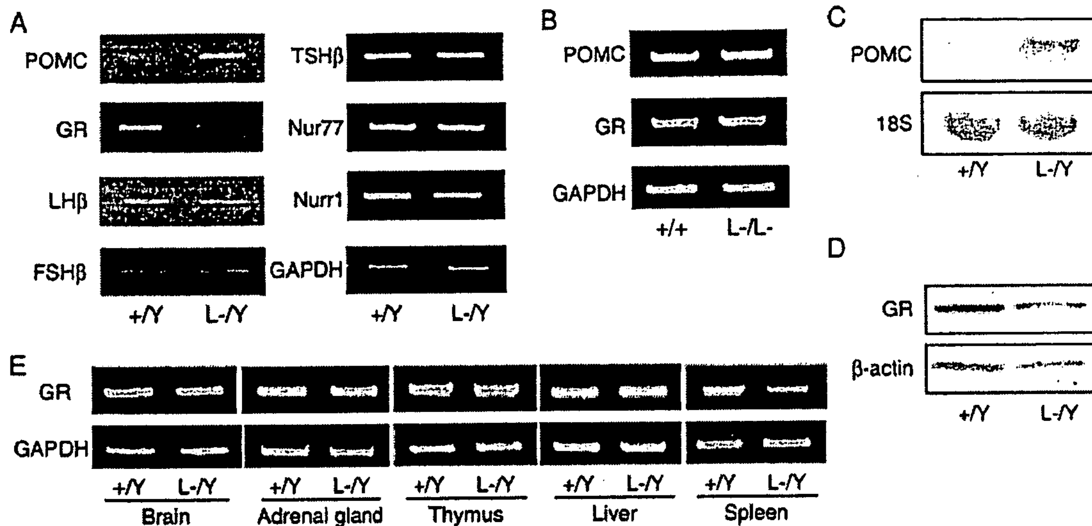

FIG. 5. Altered expression levels of gene transcripts involved in the HPA axis. (A) Increased POMC and decreased GR expression levels of transcripts in ARKO pituitary by semiquantitative RT-PCR. LHβ, luteinizing hormone β; FSHβ, follicle-stimulating hormone β; TSHβ, thyroid-stimulating hormone β. (B) No significant alterations of POMC and GR mRNA levels in the pituitary glands of female ARKO ($AR^{L-/L-}$) mice. (C and D) Northern blot analyses showing clear up-regulation of POMC mRNA levels and down-regulation of GR mRNA levels in the ARKO pituitary. (E) Tissue-specific reduction of GR transcripts in ARKO mice. GR expression levels are down-regulated only in the spleen and pituitary in male ARKO mice.

The hypertrophic and hyperplastic adrenal glands in the ARKO mice probably resulted from high levels of serum ACTH, derived from high POMC transcript levels in the pituitaries of ARKO mice. Studies with transgenic mice expressing antisense RNA against the GR in the brain and anterior pituitary demonstrate that the GR mediates the negative feedback regulation of glucocorticoid production through HPA axis activity (26, 36). Consistent with this observation, the male ARKO mice had low pituitary GR mRNA levels but no difference in the distribution of pituitary hormone-producing cells compared to WT animals. Thus, our findings suggest that the activated AR in the pituitary gland is needed to express pituitary GR at a sufficiently high level to participate in the negative feedback regulation of glucocorticoid production. The X-zone, which is considered a fetal zone, regresses during sexual maturation and reappears after gonadectomy (11). The molecular basis underlying X-zone regression during sexual maturation remains to be investigated. However, our results raise the possibility that the activated AR in adrenal glands induces X-zone repression by the induction of apoptosis. Consequently, the identification of AR target genes expressed in the X-zone is another interesting direction to pursue.

Liganded AR augments GR gene expression in the pituitary gland. We found that GR gene expression was impaired in the pituitary glands of ARKO males. We presumed that the reduced GR levels led to increased expression of the POMC gene, with subsequent high levels of serum ACTH. This idea was supported by the observation that the suppression of ACTH production by exogenous glucocorticoids was partially impaired in the ARKO mice. Moreover, the DHT-activated AR enhanced the GR mRNA levels in a pituitary cell line but not in 3T3-L1 preadipocytes. The effect of DHT was most likely mediated by a response element in an upstream region of the GR promoter exon 1B (33). Thus, the activated AR directly induces the pituitary GR in a cell-specific manner. How this is accomplished on a molecular level remains to be elucidated.

Do androgen/AR signaling disorders link with an ACTH-dependent hypercorticoid state? A hypercorticoid state in humans is well known to cause Cushing's syndrome, in which patients suffer from a number of disorders such as centripetal obesity, facial rounding, glucose intolerance, hyperinsulinemia, and impaired lipid and bone metabolism (23). Most of these lesions are a reflection of glucocorticoid-driven gluconeogenesis. The hypercorticoid state may result from either endogenous disorders or chronic treatment with exogenous glucocorticoid. Endogenous causes of Cushing's syndrome are further classified as ACTH dependent or independent (18). The ACTH-dependent syndrome is characterized by up-regulated levels of ACTH; however, the molecular basis underlying the ACTH overproduction remains to be investigated. It is possible that sex steroids are involved, but this has not yet been fully addressed.

The male ARKO mice exhibited abnormalities similar to those seen for ACTH-dependent Cushing's syndrome patients. Since we detected up-regulation of the pituitary POMC transcript, other POMC-derived peptides might have contributed to the onset of obesity in male ARKO mice. For example, α-MSH in the neurons of the hypothalamus plays a central role in appetite control and energy homeostasis (3, 4). Although we detected no clear alteration in α-MSH immunoreactivity in the arcuate nuclei of the hypothalami of male ARKO mice, it will be of interest in future experiments to examine the melanocortin receptor system in ARKO brain. In contrast to the male ARKO mice, ARKO females did not display some of the abnormalities, such as obesity. It is possible that the lack of obesity in female ARKO mice may result from activation of estrogen receptors (ERs). ERs activated by high physiological

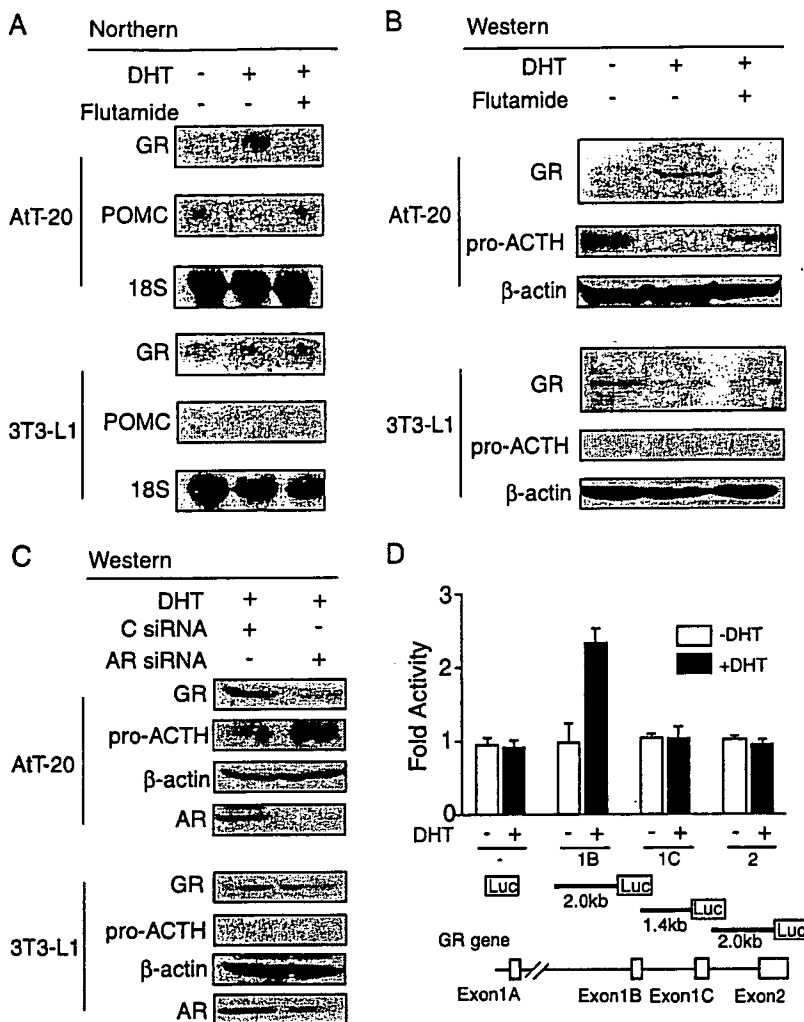

FIG. 6. Cell-type-specific regulation of the GR by activated AR. (A) Regulation of GR and POMC gene expression by treatment with either DHT or an AR antagonist (Flutamide) in the cultured cells as analyzed by Northern blot analysis. (B) Expression of the GR and pro-ACTH proteins was analyzed by Western blot analysis. (C) The significance of AR in the GR gene regulation was tested by AR RNA interference (with small interfering RNA [siRNA]) in the cultured cells. C siRNA, control siRNA. (D) Luciferase assay was performed with a series of the GR promoter regions in AtT-20 cells. After transfection with each of the promoter tk-luciferase vectors, the transfected cells were incubated with or without 10^{-7} M DHT.

levels of endogenous estrogens are effective in maintaining the proper levels of pituitary GR mRNAs needed to control POMC gene expression. This idea is indeed supported by the finding of unaltered levels of GR and POMC transcripts in the pituitary glands of the female ARKO mice. Moreover, estrogen treatment in female rats is shown to suppress serum levels of ACTH (27, 41). The common but gender-specific putative functions of the AR and ER in the brain have already been described in the context of mouse sexual behavior (24, 29). Though the possible ER functions remain to be studied for female ERKO mice, the present study suggests that the activated AR potentiates the negative HPA feedback regulation of glucocorticoid production through up-regulation of GR expression levels. Our study implies that the AR may be a potential therapeutic target for ACTH-dependent Cushing's syndrome.

In conclusion, the present study suggests that the androgen/AR signaling system is a negative pathway for glucocorticoid secretion in adult male mice. ARKO mice showed decreased GR expression in the pituitary glands and increased circulating ACTH and glucocorticoid. Androgens may increase the sensitivity of the HPA negative feedback loop to glucocorticoids by increasing GR expression in the pituitary gland, leading to suppression of adrenal cortical function. Thus, we presume that activated AR in the pituitary gland is a component of the negative feedback system for glucocorticoid production.

