraction assay, we verified that inhibitor A (100 μM), inhibitor B (100 μM) and CBX (1 μM), all potently inhibited 11β-HSD1 activity as little as 25% vs. control, respectively (Fig. 2A). In J774.1 cells, treatment of inhibitor A (10 μM), inhibitor B (100 μM), and CBX (100 μM) markedly inhibited 11β-HSD1 activity (48 ± 23% [$P < 0.05$], 30 ± 1% [$P < 0.01$], and 80 ± 7% [$P < 0.01$] of reduction vs. LPS-treated cells, respectively), confirming that these compounds serve as potent inhibitors against 11β-HSD1 in J774.1 cells (Fig. 2B).

Noteworthy is that treatment of J774.1 cells with inhibitor A (10 μM), inhibitor B (100 μM) and CBX (100 μM) reduced IL-1β mRNA level (27 ± 5%, 38 ± 10%, 88 ± 2% of reduction vs. LPS-treated cells without compounds, respectively, $P < 0.01$) (Fig. 3A, upper). Consequently, IL-1β concentration in the media was markedly decreased (inhibitor A; 34 ± 10%, CBX; 75 ± 3% of reduction vs. LPS-treated cells, respectively, $P < 0.01$) (Fig. 3A, lower). TNF-α mRNA (inhibitor B; 20 ± 11.7% [$P < 0.05$], CBX; 34 ± 5% [$P < 0.01$] of reduction vs. LPS-treated cells, respectively) (Fig. 3B, upper) and its concentration in the media (inhibitor B: 41 ± 8%, CBX: 57 ± 4% of reduction vs. LPS-treated cells, respectively, $P < 0.01$) (Fig. 3B, lower) were also significantly suppressed by the treatment of 11β-HSD1 inhibitors. In MCP-1, only CBX exerted

