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***Note***

# Resveratrol Down-Regulates the Androgen Receptor at the Post-Translational Level in Prostate Cancer Cells

Naoki HARADA, Yohei MURATA, Ryoichi YAMAJI*, Takumi MIURA, Hiroshi INUI and Yoshihisa NAKANO

*Division of Applied Life Sciences, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Sakai, Osaka 599–8531, Japan*

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***Summary*** Androgen receptor (AR) functions as a transcriptional factor for the development and progression of prostate cancer. Resveratrol is known to inhibit the function of AR and to repress AR expression at the transcriptional level. This study focuses on the effects of resveratrol on the AR function and the post-translational AR level. Resveratrol repressed the transcriptional activities of a mutant AR lacking the ligand-binding domain, a constitutive active form of AR, and wild-type AR in a concentration-dependent manner in human prostate cancer PC-3 cells, indicating that resveratrol does not inhibit the transcriptional activity of AR through binding to the ligand-binding domain of AR. Furthermore, the half-life of AR protein was approximately 4 h in resveratrol-treated AR-positive prostate cancer LNCaP cells, compared to approximately 13 h in control cells, as determined by cycloheximide chase. These results indicate that resveratrol down-regulates AR protein through a post-translational mechanism and suggest that the inhibitory effect of resveratrol on AR function is partly attributable to a decrease in the post-translational AR level.

***Key Words*** resveratrol, androgen receptor, prostate cancer, phytoestrogen

Resveratrol (3,4′,5-trihydroxystilbene) is a natural phytoalexin that is synthesized in several plants as a defensive response against adverse conditions such as environmental stress or fungal infection (*1*). Resveratrol is a constituent of a variety of edible plant products such as peanuts and grape skin, and as a consequence, red wine (*2*). Several lines of evidence suggest that resveratrol plays a role in the prevention of human pathological processes such as inflammation (*3*), atherosclerosis (*4*) and carcinogenesis including prostate cancer (*2*, *5*, *6*).

Prostate cancer is the most commonly diagnosed malignant carcinoma in American men (*7*). It has a high morbidity and mortality and is the second leading cause of cancer death among American men. Prostate cancer develops and progresses under the influence of androgens such as testosterone and 5$\alpha$-dihydrotestosterone (DHT). DHT has a higher affinity for the androgen receptor (AR) and acts as the primary androgen to exert androgen signaling. The AR is a member of the steroid hormone nuclear receptor superfamily and is composed of three major domains: an N-terminal domain, a central DNA-binding domain and a C-terminal ligand-binding domain (LBD) (*8*). The N-terminal domain is variable, and the other two domains are highly conserved among the steroid hormone nuclear receptors. The ligand-free AR exists in the cytosol as an inactive state in a heterometric complex that includes at least two heat-shock proteins (HSP90 and HSP70) (*9*). However, the binding of DHT to AR causes AR to undergo a conformational change from the inactive form to the active form, followed by dissociation of AR from the HSPs. The AR translocates into the nucleus and binds to an androgen response element in the promoter region of androgen-responsive genes to activate transcription of these genes as a transcriptional factor.

Preventive strategies are the only available means to reduce the morbidity and mortality associated with prostate cancer because no effective therapy for prostate cancer has been found. Plant-based dietary factors may have chemopreventive effects on human carcinogenesis. Recently, considerable attention has been focused on the role of phytoestrogens in preventing prostate cancer. Resveratrol, which possesses structural similarities to the synthetic estrogen diethylstilbestrol, not only exerts an estrogen-like activity through the estrogen receptor (ER) as a phytoestrogen (*10*), but also inhibits the function of the AR in androgen-dependent prostate cancer cells (*11*, *12*). Because resveratrol reduces the transcriptional activity of the AR promoter in resveratrol-treated cells, the decrease of AR mRNA expression appears to contribute to the depression of AR function. However, the mechanism by which resveratrol inhibits AR function remains unclear. In the

*To whom correspondence should be addressed.
E-mail: yamaji@biochem.osakafu-u.ac.jp

Abbreviations: AR, androgen receptor; CHX, cycloheximide; DHT, 5$\alpha$-dihydrotestosterone; ER, estrogen receptor; LBD, ligand-binding domain; RLU, relative light units.

present study, we report that resveratrol does not inhibit AR transactivation through binding to the LBD of AR. Furthermore, we demonstrate that resveratrol down-regulates AR protein at the post-translational level.