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Herbicide atrazine activates SF-1 by direct affinity and concomitant co-activators recruitments to induce aromatase expression via promoter II

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Abstract

The popular herbicide atrazine is an endocrine disruptor that demasculinizes and feminizes several species of animals, and co-relates with breast and reproductive disorders in mammalians. We recently reported that atrazine induces human *aromatase* gene expression via promoter II (ArPII) in a steroidogenic factor 1 (SF-1)-dependent manner. Here, we show that knockdown of SF-1 abolishes ArPII induction by atrazine in H295R cells, which harbor high SF-1 expression and are originally atrazine-responsive. Conversely, exogenous SF-1 enables atrazine to induce ArPII in the otherwise non-responsive KGN cells. Atrazine's effect is independent from protein kinase A and LRH-1, a close relative of SF-1. However, it binds directly to the SF-1, and concomitantly, enhances interactions of SF-1 with co-activator TIF2, and renders more SF-1 binding to ArPII chromatin. Intriguingly, LBD mutations do not alter SF-1's ability to mediate atrazine stimulation, suggesting that atrazine interacts with SF-1 via a region(s) other than the ligand binding pocket. These data suggest that atrazine binds to and activates SF-1 to induce ArPII.
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Keywords: Aromatase; Steroidogenic factor 1; Atrazine; Endocrine disruption

Atrazine is one of the most commonly used herbicides in the world, and is the most common contaminant of ground and surface water [1]. Atrazine poses concerns because it is a potent endocrine disruptor. Studies in every vertebrate class examined show that atrazine inhibits androgen-mediated development and simultaneously produces estrogen-like effects in exposed individuals [2]. Atrazine induces feminization in male tadpoles, renders some males infertile, and may thus contribute the recent global amphibians decline [1,3]. It is also reportedly associated with decreased semen quality and fertility in men living in agricultural areas [4]; and increased breast cancer incidence in women whose well water is contaminated with atrazine [5].

Atrazine does not act as an androgen receptor antagonist or an estrogen receptor agonist due to lack of affinity for both hormone receptors [6]. However, it reportedly inhibits phosphodiesterase [6–8], leads to elevated cAMP

Abbreviations: ArPII, aromatase promote II; DMSO, dimethyl sulfoxide; LBD, ligand binding domain; LRH-1, liver receptor homolog-1; PKA, protein kinase A; QCM, quartz crystal microbalance; SF-1, steroidogenic factor 1; SPR, surface plasmon resonance.

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levels, which result in elevated transcription of *CYP19* (the gene for human aromatase), increased aromatase activity, and subsequent estrogen production.

Although this mechanism is consistent with the demasculinization and feminization effects of atrazine, some studies suggest that atrazine does not induce aromatase in some species and/or in certain cell lines [9]. For example, although atrazine induces aromatase in human H295R adrenal carcinoma cells, the current authors failed to reproduce this result in ovarian granulosa KGN cells [10]. We recently reported that this apparent discrepancy is due to the requirement of the nuclear receptor, steroidogenic factor 1 (SF-1) in atrazine-enhanced aromatase expression [2].

SF-1, which specifically expresses in steroidogenic tissues, is essential for steroidogenesis, steroidogenic tissues development, and normal sex differentiation [11,12]. SF-1 is a critical factor in the induction of aromatase via the aromatase promoter II (ArPII); and is required for ArPII to be transcriptionally regulated by the cAMP-protein kinase A (PKA) pathway [13]. Our present study confirmed that atrazine induces aromatase expression via promoter II in a SF-1-dependent manner. We also demonstrated that the chemical binds directly to the receptor, and that the affinity is most likely independent of the LBD. Concomitantly, atrazine significantly enhanced interactions of SF-1 with its co-activator, TIF2; and also induces binding of SF-1 to native chromatin of ArPII. These data suggest that the herbicide atrazine is potentially an artificial ligand for SF-1; and highlight the importance of atrazine as a risk factor in endocrine disruption in wildlife and reproductive cancers in rodents and humans.

Materials and methods

Materials. The human ovarian granulosa-like tumor cell line KGN was established and maintained as previously reported [14]. H295R and NIH-3T3 cells were obtained from the American Type Culture Collection. Atrazine and simazine standards were obtained from Wako Pure Chemical Co. (Osaka, Japan). H-89 dihydrochloride hydrate, a selective, potent inhibitor of cAMP-dependent protein kinase, was purchased from Sigma–Aldrich, Inc. (St. Louis, MO). A 4.0 kb human *CYP19* promoter II luciferase reporter (pGL3-ArPII4.0), and a 4.0 kb human SF-1 promoter reporter were constructed previously [15,16]. The adenovirus construct expressing bovine SF-1, Adeno-SF-1 and the one expressing β-galactosidase, Adeno-LacZ, were prepared as previously described [2].

Western blotting and mRNA quantification. The anti-SF-1 antibody, used for both the ChIP assay and Western blotting, was kindly provided by Professor K. Morohashi (National Institution for Basic Biology, Okazaki, Japan). Relative mRNA copy numbers to β-actin of endogenous LRH-1 in H295R and KGN cells were analyzed by real-time PCR. Primers for human LRH-1 were, forward: 5′CTG ATA CTG GAA CTT TTG AA3′; reverse: 5′CTT CAT TTG GTC ATC AAC CTT3′. Those for human β-actin were, forward: 5′AAA CTA CCT TCA ACT CCA TC3′; reverse: 5′ATG ATC TTG ATC TTC ATT GT3′.

Human SF-1 RNA interference. To knockdown endogenous SF-1 expression, a pool of three human SF-1-targeting siRNA oligonucleotides (Santa Cruz, Cat#: sc-37901) or a control scrambled siRNA pool (Santa Cruz, Cat#: sc-36869) was transfected to H295R cells pre-seeded in 6-well plate by X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics GmbH, Germany). The final siRNA concentration in medium was 100 nM. Three, five or seven days after transfection, cells were harvested, and expressions of SF-1 were determined by Western blotting. The anti-SF-1 siRNA substantially lowered SF-1 protein levels from the 5th day, and the effect sustained at the 7th day. For combined luciferase reporter assays, ArPII reporter together with an internal control vector (pRL-CMV) were transfected to H295R cells on the 6th day post siRNA transfection, chemicals applied on the 7th day, and luciferase assays were performed 48 h later. SF-1 knockdown was also achieved by using a pool of four SF-1-targeting siRNA oligonucleotides (ON-TARGET SMART-pool; Cat#: L-003429-00-0005 Dharmacon, Lafayatte, CO) and the subsequent reporter assays were replicated.

Relative luciferase reporter assays and mammalian two-hybrid assays were performed essentially as previously described [17].

In vitro atrazine–SF-1 interaction assay. Baculovirus was used to express the Flag containing SF-1 fusion protein in insect sf21 cells [2]. The binding affinity of atrazine to SF-1 was measured by using a 27-MHz Quartz Crystal Microbalance (QCM) (Initium Co., Tokyo, Japan). Chemical of interest was immobilized onto a QCM electrode, which was soaked in PBS buffer (8 mL, pH 7.4), and monitored continuously for QCM frequency change at 25 °C. After the frequency change was stabilized, recombinant SF-1 protein was added to the solution, and the subsequent time-course of frequency change was assessed.

Statistical analysis. Data are expressed as means ± SD and was evaluated by one-way ANOVA or Student's two-tail *t*-test, followed by posthoc comparisons with Fisher's protected least significant difference test, as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results and discussion

ArPII responds to atrazine SF-1 dependently

By applying a luciferase reporter system, in which a 4.0 kb human ArPII luciferase reporter and a human SF-1 expression vector are co-expressed in NIH-3T3 fibroblast cells, which lack endogenous SF-1, we previously showed that of 55 known environmental hormone chemicals screened, atrazine and its analog simazine were found to stimulate SF-1-mediated ArPII activity by a factor of 2–3; while neither chemical affected ArPII in the absence of SF-1 co-expression in these cells [2]. As introduced above, atrazine is reportedly able to induce aromatase expression in H295R adrenocortical carcinoma cells, but not in KGN granulosa-like cancer cells. We proposed that the difference is due to an over fiftyfold higher of endogenous SF-1 mRNA expression in H295R cells as compared to KGN cells. Upon exogenous SF-1 over-expression, both aromatase mRNA expressions and enzymatic activities became responsive to atrazine in KGN cells [2]. Here, we show that the ArPII reporter readily responded to atrazine without exogenous SF-1 co-expression in H295R cells (Fig. 1a). The atrazine stimulation was dose-dependant, and effective at concentrations as low as 10^{-7} M (21.6 ppb), which is ecologically relevant [3]. To further confirm that SF-1 is essential for atrazine stimulation, we knocked down endogenous SF-1 in H295R cells by RNAi. Fig. 1b shows that siRNA mediated down-regulation of SF-1 in the cells resulted in a reduced basal promoter activity, and importantly, a compete loss of responsiveness of ArPII to either atrazine or simazine. In KGN cells, consistent with the lack of effect on aromatase activity [10], ArPII, the dominant aromatase promoter the cells use,

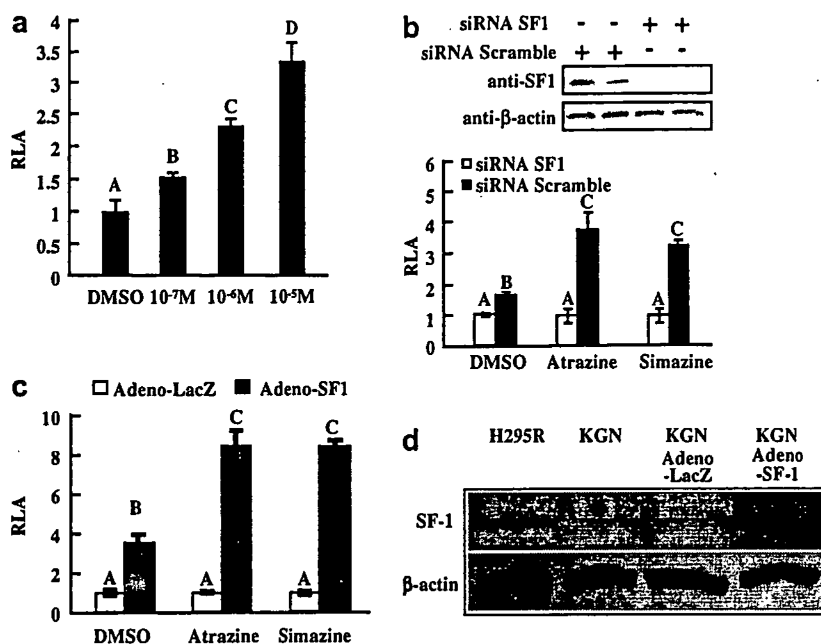

Fig. 1. ArPII responds to atrazine SF-1 dependently. (a) H295R cells with pGL3-ArPII4.0 and pRL-CMV internal control co-transfected were incubated for 48 h in DMSO or increasing concentrations of atrazine before luciferase assay. (b) Anti-SF-1 and anti-β-actin immunoblottings in H295R cells on the 5th day post siRNA transfection (upper panel). siRNA pre-treated H295R cells were co-transfected with pGL3-ArPII4.0 and pRL-CMV, and were subsequently incubated for 48 h in 10^{-5} M of atrazine, simazine or DMSO before relative luciferase assay (lower panel). (c) ArPII reporter were transfected in KGN cells pre-infected with either Adeno-LacZ or Adeno-SF-1, the cells were then incubated for 48 h in 10^{-5} M atrazine, simazine or the solvent DMSO before luciferase assay. (d) Anti-SF-1 immunoblottings in H295R cells, native KGN cells and KGN cells infected with Adeno-LacZ or Adeno-SF-1. Letters above bars show statistical groups (ANOVA, $P < 0.05$). RLA, relative luciferase activity.

was unchangeable by either chemical (Fig. 1c). However, once SF-1 was delivered by adenovirus, there was a near fourfold increase in basal activity and enhanced responsiveness to the chemicals; both atrazine and simazine further increased SF-1-enhanced ArPII activity by two- to threefold (Fig. 1c). Fig. 1d shows markedly higher endogenous SF-1 protein levels in H295R cells compared with KGN cells, and a successful SF-1 over-expression mediated by adenovirus vector in KGN cells. Thus atrazine or simazine induction of ArPII is SF-1 dependent.