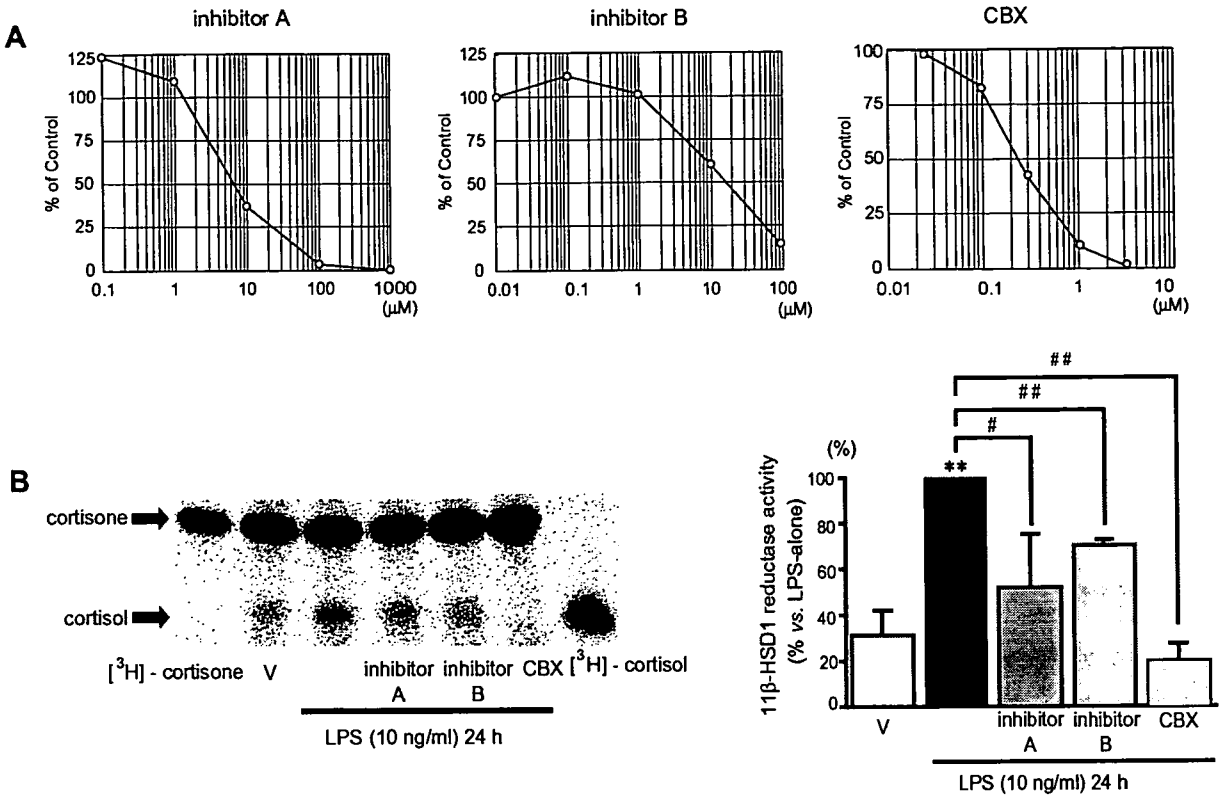

Fig. 2. 11β-HSD1 activity assay for validation of 11β-HSD1 inhibitors. (A) Microsome fraction 11β-HSD1 activity assay: inhibitor A (0.01–100 μM), inhibitor B (0.1–100 μM), CBX, carbenoxolone (0.01–10 μM). The *x*-axis shows the log concentration of each inhibitor and the *y*-axis shows log% inhibition of 11β-HSD1 activity compared to control. (B) Intact cell assay for 11β-HSD1 reductase activity. J774.1 cells were incubated for 24 h in serum-free RPMI 1640, adding 250 nM of cortisone with tritium labeled cortisone (left): a representative autoradiograph of TLC in 11β-HSD activity assay and (right): quantification of 11β-HSD activities. Intensities of signals at cortisol correspond to the enzyme activity of reductase. The *y*-axis shows the percentage of 11β-HSD1 reductase activity compared to LPS (10 mg/ml) treated cells per se. Treatment of inhibitor A (10 μM), inhibitor B (100 μM), and CBX (100 μM) substantially reduced 11β-HSD1 activity in J774.1 cells (inhibitor A and inhibitor B as mentioned in Section 2).

significant effect on the mRNA level (48 ± 11% of reduction vs. LPS-treated cells. $P < 0.01$) (Fig. 3C, upper) and concentration in the media (57 ± 4% of reduction vs. LPS-treated cells. $P < 0.01$) (Fig. 3C, lower).

The mRNA level of IL1R1 (receptor for IL-1β), TNFR1 (receptor for TNF-α), CCR2 (receptor for MCP-1), toll-like receptor 4 (TLR4) (receptor for LPS) and GR (receptor for glucocorticoid) was not significantly changed with these 11β-HSD1 inhibitors (data not shown).

3.3. *Effect of dexamethasone on inflammatory properties in J774.1 macrophages*

We examined the effect of glucocorticoid replenishment on the secretion of pro-inflammatory cytokine and chemokine in J774.1 macrophages. Treatment of J774.1 cells with dexamethasone (DEX) (10^{-10}–10^{-7} M, 24 h) suppressed the mRNA expression and secretion of IL-1β, TNFα and MCP-1 in a dose-dependent manner (supplementary figure A–F). Furthermore, co-treatment of LPS (10 ng/ml) with DEX markedly reduced the expression and secretion of IL-1β, TNFα and MCP-1 dose-dependently (supplementary figure A–F).

Treatment of J774.1 cells with DEX (10^{-7} M) reduced IL-1β mRNA level (85 ± 8% of reduction vs. vehicle, 91 ± 0.1% of reduction vs. LPS-treated cells, respectively, $P < 0.01$). Consequently, IL-1β concentration in the media was decreased (83 ± 8% of reduction vs. LPS-treated cells, $P < 0.01$) (supplementary figure A, D). Regarding TNF-α, mRNA (55 ± 8% of reduction vs. vehicle, 47 ± 14% of reduction vs. LPS-treated cells, respectively, $P < 0.01$) and concentration in the media (51 ± 14% of reduction vs. vehicle, 43 ± 8% of reduction vs. LPS-treated cells, respectively, $P < 0.01$) were markedly suppressed by DEX (supplementary figure B, E). Regarding MCP-1, DEX also decreased the mRNA level (65 ± 4% of reduction vs. vehicle [$P < 0.05$], 83 ± 3% of reduction vs. LPS-treated cells [$P < 0.01$], respectively) and concentration in the media (81 ± 10% of reduction vs. vehicle, 68 ± 5% of reduction vs. LPS-treated cells, respectively, $P < 0.01$) (supplementary figure C, F).