## *Materials and Methods*

*Cell culture.* Human prostatic carcinoma cell lines (AR-positive LNCaP and AR-negative PC-3) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 $\mu$g/mL streptomycin at 37˚C in a 5% $CO_2$/95% air atmosphere at 100% humidity unless otherwise indicated. PC-3 and LNCaP cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Miyagi, Japan).

*Plasmids.* The mammalian expression vectors for human wild-type AR and mutant AR (AR$\Delta$C-Nuc) lacking the ligand-binding domain (pcDNA3.1-AR and pcDNA3.1-AR$\Delta$C-Nuc, respectively) and luciferase reporter vector (pARE2-TATA-Luc) have been described previously (*13*).

*Reporter assay.* PC-3 cells were grown to confluence in phenol red-free RPMI 1640 medium supplemented with 10% dextran-coated charcoal-stripped fetal bovine serum (steroid-free RPMI 1640 medium). Cells were seeded on 12-well dishes and cultured to 90% confluence in steroid-free RPMI 1640 medium. Cells were transiently co-transfected with pcDNA3.1-AR or pcDNA3.1-AR$\Delta$C-Nuc, pARE2-TATA-Luc and pRL-SV40 vector (*Renilla* luciferase expression vector: Promega Corp., Madison, WI, USA) using Metafectene reagent (Biontex Laboratories GmbH, Martinsried/Planegg, Germany) for 24 h. The medium was replaced with fresh steroid-free RPMI 1640 medium supplemented with 10 nmol/L DHT and various concentrations of resveratrol or 10 $\mu$mol/L hydroxyflutamide, and cells were cultured for an additional 24 h. Cell lysates were prepared, and firefly and *Renilla* luciferase activities were determined with the Dual-Luciferase reporter assay kit and GloMax 20/20 Luminometer (Promega, Madison, WI, USA). Transfection efficiency was normalized with *Renilla* luciferase expression vector (pRL-SV40), and data are expressed as relative light units (firefly luciferase divided by *Renilla* luciferase).

*Western blot.* For the endogenous AR protein level, LNCaP cells were cultured in steroid-free RPMI 1640 medium for 48 h, and incubated with various concentrations (1–50 $\mu$mol/L) of resveratrol in the presence of 10 nmol/L DHT for an additional 24 h, followed by preparation of cell lysates in lysis buffer (20 mmol/L Hepes-NaOH, pH 7.5, containing 0.5% NP-40, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 100 $\mu$mol/L 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 $\mu$g/mL leupeptin and 1 $\mu$g/mL aprotinin). For the cycloheximide (CHX) treatment experiment, LNCaP cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics and incubated with 50 $\mu$mol/L resveratrol in the presence of 10 $\mu$g/mL CHX for the indicated time periods, followed by preparation of cell lysates in lysis buffer. CHX was added to the medium 30 min before addition of resveratrol. Cell lysates were subjected to SDS-PAGE in 9% gel and analyzed by Western blotting with polyclonal anti-AR (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and monoclonal anti-$\alpha$-tubulin antibodies (Sigma-Aldrich, St. Louis, MO, USA), followed by immunoreaction with the horseradish peroxidase-conjugated secondary anti-rabbit and anti-mouse antibodies, respectively. The immunoreactive bands were detected using the Super Signal chemiluminescent substrate (Pierce Biotechnology, Rockford, IL, USA), and exposed to Kodak BioMax XAR film. The intensity of immunoreactive proteins was quantified by densitometry using a computer image analysis program (National Institutes of Health Image, version 1.60).

*Statistical analysis.* The effects of resveratrol on AR transactivation were evaluated by one-way ANOVA, and a post-hoc analysis was performed by Tukey's test. Analysis was performed with GB-Stat 5.4 software (Dynamic Microsystems, Silver Spring, MD, USA). Each data value is shown as means±SD, and differences are considered significant when $p<0.05$.