Besides its vital roles in the reproductive system, ArPII is also critically involved in oncogenesis of breast cancer [15]. Importantly, in addition to reproduction disrupting effects, atrazine also increases the incidence of mammary cancer in rodents [2]; and is associated with breast cancer in women [5].

PKA and LRH-1 is not essential for atrazine stimulation

Both chemicals at concentration of 10^{-5} M were not cyto-toxic (Supplementary Fig. 1a), and didn't affect *SF-1* gene expression (Supplementary Fig. 1b and c).

SF-1 is required for conveying cAMP-PKA-dependent *CYP19* transcription via ArPII [13]. PKA directly enhances SF-1 transactivation by recruiting co-activators such as GCN5, TRRAP [13] and p300 [18], and disassociations of repressor DAX-1 [13]; PKA also reportedly removes an inhibitory ligand, sphingosine, from SF-1 [19]. On the other hand, Atrazine increases cellular cAMP levels by inhibiting phosphodiesterase as above-mentioned. Thus, activation of PKA is likely a mechanism by which atrazine may enhance SF-1 function. Consistently, a CRE (cAMP responsive element) luciferase reporter was found activated by either atrazine or simazine in H295R cells (ANOVA < 0.05, Fig. 2a); suggesting that the herbicides truly activate PKA in these cells. We then elucidated whether atrazine is still effective when PKA signaling is eliminated by H89. Fig. 2b shows that 10^{-5} M atrazine-induced PKA signaling was sufficiently blocked by 10^{-5} M H89. Fig. 2c shows that the basal levels of ArPII activities were dose-dependently decreased by H89 as expected, but surprisingly, atrazine stimulation of the promoter was not significantly changed by either 10^{-6} or 10^{-5} M H89. In an independent experiment, we also found that while responsiveness of ArPII to 10^{-6} M forskolin (a specific PKA agonist) in H295R cells was dose-dependently reduced by H89 as expected, responsiveness to atrazine was not altered (Fig. 2d). These data suggest that PKA signaling is not the only mechanism by which atrazine induces aromatase. Actually, neither chemical altered intracellular cAMP production or CRE-Luc activities in KGN cells (Supplementary Fig. 2a and b), even with Adeno-SF-1 infection.

Given that liver receptor homolog-1 (LRH-1), a close relative of SF-1, also regulates ArPII, and is also linked

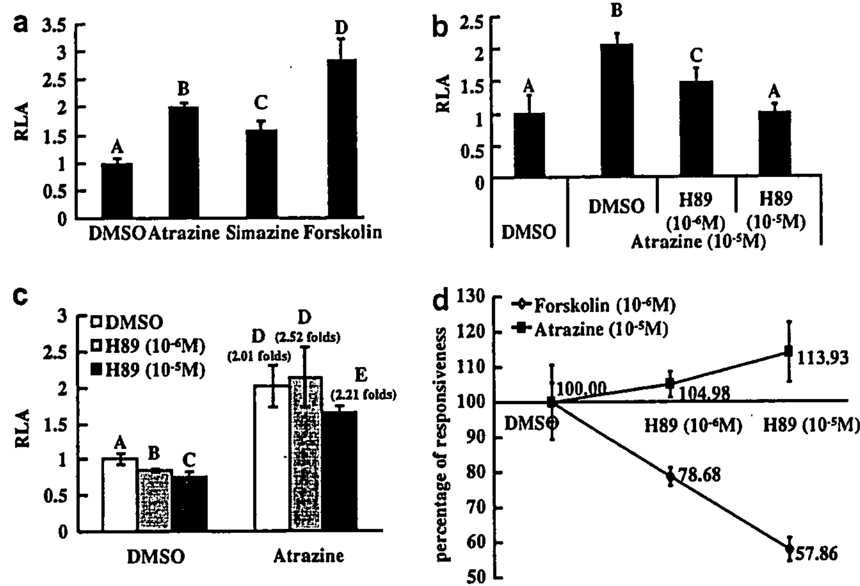

Fig. 2. PKA is not essential for atrazine stimulation. (a) CRE-Luc was transfected to H295R cells to sensitively monitor intracellular PKA signaling induced by 48 h of atrazine, simazine or forskolin. (b) CRE-Luc transfected H295R cells were treated with 10^{-5} M atrazine together with increasing concentrations of H89 as indicated. (c) pGL3-ArPII4.0 was transfected to H295R cells, which were subsequently treated with 10^{-5} M of atrazine together with increasing concentrations of H89, as indicated. (d) H295R cells were transfected with pGL3-ArPII4.0, and were subsequently exposed to 10^{-6} M forskolin or 10^{-5} M atrazine in combination with increasing concentrations of H89. Folds induction (responsiveness) of ArPII by forskolin or atrazine in the absence of H89 (DMSO) was set to 100. Note that H89 dose-dependently reduced ArPII responsiveness to forskolin but not that to atrazine. Letters above bars show statistical groups (ANOVA, $P < 0.05$). RLA, relative luciferase activity.

to the PKA-mediated induction of ArPII [20]. We therefore analyzed the role LRH-1, if any, in mediating the atrazine stimulation of ArPII. NIH-3T3 cells pre-cotransfected with ArPII reporter and expression vectors for SF-1 or LRH-1 or blank pcDNA3.1, were exposed to 48 h of 10^{-5} M atrazine, 10^{-6} M forskolin or the solvent DMSO. Fig. 3a shows that although SF-1 rendered ArPII responding to both forskolin and atrazine, the promoter was responsive to only forskolin, but not atrazine, in the presence of LRH-1. Furthermore, the endogenous mRNA expression of LRH-1 in the atrazine-non-responsive KGN cells was several hundredfold higher than that in the atrazine-responsive H295R cells (Fig. 3b), further suggesting a lack of LRH-1 involvement in atrazine stimulation of ArPII. Given that LRH-1 is sufficient to mediate PKA stimulation over ArPII, the abundant expression of the receptor in the atrazine non-responsive KGN cells serves as indirect evidence that atrazine stimulation is PKA independent.

Physical interaction between atrazine and SF-1 in vitro

We then studied whether atrazine binds to recombinant SF-1 protein *in vitro* using 27-MHz QCM. Atrazine, phospholipid 16PC (a known ligand for SF-1 [21]), or 2,4,5T (a chemical that dose not affect SF-1 function [2] were first fixed to individual QCM electrodes. Interactions were monitored by the linear decrease of the emitted fre-

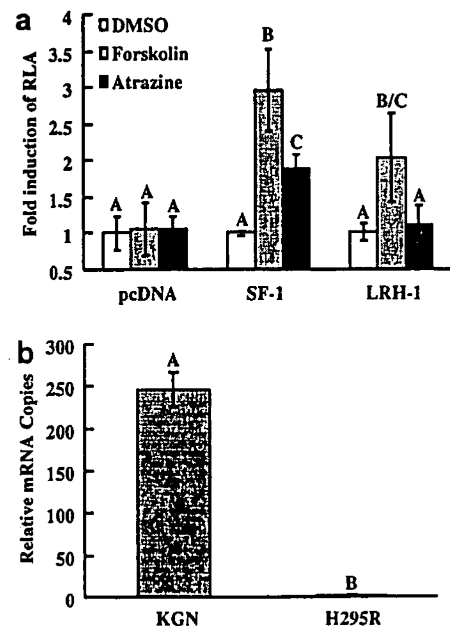

Fig. 3. Lack of role of LRH-1 in mediating atrazine responsiveness. (a) Responsiveness of ArPII to 48 h of 10^{-5} M atrazine, 10^{-6} M forskolin or the solvent DMSO in NIH-3T3 cells co-transfected with either pcDNA3.1, pcDNA3.1-hSF-1, or pcDNA3.1-hLRH-1. RLA, relative luciferase activity. (b) Real-time PCR quantification of endogenous LRH-1 mRNA in H295R and KGN cells. The relative copies of LRH-1 to that of β-actin in H295R cells were set to 1. Letters above bars show statistical groups (ANOVA, $P < 0.05$).

quency (ΔF) with increasing mass present on the QCM electrode. As shown in Fig. 4a, the frequency of 16PC immobilized on the QCM electrode decreased when SF-1 was added to reaction solution. SF-1 also decreased atrazine frequency, while exhibited no effect on the negative control 2,4,5T, indicating that there was a direct and specific interaction between atrazine and SF-1. The affinity data are significant because SF-1 has, for a long time since its discovery, been considered as an orphan nuclear receptor, until the recent discovery of phospholipids as endogenous ligands for SF-1 and the closely related receptor LRH-1 [21,22]; although the physiological significance and functional role of phospholipids in the regulation of receptor activity remains to be elucidated and is seems to be confounded by the absence of a lipid in the pocket of the mouse LRH-1 [23].

Atrazine enhances SF-1–TIF2 interaction

To address whether the affinity between SF-1 and atrazine is followed by co-activator recruitment, we further performed mammalian two hybrid assay-based interaction assays in which full length human TIF2 (an established co-activator for SF-1 [21] was fused to VP16 activation domain to produce pACT-TIF2 and full length of human SF-1 was fused to DBD of GAL4 to produce pBIND-SF-1, respectively. As shown in Fig. 4b, transfection of pACT-TIF2 or pBIND-SF-1 had either no or very mild effects on the pG5-Luc reporter in KGN cells; co-transfection of both pACT-TIF2 and pBIND-SF-1 significantly stimulated the reporter, suggesting that the two proteins interacted and the system worked effectively. In cells co-transfected with both fusion proteins, atrazine was found to further stimulate the reporter activities in a dose-dependent manner (Fig. 4c), suggesting that the chemical enhanced the interaction between SF-1 and its co-activator TIF2. We also made alternative fusion constructs of pACT-SF-1 and pBIND-TIF2 and similar results were obtained (Supplementary Fig. 3a and b). While being less dramatic, atrazine was also enhanced SF-1–SRC-1 interactions (Supplementary Fig. 3c). Thus the atrazine–SF-1 affinity is coupled with enhanced receptor–co-activators interactions. Functional augmentation of SF-1 by atrazine is also evidenced by enhanced SF-1–ArPII chromatin association [2].