4. Discussion

Here, we demonstrated that J774.1 macrophages expressed considerable amount of mRNA and reductase activity of 11β-HSD1. Our data is the first to demonstrate that the cell activation with LPS markedly augmented mRNA expression and reductase activity of 11β-HSD1, accompanied by a drastic morphological change and increased secretion of pro-inflam-

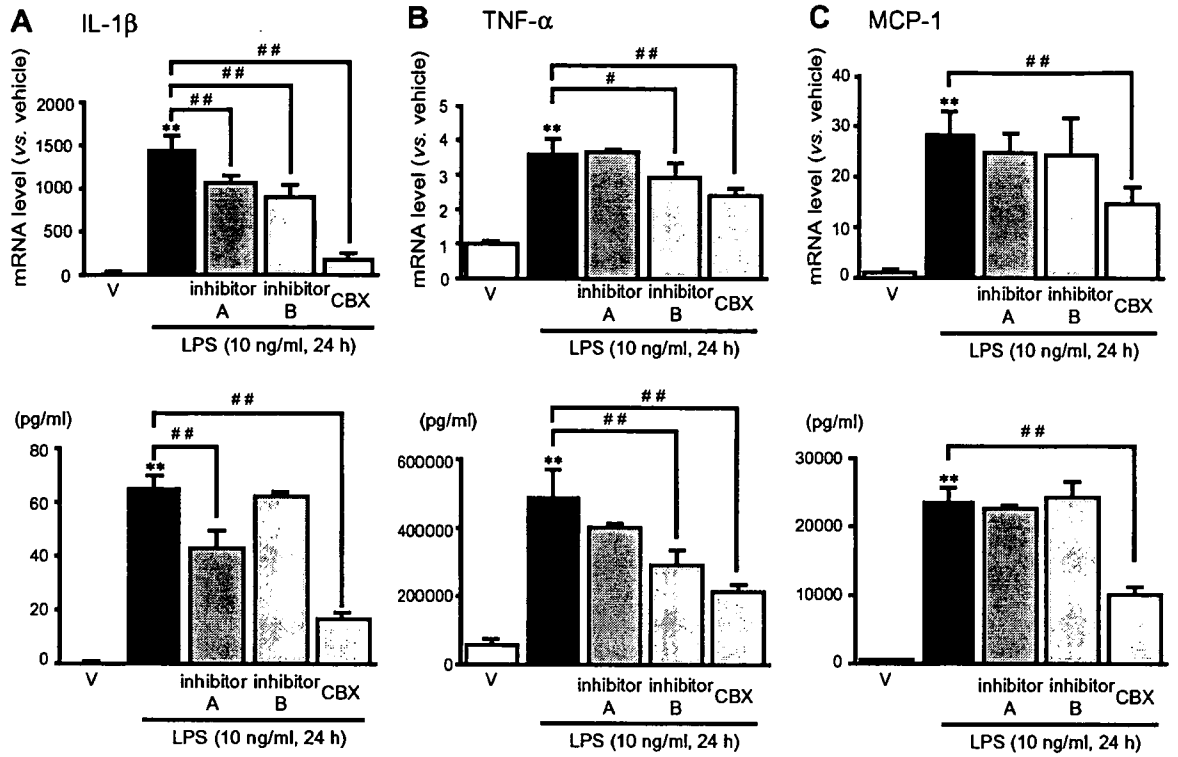

Fig. 3. Pharmacological inhibition of 11β-HSD1 considerably reduced IL-1 β, TNF-α or MCP-1 expression in and secretion from LPS-activated J774.1 macrophages. Effect of pharmacological inhibition of 11β-HSD1 on (A) IL-1β, (B) TNF-α and (C) MCP-1 mRNA in and secretion from LPS-activated J774.1 macrophages: J774.1 cells were activated by LPS (10 ng/ml) and co-treated with inhibitor A (10 μM), inhibitor B (100 μM) or CBX (100 μM) for 24 h. (upper); mRNA for IL-1β, TNF-α and MCP-1 determined by real-time PCR. Level of each mRNA was normalized to that of 18S rRNA and expressed as a relative value vs. control (lower). Concentrations of IL-1β, TNF-α and MCP-1 in the media measured by ELISA. Data are expressed as means ± S.E.M. of triplicate experiments. $^{**}P < 0.01$ vs. vehicle (V) treated group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. LPS-treated cells.

matory cytokines [13]. Recent studies highlighted the concept that not only LPS but non-microbial molecules such as fatty acids can also activate macrophages through TLR4 [21]. It is also suggested that endogenous, intestine-derived LPS contributes to the pathophysiology of obesity-associated fatty liver diseases [22]. In this context, potential mechanism of LPS-mediated dysfunction of adipose tissue stimulates much interest in future studies.