## *Results*

To assess whether resveratrol functions as an antagonist through binding to the LBD of AR, PC-3 cells were co-transfected with wild-type AR or mutant AR (AR$\Delta$C-Nuc) expression vector and luciferase reporter vector, and cultured in the presence of DHT and resveratrol.

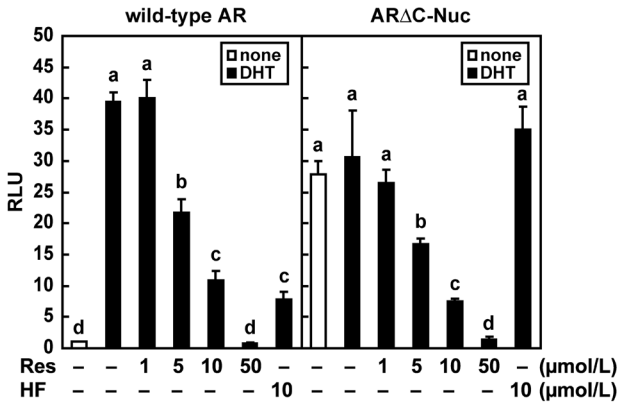


Fig. 1. Effect of resveratrol on the transcriptional activities of AR and AR$\Delta$C-Nuc. PC-3 cells that had been cultured in steroid-free RPMI1640 medium were co-transfected with wild-type AR (left panel) or AR$\Delta$C-Nuc (right panel) expression vector, pARE2-TATA-Luc and pRL-SV40 for 24 h, followed by incubation in the presence of 10 nmol/L DHT and various concentrations of resveratrol (Res) or 10 $\mu$mol/L hydroxyflutamide (HF) for a further 24 h. Luciferase activities were determined. Firefly luciferase activity was divided by *Renilla* luciferase activity, and data are expressed as relative light units. Experiments were repeated three times in triplicate, and values are indicated as means±SD. Each graph is representative of three independent experiments. Values with different letters are significantly different ($p<0.05$).