Mutations in DBD, but not LBD of SF-1 alter atrazine stimulation

To address the corresponding functional domain of SF-1 in mediating atrazine stimulation over ArPII, we introduced mutations to the DBD (G35E, DBD mutation, abolishes SF-1 transactivation dramatically [13]) or LBD (G341E and Y436F-K440A, pocket mutations that abolish

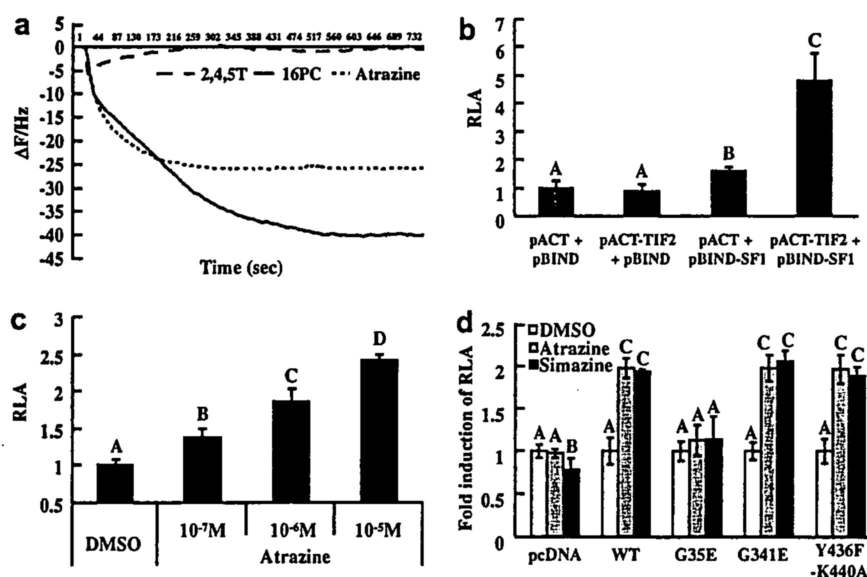

Fig. 4. Atrazine is potentially a ligand for SF-1. (a) Detection of direct atrazine–SF-1 interaction by quartz-crystal microbalance. Chemicals of interest were immobilized on the electrode individually and purified SF-1 was then added to the reaction solution. (b) Mammalian two-hybrid assays for in vivo SF-1–TIF2 interaction. KGN cells were co-transfected with a DNA mixture consisting of pG5-LUC together with equimolar cross-combinations of pACT or pACT-TIF2 and pBIND or pBIND-SF-1 as indicated. The co-existence of pACT-TIF2 and pBIND-SF-1 significantly induced the reporter, suggesting interaction. (c) KGN cells co-transfected with pG5-LUC, pACT-TIF2, and pBIND-SF-1 were treated with increasing concentrations of atrazine for 48 h before the luciferase assay. (d) Effects of domain specific mutations of SF-1. Responsiveness of ArPII to 48 h of 10^{-5} M atrazine, simazine or the solvent DMSO in NIH-3T3 cells co-transfected with either pcDNA3.1 or variants of pcDNA3.1-hSF-1 (WT, wild type; G35E, DBD mutation that abolishes SF-1 transactivation dramatically; G341E and Y436F-K440A, pocket mutations that abolishes SF-1-phospholipid interaction partially or near-completely, respectively). Letters above bars show statistical groups (ANOVA, $P < 0.05$). RLA, relative luciferase activity.

interaction of SF-1 and its endogenous phospholipid ligand partially and near-completely, respectively [22]. The effects of these mutants in mediating the responsiveness of ArPII to atrazine were analyzed by an experiment similar to Fig. 3a. Fig. 4d shows that, while G35E SF-1 lost its ability to convey the responsiveness, effects of both LBD pocket mutants were indistinguishable from that of wild type. These data indicate that functional LBD is not critically required for SF-1 to mediate the atrazine stimulation, suggesting the binding is unlikely to be within the hydrophobic ligand binding pocket, and atrazine may interact with SF-1 via regions other than the LBD.

While the precise domain(s) responsible for the affinity awaits further study, our data suggest an alternative mechanism by which atrazine induces *CYP19* expression. Atrazine may not only elevate cellular cAMP levels as a result of phosphodiesterase inhibition; but also binds to and activates the receptor SF-1, and thus induces *CYP19* expression via promoter II (a proposed model is depicted in Supplementary Fig. 4). This may serve as a common mechanism for the various adverse effects reported for atrazine (including reproductive toxicity, sexual differentiation disruption and carcinogenicity in wildlife, laboratory animals, and humans) and may also have important impacts on the current debate over atrazine.

Acknowledgments

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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.bbrc.2007.02.062.

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Effect of rosuvastatin 5–20 mg on triglycerides and other lipid parameters in Japanese patients with hypertriglyceridemia

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Abstract

To evaluate the potential dose effect of rosuvastatin on triglyceride (TG) levels in Japanese hypertriglyceridemic patients, we randomized 154 patients with TG levels of ≥200 and <800 mg/dL to 8 weeks of treatment with rosuvastatin 5, 10 or 20 mg once daily; bezafibrate 200 mg twice daily; or placebo. Compared with placebo, TG was reduced by 30.1% with rosuvastatin 5 mg, 30.1% with 10 mg and 32.3% with 20 mg (all $p \leq 0.0001$), with no evidence of a dose effect. Changes in TG were evident after 2 weeks of treatment and maintained thereafter. In a benchmark comparison, rosuvastatin across its dose range reduced TG by 29.1–31.1% from baseline versus 45.4% for bezafibrate. Compared with bezafibrate, rosuvastatin was superior with respect to changes in non-high-density lipoprotein cholesterol (non-HDL-C, −36.8 to −44.3% for rosuvastatin versus −2.0% for bezafibrate), low-density lipoprotein cholesterol (−31.9 to −41.0% versus +29.3%), total cholesterol (−27.1 to −33.3% versus +2.1%), although smaller improvements in HDL-C (12.4–16.7% versus 19.6%) were observed. Rosuvastatin also produced superior dose-related decreases in median high-sensitivity C-reactive protein (22.9–38.5%). Treatment was well tolerated in both rosuvastatin and bezafibrate patients, with clinically important increases in alanine aminotransferase being rare, no adverse effect on renal function being observed and no cases of myopathy or rhabdomyolysis being reported. The current study does not suggest a dose-related effect of rosuvastatin in lowering TG in hypertriglyceridemic Japanese patients, although dose-related improvements in other elements of the atherogenic lipid profile were observed.
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Keywords: Triglycerides; Lipoproteins; Hypertriglyceridemia; Rosuvastatin; Bezafibrate; Japanese

1. Introduction

There is evidence that elevated triglyceride (TG) levels constitute an independent risk factor for coronary heart disease [1,2], and the importance of reducing elevated TG in the context of reducing overall cardiovascular risk has been emphasized in recent lipid-lowering guidelines [3]. For patients with very highly elevated TG (>800 mg/dL), the primary focus of lipid-lowering treatment is TG reduction to prevent pancreatitis; such patients were not included in this study. More moderate elevations in TG are frequently seen as part of the metabolic syndrome, itself a condition posing increased risk for cardiovascular disease [3]. Moderate TG elevation can occur alongside cholesterol elevation (Fredrickson type IIb dyslipidemia) or in the presence of normal cholesterol levels (type IV dyslipidemia).

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Statins can have robust effects in reducing TG levels, with reductions ranging from 22 to 45%, especially in patients with mild-to-moderate hypertriglyceridemia (TG levels >250 and <400 mg/dL) [4], and are attractive options compared with fibrates or nicotinic acid because they exert strong beneficial effects on a wide range of lipid parameters. In a study of Western hypertriglyceridemic patients, rosuvastatin 5–80 mg reduced mean TG levels by 18–40% [5]. Another study of rosuvastatin demonstrated that it helped more Western hypercholesterolemic patients achieve Japan Atherosclerosis Society low-density lipoprotein cholesterol (LDL-C) goals than did atorvastatin, pravastatin or simvastatin [6]. Rosuvastatin has also been investigated in hypercholesterolemic Japanese patients and found to produce large dose-related decreases in LDL-C and beneficial changes in other atherogenic and anti-atherogenic lipid profiles [7,8]. The importance of effective lipid-lowering therapy in Japanese patients is supported by a recent study of the Japan Lipid Assessment Program, which found that many Japanese patients undergoing lipid-lowering therapy to reduce the risk of coronary heart disease achieve insufficient reductions in TG, total cholesterol (TC) and LDL-C levels [9]. The current study was conducted to evaluate the effects of rosuvastatin at 5–20 mg on TG levels and other lipid profiles in hypertriglyceridemic Japanese patients.

2. Patients and methods

This was a multicenter, randomized, parallel-group, double-blind study conducted in accordance with the Declaration of Helsinki and in compliance with the ethical principles of good clinical practice. Appropriate ethics committees or institutional review boards approved the trial, and all patients provided written, informed consent before any study procedure. Eligible patients were male and female patients aged 20–75 years with fasting TG levels of ≥200 to <800 mg/dL. Exclusion criteria included presence or suspicion of pancreatitis; use of glitazones within the 3 prior months; pregnancy, breast-feeding or childbearing potential in the absence of use of adequate contraceptive techniques; active arterial disease; uncontrolled hypertension; fasting serum glucose of ≥140 mg/dL or glycated hemoglobin (HbA_{1c}) ≥8%; heterozygous or homozygous familial hypercholesterolemia; serum creatine kinase >3 times the upper limit of normal (ULN); active liver disease or hepatic dysfunction defined by alanine aminotransferase (ALT), aspartate aminotransferase (AST) or bilirubin ≥1.5 times ULN; and serum creatinine >1.5 mg/dL. All lipid-lowering drugs were discontinued at the first study visit. After a 6–9-week dietary lead-in period, eligible patients were randomized to receive rosuvastatin 5, 10 or 20 mg once daily; bezafibrate 200 mg twice daily; or placebo for 8 weeks. For entry into the treatment phase, patients had to have pre-treatment TG measurements within 30% of each other on consecutive occasions.