Glucocorticoids are widely used as anti-inflammatory agents in human clinics [23]. Consistent with the notion that glucocorticoids act as anti-inflammatory hormones, our data demonstrated that treatment of J774.1 cells with dexamethasone significantly suppressed the mRNA expression and secretion of IL-1β, TNF-α and MCP-1 in a dose-dependent manner (supplementary figure). On the other hand, physiological effects of endogenous glucocorticoids are distinct from those in pharmacological or therapeutic concentrations of synthetic cortisol analogues [24–27]. A recent study showed that 11β-HSD1-mediated intracellular glucocorticoid reamplification in macrophages enhanced phagocytic activity [28]. However, it still remains controversial whether the activation of 11β-HSD1 is involved in pro-inflammatory properties of macrophages. To test the possibility, we assessed the potential impact of pharmacological inhibition of 11β-HSD1 on secretion of pro-inflammatory cytokine and chemokine. Our data demonstrated for the first time that treatment of J774.1 cells with three structurally distinct 11β-HSD1 inhibitors considerably suppressed the mRNA expression and secretion of IL-1β, TNF-α or MCP-1, clearly indicating that intracellular regeneration of active glucocorticoid via 11β-HSD1 does exert pro-inflammatory effects on activated macrophages. Taking structural and pharmacological differences of compounds into consideration, further investigation is required to reinforce the result.

Macrophages play pathophysiologic roles in adipose dysfunction, arteriosclerosis [16] and inflammatory fatty liver diseases [22]. A recent study showed that pharmacologic inhibition of 11β-HSD1 ameliorated diabetes, dyslipidemia [29] and arteriosclerosis [16] in mouse models. It should also be noted that, reminiscent of adipocytes [16]. 11β-HSD1 expression and reductase activity is predominated in macrophages (Fig. 1), while that of 11β-HSD2 and dehydrogenase activity were not detected in our assays (data not shown). Taken together, our results suggest that 11β-HSD1 in activated macrophages may be a novel target for the treatment of metabolic diseases.

In adipose tissue, 11β-HSD1 was expressed not only in mature adipocytes but also stromal vascular fraction (SVF) cells which include infiltrated macrophages [30]. Number of cells contained in SVF was estimated to be two-thirds of that in whole adipose tissue [31]. Our data in human adipose tissue biopsies also showed that SVF from adipose tissue expressed 11β-HSD1 to a considerable extent (approximately 20% of that in floating matured adipocytes) (Yasue, S. et al. manuscript in preparation). Thus, 11β-HSD1 in activated macrophages may play a role in the pathophysiology of adipose

inflammation and dysfunction. Further investigations in vivo should validate this notion and may open a fresh avenue for molecular and cellular mechanism of adipose inflammation and dysfunction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.11.032.

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Short-term Effects of Atorvastatin on Bone Turnover in Male Patients with Hypercholesterolemia

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Abstract. No consensus has been reached on whether the 3-hydroxy 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, known as statins, have beneficial effects on bone health. The purpose of our study was to evaluate the effects of atorvastatin on bone metabolism by means of measuring bone turnover markers in male patients with hypercholesterolemia both at diagnosis and prospectively after 3 months of treatment. Twenty-two Japanese male patients (mean age 62.36 ± 10.1 years) with untreated hypercholesterolaemeia were selected for this study. After 3-months treatment of atorvastatin, total cholesterol and low density lipoprotein cholesterol significantly decreased as expected ($p<0.001$ for both parameters). Bone-specific alkaline phosphatase (BAP) did not change significantly ($p = 0.444$). However, serum N-terminal telopeptide of type I collagen (NTx) significantly decreased by −19.86 ± 26.4% ($p = 0.020$). In addition, ΔNTx during the course of this study was negatively correlated with NTx at baseline ($r = -0.645$, $p = 0.0008$). Although there was a tendency of positive correlations of ΔNTx with Δtotal cholesterol, Δtriglycerides, and Δlow density lipoprotein cholesterol, and of negative correlations of ΔNTx and ΔBAP with Δhigh density lipoprotein cholesterol, none of them reached statistical significance. Our findings suggest that atorvastatin may have potentially beneficial effects on bone metabolism in patients with hypercholesterolemia mostly by reducing bone resorption rather than by stimulating bone formation. Further studies with more patients and longer duration are warranted to evaluate its effects, if any, on prevention of osteoporosis and subsequent fractures.

