sequences in the *in vivo* dispositions of coumarin and nicotine. However, such report did not identify the health status of subjects and smokers were also recruited in their study. Moreover, the probe drugs used were not standardized. Thus, to give a strong support to the existence of the discordance between coumarin and nicotine dispositions, we provided the data and information in this regard by performing the study in a larger scale population. Inter-individual differences in the postulated enzymatic activity of CYP2A6 proved the existence of the close association between the phenotype and genotype.

Although the *CYP2A6* genotype-phenotype correlation was found to be rather clear, the homozygotes of the *CYP2A6*4* allele were totally deficient in cotinine formation, whereas the amounts of 7-OHC excreted in the urine in these subjects were, though, extremely low but still detectable (**Fig. 2**). Taken together with *in vitro* studies employing genetically expressed human *CYP2A13* gene system,[33,34] our data confirmed the role of other enzymes in the *in vivo* 7-OHC formation. However, the contribution of CYP2A13 to the *in vivo* dispositions of coumarin and nicotine is obviously minimal since the *in vitro* intrinsic activity of the enzyme toward coumarin is about 10-fold less than that of CYP2A6.[35] Moreover, the subjects with *CYP2A6*4/*4* showed total absence of the *in vivo* metabolism toward nicotine.

The discordance between CYP2A6 catalytic activities toward coumarin and nicotine did exist at a large sample population. The change in substrate specificity of the *CYP2A6*7* allele has been hypothesized to account for the discordance in the rates of coumarin 7-hydroxylase and nicotine *C*-oxidase.[36] Since the I471T variant of CYP2A6.7 is not located in the substrate-recognition site,[37] the mechanism(s) of functional changes dependent of substrates such as coumarin and nicotine needs further investigation. Despite an exception for the *CYP2A6*7* allele, the correlation study ($R=0.92$, $p<0.001$) indicated that in human both 7-OHC and cotinine formations were mostly catalyzed by CYP2A6 (**Fig. 2**). This means that both coumarin and nicotine could be clinically and experimentally used as probe drugs in a routine CYP2A6 phenotyping test.

Even though no subject genotyped as a homozygote for *CYP2A6*10* allele was found in the present study, the effects of *CYP2A6*10* allele on the rates of 7-OHC and cotinine formation were observed (**Table 3**). The frequency of *CYP2A6*9* allele found in this study was corresponding to that of Japanese (**Table 4**). Individuals possessing *CYP2A6*9* allele showed a lower rate of 7-OHC excretion and cotinine formation (**Table 3**). The data were consistent with the *in vivo* studies performed in healthy volunteers that displayed reduced enzyme activities in the subjects genotyped as *CYP2A6*1/*9* and *CYP2A6*9/*9*.[27] Taken together, this confirmed the effect of *CYP2A6*9* allele on the decrease in the CYP2A6 activity *in vivo*. Our classification of the subjects into four phenotypic groups; extensive, intermediate, poor and very poor phenotypic group, was more advantageous, reliable, and useful for clinical application. As can be seen in **Fig. 3**, the average amounts of 7-OHC excreted in the urine and cotinine/nicotine ratio in the plasma of the subjects in each phenotypic group was significantly different from the other phenotypic groups.

Since CYP2A6 is capable of bioactivating tobacco-specific procarcinogens, CYP2A6 PM could be theoretically protected from developing cancer at the target sites mainly the lungs.[36] Results reported by Fujieda *et al.*[6] clearly indicated that Japanese smokers with *CYP2A6* variant alleles had significantly lower risk of lung cancer. However, a meta-analysis conducted by Carter *et al.*[7] showed that CYP2A6 mutation had no protective effect toward the risk of lung cancer. The discordance in the literature may, therefore, be resulted from various aspects of study design such as broad definitions of smokers, ethnic stratification, genotype groups, and genotyping assays.

We, hereby, confirmed that the frequency distribution of *CYP2A6* variant alleles was consistent over Mongolians, and these ethnic groups may, therefore, have similar potential tobacco-related lung cancer risk, pharmacological as well as toxicological responses to some anticancer agents such as coumarin, fadrozole, and tegafur. The data presented in the present study, therefore, may be applicable to drug development and therapy with drugs metabolized mainly by CYP2A6.

Since there are still large CYP2A6-activity variations among the subjects with the same genotype, particularly *CYP2A6*1/*1* (**Fig. 3**), it appears that other factors may also contribute to the CYP2A6 enzyme activity. Among those, gender and environmental contaminants have been found to influence CYP2A6. The average amounts of 7-OHC excreted in the urine in females was higher than that in males,[32,38] while the exposure to cadmium from environmental pollutant and diet resulted in the induction of the enzyme activity.[39] In contrast, plant substances seemed to have a minimal influence on CYP2A6 activity.[40] In addition to those host and/or environmental factors, the intra-genotypic variation of CYP2A6 activity among the subjects may possibly be marked due to the presence of unknown lack-functioned or gain-functioned mutations in the *CYP2A6* gene. The complex etiology of the intra-genotypic variation of CYP2A6 activity may also be resulted from the potential consideration of epigenetics.

In conclusion, we performed a population study of *CYP2A6* genotype-phenotype analysis in 120 Thais who took both coumarin and nicotine sequentially. This

procedure provided a precise evaluation of *CYP2A6* genetic polymorphism in the metabolism of coumarin and nicotine. The CYP2A6 activity toward coumarin was markedly low in the subjects homozygous for either *CYP2A6*4* or *CYP2A6*9*, or heterozygous for these alleles, whereas the enzyme activity toward nicotine was dramatically reduced in the homozygotes for *CYP2A6*4*, *CYP2A6*7*, and *CYP2A6*9* or the heterozygotes with these alleles. The presence of *CYP2A6*4*, *CYP2A6*7*, *CYP2A6*9*, or *CYP2A6*10* was a critical factor determining inter-individual variations in drug oxidation of CYP2A6 substrates. We, therefore, propose that nicotine is a better probe according to its specificity, while coumarin is still valuable to be used for a routine CYP2A6 phenotyping since the test employs a non-invasive method.

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Activation of p53 as a causal step for atherosclerosis induced by polycyclic aromatic hydrocarbons

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Abstract **This study was performed to prove our hypothesis that the metabolite(s) of polycyclic aromatic hydrocarbons (PAHs) caused the activation or phosphorylation of p53 via DNA damage to suppress the liver X receptor (LXR)-mediated signal transductions as a probably more direct mechanism. We found that LXR-mediated *trans*-activation was inhibited by 3-methylchoranthrene (MC) and doxorubicin (Dox) in HepG2 cells carrying wild-type p53, but not in Hep3B cells possessing mutant p53. The exogenous expression of wild-type p53 suppressed the LXR-mediated *trans*-activation in Hep3B cells. The expression of mRNA for ATP binding cassette A1 was suppressed by MC and Dox in HepG2 cells. The protein expression of retinoid X receptor (RXR), a partner of LXR to form a heterodimer, was suppressed by MC and Dox in HepG2 cells.**

Keywords: ABCA1; AHR; Atherosclerosis; DNA damage; Luciferase assay; MC; Quantitative RT-PCR

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]-pyrene (B[*a*]P) and 3-methylcholanthrene (MC), are ubiquitous contaminants in the environment. PAHs are detected in multiple sources, including cigarette smoke, exhaust emissions, industrial wastes and the pyrolysates of foods [1–3]. They cause a wide variety of toxicities, including carcinogenesis, atherogenesis and teratogenesis [4]. These toxic effects are known to be mediated by aryl hydrocarbon receptor (AHR), a ligand-dependent basic helix–loop–helix transcription factor [5–7]. A ligand-activated AHR translocates into the nucleus, forms a heterodimer complex with AHR nuclear translocator (ARNT), and finally interacts with xenobiotic responsive elements in the 5′-flanking regions of the AHR-target genes [8]. As one of the important mechanisms of PAH-induced toxicities, AHR up-regulates drug metabolizing enzymes such as cytochrome P450 (CYP), especially CYP1A1, which metabolizes PAHs to yield reactive intermediates causing DNA damage [9].

Tumor suppressor p53 is also known to be a transcription factor activated or phosphorylated by many types of stresses, including DNA damage [10,11]. The activated p53 subsequently *trans*-activates target-genes responsible for growth arrest or apoptosis [10,11]. Recently, it has been reported that p53 interacts with nuclear receptor, glucocorticoid receptor (GR), which is activated by glucocorticoids and maintains homeostasis in response to internal or external stresses [12]. The interaction results in the promotion of the proteosomal degradation of both proteins [12]. In addition, p53 is reported to inhibit the specific binding of nuclear receptor, androgen receptor (AR), which binds to androgens and is critical for the development, growth and maintenance of the male reproductive system, to DNA as a result of the inhibition of AR dimerization [12]. These studies provide evidence for a negative cross-talk between p53 and nuclear receptors.

PAHs, including B[*a*]P and MC, was reported to induce the atherosclerosis in several experimental animals [13–15]. Previously, we reported that MC inhibited liver X receptor (LXR)-mediated signal transductions, which are known to maintain cholesterol homeostasis, through AHR to cause atherosclerosis [16]. We also reported that the metabolism, probably the metabolic activation, of MC by CYP1A1 was a necessary step to repress the LXR-originated signal transductions by MC [17]. In the present study, we hypothesized that p53 activated by the metabolite(s) of PAHs suppressed LXR as well as GR or AR [12]. In this paper, we show evidence supporting our idea that p53 activated by PAHs acts as a negative regulator of LXR-mediated signal transductions to cause atherosclerosis via suppression of retinoid X receptor (RXR) expression, which is a partner of LXR to form a heterodimer.

2. Materials and methods

2.1. Cell culture

Human hepatoma-derived HepG2 and Hep3B cells were purchased from RIKEN (Tsukuba, Japan). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Nissui Pharmacy, Tokyo, Japan) supplemented with 10% fetal bovine serum (Bio Whittaker, Warkersville, MD), non-essential amino acids (ICN, Aurora, OH) and 1 mM sodium pyruvate (Gibco-BRL, Rockville, MD) in 5% CO_2 at 37 °C.

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Abbreviations: ABCA1, ATP binding cassette A1; AHR, aryl hydrocarbon receptor; AR, androgen receptor; ARNT, AHR nuclear translocator; B[*a*]P, benzo[*a*]pyrene; CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; Dox, doxorubicin; GR, glucocorticoid receptor; LXR, liver X receptor; LXRE, LXR response element; MC, 3-methylcholanthrene; PAH, polycyclic aromatic hydrocarbon; RT-PCR, reverse transcriptase-polymerase chain reaction; RXR, retinoid X receptor; T1317, TO-901317; TK, thymidine kinase

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2.2. Plasmids

The p(LXRE)$_2$–thymidine kinase (TK)–Luc was constructed as described previously [16]. Full-length human p53 cDNA was obtained by PCR with a sense primer, p53-*Xho*I-S (5′-CGGGCTCGAGCCATGGAGGAGCCGCAGTC-3′), and an antisense primer, p53-*Xho*I-AS (5′-GTGGCTCGAGTCAGTCTGAGTCAGGCCCT-3′). The resultant fragment was digested with *Xho*I, and inserted into the *Xho*I site of the pcDNA 3.1 mammalian expression vector (pcDNA-p53) (Invitrogen, Carlsbad, CA).