The primary objective of the study was to investigate the dose–response relationship between rosuvastatin doses and percent reduction in TG from baseline with respect to placebo. Secondary efficacy variables included changes in other lipids/lipoproteins, lipoprotein fractions, lipid ratios and high-sensitivity C-reactive protein (hs-CRP). To predict whether rosuvastatin had a TG-lowering effect similar to drugs commonly prescribed to Japanese hypertriglyceridemic patients, a commonly used drug, bezafibrate, was included as one of the treatment groups. Baseline values for TG, TC, non-high-density lipoprotein cholesterol (non-HDL-C), LDL-C, HDL-C and apolipoprotein (apo) B were the average of three measurements prior to the start of study treatment; baseline values for all other efficacy variables were values on the final pre-treatment visit. TG, TC, HDL-C, non-HDL-C and LDL-C were measured at 2, 4, 6 and 8 weeks; all other variables were measured at 4 and 8 weeks. VLDL-TG, HDL-TG, and LDL-TG fractions were measured by the ultracentrifugation method, including the use of sequential fractioning of the lipid components in solutions of varying relative density. All laboratory measurements were performed at a central clinical laboratory (Mitsubishi Chemical Bio-Clinical Laboratories, Inc., Tokyo, Japan). Percentage changes in TG, TC, HDL-C, non-HDL-C and LDL-C at week 8 were evaluated using an analysis of variance (ANOVA) including treatment (placebo and rosuvastatin 5, 10 and 20 mg) as a fixed effect. Analyses were carried out in the full analysis set population with the last observation carried forward (LOCF). Dunnet's method was used to adjust for multiple comparisons. For the above variables, mean differences between treatment and two-sided 95% confidence interval (CI) were estimated for rosuvastatin 5, 10 and 20 mg versus placebo. The p-value from ANOVA was also determined for percentage change in TG at week 8. The differences between groups for changes in very low-density lipoprotein (VLDL)-TG, LDL-TG and HDL-TG fractions; apo B, apo A-I and apo C-III; lipid and lipoprotein ratios; and hs-CRP were assessed using descriptive statistics (mean or median percentage changes) in each group.

Safety evaluations included monitoring of adverse events, clinical chemistry, hematology and urinalysis. The safety population included all patients receiving at least one dose of study medication.

3. Results

In total, 154 patients were randomized to rosuvastatin 5, 10 or 20 mg once daily; bezafibrate 200 mg twice daily; or placebo. Mean ages ranged from 50 to 56 years, most patients were male, and most in each treatment group had Fredrickson type IV dyslipidemia (Table 1). All 154 patients were included in efficacy and safety analyses. A total of 23 patients discontinued treatment, consisting of 8 in the rosuvastatin 5 mg group, 5 in the rosuvastatin 10 mg group, 4 in the rosuvastatin 20 mg group, 3 in the bezafibrate group and 3 in the

Table 1
Demographics and baseline characteristics

	Placebo (n = 35)	Rosuvastatin			Bezafibrate 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
Age (years), mean (S.D.)	51.6 (10.2)	50.4 (11.2)	51.6 (11.5)	55.8 (11.4)	50.5 (12.2)
Male/female, n (%)	21 (60.0)/14 (40.0)	22 (68.8)/10 (31.3)	26 (76.5)/8 (23.5)	20 (76.9)/6 (23.1)	21 (77.8)/6 (22.2)
Body mass index (kg/m^2), mean (S.D.)	26.0 (3.0)	25.0 (2.8)	26.1 (3.1)	25.8 (3.1)	26.0 (3.0)
Fredrickson type, n (%)					
IIb	15 (42.9)	13 (40.6)	12 (35.3)	8 (30.8)	10 (37.0)
IV	20 (57.1)	19 (59.4)	22 (64.7)	18 (69.2)	17 (63.0)
Concomitant disease, n (%)					
Hypertension	11 (31.4)	10 (31.3)	14 (41.2)	12 (46.2)	10 (37.0)
Diabetes mellitus	2 (5.7)	4 (12.5)	2 (5.9)	4 (15.4)	1 (3.7)
Coronary artery disease	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)

placebo group. Five of these patients (three in the rosuvastatin 5 mg group, one in the rosuvastatin 10 mg group and one in the bezafibrate group) discontinued treatment because of adverse events. A total of 15 patients were withdrawn according to study-specific discontinuation criteria (5 due to increased TG levels to ≥800 mg/dL, 9 due to decreased LDL-C levels to ≤50 mg/dL, and 1 due to both), 1 patient did not fulfill eligibility criteria, and 2 patients were not willing to continue in the study.

3.1. *Efficacy*

Mean baseline TG levels ranged from 334 to 398 mg/dL. Compared with placebo, TG was reduced by 30.1% with rosuvastatin 5 mg, 30.1% with rosuvastatin 10 mg and 32.3% with rosuvastatin 20 mg (all $p \leq 0.0001$), with no evidence of a dose–response (Table 2; Fig. 1). Reductions in TG were evident by week 2 and were sustained through week 8 without marked change. In terms of raw means, rosuvastatin across its dose range reduced TG by 29.1–31.1% from baseline, compared with 45.4% for bezafibrate. LDL-TG and VLDL-TG were reduced in all rosuvastatin groups with no apparent dose–response, and no change in HDL-TG was observed (Table 2).

Non-HDL-C, LDL-C, and TC were all significantly reduced with rosuvastatin, compared with placebo, with evidence of a dose effect for each (Table 3); HDL-C was significantly increased at each rosuvastatin dose with no evidence of dose-related effects. Apo B was reduced in all rosuvastatin groups with evidence of a dose effect, while apo A-1 was increased in all rosuvastatin groups with no evidence of a dose effect; however, statistical comparisons with placebo were not performed for these parameters (Table 4). Lipid ratios were all markedly reduced in an apparent dose-related manner with rosuvastatin treatment. The inflammatory marker hs-CRP was reduced by medians of 22.9–38.5% with rosuvastatin treatment, compared with an increase of 3.5% in the placebo group.

Table 2
Changes in TG levels from baseline at 8 weeks

	Placebo (n = 35)	Rosuvastatin			Bezafibrate[a] 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
TG					
Baseline (mg/dL), mean (S.D.)	334 (118)	336 (125)	338 (151)	398 (120)	355 (126)
% Change, mean (S.D.)	+1 (30)	−29 (22)	−29 (23)	−31 (39)	−45 (21)
Difference with placebo, estimate [95% CI][b]	–	−30 [−47, −13] P = 0.0001	−30 [−47, −13] P < 0.0001	−32 [−50, −14] P = 0.0001	–
VLDL-TG					
Baseline (mg/dL), mean (S.D.)	197.7 (85.6)	201.3 (92.1)	207.5 (145.3)	243.7 (94.5)	225.0 (117.5)
% Change, mean (S.D.)	+11.7 (45.8)	−22.6 (33.4)	−19.9 (41.7)	−24.7 (54.6)	−48.0 (24.5)
LDL-TG					
Baseline (mg/dL), mean (S.D.)	51.5 (16.9)	54.1 (20.2)	56.6 (24.3)	57.3 (22.7)	54.5 (18.3)
% Change, mean (S.D.)	+12.3 (49.9)	−25.6 (22.4)	−34.0 (24.7)	−31.7 (16.7)	−15.3 (23.1)
HDL-TG					
Baseline (mg/dL), mean (S.D.)	22.5 (7.2)	24.3 (9.6)	22.9 (9.9)	26.4 (9.9)	24.8 (11.0)
% Change, mean (S.D.)	+3.2 (29.8)	−1.9 (29.0)	−0.4 (29.2)	+1.7 (40.5)	−22.0 (30.2)

[a] Results for bezafibrate provided as a benchmark comparator; only raw means available in the table.
[b] Results of ANOVA model including treatment (placebo and rosuvastatin 5, 10 and 20 mg) as fixed effect. Changes are shown as differences between rosuvastatin and placebo. p-Values were calculated only for TG.

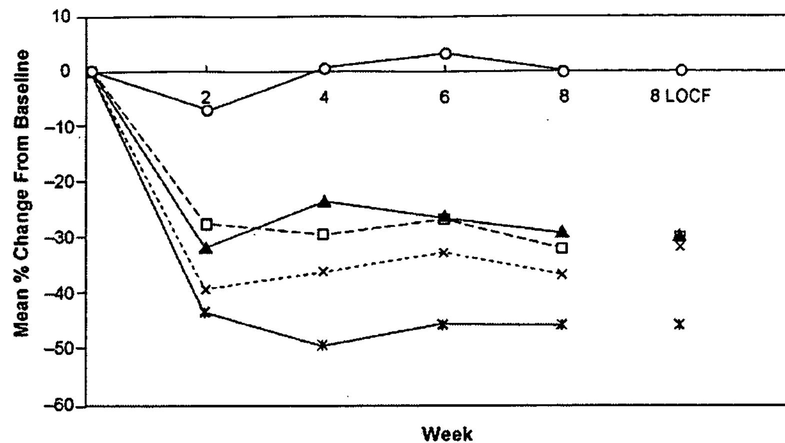

Fig. 1. Mean % change in TG from baseline at 2, 4, 6 and 8 weeks and at 8 weeks with last observation carried forward (LOCF) in patients receiving placebo or rosuvastatin 5, 10 or 20 mg once daily, or bezafibrate 200 mg twice daily (○, placebo; □, rosuvastatin 5 mg qd; ▲, rosuvastatin 10 mg qd; ×, rosuvastatin 20 mg qd; ✱, bezafibrate 200 mg bid).

When compared with bezafibrate (raw means), rosuvastatin produced superior results with respect to changes in non-HDL-C and LDL-C, while generating smaller improvements in HDL-C (12.4–16.7% for rosuvastatin across its dose range versus 19.6% for bezafibrate) (Tables 3 and 4). Hs-CRP also exhibited dose-related reduction with rosuvastatin treatment (22.9–38.5%).