Key words: Atorvastatin, Bone turnover, Osteoporosis, Hypercholesterolemia

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IN addition to their cholesterol-lowering properties, statins, the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are known to have various so-called pleiotropic effects including improvement of endothelial function, increased nitric oxide (NO) bioavailability, antioxidant properties, stabilization of atherosclerotic plaques, regulation of progenitor cells, inhibition of inflammatory responses and immunomodulatory actions [1]. However, no consensus has been reached yet as to whether statin has beneficial effects on bone health [2]. Experimental studies have shown that statins stimulate osteoblast-derived bone morphogenetic protein 2 (BMP-2) expression and subsequently enhance osteoblastic bone formation [3, 4]. In addition, it has also been suggested that statins directly affect osteoclasts through mechanisms analogous to those of bisphosphonates, because bisphosphonates and statins exert their effects by inhibiting the same mevalonate pathway [5, 6]. These findings raise the hope that statins might have significant bone-formative and antiresorptive effects on bone metabolism in humans.

However, although there have been a growing number of clinical studies examining the effects of statins

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Table 1. Clinical trials examining effects of statins on bone turnover, cited in the discussion

Authors and Year	Patients with Hyperlipidemia		Statins	Duration (month)	Bone Turnover Markers	
	Number	Gender (M/F)			Bone Formation Markers	Bone Resorption Markers
Stein *et al.*[13], 2001	390	170/220	Simvastatin	3	BAP decreased.	s-CTx unchanged
	404	157/247	Atorvastatin		BAP unchanged.	
Watanabe *et al.*[16], 2001	12	0/12	Fluvastatin	1	OC increased. BAP unchanged.	u-NTx decreased.
				6	OC unchanged. BAP unchanged.	u-NTx unchanged.
Bjarnason *et al.*[12], 2001	45	0/45	Fluvastatin	3	OC unchanged. ALP unchanged.	u-CTx decreased. s-CTx decreased.
Rejnmark *et al.*[7], 2002	140	0/140	Simvastatin, Atorvastatin, Lovastatin, Pravastatin, Fluvastatin, Cerivastatin	Cross Sectional	OC was lower. BAP was lower.	s-CTx was lower.
Kajinami *et al.*[8], 2003	35	23/12	Atorvastatin	3	OC unchanged. BAP unchanged.	u-NTx unchanged.
				6	OC unchanged. BAP increased.	
Kuzuya *et al.*[9], 2003	16	3/13	Atorvastatin	3 6	BAP unchanged.	u-NTx decreased.
Braatvedt *et al.*[11], 2004	25	9/16	Atorvastatin	3	OC unchanged. BAP unchanged.	β-CTx unchanged.
Berthold *et al.*[10], 2004	24	0/24	Atorvastatin	2	OC unchanged. BAP unchanged.	s-CTx unchanged.
Rejnmark *et al.*[14], 2004	39	0/39	Simvastatin	12	OC unchanged. BAP unchanged.	s-CTx unchanged.
Rosenson *et al.*[15], 2005	12	not shown	Pravastatin	2	OC unchanged. BAP unchanged.	u-NTx unchanged.
	14		Simvastatin		OC unchanged. BAP unchanged.	
	15		Simvastatin		OC unchanged. BAP decreased.	
Own study, 2006	22	22/0	Atorvastatin	3	BAP unchanged.	s-NTx decreased.

BAP, bone-specific alkaline phosphatase; OC, osteocalcin; ALP, alkaline phosphatase; s-, serum; u-, urinary; CTx, C-terminal telopeptide of type I collagen.; NTx, N-terminal telopeptide of type I collagen.

on bone metabolism [7–16], most of them could not find increase of bone formation markers [7, 9–15] (Table 1). Likewise, reduction of bone resorption markers was found in some studies [7, 9, 12, 16], but not in others [8, 10, 11, 13–15]. Moreover, higher bone mineral density (BMD) [16] and lower fracture rates [16, 17] in patients treated with statins have been demonstrated in some clinical studies, but not in others [7, 12, 18, 19]. One of the possible reasons for these discrepancies among previous clinical studies might be the difference in the statin used [3–5, 13, 20]. Therefore, in the present study, we used the same statin, atorvastatin, which is a relatively new product with powerful lipid-lowering potency. In addition, the pos-

sibility has been suggested that it may have greater ability to affect bone [4, 5, 19, 20].