2.3. Transient transfection and luciferase assay

One day before transfection, cells were plated at a density of 1×10^5 cells/well in a 12-well plate. HepG2 cells were transfected with 350 ng of p(LXRE)$_2$–TK–Luc, 100 ng of pcDNA-hLXRα and 50 ng of pRL-TK vector (as an internal control for transfection) by using Fugene6 (Roche Diagnostics, Indianapolis, IN). After the transfection, the medium was changed to fresh DMEM containing 1 μM TO-901317 (T1317), a LXR ligand (Sigma–Aldrich, St. Louis, MO), 1 μM MC, an AHR ligand (Sigma–Aldrich) and 1, 10 or 100 nM doxorubicin (Dox), a known p53 activator (Sigma–Aldrich). Hep3B cells were transfected with 350 ng of p(LXRE)$_2$–TK–Luc, 100 ng of pcDNA-hLXRα, 50 ng of pRL-TK vector and 0.1, 1 or 10 ng of pcDNA-p53. After the transfection, the medium was changed to fresh DMEM containing 1 μM T1317 and 0.1 or 1 μM MC. Cells were harvested after incubation for 36 h. Luciferase activity was measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI).

2.4. Real-time RT-PCR analysis

HepG2 cells were treated with 10 μM T1317, 10 μM MC and 100 nM Dox. After incubation for 24 h, total RNA from these cells was prepared using a GenElute Mammalian Total RNA Miniprep Kit (Sigma–Aldrich). Reverse transcription reaction was performed by using a First Strand cDNA Synthesis Kit for reverse transcriptase-polymerase chain reaction (RT-PCR) (AMV) (Roche Diagnostics). Quantitative real-time PCR was performed as described previously [16].

2.5. Western blot analysis

HepG2 cells were treated with 10 μM T1317 and 10 μM MC or 100 nM Dox. Nuclear extracts from these cells were prepared after incubation with MC for 0, 6, 12 or 24 h and with Dox for 24 h according to the method of Dignam et al. [18]. Protein concentration was determined using bovine serum albumin as a standard by BCA Protein Assay Reagent Kit (Pierce Biotechnology, Rockford, IL). Western blot analysis was performed using antibodies to LXRα (C-19), RXRα (D-20), p-p53 (Ser 15), p21 (C-19) (Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (AC-15) (Abcam, Cambridge, MA).

3. Results and discussion

To examine a possibility of whether the activation of p53 was a process critical for the suppression of LXR-mediated signal transductions, the effects of Dox, one of the representative p53 activators, on LXR-mediated transcriptional activity were investigated by a luciferase reporter assay using a reporter plasmid, p(LXRE)$_2$–TK–Luc (Fig. 1). When T1317, a LXR ligand, was added to a culture containing HepG2 cells transfected with the pcDNA-hLXRα,the luciferase activity seen with p(LXRE)$_2$–TK–Luc was elevated to a level of 12-fold higher than that of control (Fig. 1). The luciferase activity seen with p(LXRE)$_2$–TK–Luc in the presence of T1317 was decreased to a level of approximately 20–30% by co-treatment with MC (Fig. 1). Similarly, the luciferase activity was decreased by co-treatment with Dox in a dose-dependent manner (Fig. 1). These results support the idea that the activation of p53 is a process responsible for the suppression of LXR-mediated signal transductions by PAHs.

To further support the idea that p53 was involved in the suppression of LXR-mediated signal transductions by PAHs, the effects of MC and the exogenous expression of p53 on LXR-mediated transcriptional activity were investigated in Hep3B cells, a human hepatoma-derived cell line lacking wild-type p53 [19]. When T1317 was added to a culture containing Hep3B cells transfected with the pcDNA-hLXRα, the luciferase activity seen with the p(LXRE)$_2$–TK–Luc was increased to a level 15-fold higher than that of control (Fig. 2A). The co-treatment of Hep3B cells with MC did not affect the luciferase activity seen with the p(LXRE)$_2$–TK–Luc (Fig. 2A). When Hep3B cells were transfected with increasing amounts

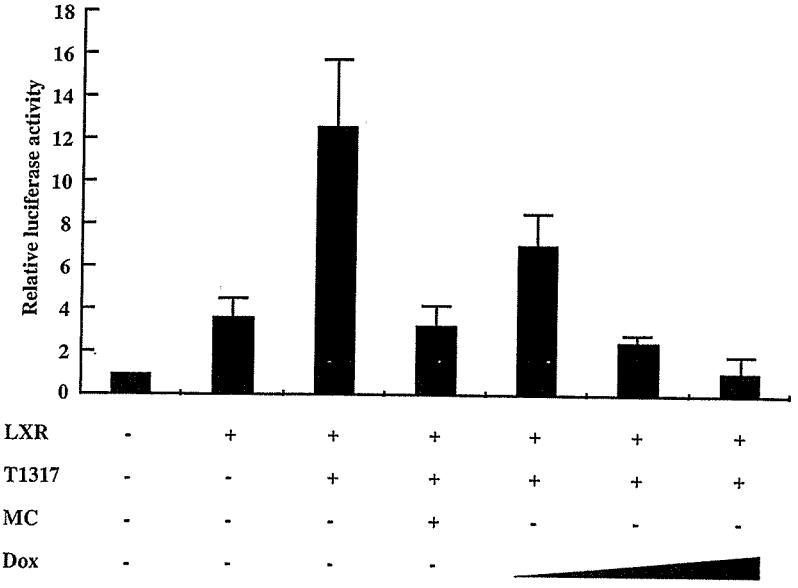

Fig. 1. Inhibition of LXR-mediated transcriptional activity by MC and Dox, which is the activator of p53. HepG2 cells were transfected with p(LXRE)$_2$–TK–Luc and pcDNA-hLXRα, and to the culture were added 1 μM T1317, 1 μM MC and 1, 10 or 100 nM Dox. Luciferase activity was measured after incubation for 36 h. Values in the figure represent the average ± S.D. from three independent experiments.

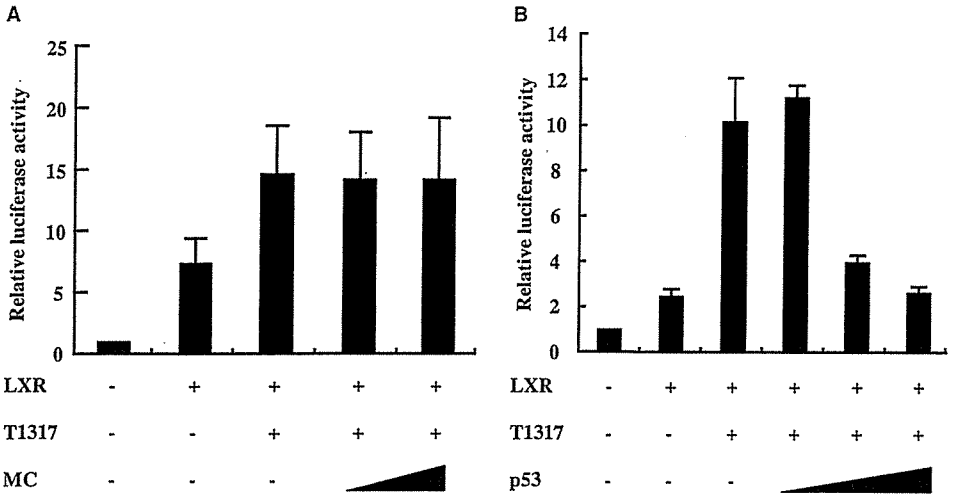

Fig. 2. Effects of MC and the exogenous expression of p53 on LXR-mediated transcriptional activity in Hep3B cells, hepatoma-derived cells carrying mutant p53. (A) A luciferase reporter plasmid, $p(LXRE)_2$–TK–Luc, was co-transfected into Hep3B cells with pcDNA-hLXRα. The Hep3B cells were treated with MC (0.1 or 1 μM). The luciferase activity was measured after incubation for 36 h. Values in the figure represent the average ± S.D. from three independent experiments. (B) A luciferase reporter plasmid, $p(LXRE)_2$–TK–Luc, was co-transfected into Hep3B cells with pcDNA-hLXRα and pcDNA-p53 (0.1, 1 or 10 ng). The luciferase activity was measured after incubation for 36 h. Values in the figure represent the average ± S.D. from three independent experiments.

of pcDNA-p53, the luciferase activity seen with $p(LXRE)_2$–TK–Luc was decreased depending on the amount of pcDNA-p53 (Fig. 2B). These results suggest that p53 plays a key role in the suppression of LXR-mediated signal transductions by PAHs.

To further support the results of reporter gene experiments, in which p53 was involved in the transcriptional down-regulation of the LXR-target genes, we examined the effects of Dox on the expression of mRNA for ATP binding cassette A1 (ABCA1), one of the LXR-target genes [20] (Fig. 3). The expression of ABCA1 mRNA was induced by treatment of HepG2 cells with T1317 and was suppressed by co-treatment with MC and Dox (Fig. 3).

It has been reported that p53 interacts with GR to promote the degradation of GR [12]. To examine the possibility of whether p53 promoted the degradation of LXR or its heterodimeric partner, RXR, the expression of LXR and RXR was investigated by western blot analysis (Fig. 4). Nuclear extracts were prepared from HepG2 cells after incubation for 0, 6, 12 or 24 h with MC, and 24 h with Dox. The increase in the amounts of phosphorylated p53 and p21 expression, which is known to be a typical p53-target gene, was seen after incubation for 6, 12 or 24 h with MC, indicating that p53 was activated by MC (Fig. 4). The expression of LXR was not decreased after incubation for 12 or 24 h with MC, while the notable decrease of RXR expression was found after incubation for 12 or 24 h with

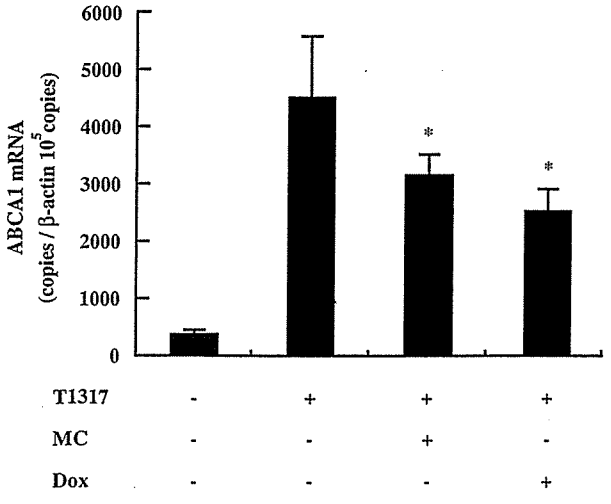

Fig. 3. Suppression of the expression of mRNA for ABCA1 by MC and Dox. The expression of mRNA for ABCA1 was quantified by a real-time RT-PCR. HepG2 cells were incubated with 10 μM T1317 and 10 μM MC or 100 nM Dox. After incubation for 24 h, total RNA was prepared from the cells and subjected to the real-time RT-PCR. Values in the figure represent the average ± S.D. from three independent experiments. *, Statistically different ($P < 0.05$) relative to the cells treated with T1317 alone.