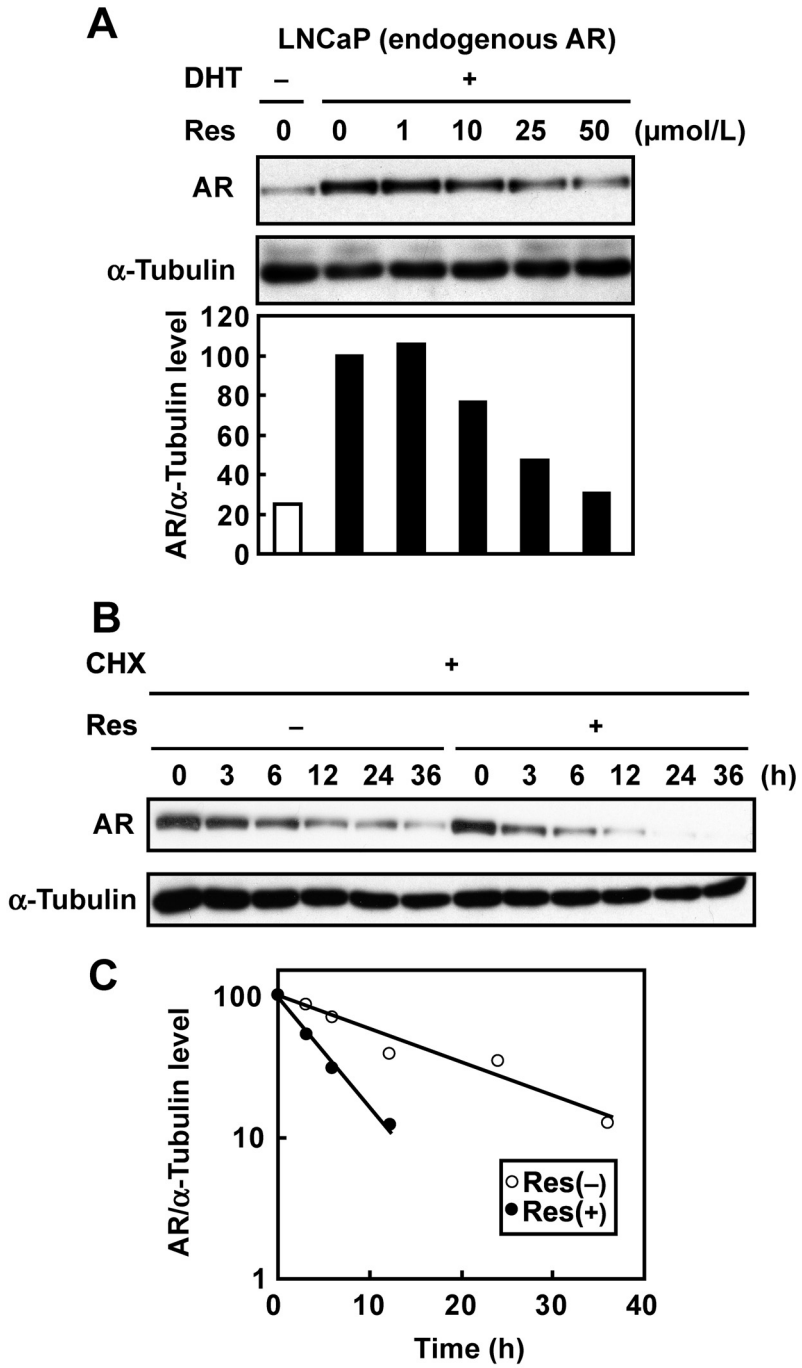


Fig. 2. Effect of resveratrol on AR protein at the post-translational level. (A) LNCaP cells that had been cultured in steroid-free RPMI 1640 medium for 48 h were incubated in the presence of 10 nmol/L DHT and various concentrations (1–50 $\mu$mol/L) of resveratrol (Res) for an additional 24 h, and cell lysates were prepared. Intensities of immunoreactive bands for AR and $\alpha$-tubulin were quantified by densitometry, and the band intensities of AR were normalized with those of $\alpha$-tubulin. Standard curves were prepared by plotting the ratio of immunoreactive bands for AR normalized with $\alpha$-tubulin level. (B) LNCaP cells that had been cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics were incubated in the presence of 50 $\mu$mol/L resveratrol (Res) and 10 $\mu$g/mL CHX for the indicated time periods, followed by preparation of cell lysates. CHX was added to the medium 30 min before addition of resveratrol. Cell lysates (10 $\mu$g of proteins) were analyzed by Western blot analysis with anti-AR or anti-$\alpha$-tubulin antibody. (C) Immunoreactive bands obtained in (B) were quantified by NIH image, and the ratio of protein level of AR divided by that of $\alpha$-tubulin was logarithmically plotted. The results are representative of two independent experiments.

AR$\Delta$C-Nuc is an LBD-deleted mutant of AR corresponding to the previously reported AR1-660 (*14*, *15*) and is constitutively localized in the nucleus as a constitutively active form of AR even in the absence of ligand. In fact, AR$\Delta$C-Nuc exhibited constitutive transcriptional activity even in the absence of DHT (Fig. 1, right panel) although wild-type AR functioned as a ligand-dependent transcription factor (Fig. 1, left panel), as has been reported previously (5). Resveratrol suppressed the transcriptional activities of both AR$\Delta$C-Nuc and wild-

type AR in a concentration-dependent manner (Fig. 1). The inhibitory effect of resveratrol on AR transactivation was observed at concentrations of 5 $\mu$mol/L or more, and resveratrol at a concentration of 50 $\mu$mol/L completely abolished DHT-activated AR transactivation. On the other hand, hydroxyflutamide, which antagonizes the function of AR through binding to the LBD of AR (*16*), inhibited the transcriptional activity of wild-type AR, but not that of AR$\Delta$C-Nuc. These results indicate that resveratrol does not contribute to the repression of AR transactivation through binding to the LBD of AR.