Another possible reason may be the gender difference in the previous clinical studies, because interpretation of the effects of statins on bone metabolism is hampered by the background of involutional osteoporosis in female patients [21]. In addition, there is ample evidence to indicate that osteoporosis in men is already a public health problem. Therefore, our assessment of the potential effects of atorvastatin on bone metabolism was limited to male patients in an effort to largely eliminate the influence of involutional osteoporosis.

The purpose of our study was thus to examine and assess the effects of atorvastatin on bone turnover in male patients with hypercholesterolemia, both at diagnosis and prospectively after 3 months of treatment.

Subjects and Methods

Subjects

Twenty-two Japanese male patients (mean age 62.36 ± 10.1 years) with untreated hypercholesterolemia, who attended the clinic of Rakuwakai Otowa Hospital between January 2005 and January 2006, were selected for this study. The diagnosis of hypercholesterolemia was established on the basis of laboratory findings, including an elevated serum total cholesterol (TC) level (>220 mg/dl) and an elevated serum low density lipoprotein cholesterol (LDL) level (>140 mg/dl). Hypercholesterolemia in all patients was treated with atorvastatin (10 mg/day) alone. During the course of this study, the dose of atorvastatin remained unchanged. This study involved a 3-month (at baseline, and 3 months after the beginning of the treatment) longitudinal examination of these 22 patients. Their clinical data at baseline are shown in Table 2.

All subjects completed a questionnaire administered by the doctor or nurse prior to entry into the study, and underwent laboratory blood and urinary tests. We excluded subjects who had a history of fractures and/or of other diseases (type 1 diabetes mellitus, liver disease, renal dysfunction, malignancy, hyperthyroidism, hyperparathyroidism, hypercorticoidism, or hypogonadism) and those taking medications (active vitamin D3, bisphosphonates, calcitonin, selective estrogen receptor modulators, estrogens, testosterones, steroids, thyroid hormones, diuretics, heparin or anticonvulsants) that could influence bone metabolism. We also excluded those with triglycerides (TG) level >500 mg/dl, because their LDL cannot be calculated adequately using the Friedewald equation. None of the subjects were smokers or substance abusers.

Table 2. Means ± SD of the variables assessed in patients with hypercholesterolemia

	Patients with hypercholesterolemia (n = 22)		*P*-value
	Baseline	3 months later	
Age (years)	62.36 ± 10.1	—	—
Height (cm)	163.44 ± 7.6	—	—
Weight (kg)	66.17 ± 12.9	—	—
BMI (kg/m^2)	24.63 ± 3.6	—	—
TC (mg/dL)	247.68 ± 25.1	179.23 ± 32.3**	<0.001
TG (mg/dL)	165.59 ± 77.7	126.23 ± 61.4*	0.033
HDL (mg/dL)	51.59 ± 11.4	52.96 ± 10.9^{NS}	0.337
LDL (mg/dL)	162.97 ± 23.3	101.03 ± 25.9**	<0.001
Ca (mg/dL)	9.58 ± 0.5	9.44 ± 0.4^{NS}	0.113
ALP (IU/L)	249.05 ± 75.3	238.68 ± 59.8^{NS}	0.31
BAP (IU/L)	22.42 ± 5.6	21.76 ± 5.7^{NS}	0.444
NTx (nmolBCE/L)	15.84 ± 4.0	12.20 ± 3.7*	0.013

Data represent mean ± SD.

BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; Ca, calcium; ALP, alkaline phosphatase; NTx, N-terminal telopeptide of type I collagen; BAP, bone-specific alkaline phosphatase.

P-values for comparisons of the parameters between at baseline and 3 months: $^{NS}P>0.05$; $^{*}P<0.05$; $^{**}P<0.01$.

This study was performed in accordance with the recommendations of the Declaration of Helsinki, and the protocol was approved by the Ethical Committee of Rakuwakai Otowa Hospital. All the subjects gave their informed consent before they were enrolled.

Biochemical measurements

All subjects underwent laboratory blood tests at baseline, and at 3 months. Serum samples were obtained before 8:00 AM after an overnight fast, and were immediately processed and kept frozen at –20°C until the assays were carried out. Serum TC, TG, high density lipoprotein cholesterol (HDL), calcium (Ca), and alkaline phosphatase (ALP) were measured with standard laboratory methods. LDL was calculated by Friedewald equation (LDL = TC – [HDL + TG/5]). Se-

rum bone-specific alkaline phosphatase (BAP) was measured with an enzyme immunoassay kit (Osteolinks-BAP; Sumitomo Pharmaceuticals Inc., Tokyo, Japan; reference range: 13.0–33.9 U/L) as a marker of bone formation. Serum N-terminal telopeptide of type I collagen (NTx) was measured by means of an enzyme-linked immunosorbent assay (OSTEOMARK; Mochida Pharmaceutical Co., Tokyo, Japan; reference range: 9.5–17.7 nmolBCE/L) as a marker of bone resorption. The intra-assay coefficient of variation for BAP and NTx is 3.8% and 7.8% respectively, while the inter-assay coefficient of variation is 1.6% and 3.7%, respectively.