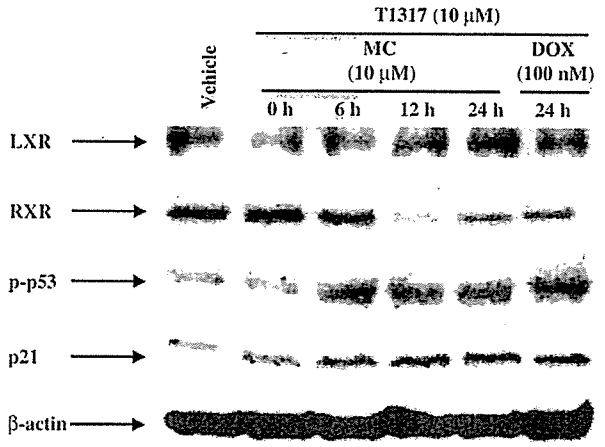

Fig. 4. Effects of MC and Dox on the protein expression of LXR and RXR. HepG2 cells were treated with 10 μM T1317 and 10 μM MC or 100 nM Dox. Nuclear extracts were prepared after incubation for 0, 6, 12 or 24 h with MC, or 24 h with Dox as indicated in the figure. Nuclear extracts (50 μg) prepared from the cells were subjected to SDS-PAGE and analyzed by western blot using antibodies to LXR, RXR, p-p53, p21 and β-actin.

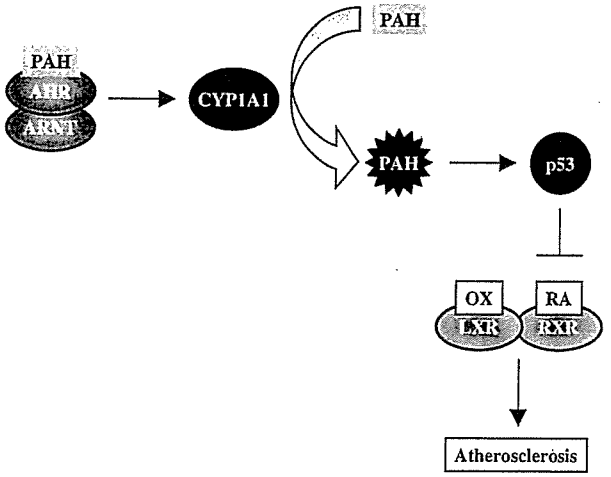

Fig. 5. Proposed mechanism(s) for the PAH-induced suppression of LXR-mediated signal transductions. PAH, polycyclic aromatic hydrocarbon; AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; OX, oxysterols; RA, retinoic acid; LXR, liver X receptor; RXR, retinoid X receptor.

MC (Fig. 4). When HepG2 cells were treated with Dox for 24 h, the expression of RXR, but not LXR, was decreased (Fig. 4). These results suggest that the activated p53 suppresses the expression of RXR, which is a heterodimeric partner of LXR.

In the present study, we found that the activated p53 suppressed the expression of RXR to cause the suppression of LXR-mediated signal transductions. Yahagi et al. [21] reported that p53 and its target genes in adipocytes of *ob/ob* mice, which develop obesity, insulin resistance and glucose intolerance owing to an inherited deficiency of the appetite-suppressing hormone, were highly induced. They also found that the activation of p53 was responsible for the suppression of the lipogenic genes which were regulated by LXR. In addition, p53 is reported to negative-regulate nuclear receptors including GR and AR [12]. Together with these results, it may be possible to assume the mechanism of atherosclerosis induced by PAHs as follows (Fig. 5). First, PAHs bind to AHR and induce the expression of CYP1A1. Second, PAHs are metabolized by CYP1A1 to generate a reactive intermediate(s) and the resultant PAH-metabolites cause DNA damage to activate p53. Third, the activated p53 suppresses the protein expression of RXR, which is a heterodimeric partner of LXR. Finally, the expression of the LXR-target genes is suppressed to cause atherosclerosis.

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Dose dependent inhibitory effects of dietary 8-methoxypsoralen on NNK-induced lung tumorigenesis in female A/J mice

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Abstract

We have reported that pretreatment by stomach tube with 8-methoxypsoralen (methoxsalen; 8-MOP), a potent human CYP2A6 inhibitor, strongly suppresses lung tumorigenesis by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in female A/J mice (Cancer Res. 2003). Here, we examined inhibitory effects with adminstration in the diet. When the mice were 7 weeks of age, they received dietary supplementation with 8-MOP at concentrations of 1, 10 or 100 ppm for 3 days prior to a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) or an equal volume of saline (vehicle control). The experiment was terminated 16 weeks after the first 8-MOP treatment and lung proliferative lesions were analyzed. The incidences and multiplicities in the 8-MOP 100 ppm-treated group were significantly reduced as compared with values for the NNK alone group ($P<0.001$). Multiplicities of NNK-induced lung proliferative lesions were also reduced in a dose dependent manner (Spearman rank correlation coefficient; $\rho=-0.806$, correction $P<0.0001$). Mouse CYP2A4 and CYP2A5 differ from each other only 11 amino acids, and are closely related to the human CYP2A6. One hour after the last of three daily doses of 8-MOP (0.5, 5 or 50 mg/kg body weight in 0.2 ml corn oil, given by stomach tube) or an equal volume of corn oil (vehicle control), given to the mice at 7 weeks of age, isolation of lung and liver RNAs demonstrated no effects on CYP2A4 and CYP2A5 mRNA levels with 8-MOP. In conclusion, the results of this study showed that clear dose response inhibitory effects of 8-MOP on NNK-induced lung tumorigenesis in female A/J mice fed diets containing 8-MOP, due to inhibition of enzyme activity of CYP2A4 and CYP2A5, rather than their gene expression.

Keywords: A/J mice; Lung carcinogenesis; NNK; CYP2A6; 8-Methoxypsoralen

1. Introduction

Lung cancer is one of the most common causes of cancer mortality in the world, with cigarette smoking

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generally regarded as the most important risk factor. Adenocarcinoma of the lung is second in frequency, after squamous cell lung cancer, and recently there appears to have been a shift to this histologic type, with an absolute increase, especially in females [1–3]. The incidence of squamous cell carcinoma, in contrast has been decreasing. The most likely explanation is the shift from high-tar non-filter to low-tar filter cigarettes. A case control study has shown that the risk of squamous cell carcinoma for smokers is lower with filter than non-filter cigarettes, but this is not the case for adenocarcinomas [4]. The change in cigarette type has led to a reduction in exposure to polycyclic aromatic hydrocarbons (PAHs) and an increase in nitrogen oxides and tobacco-specific *N*-nitrosamines, especially NNK, in the inhaled smoke [5]. Thus, the increment in adenocarcinomas may be *N*-nitrosamine related. The conclusion that NNK conceivably plays an important role in tobacco-related human lung cancer is supported by its strong potential to induce lung tumorigenesis in rodents [6].

Japanese male smokers with a cytochrome P450 2A6 (CYP2A6) gene deletion-type polymorphism have been shown to be at reduced lung cancer risk in a hospital-based case control study [7,8]. CYP2A6 is in fact recognized as being involved in the mutagenic activation of promutagens such as tobacco-specific *N*-nitrosamines [9], and we earlier demonstrated that pretreatment with 8-MOP, a potent human CYP2A6 inhibitor, strongly inhibits lung tumorigenesis by NNK in female A/J mice. In the previous study, we administrated 8-MOP by the stomach tube, but this is impractical for chemoprevention in human lung cancer. Therefore, in the present investigation, we examined effects of dietary 8-MOP on NNK-induced lung tumorigenesis in female A/J mice.

Mouse CYP2A4 and CYP2A5 differ from each other in only 11 amino acids [10], and these enzymes are closely related to the human CYP2A6 [11]. Since mouse CYP2A5 is a specific and efficient catalyst of coumarin 7-hydroxylation, while CYP2A4 is not [12], the former is considered to be the mouse ortholog of human CYP2A6. However, both isoforms catalyzed methylene hydroxylation of NNK [13], considered to be responsible for carcinogen activation in the A/J mouse lung [14]. It has already been shown that 8-MOP inhibits the coumarin 7-hydroxylase activity of human CYP2A6 [15,16] and mouse CYP2A5 [17], presumably in a mechanism-based, non-competitive fashion [16]. To determine whether 8-MOP might also influence the mRNA expression of CYP2As in vivo, an assessment of CYP2A4 and CYP2A5 mRNA induction was included in the present study.

2. Materials and methods

2.1. Chemicals

8-MOP was purchased from Sigma (St Louis, MO) and NNK from Toronto Research Chemicals (Toronto, Canada).

2.2. Animals

Female A/J mice (5 weeks of age), purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan), were maintained in the Narita Laboratory (Kanagawa, Japan) Animal Facility, according to the institutional animal care guidelines. All animals were housed in polycarbonate cages with white wood chips for bedding, and given free access to drinking water and a basal diet, Labo MR (Narita Laboratory, Kanagawa, Japan), under controlled conditions of humidity ($60 \pm 10\%$), lighting (12 h light/dark cycle) and temperature (24 ± 2 °C).