Table 3
Changes in lipid/lipoprotein profiles and hs-CRP from baseline at 8 weeks

	Placebo (n = 35)	Rosuvastatin			Bezafibrate[a] 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
Non-HDL-C					
Baseline (mg/dL), mean (S.D.)	202.7 (52.1)	190.1 (34.5)	192.3 (49.6)	191.9 (33.9)	192.7 (35.6)
% Change, mean (S.D.)	+1.4 (9.3)	−36.8 (13.7)	−41.0 (16.0)	−44.3 (17.6)	−2.0 (12.1)
vs. placebo, estimate [95% CI][b]	–	−38.1 [−46.5, −29.8]	−42.3 [−50.5, −34.1]	−45.6 [−54.5, −36.8]	–
LDL-C					
Baseline (mg/dL), mean (S.D.)	138.1 (49.7)	124.4 (35.8)	125.9 (43.1)	115.9 (29.6)	122.8 (34.9)
% Change, mean (S.D.)	+3.6 (15.5)	−31.9 (17.8)	−38.1 (18.6)	−41.0 (18.3)	+29.3 (30.2)
vs. placebo, estimate [95% CI][b]	–	−35.5 [−45.7, −25.2]	−41.7 [−51.8, −31.6]	−44.6 [−55.5, −33.7]	–
TC					
Baseline (mg/dL), mean (S.D.)	246.0 (54.9)	231.9 (35.2)	232.2 (52.6)	234.2 (36.9)	234.3 (34.3)
% Change, mean (S.D.)	+1.3 (8.2)	−27.1 (11.3)	−31.4 (13.1)	−33.3 (12.8)	+2.1 (9.9)
vs. placebo, estimate [95% CI][b]	–	−28.4 [−35.1, −21.7]	−32.7 [−39.3, −26.1]	−34.7 [−41.8, −27.6]	–
HDL-C					
Baseline (mg/dL), mean (S.D.)	43.2 (7.4)	41.8 (7.4)	39.8 (7.2)	42.3 (8.5)	41.6 (9.0)
% Change, mean (S.D.)	+1.8 (10.7)	+14.5 (16.9)	+12.4 (16.3)	+16.7 (15.7)	+19.6 (13.6)
vs. placebo, estimate [95% CI][b]	–	+12.7 [+3.9, +21.5]	+10.7 [+2.0, +19.3]	+15.0 [+5.7, +24.4]	–
RLP-C					
Baseline (mg/dL), mean (S.D.)	14 (6)	15 (11)	17 (16)	17 (10)	15 (9)
% Change, mean (S.D.)	+8 (58)	−48 (25)	−49 (24)	−50 (46)	−43 (27)
SD-LDL					
Baseline (mg/dL), mean (S.D.)	13 (5)	11 (6)	11 (8)	13 (4)	12 (6)
% Change, median	−7	−16	−22	−34	−33
hs-CRP					
Baseline (mg/dL), mean (S.D.)	0.115 (0.139)	0.120 (0.127)	0.138 (0.136)	0.166 (0.161)	0.096 (0.105)
% Change, median	+3.5	−22.9	−37.8	−38.5	−1.7

[a] Results for bezafibrate provided as a benchmark comparator; only raw means available in the table.

[b] Results of ANOVA model including treatment (placebo and rosuvastatin 5, 10 and 20 mg) as fixed effect. Changes are shown as differences between rosuvastatin and placebo. p-Values were calculated only for TG.

Table 4
Changes in apolipoprotein profiles and lipid ratios from baseline at 8 weeks

	Placebo (n = 35)	Rosuvastatin			Bezafibrate[a] 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
Apo B					
Baseline (mg/dL), mean (S.D.)	134.2 (33.4)	125.5 (23.0)	128.5 (37.6)	125.2 (19.6)	127.5 (21.7)
% Change, mean (S.D.)	+2.8 (10.1)	−29.1 (12.7)	−34.3 (14.0)	−35.3 (15.3)	+2.4 (12.7)
Apo A-I					
Baseline (mg/dL), mean (S.D.)	142.1 (22.4)	140.0 (19.2)	134.6 (16.8)	145.3 (26.3)	144.3 (36.8)
% Change, mean (S.D.)	+1.3 (7.3)	+7.8 (9.7)	+7.9 (15.9)	+9.8 (11.3)	+7.5 (9.1)
Apo C-III					
Baseline (mg/dL), mean (S.D.)	18.5 (6.2)	18.0 (4.8)	17.6 (5.8)	20.5 (5.2)	19.9 (7.8)
% Change, mean (S.D.)	+3.9 (19.2)	−18.7 (19.4)	−17.5 (26.7)	−16.6 (25.7)	−32.6 (13.6)
Non-HDL-C:HDL-C					
Baseline, mean (S.D.)	4.74 (1.15)	4.67 (1.15)	4.90 (1.25)	4.66 (1.04)	4.88 (1.51)
% Change, mean (S.D.)	+0.5 (13.1)	−43.7 (14.5)	−46.2 (17.6)	−50.2 (21.1)	−17.0 (13.8)
LDL-C:HDL-C					
Baseline, mean (S.D.)	3.21 (1.08)	3.03 (0.92)	3.17 (0.95)	2.79 (0.72)	3.12 (1.16)
% Change, mean (S.D.)	+1.9 (12.0)	−39.6 (17.4)	−43.9 (18.6)	−47.7 (20.7)	+8.7 (24.8)
TC:HDL-C					
Baseline, mean (S.D.)	5.74 (1.15)	5.67 (1.15)	5.90 (1.25)	5.66 (1.04)	5.88 (1.51)
% Change, mean (S.D.)	+0.2 (10.6)	−35.5 (11.5)	−38.0 (14.2)	−41.2 (17.1)	−13.9 (10.9)
Apo B:apo A-I					
Baseline, mean (S.D.)	0.96 (0.26)	0.92 (0.21)	0.96 (0.26)	0.88 (0.18)	0.94 (0.28)
% Change, mean (S.D.)	+1.6 (9.0)	−33.9 (12.9)	−37.9 (15.8)	−40.0 (18.4)	−3.6 (17.4)

[a] Results for bezafibrate provided as a benchmark comparator; only raw means available in the table.

3.2. Safety

Treatment-emergent adverse events occurred in 57.1% of placebo recipients, 51.1% of rosuvastatin recipients and 59.3% of bezafibrate recipients (Table 5). Adverse events considered related to study treatment occurred in 8.6% of placebo recipients, 10.9% of rosuvastatin recipients and 14.8% of bezafibrate recipients. Serious adverse events occurred in five patients receiving rosuvastatin. One patient receiving rosuvastatin 5 mg had abnormal liver function, which was later confirmed by biopsy to be due to autoimmune hepatitis. In the rosuvastatin 10 mg group, one patient had acute cholecystitis, one had small cell lung cancer, and one had a rib fracture. One rosuvastatin 20 mg patient had a benign salivary gland neoplasm. Four serious adverse events other than abnormal liver function were considered unrelated to study treatment.

No serious adverse events were seen in the bezafibrate patients. The four rosuvastatin patients discontinuing treatment because of adverse events included the patient with acute cholecystitis and the patient with abnormal liver function noted above. One patient receiving rosuvastatin 5 mg discontinued treatment because of worsening constipation, which was judged related to study treatment. Another patient receiving rosuvastatin 5 mg discontinued treatment because of numbness in the hand, which was considered unrelated

Table 5
Treatment-emergent adverse events (AEs)

	Placebo (n = 35)	No. (%) of patients rosuvastatin				Bezafibrate 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	All (n = 92)	
Any AE	20 (57.1)	14 (43.8)	17 (50.0)	16 (61.5)	47 (51.1)	16 (59.3)
Serious AE leading to death	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Serious AE	0 (0.0)	1 (3.1)	3 (8.8)	1 (3.8)	5 (5.4)	0 (0.0)
Discontinuation of study treatment due to AE	0 (0.0)	3 (9.4)	1 (2.9)	0 (0.0)	4 (4.3)	1 (3.7)
Treatment-related AE	3 (8.6)	3 (9.4)	2 (5.9)	5 (19.2)	10 (10.9)	4 (14.8)
Most frequent AEs, reported for >4% of patients in any treatment group						
Nasopharyngitis	7 (20.0)	3 (9.4)	3 (8.8)	3 (11.5)	9 (9.8)	4 (14.8)
Arthralgia	0 (0.0)	0 (0.0)	2 (5.9)	2 (7.7)	4 (4.3)	0 (0.0)
Constipation	1 (2.9)	2 (6.3)	1 (2.9)	1 (3.8)	4 (4.3)	1 (3.7)
Myalgia	1 (2.9)	0 (0.0)	2 (5.9)	2 (7.7)	4 (4.3)	0 (0.0)

Table 6
Changes in mean creatinine values and number of subjects with an increase in creatinine ≥30% from baseline at 8 weeks

	Placebo (n = 35)	Rosuvastatin			Bezafibrate 200 mg bid (n = 27)
		5 mg (n = 32)	10 mg (n = 34)	20 mg (n = 26)	
Creatinine					
Baseline (mg/dL), mean (S.D.)	0.759 (0.182)	0.794 (0.186)	0.804 (0.158)	0.813 (0.150)	0.804 (0.175)
Change (mg/dL), mean (S.D.)	−0.012 (0.072)	0.008 (0.058)	−0.006 (0.075)	−0.004 (0.067)	0.083 (0.094)
≥30% increase (n, %)	0	0	0	0	0

to study treatment. The most common adverse events (>4% frequency) in rosuvastatin patients irrespective of causality assessment were nasopharyngitis, arthralgia, constipation and myalgia. The most frequent adverse event (>4% frequency) in bezafibrate patients was nasopharyngitis.

Clinically significant increases in ALT (defined as increases >3 times ULN on two separate occasions at least 2 days apart) were observed in two rosuvastatin patients, including the patient described above who was subsequently diagnosed with autoimmune hepatitis, and in one patient receiving rosuvastatin 20 mg (increases at weeks 2 and 6). No patients had serum creatine kinase elevations >10 times ULN, and no cases of myopathy or rhabdomyolysis were observed. No patients had increases in creatinine of ≥30% from baseline, and there were no notable differences among treatment groups with regard to changes in mean creatinine levels (Table 6). No proteinuria (defined as shift from "none/trace" at baseline to "≥++") was observed in study patients at weeks 4, 8, or at the time of withdrawal. Transient hematuria was observed in three patients receiving rosuvastatin 5 mg, one receiving 10 mg and one receiving 20 mg.

4. Discussion

In this study, rosuvastatin 5–20 mg reduced TG levels in hypertriglyceridemic Japanese patients by 30–32% over 8 weeks, compared with placebo, with no evidence of a dose-related effect. Reductions were observed after 2 weeks of treatment and were maintained throughout the 8 weeks. Rosuvastatin treatment also produced significant beneficial changes in other lipid parameters, compared with placebo, including non-HDL-C reductions of 38–46%, LDL-C reductions of 35–45%, TC reductions of 28–35% and HDL-C increases of 11–15%, as well as marked reductions in VLDL- and LDL-TG fractions and lipid ratios from baseline. Median levels of the inflammatory marker hs-CRP were reduced by 22.9–38.5% with rosuvastatin treatment. A bezafibrate arm was included in the current study to provide guidance in the design of a phase III trial of rosuvastatin in a hypertriglyceridemic population. Treatment with bezafibrate 400 mg/day produced greater improvement in TG and HDL-C than rosuvastatin but, as expected, had substantially less beneficial effects on other atherogenic markers, including a 2.0% decrease in non-HDL-C from baseline, and a 29% increase in LDL-C.