Statistical analysis

Data were analyzed by paired t-test for longitudinal differences between at baseline and at 3 months, and by Pearson's correlation test for determining correlations. Statistics were calculated with StatView version 5.0 (Abacus Concepts, Inc., Berkeley, CA, USA). A P value <0.05 was considered statistically significant.

Results

Fortunately, the atorvastatin treatment was well tolerated by our patients, and none of them dropped out during the course of this study. Table 2 shows the longitudinal characteristics of the patients. NTx, but not BAP, was significantly decreased after 3-months treatment of atorvastatin ($p = 0.013$) (Fig. 1). NTx was decreased in as many as 18 (81.8%) of the patients, while BAP only in 11 (50.0%) of them. The percentage change of the reduction of NTx was significant compared with the baseline (–19.86 ± 26.4%, $p = 0.020$), while that of BAP was not significant (–1.32 ± 19.1%, $p = 0.749$). The change of NTx and BAP was over the so-called minimum significant change (14.2% and 23.1%, respectively) in 14 and 3 patients, respectively. TC and LDL were also significantly decreased after 3-months treatment, as expected. The other biochemical parameters (TG, HDL, Ca, and ALP) were unaltered after 3-months treatment of atorvastatin.

Table 3 shows correlations of ΔNTx and ΔBAP with age, height, weight, BMI and the biochemical parameters at baseline. ΔBAP was positively correlated with HDL at baseline (r = 0.543, $p = 0.0079$). ΔNTx was negatively correlated with NTx at baseline (r = –0.645, $p = 0.0008$) (Fig. 2).

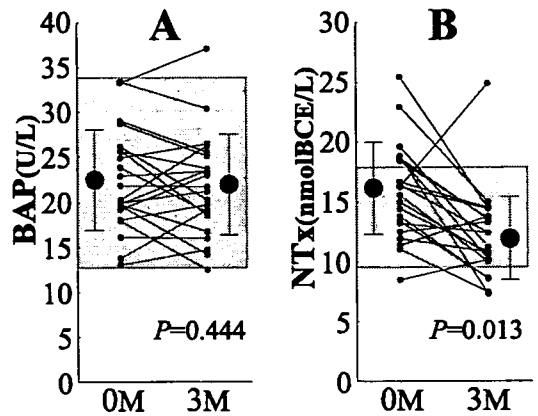

Fig. 1. Comparison of bone turnover markers between at baseline and at 3 months. BAP (A) and NTx (B) levels at baseline (0 M) and at 3 months (3 M) in patients with hypercholesterolemia are plotted. The bold circles represent the mean, and the vertical lines represent the SD for each marker. The shaded areas represent reference range. P-values for comparison of the parameters between at baseline and the 3 months.

Table 4 shows correlations of ΔNTx and ΔBAP with Δage, Δheight, Δweight, ΔBMI and the Δbiochemical parameters. Although there was a tendency of positive correlations of ΔNTx with ΔTC, ΔTG, and ΔLDL, and of negative correlations of ΔNTx and ΔBAP with ΔHDL, none of these trends reached statistical significance.

Discussion

Concerning the biological effects of statins on bone metabolism, Mundy *et al.* [3] first reported that statins stimulated osteoblast-derived BMP-2 expression and subsequently enhanced osteoblastic bone formation. Since then, this enhancing effect of statins on bone formation has been repeatedly confirmed by numerous *in vitro* studies [4]. In addition, animal model studies also showed that bone formation rate was increased in rats given statins [22]. These findings strongly suggest the possibility that statins could potentially be useful as an anabolic therapeutic agent for osteoporosis.