2.3. Experimental design

The design for the lung tumorigenesis experiment is outlined in Fig. 1. When the mice were 7 weeks of age, they were fed diets supplemented with 8-MOP at concentrations of 100, 10 or 1 ppm (Groups 1, 2 and 3) for 3 days. 3 days after the first treatment, each group was given a single dose of NNK (2 mg/0.1 ml saline/mouse, i.p.) or an equal volume of saline (vehicle control). Group 4 was treated with NNK alone and Group 5 with 8-MOP 100 ppm + saline. They were then fed basal diets and maintained without further treatment until the termination after 16 weeks, when all surviving mice were killed under ether anesthesia. At autopsy, their lungs were excised and weighed and then infused with 10% neutral buffered formalin, and carefully inspected grossly. All macroscopically detected lung nodules were counted under

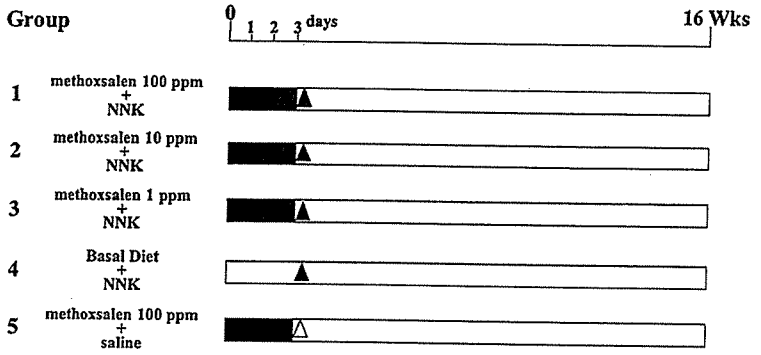

Fig. 1. Experimental design. Animals: A/J female mice, 7 weeks age ■; 8-MOP (100, 10, 1 ppm, p.o.), □: basal diet (vehicle control; Labo. MR, p.o.), ▲: NNK (2 mg/mouse, i.p.), △: saline (vehicle control; 0.1 ml/mouse, i.p.).

stereomicroscope, and each lung lobe was examined histopathologically.

2.4. Subjects for mRNA quantitation

In a separate experiment, mice at 7 weeks of age were treated with 8-MOP (50, 5 or 0.5 mg/kg body weight in 0.2 ml corn oil, given by stomach tube) or an equal volume of corn oil (vehicle control) daily for 3 days. All mice were killed at one hour following final 8-MOP treatment for lung and liver RNA isolation and quantitative analysis of CYP2As.

2.5. RNA isolation

Total RNA was isolated from 30 mg aliquots of whole lung and liver tissues using RNAlater RNA Stabilization Reagent (Qiagen Corp., Hilden, Germany) and an RNeasy Mini Kit (Qiagen Corp., Hilden, Germany). The concentration of RNA was measured from the absorbance at 260 nm and first strand cDNA was synthesized from 400 ng of total RNA using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster city, CA) according to the manufacturer's instructions.

2.6. Quantitative real-time RT-PCR

Optimal primers and probes were selected using Software Primer Express Ver.1.7 (Applied Biosystems, Foster city, CA). The TaqMan Rodent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) Control Reagent (Applied Biosystems, Foster City, CA) was used for PCR of GAPDH mRNA as an internal control. The sequences of primers and TaqMan probes for mouse CYP2A4 and CYP2A5 mRNAs were as follows: CYP2A4, forward 5′-GCTATGGCTTTCTGTTGCTCATG-3′, reverse 5′-ATCACCCGATCAATCTCCTCAT-3′, and probe, 5′-TTGAGGCCAAGGTC-3′; mouse CYP2A5, forward 5′-CCAACGTTATGGTCCTGTATTCAC-3′, reverse 5′-TCCACCAGAGCTTCCTTGACT-3′, and probe 5′-ACAGCACCACAATTC-3′. The sequences of primers and TaqMan probes for GAPDH mRNA were no known because of purchasing from Assays-on-demand system of ABI (Applied Biosystems, Foster city, CA).

Quantitative real-time RT-PCR was performed in an ABI PRISM 7000 Sequence Detection System using specific primers and TaqMan probes for mouse CYP2A4 and CYP2A5. PCR was carried out in 50 uL reaction mixtures containing 25 uL of 2×TaqMan Universal PCR Master Mix, 50 ng of cDNA, 100 nM of each primer, and 200 nM of TaqMan probe. Cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and then 40 cycles of 15 s at 95 °C followed by 1 min at 60 °C. PCR amplification of GAPDH mRNA was similarly carried out. TaqMan PCR products were detected as an increase in fluorescence from cycle to cycle. The amplification plots of the PCR reaction were used to determine the threshold cycle (Ct). The Ct value represented the PCR cycle at which an increase in reporter fluorescence (ΔRn) above the line of the optimal value was

first detected. The initial copy number of the target mRNA was calculated by a plot of the Ct against the input target quantity.

Both the precise amount and quality of total RNA are difficult to assess. Therefore, we also quantified transcripts of GAPDH gene as an internal control according to quantitative RT-PCR. Normalization of the data was achieved by quantitating the cycle number at an arbitrary fluorescence intensity in the linear exponential phase and calculating the ratio of the cycle number of each enzyme relative to that of GAPDH.

2.7. Statistical analysis

The data for final body weights were analyzed by the Student's *t*-test. The incidences of lung proliferative lesions were analysed by the Fisher's exact probability test and data for multiplicity by the Student's *t*-test. Dose dependence of the inhibitory effects of 8-MOP was assessed with the Spearman's rank correlation coefficient. Mouse CYP2A4 and CYP2A5 mRNA levels were analyzed by the Kruskal–Wallis test.

3. Results

The results for final body weights and intake of 8-MOP in A/J mice fed diets containing 8-MOP are shown in Table 1. Final body weights did not significantly differ among the groups. The average of daily 8-MOP intake in groups 1–5 was 21.39, 2.45, 0.24, 0 and 23.95 mg/kg b.w./day, respectively, increasing in proportion with the compound in the diet. Lung whitish nodules were detected in all groups macroscopically, but were very rare in the 8-MOP 100 ppm+NNK group. Lung proliferative lesions were hyperplasias and adenomas, diagnosed according to the criteria of 'International Classification of Rodent Tumors: The Mouse [18]', and their numbers were counted under a microscope. Lung carcinomas were not found in any of the animals. Incidences and multiplicities of lung proliferative lesions are summarized in Table 2. The values in the 8-MOP 100 ppm-treated Group 1 were significantly lower than in the NNK alone group ($P<0.001$). 8-MOP reduced the multiplicities of NNK-induced lung proliferative lesions in a dose dependent manner (Spearman rank correlation coefficient; $\rho=-0.806$, correction $P<0.0001$).

Data for relative-quantification of CYP2A mRNAs in livers and lungs of A/J mice are summarized in Fig. 2. Expression of mouse CYP2A4 and CYP2A5 mRNAs in liver and lung was not influenced by the 8-MOP treatment.

4. Discussion

The present investigation demonstrated clear inhibitory effects of 8-MOP in the diet on NNK-induction of lung tumorigenesis in female A/J mice. In contrast to our previous study [10], dose dependence was here apparent for multiplicity. Our earlier experiments showed strong inhibition of lung tumorigenesis at 12.5 mg/kg of 8-MOP by gavage. Daily 8-MOP intake in Group 1 fed the diet containing 100 ppm of 8-MOP was calculated to be 21.39±3.16 mg/kg/day, but it is supposed that plasma 8-MOP concentrations achieved were low compared to that with 12.5 mg/kg 8-MOP by gavage. The results suggest that administration of a diet supplemented with CYP2A6 inhibitors have practical potential for chemoprevention of tobacco related lung cancer.

In 16 week bioassays of NNK-induced lung tumorigenesis in A/J mice, multiplicities of lung

Table 1
Final body weights and intake of methoxsalen in A/J mice fed diets containing 8-MOP

Group	Treatment	No.[a]	Final body weight (g)[b]	Daily 8-MOP intake (mg/kg b.w./day)
1	8-MOP 100 ppm+NNK	20	26.54±6.14	21.39±3.16
2	8-MOP 10 ppm+NNK	20	26.86±5.77	2.45±0.31
3	8-MOP 1 ppm+NNK	20	26.24±5.43	0.24±0.02
4	NNK alone	20	27.33±5.97	0
5	8-MOP 100 ppm+saline	20	28.22±2.53	23.95±2.70

[a] Number of mice examined.
[b] Mean±SD.

Table 2
Data for incidences and multiplicities of NNK-induced lung proliferative lesions in A/J mice treated with 8-MOP

Group	Treatment	No.[a]	Hyperplasia		Adenoma		Hyperplasia + adenoma	
			Incidence (%)[b]	Tumors/ mouse[c]	Incidence (%)	Tumors/ mouse	Incidence (%)	Tumors/ mouse
1	8-MOP 100 ppm + NNK	20	5/20 (25.0)[d]	0.25 ± 0.44[e]	10/20 (50.0)[f]	0.60 ± 0.68 [g]	13/20 (65.0)[d]	0.85 ± 0.75[e]
2	8-MOP 10 ppm + NNK	20	16/20 (80.0)	1.50 ± 1.00	14/20 (70.0)	1.35 ± 1.39	17/20 (85.0)	2.85 ± 1.73
3	8-MOP 1 ppm + NNK	20	15/20 (75.0)	1.55 ± 1.43	17/20 (85.0)	2.15 ± 1.53	19/20 (95.0)	3.70 ± 2.54
4	NNK alone	20	19/20 (95.0)	1.85 ± 1.18	15/20 (75.0)	1.85 ± 1.57	20/20 (100)	3.70 ± 2.23
5	8-MOP 100 ppm + saline	20	2/20 (10.0)	0.10 ± 0.31	1/20 (5.0)	0.05 ± 0.22	3/20 (15.0)	0.15 ± 0.37

[a] Number of mice examined.
[b] Number of mice observed each lesion (%).
[c] Mean ± SD.
[d] Significantly different from Group 4 by Fisher's exact probability test ($P < 0.01$).
[e] Significantly different from Group 4 by Student's-t test ($P < 0.0001$).
[f] Significantly different from Group 3 by Fisher's exact probability test ($P < 0.05$).
[g] Significantly different from Group 4 by Student's-t test ($P < 0.001$).