A trial of Western hypertriglyceridemic patients [5] showed that 10 mg of rosuvastatin provided greater TG reductions than did 5 mg (37% versus 18%), although there was little difference in treatment effect at doses above 10 mg (37% reduction with 20 mg, 40% with 40 mg and 40% with 80 mg). However, median decreases in TG for the 5-, 10-, 20-, 40- and 80-mg doses were 21, 37, 37, 43 and 46%, respectively, suggesting a modest dose–response relationship. Data from the current study in Japanese patients do not suggest a dose effect in TG reduction, with very similar magnitudes of reduction being observed at 5-, 10- and 20-mg doses.

This study showed that rosuvastatin 5 mg produced beneficial reductions in TG levels, although higher doses did not provide greater TG reductions. However, dose-related improvements in other atherogenic lipids – including non-HDL-C, LDL-C, apo B, and atherogenic:anti-atherogenic lipoprotein ratios – were observed in these hypertriglyceridemic patients, which is consistent with results from previous studies of rosuvastatin in hypercholesterolemic Japanese patients [7,8].

Although bezafibrate did not provide a marked beneficial effect on lipid parameters such as LDL-C and non-HDL-C, it produced substantial reductions in TG and increase in HDL-C. These findings are consistent with other major studies of fibrates, which demonstrated that fibrates do not appreciably change the concentration of LDL-C [10–12], but rather change particle size distribution, reducing small LDL subfractions and increasing peak particle size [13–15].

The differences between rosuvastatin and bezafibrate in reducing non-HDL-C levels are of particular importance for patients with elevated TG. Non-HDL-C provides a measure of all atherogenic (apo B-containing) lipoproteins, including VLDL remnant lipoproteins, and is more widely available than apo B measurement; there are some data to suggest that non-HDL-C may improve prediction of cardiovascular disease risk over LDL-C [16,17]. The US National Cholesterol Education Program Adult Treatment Panel III guidelines [3] recommend the reduction in non-HDL-C as a secondary target of lipid-lowering therapy after LDL-C goals have been met. Others have suggested that apo B is also an important indicator of cardiovascular risk and in fact may be superior to either LDL-C or non-HDL-C in this regard [18–20].

The coefficients of variation for percentage change of HDL-C, HDL-TG and apo A-I are relatively high. However, the data tends to suggest qualitative differences in the way the two drugs affect HDL. HDL-TG was reduced by about

20% with bezafibrate but rosuvastatin had little or no effect on this parameter. Moreover, the percentage increases in apo A-I with rosuvastatin seem to be greater than with bezafibrate relative to the changes in HDL-C. These compositional changes may reflect differences in the mechanisms by which statins and fibrates elevate HDL.

Complementing previous studies of rosuvastatin in a Japanese population [7,8], the present investigation further supports the comparable efficacy of rosuvastatin in lowering lipid levels in this ethnic group compared with others. These observations are particularly noteworthy in light of the well-documented population differences in rosuvastatin plasma exposure between Western and Asian subjects (1.6–2.3-fold greater rosuvastatin exposure in Asians compared with Western subjects) [21].

The ethnic differences in rosuvastatin disposition also appeared to have no effect on susceptibility to known adverse events. Rosuvastatin was well tolerated in the study, with no indication of dose-related increases in adverse events or laboratory abnormalities. Liver function abnormalities attributable to rosuvastatin treatment were rare, there were no cases of proteinuria and no evidence of renal impairment, and no cases of myopathy or rhabdomyolysis were observed.

In summary, rosuvastatin 5–20 mg reduced TG by 30–32% in Japanese hypertriglyceridemic patients, with no evidence of a dose-related effect on TG reduction. Rosuvastatin treatment also provided large dose-related decreases in LDL-C, robust increases in HDL-C and large improvements in other atherogenic lipid parameters. Many hypertriglyceridemic patients require improvements in other lipid parameters, and these parameters should guide selection of rosuvastatin dose. Rosuvastatin was markedly superior with respect to changes in non-HDL-C and LDL-C improvements, while bezafibrate demonstrated greater improvemnets in TG and HDL-C levels. Rosuvastatin treatment was well tolerated and raised no specific safety concerns in hypertriglyceridemic patients.

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Insulin-like Growth Factor 1/Insulin Signaling Activates Androgen Signaling through Direct Interactions of Foxo1 with Androgen Receptor*[S]

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The androgen-androgen receptor (AR) system plays vital roles in a wide array of biological processes, including prostate cancer development and progression. Several growth factors, such as insulin-like growth factor 1 (IGF1), can induce AR activation, whereas insulin resistance and hyperinsulinemia are correlated with an elevated incidence of prostate cancer. Here we report that Foxo1, a downstream molecule that becomes phosphorylated and inactivated by phosphatidylinositol 3-kinase/Akt kinase in response to IGF1 or insulin, suppresses ligand-mediated AR transactivation. Foxo1 reduces androgen-induced AR target gene expressions and suppresses the *in vitro* growth of prostate cancer cells. These inhibitory effects of Foxo1 are attenuated by IGF1 but are enhanced when it is rendered Akt-nonphosphorylatable. Foxo1 interacts directly with the C terminus of AR in a ligand-dependent manner and disrupts ligand-induced AR subnuclear compartmentalization. Foxo1 is recruited by liganded AR to the chromatin of AR target gene promoters, where it interferes with AR-DNA interactions. IGF1 or insulin abolish the Foxo1 occupancy of these promoters. Of interest, a positive feedback circuit working locally in an autocrine/intracrine manner may exist, because liganded AR up-regulates IGF1 receptor expression in prostate cancer cells, presumably resulting in higher IGF1 signaling tension and further enhancing the functions of the receptor itself. Thus, Foxo1 is a novel corepressor for AR, and IGF1/insulin signaling may confer stimulatory effects on AR by attenuating Foxo1 inhibition. These results highlight the potential involvement of metabolic syndrome and hyperinsulinemia in prostate diseases and further suggest that intervention of IGF1/insulin-phosphatidylinositol 3-kinase-Akt signaling may be of clinical value for prostate diseases.

Androgen receptor (AR)[2] is a member of a nuclear receptor superfamily and functions as a ligand-dependent transcription factor. The androgen-AR system mediates male sexual differentiation in the uterus, sperm production at puberty, prostate development in the adult, and primary prostate cancer (PC) growth in patients with PC (1). PC is the most common malignancy in men worldwide and the second leading cause of cancer-related mortality in the United States (2). The fact that over 70% of PCs rely on androgen stimulation for growth sets the basis for androgen ablation therapy, which is initially effective but invariably results in treatment resistance after a period of time (3). The disease is then referred to as androgen-independent PC and progresses to a fatal outcome. Recent loss-of-function studies have revealed that AR still plays a key role in hormone-refractory progression of PC (4, 5). An adaptation of AR signaling in order to function under low or absent androgen levels may occur (6). Among the various suggested mechanisms by which AR may be reactivated in a low androgen environment (7), signaling by growth factors, especially insulin-like growth factor 1 (IGF1), is reportedly of significant importance (8–11). High IGF1 serum levels are correlated with an increased risk of PC (8, 9), whereas IGF1 enhances AR transactivation under very low or absent androgen levels (12, 13) and promotes PC cell proliferation (10). Recent studies have also revealed that high serum insulin levels are associated with an increased incidence of PC (14, 15), although there is a lack of mechanistic studies implicating insulin signaling in the regulation of AR function.

Foxo1, also known as FKHR, together with other two homologs, FKHRL1 and AFX, belong to the Foxo subfamily of the forkhead transcription factor family, which includes a large array of transcription factors characterized by the presence of a

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. 1–9.

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[2] The abbreviations used are: AR, androgen receptor; hAR, human AR; ARE, androgen-response element; ChIP, chromatin immunoprecipitation; DHT, 5α-dihydrotestosterone; ER, estrogen receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GR, glucocorticoid receptor; IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; MMTV, mouse mammary tumor virus; PC, prostate cancer; PI3K, phosphatidylinositol 3-kinase; PR, progesterone receptor; PSA, prostate-specific antigen; PTEN, human phosphatase and tensin homolog (mutated in multiple advanced cancers 1; EIA, enzyme immunoassay; PBS, phosphate-buffered saline; ANOVA, analysis of variance; HI, heterogeneity index.

conserved 110-amino acid winged helix DNA-binding domain (16). Foxo subfamily members play important roles in cell cycle regulation and apoptosis, as well as in metabolic homeostasis (17).

Phosphatidylinositol 3-kinase (PI3K)/Akt signaling, which is activated by both liganded IGF1 receptor (IGF1R) and insulin receptor, phosphorylates each of the Foxo proteins at three different Ser/Thr residues (17). The phosphorylated Foxo proteins become inactive and are exported from the nucleus. Subsequently, they become sequestered in the cytoplasm, where they interact with 14-3-3 protein.

In this study, we observe that Foxo1, which is endogenously expressed in PC cells, can interact with AR via the C terminus of the receptor in a ligand-dependent manner and suppress ligand-induced AR transactivation. Foxo1 impaired AR signaling by interfering with ligand-induced AR nuclear translocation and subnuclear compartmentalization as well as receptor-target gene promoter interactions. Furthermore, IGF1/insulin-PI3K/Akt pathway-induced phosphorylation of Foxo1 ameliorated the suppression. Intriguingly, liganded AR stimulated IGF1R expression, suggesting the presence of local positive feedback between IGF1 and AR signaling in PC cells.

EXPERIMENTAL PROCEDURES

Materials—The human PC cell lines LNCaP, DU145, ALVA41, and PC3 were maintained as described previously (18). HEK293 and COS7 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10 units/liter penicillin, and 10 μg/ml streptomycin in 75-cm^2 flasks in a humidified 5% CO_2 incubator at 37 °C. The following items were obtained commercially: 5α-dihydrotestosterone (DHT; Sigma); IGF1 (R&D Systems, Minneapolis, MN), insulin (Sigma); anti-AR N-terminal (N-20) antibody (Santa Cruz Biotechnology, Santa Cruz, CA); anti-FLAG M2 monoclonal antibody; and anti-FKHR (Foxo1) monoclonal antibody (Sigma).

The firefly luciferase reporter plasmids pGL3-MMTV, pPSA-LUC, GRE-Luc, and pERE2-tk109-LUC, as well as expression vectors for human AR (pCMV-hAR), estrogen receptor (ER) (pSG5-Erα and pSG5-Erβ), GRα (pSG5-GRα), PR (pSG5-PRA and pSG5-PRB), an AR-EGFP chimera (pCMV-AR-GFP), and expression vectors for FLAG-mFoxo1 (mouse) and FLAG-mFoxo1-3A (T24A, S253A, and S316A) chimeras were described previously (18–21). Full-length cDNAs encoding wild-type and mutant mFoxo1 were subcloned in-frame into pEYFP-C2 (Clontech) to generate EYFP-Foxo1 fusion vectors.