The effects of resveratrol on the endogenous level of AR protein were determined. LNCaP cells were incubated in the presence of various concentrations of resveratrol, and cell lysates were prepared, followed by Western blot analyses with anti-AR and anti-$\alpha$-tubulin antibodies. DHT caused an increase in the AR protein level, consistent with the previous report that DHT stabilizes AR protein (*17*, *18*). However, resveratrol repressed the DHT-increased AR protein level at concentrations greater than 10 $\mu$mol/L, but not the $\alpha$-tubulin protein level (Fig. 2A). Furthermore, to determine whether a decrease in AR protein caused by resveratrol occurs at the post-translational level, LNCaP cells were treated with resveratrol in the presence of CHX, and the endogenous AR protein was analyzed by Western blot. Following protein synthesis inhibition by CHX treatment, the endogenous AR protein levels in both resveratrol-treated and -untreated cells decreased steadily in a time-dependent manner, whereas $\alpha$-tubulin protein remained intact (Fig. 2B). The intensities of AR protein levels that had been normalized with those of $\alpha$-tubulin protein levels were plotted logarithmically to calculate the half-life of AR protein. The AR in resveratrol-treated cells was degraded more rapidly than that in control cells, and the half-lives of AR proteins in resveratrol-treated and -untreated cells were 4 (correlation coefficient=0.995) and 13 h (correlation coefficient=0.951), respectively (Fig. 2C).

## *Discussion*

Resveratrol exerts an estrogen-like activity through binding to the LBD of ER in a concentration-dependent manner up to 50 $\mu$mol/L, although 100 $\mu$mol/L resveratrol suppresses the estrogen-like action (*10*). On the other hand, resveratrol inhibits the transcriptional activity of AR at concentrations of 50–100 $\mu$mol/L (*11*, *12*) and down-regulates AR mRNA expression in prostate cancer cells at concentrations greater than 50 $\mu$mol/L (*11*). In the present study, to obtain information concerning the mechanism by which resveratrol inhibits AR function, we analyzed the effects of resveratrol on the transcriptional activity of AR$\Delta$C-Nuc and the post-translational AR expression level.

Our finding that resveratrol inhibited the transcriptional activities of AR$\Delta$C-Nuc and wild-type AR (Fig. 1) indicates that resveratrol does not attenuate the function of AR through binding to the LBD, even though the structure of the LBD is highly conserved between AR and ER. Genistein, a soya isoflavone, which exhibits an estrogen-like activity through binding to the LBD of ER, inhibits the transcriptional activity of AR without direct interaction with the LBD of AR (*19*). Quercetin, a naturally occurring flavonoid compound, also represses the function of AR (*20*). These phytochemicals including resveratrol inhibit the function of AR, at least in part, by decreasing AR expression at the transcriptional level. Our finding that resveratrol shortened the half-life of AR protein in LNCaP cells (Fig. 2C) indicates that resveratrol decreases AR expression at the post-translational level. Resveratrol seems to destabilize AR protein or activate its degradation. Androgen binding causes a conformational change, leading to stabilization of AR protein. Hydroxyflutamide antagonizes the DHT-induced AR transactivation and abrogates the DHT-induced stabilization of the AR protein through binding to the LBD of AR, indicating that the antagonistic action of hydroxyflutamide on AR transactivation is exerted through, at least in part, destabilization of AR protein (*17*, *18*). Although the mechanism by which the hydroxyflutamide-bound AR is degraded remains unclear, the AR protein level is regulated by at least two proteolytic pathways, one of which depends on the 26S proteasome (*21*). The other relies on PTEN and caspase 3 (*22*), independent of the proteasome. Because resveratrol did not bind to the LBD of AR, the mechanism by which resveratrol down-regulates AR protein at the post-translational level may be different from the mechanism by which antiandrogens such as hydroxyflutamide destabilize AR protein.

Endocrine therapies such as reducing the levels of circulating androgens, blocking agonist activation with antagonists or both are the standard treatment for prostate cancer. However, prostate cancer eventually returns in the form of more aggressive tumors that are hormone-refractory or androgen-independent, making androgen ablation therapy ineffective. Most androgen-independent prostate tumors show high levels of AR expression and expression of androgen-regulated genes such as prostate-specific antigen (*23*), indicating the reactivation of AR transactivation in hormone-resistant prostate cancer. Because decreasing the AR protein level to minimize or eliminate the function of AR is expected to be an effective strategy for repressing the development and progression of prostate cancer, resveratrol may be useful as a chemopreventive and/or chemotherapeutic agent for prostate cancer.

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