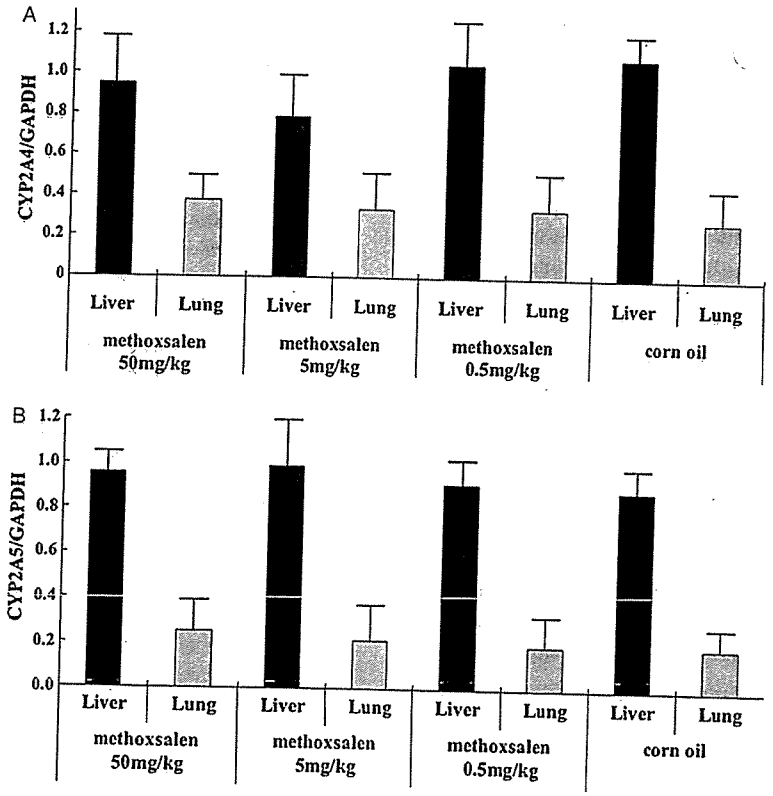

Fig. 2. Quantitative real-time RT-PCR analyses of mouse CYP2A4 and CYP2A5 mRNA expression in the livers and lungs. Data are mean ± SD values, normalized with respect to GAPDH.

tumors were 7.2–11.9 tumors/mouse [20–24]. In our previous study [19], multiplicity of NNK-induced lung adenomas and hyperplasia + adenomas were 5.97 and 8.16/mouse, respectively. In another unpublished study in our laboratory, the values were 13.4 and 15.6 tumors/mouse. However, in the present study, the corresponding figures were 1.85 and 3.70/mouse. It is difficult to explain these differences, but the basal diet used might be an important factor. In our previous study, we used Oriental MF diet (Oriental Yeast Co., Ltd, Tokyo, Japan) which is cereal based closed formula diet as the basal diet, whereas in the present study, we used Labo MR diet, another cereal-based closed formula diet. Hecht has reported that lung tumor multiplicity in NNK-treated mice is greater when maintained on the AIN-76A than the NIH-07 diet [20], respectively semi-synthesized and cereal based open formula diets. The suggestion was made that a component of the NIH-07 diet may inhibit tumor initiation by NNK. This is also as possible influence with our basal diets.

One other possibility is variation in viral infection. It is well established that bacteria or viruses play important roles in carcinogenesis in several organs. For example, H. pylori is considered a gastric carcinogen [25], the human papilloma virus causes cancer of the uterine cervix [26], and hepatitis B and C viruses causes hepatocellular carcinoma development [27,28]. Similarly, lung carcinogenesis may also be influenced by infection with viruses [29,30]. Furthermore, administration of antibiotics has been found to inhibit progression of rat lung carcinogenesis by suppressing chronic inflammation [31]. In the present study, mice were maintained under specific pathogen free (SPF) conditions, but in our previous study the animal laboratories were conventional (non-SPF).

8-MOP is a proven mechanism based inhibitor of human CYP2A6 [16] and mouse CYP2A4 and CYP2A5 [17], reducing enzyme activity. In the present study, we could not obtain any evidence that 8-MOP inhibits mRNA expression of the two isoforms. In conclusion, the results of this study showed clear dose dependent inhibitory effects of 8-MOP on NNK-induction of lung tumorigenesis in female A/J mice fed diet containing 8-MOP, apparently due to inhibition of activity of CYP2A4 and CYP2A5, rather than their mRNA expression.

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PSPC1, NONO, and SFPQ Are Expressed in Mouse Sertoli Cells and May Function as Coregulators of Androgen Receptor-Mediated Transcription[1]

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ABSTRACT

In Sertoli cells of testis, androgen receptor-regulated gene transcription plays an indispensable role in maintaining spermatogenesis. Androgen receptor activity is modulated by a number of coregulators which are associated with the androgen receptor. Non-POU-domain-containing, octamer binding protein (NONO), a member of the DBHS-containing proteins, complexes with androgen receptor and functions as a coactivator for the receptor. Paraspeckle protein 1 alpha isoform (PSPC1, previously known as PSP1) and Splicing factor, proline- and glutamine-rich (SFPQ, previously known as PSF), other members of the DBHS-containing proteins, are also found in androgen receptor complexes, suggesting that these DBHS-containing proteins may cooperatively regulate androgen receptor-mediated gene transcription. We demonstrated that PSPC1, NONO, and SFPQ are coexpressed in Sertoli cell line TTE3 and interact reciprocally. The effect of the DBHS-containing proteins on the transcriptional activity was assessed using the construct containing androgen-responsive elements followed by a luciferase gene. The results showed that all the DBHS-containing proteins activate androgen receptor-mediated transcription, and PSPC1 is the most effective coactivator among them. Furthermore, we confirmed the presence of PSPC1, NONO, and SFPQ proteins in Sertoli cells of adult mouse testis sections. These observations suggest that PSPC1, NONO, and SFPQ form complexes with each other in Sertoli cells and may regulate androgen receptor-mediated transcriptional activity.

androgen receptor, gene regulation, Sertoli cells, spermatogenesis, testis

INTRODUCTION

Spermatogenesis is a multistep process leading to the generation of highly specialized spermatozoa. The developmental process begins with spermatogonia that are committed to further differentiation by undergoing two meiotic divisions, resulting in haploid round spermatids. During spermiogenesis, haploid spermatids undergo drastic morphological changes, including formation of the acrosome and the sperm flagellum, decrease of the nuclear size due to the unique DNA packaging, and exclusion of most of the cytoplasm [1]. The complexity of the differentiation process requires a highly specialized program of gene expression of male germ cells [2, 3]. For example, cAMP responsive element modulator (CREM) has been shown to play an important role in germ cell-specific transcription by binding to CRE sequences [4, 5], and Poly (A) polymerase beta (PAPOLB) is known to adjust the timing of haploid-specific translation by controlling the cytoplasmic mRNA polyadenylation [6].

However, the endogenous gene expression program of male germ cells is not sufficient for spermatogenesis, and support from nearby Sertoli cells is indispensable. Throughout spermatogenesis, Sertoli cells interact directly with germ cells within the seminiferous tubules. Sertoli cells regulate highly organized and precisely synchronized germ cell development by nourishing germ cells via their secretion products [7–9]. These controls by Sertoli cells are also regulated by external stimuli. Androgen and androgen receptor (AR)-mediated gene transcription are important for the Sertoli cell functions [2, 10]. Mice lacking AR in Sertoli cells show spermatogenic arrest, which results in azoospermia and infertility [11, 12]. Thus, AR-mediated transcription in Sertoli cells plays an indispensable role in spermatogenesis.

AR belongs to the nuclear receptor superfamily that includes receptors for thyroid hormone, retinoic acid, estrogen, progesterone, glucocorticoid, and other hormones [13]. AR forms a homodimer and binds to androgen-responsive elements in promoters/enhancers of AR-driven genes. AR is composed of N-terminal transactivation domain (NTD), DNA-binding domain (DBD), and ligand-binding domain (LBD) at the C-terminus. NTD possesses an activation function domain 1 (AF-1) and is involved in making contact with the general transcriptional machinery [14–16]. The transcriptional activity of AR is modulated by coregulators, which include coactivators that enhance AR transactivation and corepressors that suppress AR transactivation [17–19].

Non-POU-domain-containing, octamer binding protein (NONO) is known as one of the coregulators of AR. NONO interacts directly with the AR AF-1 domain and acts as a coactivator [20, 21]. NONO contains a DBHS (Drosophila behavior, human splicing) domain characterized by two tandem RNA recognition motifs (RRMs) and a helix-turn-helix (HTH) DNA binding domain. In mammals, two other DBHS-

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containing proteins, Paraspeckle protein 1 (PSPC1, previously known as PSP1) and Splicing factor, proline- and glutamine-rich (SFPQ, previously known as PSF), have been reported [22, 23]. PSPC1 has two isoforms, alpha (referred to as PSPC1 in this paper) and beta, produced by alternative splicing. It has been reported that DBHS-containing proteins regulate nuclear receptors, such as progesterone receptor [24] and thyroid hormone receptor [25], and also participate in mRNA regulation in the nucleus, including splicing [26, 27], 3′-end cleavage [27, 28], and nuclear retention of edited RNA [29]. Since DBHS-containing proteins are identified in RNA-transporting granules [30], it is likely that these proteins also participate in RNA metabolism in the cytoplasm. In addition, DBHS-containing proteins are reported to be involved in activation of DNA topoisomerase I [31] and DNA double-strand break rejoining [32]. Therefore, these proteins are regarded as multifunctional proteins involved in various aspects of gene expression. In many cases, NONO and SFPQ are copurified [20, 27–40], suggesting that these proteins may control gene expression as a complex. We have previously confirmed the direct interaction between PSPC1 and NONO, and SFPQ by coimmunoprecipitation experiments and yeast two-hybrid assays [41], indicating that the DBHS-containing proteins interact reciprocally.

We have sought to define expression and function of DBHS-containing proteins in testis, and to elucidate the biological significance of these proteins in spermatogenesis. In this paper, we have shown the expression of the DBHS-containing proteins and reciprocal complex formations in the Sertoli cell line TTE3. These proteins enhanced AR-mediated transactivation, and we confirmed the expression of the DBHS-containing proteins in the Sertoli cells of adult mouse testis. These observations suggest that the DBHS-containing proteins may be involved in spermatogenesis by regulating AR-mediated transcription in the Sertoli cells.

MATERIALS AND METHODS

Plasmids, Cells, and Mice

The expression plasmids for Myc-tagged DBHS-containing proteins (pMyc-CMV-2-Pspc1, pMyc-CMV-2-Nono, and pMyc-CMV-2-Sfpq) were prepared by subcloning mouse *Pspc1alpha*, mouse *Nono*, and mouse *Sfpq* cDNAs (GenBank accession numbers: NM_025682, NM_023144 and NM_023603) into the pMyc-CMV-2 vector (Clontech). A PSPC1 RRM mutant (F118A, F120A, K197A, F199A), which did not bind to RNA, was generated as described previously [42] and cloned into pMyc-CMV-2. By using the same method, the expression plasmids for a NONO RRM mutant (pMyc-CMV-2-Nono RRM mutant, F113A, F115A, K192A, I194A) and a SFPQ RRM mutant (pMyc-CMV-2-Sfpq RRM mutant, F326A, F328A, K405A, I407A) were also generated. The expression vector for androgen receptor pSG5-hAR and $p(ARE)_2$-luc plasmid containing two consensus androgen-responsive elements were described earlier [43].

COS-1 cells were cultured in Dulbecco modified Eagle medium (DMEM)(Sigma) supplemented with 10% Donor Calf Serum (DCS) (Thermotrace) and maintained at 37°C in an atmosphere of 5% CO_2. TTE3 cells were cultured in DMEM containing 10% DCS on collagen type I pre-coated dishes (Celltight C-1, Sumitomo Bakelite) at the permissive temperature of 33°C or nonpermissive temperature of 37°C in an atmosphere of 5% CO_2.

Nine-week-old male BALB/cAJcl mice were purchased from CLEA Japan. Animal experiments were conducted in accordance with the National Institutes of Health standards established in the Guidelines for the Care and Use of Experimental Animals.