However, clinical studies in humans have not always succeeded in demonstrating this stimulating effect of statins on bone formation [7, 9–15]. One of the possible reasons for this discrepancy between *in vivo* and *in vitro* studies may be the differences in the dosage of

Table 3. Correlations of ΔBAP and ΔNTx with age, height, weight, BMI and the biochemical parameters at baseline in patients with hypercholesterolemia

	Age	Height	Weight	BMI	TC	TG	HDL	LDL	Ca	ALP	BAP	NTx
ΔBAP	0.196	0.156	−0.185	−0.347	0.072	−0.138	0.543**	−0.097	0.083	0.218	−0.332	0.224
ΔNTx	−0.021	0.063	0.135	0.171	−0.030	−0.039	0.251	−0.094	0.017	0.094	0.249	−0.645**

Values are correlation coefficients.
BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; Ca, calcium; ALP, alkaline phosphatase; NTx, N-terminal telopeptide of type I collagen; BAP, bone-specific alkaline phosphatase.
P-values for correlations of BAP and NTx with age, height, weight, BMI and the biochemical parameters in patients with hypercholesterolemia: $^{**}P<0.01$.

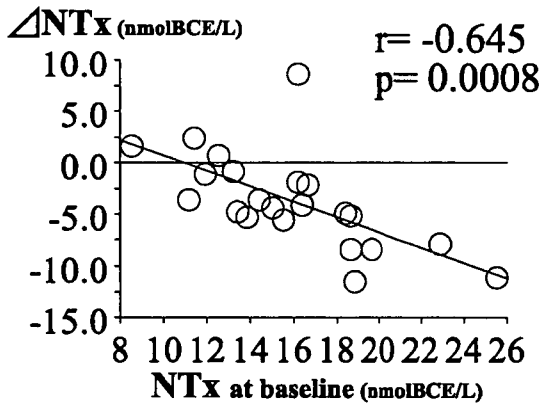

Fig. 2. Correlation of ΔNTx with NTx at baseline. ΔNTx are plotted against NTx at baseline. The line reflects the regression and r means the correlation coefficient. *P*-value for correlation between ΔNTx with NTx at baseline.

Table 4. Correlations of ΔBAP and ΔNTx with Δbiochemical parameters in patients with hypercholesterolemia

	ΔTC	ΔTG	ΔHDL	ΔLDL	ΔCa	ΔALP
ΔBAP	-0.009^{NS}	0.008^{NS}	-0.250^{NS}	0.037^{NS}	-0.064^{NS}	0.157^{NS}
ΔNTx	0.160^{NS}	0.195^{NS}	-0.211^{NS}	0.131^{NS}	0.089^{NS}	0.257^{NS}

Values are correlation coefficients.
BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; Ca, calcium; ALP, alkaline phosphatase; BAP, bone-specific alkaline phosphatase; NTx, N-terminal telopeptide of type I collagen.
P-values for correlations of ΔBAP and ΔNTx with Δbiochemical parameters in patients with hypercholesterolemia: $^{NS}P>0.05$.

statins [5, 10]. Indeed, some clinical studies reported that statins increased bone formation markers [8]. However, most of them have shown that statins either did not alter [8–16], consistent with our finding, or decreased [7, 13, 15] bone formation markers. These discrepancies among previous clinical studies and ours might be explained partly by the statin used [3–5, 13, 20]. Stein *et al.* [13] actually described the difference between the effects of different statins on bone metabolism in humans, and some *in vitro* studies showed that beneficial effects on bone were found only in lipophilic statins such as atorvastatin, but not hydrophilic pravastatin [3, 4, 20]. We are aware of 5 clinical studies [8–11, 13] examining the effects of atorvastatin, which was used in our study, on bone metabolism. In agreement with our results, 4 of them [9–11, 13] found no significant changes of bone formation markers by atorvastatin. These results and ours do not support the hypothesis that clinical use of atorvastatin exerts anabolic effects on human bone metabolism.

On the other hand, the present study revealed that NTx significantly decreased after 3 months-treatment with atorvastatin, suggesting antiresorptive effects of atorvastatin. Moreover, this antiresorptive effect of atorvastatin was found to be all the more prominent in patients with higher NTx, suggesting beneficial effects of atorvastatin on bone health. Although this negative correlation between ΔNTx and NTx at baseline may indicate that atorvastatin could exert bone protective effects more in osteoporotic patients as in the case with bisphosphonates, the most powerful antiresorptive agent available, the mechanism of this correlation is unclear from our study. However, the fact that both bisphosphonates and statins exert their effects by inhibiting the same mevalonate pathway [6] supports our hypothesis. In addition, although some controversies exist, some clinical studies examining the effects of statins on bone metabolism have actually shown significant reduction in bone resorption markers [7, 9, 12, 16], consistent with our finding. A recently published study by Kuzuya *et al.* [9] showed that 1-year treatment