PRL-SV40, pG5-Luc, pBIND, and act expression vectors were obtained from Promega (Madison, WI). A full-length cDNA encoding human phosphatase and tensin homolog (PTEN) (mutated in multiple advanced cancers 1) (GenBank™ accession number NM_000314) was obtained from OriGene Technologies, Inc. (Rockville, MD). A full-length cDNA encoding wild-type mFoxo1 was inserted in-frame into the act vector to generate a pACT-Foxo1 chimera. Expression vectors for pBIND-AR-N-(1–660) and pBIND-AR-C-(615–919) chimeras were constructed previously (18).

Relative Luciferase Reporter Assays and Transfection—Relative luciferase reporter assays were performed as described previously (22). Transfections were carried out using FuGENE 6 or FuGENE HD (Roche Applied Science). Stable clones expressing modest amounts of FLAG-Foxo1 or FLAG were established using 600 μg/ml G418 sulfate.

Cell Proliferation Assays—The proliferation of LNCaP cells stably expressing either FLAG-Foxo1 or the control FLAG tag was determined using a CellTiter 96® nonradioactive cell proliferation assay kit (Promega) according to the manufacturer's instructions.

Coimmunoprecipitation, Western Blotting, Mammalian Two-hybrid Assays, and Modified Mammalian One-hybrid Assays—Coimmunoprecipitation, Western blotting, mammalian two-hybrid assays, and modified mammalian one-hybrid assays were performed essentially as described previously (23).

Living Cell Laser Confocal Fluorescence Microscopy Assays—Living cell laser confocal fluorescence microscopy assays were performed essentially as described previously (22). Colocalization and line scan analyses were carried out using the LSM software (version 3.0).

Chromatin Immunoprecipitation (ChIP) Assays—ChIP assays were performed essentially as described previously (24). An anti-AR N-20 antibody and anti-FLAG M2 monoclonal antibodies were applied. Primer information is available upon request.

Real Time PCR—Various transcripts in proper medium-treated cells were analyzed by real time PCR using a LightCycler as described previously (25, 26). Primer information for each target transcript is available upon request.

PSA Measurements—Secretion of human PSA protein into the cell culture medium was measured using an enzyme immunoassay (EIA) at SRL Inc. (Tokyo, Japan).

Statistical Analysis—Data were expressed as means ± S.D. and evaluated by Student's two-tailed t test or ANOVA, followed by post hoc comparisons with Fisher's protected least significant difference test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Foxo1 Represses Ligand-induced AR Transactivation and Delays LNCaP Cell Proliferation—Initially, the effects of Foxo1 on the transactivation induced by ligand-bound AR were investigated using relative luciferase activity assays. COS7 cells were cotransfected with pCMV-hAR and an artificial luciferase reporter for AR (pGL3-MMTV) together with increasing doses of a Foxo1 expression vector. As shown in Fig. 1*a*, Foxo1 inhibited agonist-induced transcription from the MMTV promoter in a dose-dependent manner. Similarly, the transactivation of endogenous AR monitored by the MMTV promoter in LNCaP human PC cells was suppressed by Foxo1 in a dose-dependent manner (supplemental Fig. 1*a*). Foxo1 also inhibited liganded AR-mediated expression of the promoter of a native AR target gene, *PSA*, in both COS7 (supplemental Fig. 1*b*) and DU145 (supplemental Fig. 1*c*) human PC cells. Thus, the inhibitory effect of Foxo1 on AR, either exogenous or endogenous, is not limited to one cell line and/or promoter. Endogenous Foxo1 mRNA was readily detectable by RT-PCR in all four PC cell

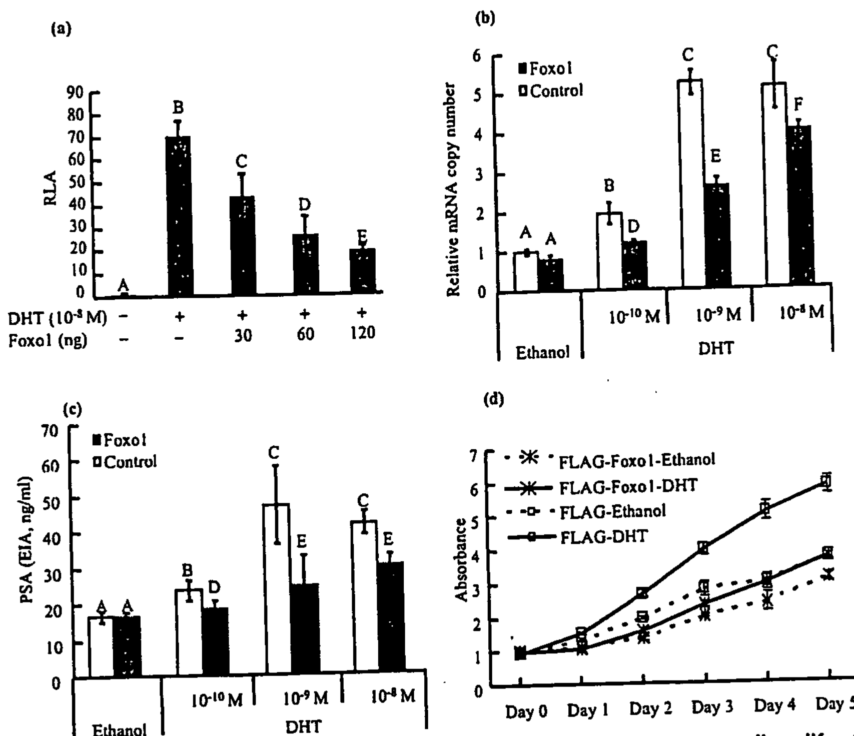

FIGURE 1. **Foxo1 represses ligand-induced AR transactivation and delays LNCaP cell proliferation.** *a*, COS7 cells growing in 24-well plates were transiently cotransfected with a DNA mixture consisting of 300 ng of pGL3-MMTV, 5 ng of pRL-CMV, 30 ng of pCMV-hAR, and increasing amounts (0–120 ng/ml) of FLAG-Foxo1. The empty pcDNA-FLAG vector was cotransfected to equalize the total amount of DNA (in terms of the molar amount) and control for artifacts of the vector DNA. The cells were then treated with 10^{-8} M DHT or the solvent (ethanol) dissolved in serum-free medium for 24 h, before being analyzed by luciferase assays. *b* and *c*, LNCaP cells stably expressing FLAG-Foxo1 or the control FLAG tag were exposed to increasing concentrations of DHT as indicated. Endogenous expression of PSA mRNA (*b*) and PSA protein secreted into the culture medium (*c*) were assayed by real time PCR and EIA, respectively. *d*, LNCaP cells stably expressing either FLAG-Foxo1 or the control FLAG tag were grown in the presence of 10^{-8} M DHT or the solvent (ethanol), and cell proliferation was dynamically evaluated using a CellTiter 96® nonradioactive cell proliferation assay kit. Data are presented as the mean ± S.D. (*a*, *c*, and *d*) or mean ± S.E. (*b*). *Letters above the bars* show statistical groups (ANOVA, $p < 0.05$). *RLA*, relative luciferase activity.

lines, namely ALVA41, DU145, LNCaP, and PC3 cells, with DU145 and LNCaP cells showing 2-fold higher expression levels than PC3 and ALVA41 cells (supplemental Fig. 1*d*).

To further explore the relevance of the inhibitory effects of Foxo1 on AR observed in the promoter assays, the androgen-mediated endogenous PSA expression levels were studied in LNCaP cells stably expressing either FLAG-Foxo1 or the control FLAG tag. As shown in Fig. 1, *b* and *c*, DHT-stimulated PSA expression, which was dose-dependent in terms of both the mRNA level (assayed by real time PCR) and the amount of protein secreted into the medium (quantified by EIA), was significantly lower in cells overexpressing Foxo1, indicating that Foxo1 down-regulates the expressions of endogenous androgen-responsive genes. Lentivirus was applied as an additional method for overexpressing Foxo1 in LNCaP cells, and similar results were obtained (supplemental Fig. 1*e*).

Foxo1 also acted on other steroid hormone receptors (supplemental Fig. 2). Specifically, it enhanced the transcription mediated by liganded PR-A, PR-B, and GRα, exhibited no dramatic effect on ERα, but suppressed ERβ function in a similar manner to that observed for AR. Thus, the inhibitory effects are relatively specific for AR and ERβ.

LNCaP cells are typical androgen-responsive PC cells, and their proliferation is largely dependent on the availability of androgen as well as functional AR. To study the effects of Foxo1 on LNCaP cell growth, the two cell lines stably expressing either FLAG-Foxo1 or the control FLAG tag were grown in medium containing charcoal-stripped serum supplemented with either DHT (10^{-8} M) or vehicle (ethanol), and a time course experiment was performed. Among the cells grown in the presence of DHT, the number of FLAG-Foxo1-expressing cells was decreased compared with control cells, even after 1 day of treatment, and then continued to decrease in a time-dependent manner (Fig. 1*d*). The basal proliferation in the presence of ethanol was also reduced to some extent in the stable FLAG-Foxo1-expressing cells, but more prominent inhibition was observed in cells treated with DHT. Thus, Foxo1 overexpression reduced the proliferation of LNCaP cells.

IGF1 Ameliorates Foxo1 Suppression over AR Transactivation—Foxo1 protein is a target of Akt kinase and negatively regulated by phosphorylation in an insulin- and/or IGF1-dependent manner, with resultant nuclear exportation and cytoplasmic sequestration. Therefore, either IGF1 or insulin is expected to attenuate the inhibitory effects of Foxo1 on AR. Initially, we observed that a constitutively active mutant, Foxo1-3A (nonphosphorylatable mutant with all three Akt target residues mutated to alanine, specifically T24A, S253A, and S316A), was more potent than Foxo1 at suppressing DHT-induced AR transactivation (Fig. 2) in LNCaP cells, in which the impaired PI3K/Akt signaling was rescued by cotransfection of PTEN (LNCaP cells lack functional PTEN, and their Akt is constitutively active (27, 28)). In these PTEN-expressing LNCaP cells, IGF1 significantly augmented liganded AR-mediated PSA promoter activities. Of importance, the IGF1-augmented DHT-induced AR transactivation was sharply suppressed by Foxo1-3A but not by wild-type Foxo1. In other words, IGF1 abolished the suppression of liganded AR by wild-type Foxo1 but not that mediated by the Akt-nonphosphorylatable Foxo1 mutant. The rescue by IGF1 was dose-dependent (supplemental Fig. 3*a*) and also found in DU145 cells, which contain intact PTEN and PI3K/Akt signaling (supplemental Fig. 3*c*). Intriguingly, insulin, another PI3K/Akt stimulator, also enhanced the DHT-induced AR transactivation in the PTEN-expressing