Mouse Monoclonal Antibody Production

Synthetic peptides corresponding to amino acids 505–523 in mouse PSPC1 (CFGRGSQGGNFEGPNKRRRY), 453–464 in mouse NONO (CPPAFNRPAPGAE), and 681–699 in mouse SFPQ (CAGYGRGREEYEGPNKKPRF) were purchased from BIO SYNTHESIS. These peptides, conjugated with KLH (Pierce), were injected twice at a 1-wk interval into BALB/cAJcl mice. Three days after the second boost, the lymph node cells were fused with the myeloma line P3U1. The culture media were screened by ELISA and following immunoblotting. The cells from the positive wells were cloned by the standard limiting dilution technique. Anti-PSPC1 (clone 1L4), anti-NONO (clone NC5), and anti-SFPQ (clone FC23) mouse monoclonal antibodies were used in this study.

Immunoblotting

Fifteen μg of TTE3 cell lysates prepared as described previously [41] were analyzed on 8% SDS-polyacrylamide gels and blotted onto Immobilon-P membrane (Millipore). After saturation with 5% skim milk (Difco) in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 137 mM NaCl) containing 0.1% Tween-20 for 1 h at room temperature, membranes were incubated with a 1:40 dilution of antibodies to either PSPC1, NONO, or SFPQ followed by an incubation with a 1:10000 dilution of HRP-conjugated goat anti-mouse IgG (ZYMED). Enzymatic activities were detected by ECL substrate (Amersham Biosciences).

Immunocytochemistry

TTE3 cells were fixed in 4% paraformaldehyde in PBS for 20 min and for an additional 30 min in methanol. After washing with PBS, coverslips were blocked with 10% goat serum. Cells were incubated with a 1:10 dilution of anti-PSPC1, anti-NONO, or anti-SFPQ antibody respectively for 1.5 h at room temperature. After washing three times with PBS, the cells were incubated with a 1:100 dilution of Alexa Fluor 546 goat anti-mouse IgG (Invitrogen), for 30 min. The expression of proteins was visualized by fluorescence microscopy (Olympus).

Immunoprecipitation

For immunoprecipitation analysis, TTE3 cells (1.8×10^7 cells) were harvested and lysed in 4.8 ml of lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% NP-40, 1 mM Na_3VO4, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM DTT) with or without 10 μg/ml RNase A and incubated at 4°C for 30 min followed by centrifugation at 15,000 rpm for 15 min. The resulting supernatants were decanted into fresh tubes and preabsorbed with Protein-A agarose beads (SIGMA) for 30 min followed by incubation with anti-PSPC1, anti-NONO, anti-SFPQ, or anti-Myc (9E10) antibody coupled to Protein A-agarose beads for 2 h. The immunoprecipitates were washed three times with lysis buffer. Immunoprecipitates were immunoblotted with each antibody.

Luciferase Assay

COS-1 cells were transiently transfected by the calcium phosphate precipitate method with 0.2 μg of $p(ARE)_2$-luc plasmid, 0.1 μg of pSG5-hAR, 0.3 μg of PSPC1, NONO, or SFPQ expression vector. The total amount of vector added to each well was adjusted by adding an empty vector pMyc-CMV-2. Six hours after transfection, the media were replaced with fresh media containing 0.2% DCS. Dihydrotestosterone (DHT, 10^{-9} M) ligand was added and cells were incubated for an additional 12 h. Luciferase activities were determined as described previously [44] using a TD-20/20 luminometer (Turner Designs). Each plasmid was assayed in triplicate at least three different times. pRL-tk (Promega) was cotransfected to normalize transfection efficiencies. All the data were analyzed by Student *t*-test using Microsoft Excel. A P value of less than 0.05 was considered to be statistically significant.

RT-PCR Analysis

COS-1 cells were transfected and treated with DHT as done in the luciferase assay with the exception that cells were cultured in 90 mm dishes. Cells were harvested, lysed in IP buffer (50 mM Tris-HCl pH 7.5, 200 mM NaCl, 5 mM EDTA, 1% Triton-X100, 0.5% NP-40, 250 mM sucrose, 1 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 50 mM NaF, 1 mM Na_3VO4, 1 mM DTT, 10 U/ml RNase inhibitor (TOYOBO), and incubated at 4°C for 20 min followed by centrifugation at 15,000 rpm for 15 min. The supernatants were decanted into fresh tubes and preabsorbed with Protein G-Sepharose 4 Fast Flow (Amersham Biosciences) for 30 min followed by incubation with anti-Myc monoclonal antibody (9E10) or anti-HA monoclonal antibody (12CA5) coupled to Protein G-Sepharose 4 Fast Flow for 1 h. After washing the immunoprecipitates five times with IP buffer, RNA was purified with an RNeasy Mini Kit (QIAGEN). Eluted RNA was reverse-transcribed using a Sensiscript RT Kit (QIAGEN) with the luciferase rv primer, (5′-CGAGTGTAGTAAACATTCCAAAACCGTGATGG-3′), and amplified

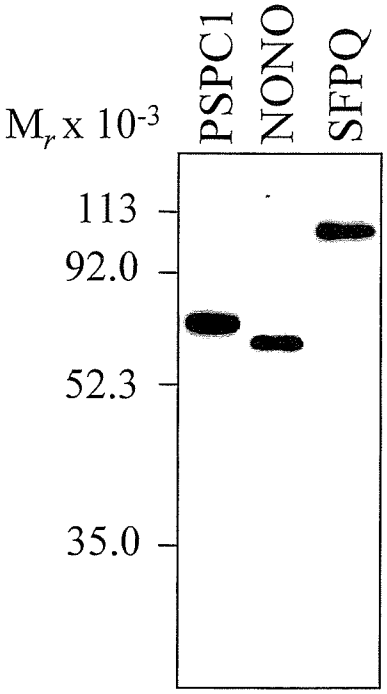

FIG. 1. PSPC1, NONO, and SFPQ expressed in Sertoli cell line TTE3. TTE3 cell extracts were electrophoresed on SDS-PAGE gels, transferred onto polyvinylidene fluoride membranes, and incubated with anti-PSPC1, anti-NONO, or anti-SFPQ antibodies.

using SP-Taq DNA polymerase (Hokkaido System Science) with the luciferase fw primer, (5′-CTAAAACGGATTACCAGGGATTTCAGTCGATG-3′), and the luciferase rv primer.

Immunohistochemistry

Testes were removed from BALB/cAJcl mice deeply anesthetized with Ketalar 50 (Sankyo) and fixed overnight in Bouin fixative. The testes were embedded in paraffin and cut into 8-μm sections. After deparaffinization and rehydration by xylene and serial dilutions of aqueous ethanol, the slides were immersed in sodium citrate buffer pH 6.0 and heated for 10 min at 121°C for antigen retrieval. The slides were washed in PBS and permeabilized with 0.2% Triton X-100 in PBS for 30 min at room temperature. After blocking with 10% goat serum and 1% BSA, cells were incubated with a 1:10 dilution of anti-PSPC1, anti-NONO, or anti-SFPQ antibody or a 1:300 dilution of anti-WT1 rabbit polyclonal antibody (Santa Cruz Biotechnology), in 2% goat serum and 1% BSA in PBS overnight at 4°C. Subsequently, slides were washed, incubated with a 1:100 dilution of Alexa Fluor 488 $F(ab')_2$ fragment of goat anti-mouse IgG (H+L) (Invitrogen) and goat anti-rabbit IgG (H+L)-TRITC (Zymed) in 10% goat serum and 1% BSA in PBS for 2 h at room temperature, and counterstained with DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride).

RESULTS

Monoclonal Antibody Production

Previously we reported that rabbit polyclonal antibody raised against bacterially expressed recombinant PSPC1 recognizes both of PSPC1alpha and its splicing variant PSPC1beta, and kidney expresses PSPC1beta exclusively [41]. However, mass spectrometric analysis showed that the immunoprecipitates from kidney actually contained one isoform of SEPT4 (previously known as M-Septin) [45], but not PSPC1beta (data not shown). These two proteins have a similar molecular mass (PSPC1beta; 45 kDa, SEPT4; 44 kDa) and share antigenic determinant EELRRXQE in PSPC1 (369–376) and SEPT4 (359–366). To avoid cross-reaction, we designed a new synthetic peptide specific to PSPC1, immunized BALB/cAJcl with the peptide, and developed a mouse anti-PSPC1 monoclonal antibody. On Western blotting, this antibody recognized a single band of 59-kDa protein in testis and kidney (see Supplemental Figure 1, available online at www.biolreprod.org). Similarly, we developed anti-NONO and anti-SFPQ mouse monoclonal antibodies. We performed a peptide competition study to verify the specificities, and the results indicated that these antibodies specifically recognized PSPC1, NONO, or SFPQ and had no cross-reactivity with other DBHS-containing proteins (see Supplemental Figure 2, available online at www.biolreprod.org). Moreover we carried out mass spectrometric analysis of the peptides immunoprecipitated from mouse testis extracts by the antibodies. MALDI profiles revealed that anti-PSPC1 antibody immunoprecipitated PSPC1 protein, anti-NONO antibody immunoprecipitated NONO protein, and anti-SFPQ antibody immunoprecipitated SFPQ protein (see Supplemental Table 1, available online at www.biolreprod.org). These data confirmed the antigenic specificities of monoclonal antibodies, and these antibodies were used for the following analysis.

DBHS-Containing Proteins Are Expressed in Sertoli Cell Line TTE3

NONO interacts with AR and enhances transcriptional activity, and PSPC1 and SFPQ, other members of the DBHS-containing proteins, coprecipitate with AR [20]. These data suggest that PSPC1 and SFPQ may also modulate AR activity. Androgen and AR-dependent gene transcription in Sertoli cells are essential for maintaining normal spermatogenesis. We investigated whether DBHS-containing proteins are expressed in Sertoli cell line TTE3, which is a conditionally immortalized testicular Sertoli cell line from transgenic mice bearing the temperature-sensitive simian virus 40 large T antigen gene [46]. Whole-cell extracts from TTE3 cells were separated by SDS-PAGE, transferred to polyvinylidene fluoride membrane, and probed with anti-PSPC1, anti-NONO, or anti-SFPQ antibody. PSPC1, NONO and SFPQ were expressed abundantly in TTE3 cells (Fig. 1). Next, we examined the intracellular distributions of DBHS-containing proteins in TTE3 cells. TTE3 cells were cultured on coverslips, fixed, and immunostained with each antibody. Strong expressions of PSPC1, NONO, and SFPQ were detected in the nucleus while faint signals for PSPC1 and NONO and an intensive signal for SFPQ were also observed in the cytoplasm (Fig. 2).

Endogenous DBHS-Containing Proteins in TTE3 Cells Form Complexes with Each Other

Complex formation among DBHS-containing proteins is a controversial issue. Fox et al. reported that PSPC1-NONO and NONO-SFPQ complexes were observed in HeLa cells, but PSPC1-SFPQ complex was not [42]. In our previous paper, all reciprocal interactions were shown by coimmunoprecipitation assays of overexpressed DBHS-containing proteins, and yeast two-hybrid assays [41]. Therefore, we investigated complex formation among endogenous DBHS-containing proteins in TTE3 cells. DBHS-containing proteins were immunoprecipitated with anti-PSPC1, anti-NONO, or anti-SFPQ antibody from TTE3 cells, and the immunoprecipitants were subjected to Western blotting with appropriate antibodies. PSPC1 coimmunoprecipitated with NONO and SFPQ, and NONO and SFPQ behaved similarly (Fig. 3). Addition of RNase to the cell extracts did not alter the immunoprecipitation, indicating that the interaction between DBHS-containing proteins is a direct protein-protein interaction. DAZAP1, which is abundantly expressed in testis, did not coimmunoprecipitate with DBHS-

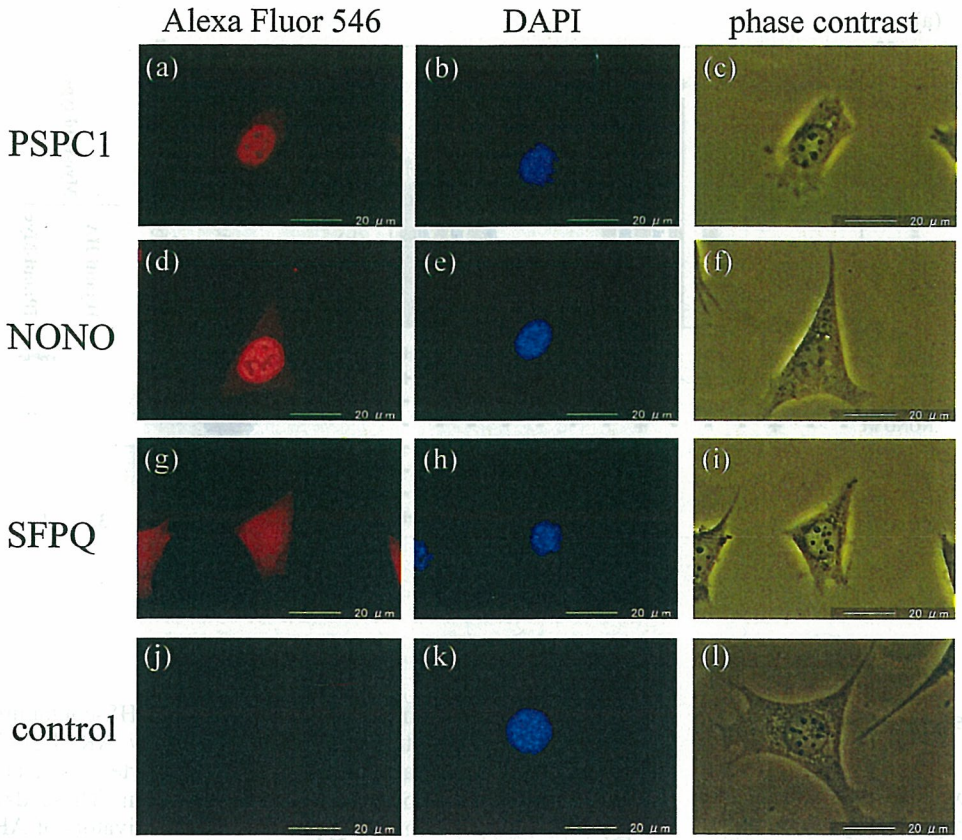

FIG. 2. Localization of the DBHS-containing proteins in TTE3 cells. TTE3 cells were grown on coverslips and labeled for immunofluorescence with antibody to PSPC1 (**a**), NONO (**d**), or SFPQ (**g**). The cells treated only with the secondary antibody were used as negative controls (**j**). **b**, **e**, **h**, and **k**) Nuclear staining by DAPI for the same samples in **a**, **d**, **g**, and **j**, respectively. **c**, **f**, **i**, and **l**) Phase contrast microscopy for the same samples in **a**, **d**, **g**, and **j**, respectively.

containing proteins [47, 48], showing that the interactions are significant. Interestingly, the amount of PSPC1 precipitated with anti-PSPC1 antibody was about the same as the amounts of NONO and SFPQ coimmunoprecipitated with PSPC1. Immunoprecipitations by anti-NONO antibody and anti-SFPQ antibody showed similar tendencies.

DBHS-Containing Proteins Regulate AR-Mediated Transcription

To investigate whether DBHS-containing proteins affect AR-mediated transcription, we planned to perform a luciferase assay with TTE3 cells. Although we tried various transfection reagents and transfection protocols repeatedly, transfection efficiency was extremely low. Therefore the luciferase assay with COS-1 cells using a reporter plasmid containing androgen-responsive elements was performed. COS-1 cells were transfected with expression plasmids for AR and DBHS-containing proteins and reporter plasmids, and treated with DHT. NONO enhanced the transactivation function of AR as previously reported [20, 21], and PSPC1 and SFPQ also enhanced the AR function. PSPC1 showed the highest transactivation of the reporter gene while NONO and SFPQ showed equally lower transactivation (Fig. 4a). DBHS-containing proteins are RNA binding proteins that share RNA recognition motifs (RRMs). We tested whether DBHS-containing proteins bind to luciferase mRNA. COS-1 cells were transfected, treated with DHT as done in the luciferase assay, and harvested. Myc-PSPC1, Myc-NONO, or Myc-SFPQ was immunoprecipitated from cell lysates with anti-Myc antibody. RT-PCR was performed for the immunoprecipitant as template with luciferase-specific primers, and weak binding of DBHS-containing proteins to luciferase mRNA was observed (Fig. 4b, lane 4). Next, to address the question whether the enhancement of luciferase activity is influenced by post-transcriptional regulation, the RRM mutants, in which the RNA binding activity was abolished without disrupting the overall structure of the RRM domain, were constructed according to the procedure of Fox et al. [42], and luciferase assays were performed. These mutants did not show RNA binding activity (Fig. 4b, lane 2) but did show activated AR-mediated transcription as well as the wild type DBHS-containing proteins in the luciferase assay (Fig. 4a). These data demonstrate that the enhancement of luciferase activity is mediated by transcriptional regulation, not by post-transcriptional regulation.

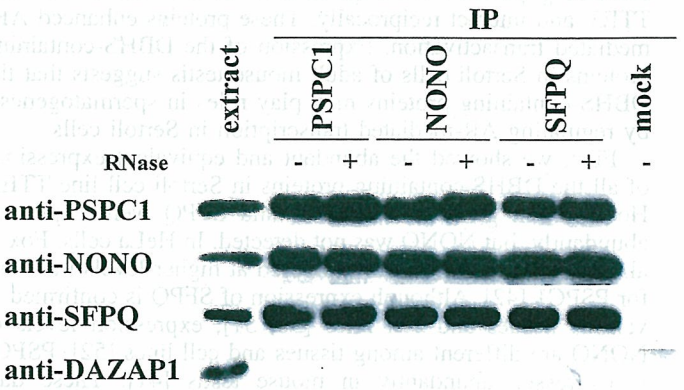

FIG. 3. DBHS-containing proteins interact reciprocally. TTE3 cell lysates treated with RNase or untreated lysates were subjected to immunoprecipitation with anti-PSPC1, anti-NONO, anti-SFPQ, or anti-Myc (9E10, for negative control) antibody. Western blotting was performed with whole extracts and immunocomplexes using an appropriate antibody.

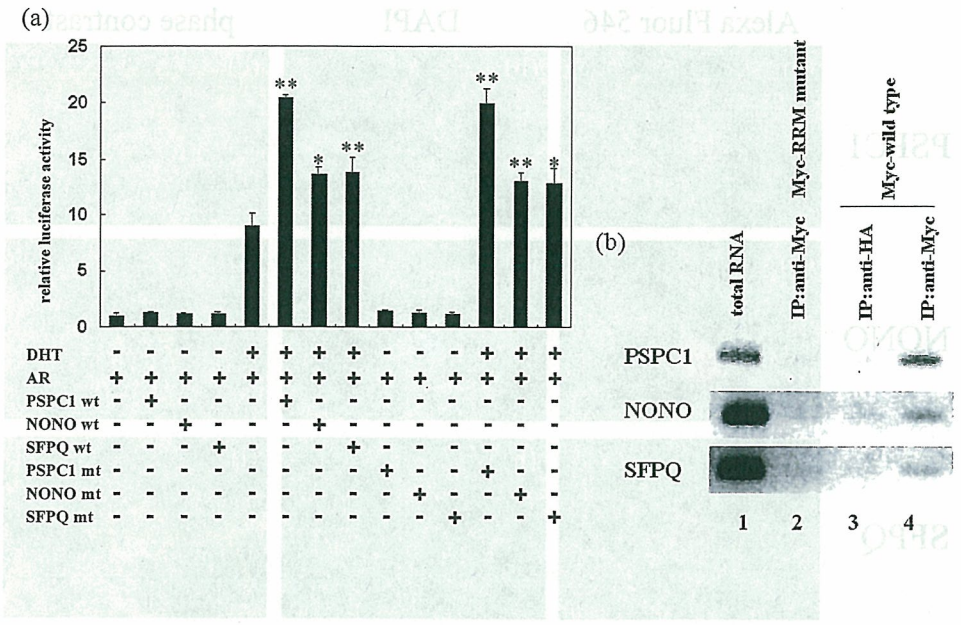

FIG. 4. DBHS-containing proteins regulate androgen receptor-mediated transcription. **a**) COS-1 cells were cotransfected with p(ARE)$_2$-luc (a luciferase reporter plasmid containing androgen-responsive elements), expression vector containing AR, and wild type (wt) or RRM mutant (mt) DBHS-containing protein expression vector in the absence (–) or presence (+) of dihydrotestosterone (DHT). The bars represent the mean ± SD. * $P < 0.05$ vs. control experiments without DBHS-containing protein expression vector; ** $P < 0.01$ vs. control experiments without DBHS-containing protein expression vector. **b**) DBHS-containing protein RRM mutants do not bind to luciferase mRNA. COS-1 cells were transfected with Myc-tagged DBHS-containing protein RRM mutant or Myc-tagged DBHS-containing protein wild type expression vectors as luciferase assay. Cell lysates were subjected to immunoprecipitation with anti-Myc or anti-HA antibody. RT-PCR was performed with immunoprecipitants as templates and luciferase-specific primers. Total cell RNA was assayed as a positive control.

DBHS-Containing Proteins Are Expressed in Sertoli Cells of Testis

DBHS-containing proteins were expressed in Sertoli cell line TTE3, along with activated androgen receptor-mediated transcription. We next examined the expression of DBHS-containing proteins in Sertoli cells of testis. Adult mouse testis sections were immunostained with anti-PSPC1, anti-NONO, or anti-SFPQ antibody. Anti-WT1 antibody was used as a marker to identify the Sertoli cells [49]. The expression of each DBHS-containing protein in seminiferous tubules displayed a distinct profile. The germ cells expressed PSPC1 and SFPQ, but not NONO (Fig. 5b, 5l and 5g, respectively). The signals of all DBHS-containing proteins were detected in the cells adjacent to the basal membrane of the seminiferous tubule. These cells also expressed WT1 (Fig. 5a, 5f and 5k), demonstrating that all the DBHS-containing proteins are expressed in the Sertoli cells.

DISCUSSION

In this study, we showed that all of the three DBHS-containing proteins are expressed in mouse Sertoli cell line TTE3, and interact reciprocally. These proteins enhanced AR-mediated transactivation. Expression of the DBHS-containing proteins in Sertoli cells of adult mouse testis suggests that the DBHS-containing proteins may play roles in spermatogenesis by regulating AR-mediated transcription in Sertoli cells.

First, we showed the abundant and equivalent expressions of all the DBHS-containing proteins in Sertoli cell line TTE3. However, in germ cells, PSPC1 and SFPQ were expressed abundantly, but NONO was not detected. In HeLa cells, Fox et al. reported that NONO is expressed at higher levels than that for PSPC1 [42]. Although expression of SFPQ is confirmed in various tissues and cell lines [50, 51], expression levels of NONO are different among tissues and cell lines [52]. PSPC1 is expressed abundantly in mouse testis [41]. These data indicate that at least expression levels of PSPC1 and NONO are different depending on tissues and cell types.

The transcriptional activity of AR is modulated by various coregulators, which include coactivators and corepressors. Luciferase assay using a reporter plasmid containing androgen-responsive elements indicated that all the DBHS-containing proteins enhance the transcription mediated by AR. PSPC1 showed the highest transactivation of the reporter gene, and NONO and SFPQ showed equal transactivation. These data suggest that DBHS-containing proteins are coactivators of AR. The coregulators can be divided into two major types. Type I coregulators, such as HMGB1 and HMGB2 [53] and CMTM2A [54], function primarily with the nuclear receptor at the target gene promoter to facilitate DNA occupancy, chromatin remodeling, or the recruitment of general transcription factors associated with the RNA polymerase II holocomplex. Type II coregulators, such as FLNC [55] and PAK6 [56], contribute to AR protein stability in the presence of ligands or influence the subcellular distribution of AR [17–19]. Although the molecular mechanism of AR transactivation by the DBHS-containing proteins remains elusive, the DBHS-containing proteins are known to have a DNA binding domain [23, 41], and we confirmed the expression of these proteins in the nucleus of the TTE3 cell line. It was previously reported that NONO enhances the association of transcription factors to their target DNA [57], suggesting that the DBHS-containing proteins enhance the association of AR to their targets, like HMGB1 and HMGB2 enhance the binding of AR, progesterone receptor, and glucocorticoid receptors to their target DNA and enhance the transactivation [53]. Alternatively, DBHS-containing proteins might activate transactivation of AR by stimulating DNA topoisomerase I to relieve torsional strain, and this is supported by the observation that NONO and SFPQ interact with DNA topoisomerase I and activate its enzymatic activity [31]. Thus we speculate that DBHS-containing proteins belong to the Type I coregulators that functions primarily at the target gene promoter site.

DBHS-containing proteins are known not only to regulate transcription but also to bind directly to RNA for post-transcriptional regulation. DBHS-containing proteins are also known to be involved in splicing, polyadenylation, nuclear retention of edited RNA, and transport of mRNA [23, 26–30]. In TTE3 cells, SFPQ was expressed not only in the nucleus but also in the cytoplasm, and weak expression of PSPC1 and NONO was observed in the cytoplasm. We also found weak but significant expression of PSPC1 in the cytoplasm of germ

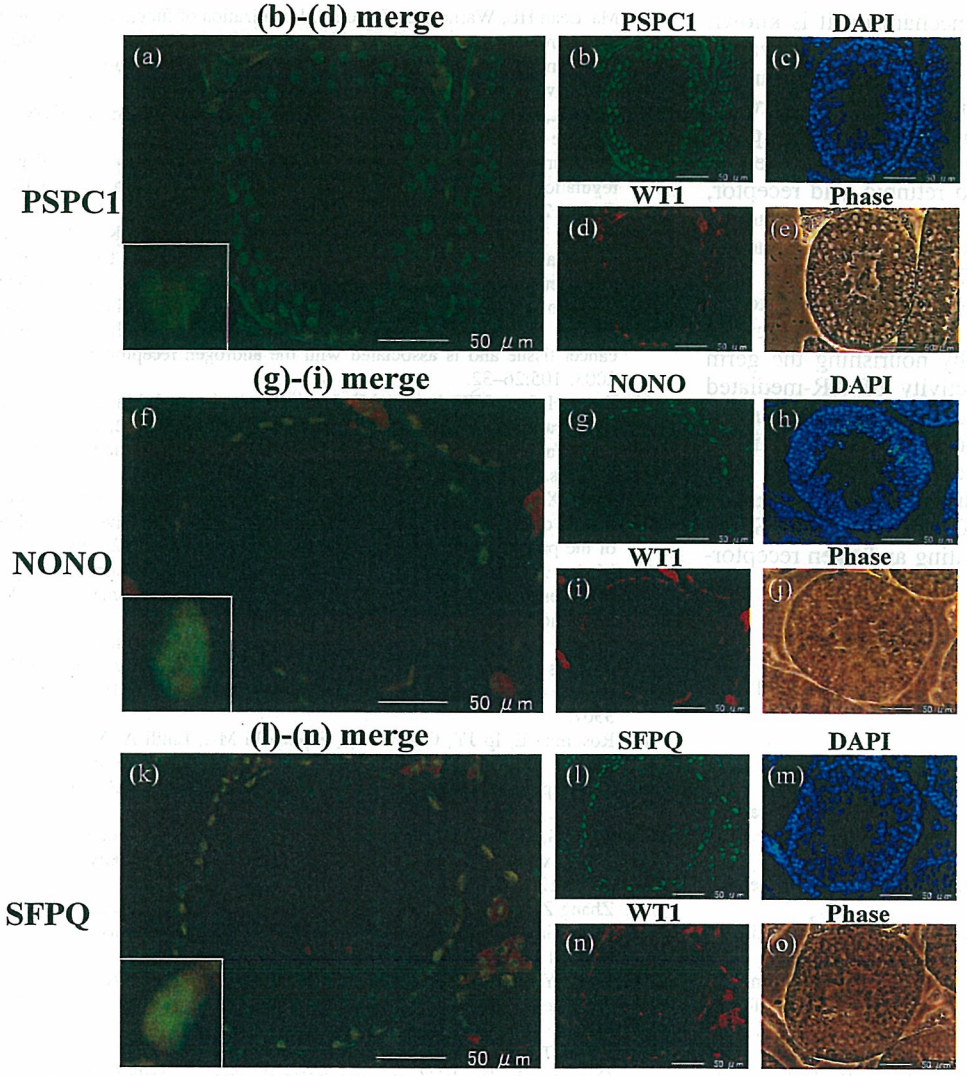

FIG. 5. Expression of DBHS-containing proteins in mouse adult testis. Sections of adult mouse testis were immunostained with anti-PSPC1 (**b**), anti-NONO (**g**), or anti-SFPQ (**l**) antibody. **d**, **i**, and **n**) Immunostaining with anti-WT1 rabbit polyclonal antibody for the same samples in **b**, **g**, and **l**, respectively. **c**, **h**, and **m**) Nuclear staining by DAPI for the same samples in **b**, **g**, and **l**, respectively. **e**, **j**, and **o**) Phase contrast microscopy for the same samples in **b**, **g**, and **l**, respectively. **a**) Merged image of **b-d**. **f**) Merged image of **g-i**. **k**) Merged image of **i-n**. The insets in **a**, **f**, and **k** show high magnifications of Sertoli cells.

cells (data not shown), suggesting that DBHS-containing proteins function also in the cytoplasm. From these findings, we speculate that DBHS-containing proteins may regulate not only transcription but also RNA metabolism such as splicing, mRNA export, and RNA nuclear retention. The DBHS-containing proteins may maintain spermatogenesis by activating AR-mediated transcription and regulating the transcribed mRNA both in the nucleus and cytoplasm of Sertoli cells.

Fox et al. reported that PSPC1 interacts with NONO, and not with SFPQ in HeLa cells [42]. However, we previously showed the interaction between overexpressed PSPC1 and NONO, and also between PSPC1 and SFPQ [41]. In this study, we investigated the complex formation among the endogenous DBHS-containing proteins in TTE3 cells, and observed all combinations of the interactions among PSPC1, NONO, and SFPQ. It has been reported that all the DBHS-containing proteins are found in the protein complex with the AR AF-1 domain [20]. Moreover, our preliminary proteomic analysis demonstrated that anti-PSPC1 antibody immunoprecipitates NONO and SFPQ together with PSPC1 in testis extract, and yeast two-hybrid assays using PSPC1 as a bait protein showed the interaction of PSPC1 with NONO and SFPQ (data not shown). Therefore PSPC1 actually interacts with both NONO and SFPQ. We assume that the inconsistency between the data by Fox et al. and by us may come from the difference in the interacting partner in the cell line used, not only from the difference in expression levels of the DBHS-containing proteins. If we assume that PSPC1 forms only heterodimers with NONO or SFPQ, PSPC1 should immunoprecipitate most abundantly among these proteins. However, the amount of PSPC1 precipitated with anti-PSPC1 antibody was almost the same as those of NONO and SFPQ coimmunoprecipitated with PSPC1. Immunoprecipitations by anti-NONO antibody and anti-SFPQ antibody also showed similar tendencies. These results raise the possibility that the DBHS-containing proteins may form multimers. It was previously reported that Hrp65 protein, *Chironomus tentans* homolog of the PSPC1/NONO/SFPQ family, can self-associate, and most of the Hrp65 protein are in complexes that consist of three to six Hrp65 protein molecules [58], suggesting that PSPC1, NONO, and SFPQ also form large complexes by interacting reciprocally. DBHS-containing proteins are known not only to regulate transcription but also to be involved in multiple regulations including RNA metabolism [23, 26–30] and DNA metabolism [31, 32]. It is likely that the differences in the composition of the DBHS-containing protein complex bring about functional diversity of DBHS-containing proteins.

We confirmed the expressions of all the DBHS-containing proteins in Sertoli cells of adult mouse testis, and these proteins exhibited enhancement of transactivation of AR. PSPC1 and SFPQ were expressed also in germ cells. Because germ cells do not express AR, PSPC1 and SFPQ expressed in germ cells may